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Sinéad T. Loughran · John Joseph Milne *Editors*

Protein Chromatography

Methods and Protocols

Third Edition

Humana Press

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Preface

Proteins play critical roles in virtually all cellular processes of living organisms. These macromolecules are to be found in roles that are enzymatic, regulatory, structural, and immunological to name but a few. To elucidate the structure and function of any protein, it is first necessary to purify it. The goal of protein chromatography is to study the structure, function, and interactions of proteins, and it is achieved by separating and purifying proteins based on properties such as size, charge, hydrophobicity, and affinity for specific ligands. Many purification schemes and chromatographic methods for the isolation of native proteins from complex sources have been developed over the years.

Every protein has its unique amino acid sequence, determined by the nucleotide sequence of a corresponding gene, which determines its specific three-dimensional structure and function. There has been a significant advancement in the field of protein biology in the last 50 years, driven by technological developments and a better understanding of the structure and function of proteins and of how we identify, isolate, and manipulate individual genes and proteins. Thus, the emergence of recombinant DNA technology, genomics, and bioinformatics means that now theoretically any protein can be expressed in a "tagged" and rapidly purifiable recombinant form from a heterologous host cell. This has resulted in a deeper understanding of the role of proteins in cellular processes and diseases and has led to the development of new treatments and therapies based on this knowledge.

The chapters in this volume cover various aspects of protein purification, including a range of techniques and approaches in protein extraction and purification as well as downstream processing issues, protein stability and quantitation, and the application of these techniques in areas such as clinical proteomics, gene therapy, and immunoprecipitation. As in any Methods in Molecular Biology™ volume, the emphasis here is on providing clear protocol-style chapters suitable for newcomers to the field. The chapters herein address topics such as tagging, storage, and lyophilization and the use of different chromatography methods, including ion-exchange chromatography, hydroxyapatite chromatography, lectin affinity chromatography, magnetic nanoparticle separation, and counter-current chromatography.

In brief, many of the chapters concern the generation and purification of recombinant proteins, reflecting the major contribution that molecular biology has made to the field. These deal with topics such as the purification of viral vectors and the tagging of proteins to enhance their solubility and simplify their purification on an individual scale or in high-throughput systems. It is of course also to be expected that a compilation such as this would include the more "classical" purification methods that are based on exploiting the physicochemical properties of the target protein. The reader will therefore find protocol-style chapters describing techniques such as membrane protein purification, immunoprecipitation, and protein extraction and purification by differential solubilization and not just the more commonly used methods but also more recently developed approaches such as single-step split intein ELP tag system purification and lectin-based affinity chromatography. We also felt that some topics necessitated treatment in an overview-style format, due to the need to encompass a substantial number of variations that have evolved within these areas (e.g., scale-up of protein purification and the tagging of recombinant proteins) or to cover emerging areas of interest (e.g., mixed-mode chromatography, continuous counter-current

chromatography, viral vector purification, and magnetic nanoparticles for protein separation and purification). These emerging areas have the potential to revolutionize the field of protein chromatography on several levels including by reducing the number of steps required to purify a protein (single-step purification and mixed-mode chromatography), by allowing higher productivity, enhanced process control, and reduced cost of purification (continuous chromatography), by facilitating purification of previously difficult-to-purify proteins (alternative ligand-based chromatography and mixed-mode chromatography) and by improving selectivity and robustness (mixed-mode chromatography). This compendium of methods in protein chromatography does not pretend to be comprehensive however, and we plead that an attempt to cover the entire potential menu in one volume would have been futile in this constantly evolving field.

We are indebted to all authors who have generously given their expertise and insight to enrich this volume. Our aims were to assemble contributions from experienced scientists who have hands-on expertise in the field of protein chromatography, and to place particular emphasis on the production of clearly presented step-by-step methodologies, tips, and associated explanatory notes as well as providing an overview of emerging areas in the field. We hope that those who use these methods will succeed in establishing them in their own laboratories and in troubleshooting any issues that arise. We wish to extend a particular thanks to the series editor, Prof. John Walker, for his patience, advice, and encouragement throughout.

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Chapter 1

A Synopsis of Proteins and Their Purification

Dermot Walls, Gary Cooney, and Sinéad T. Loughran

Abstract

The goal of protein purification is to separate a specific protein from all other biomolecules. Classical chromatographic procedures have been designed to exploit particular distinguishing features of individual target proteins, such as size, shape, physicochemical properties, and binding affinity. Advances in molecular biology and bioinformatics have positively contributed at every level to the challenge of purifying individual proteins and more recently have led to the development of high-throughput proteomic platforms. In this chapter, a synopsis of advancements in the field of protein chromatography is presented, with reference to the principal tools and resources that are available to assist with protein purification strategies.

Key words Protein, Purification, Chromatography, Proteome

The study of proteins and their associated functions is central to our understanding of virtually all fundamental biological processes. The term "protein purification" refers to a series of procedures that are designed to isolate a single protein type from a complex biological source such as tissue or a microbial/mammalian cell culture. Proteins are probably the most commonly purified type of biological molecule as they are integral components of cellular structures and many biological processes, and among other roles are to be found as enzymes, scaffold molecules, cell signal transducers, and components of gene regulatory complexes. A successful purification strategy is essential prior to performing structural and functional studies on a protein of interest. The various stages in the purification process (summarized in Table 1) may free the protein from a matrix in which it is confined, separate the protein from other nonprotein parts of the starting material, and finally separate the desired protein species from all other proteins and contaminants present. The isolation of one protein, free of all other biomolecules, is the primary objective, and separation procedures are designed to exploit any distinguishing features of the target protein, such as its size/shape, its physicochemical properties, and its binding affinity. Each stage (as outlined in Table 1) is crucial for achieving high

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Table 1
The various stages in the chromatography purification process of a protein

Purification stage	Description	Typical methods/techniques used
Cell lysis and protein extraction	Breaking open cells and extracting proteins	Sonication, homogenization, or mechanical disruption
Pre-purification	Removing cellular debris, nucleic acids, and other impurities	Centrifugation, filtration, or precipitation
Chromatography	Separating and purifying the protein from other impurities	Ion exchange, size exclusion, affinity, or hydrophobic interaction chromatography
Concentration	Concentrating the protein	Ultrafiltration, precipitation, or centrifugation
Buffer exchange and dialysis	Changing the buffer or salt conditions to ensure protein stability and activity	Dialysis or buffer exchange
Characterization	Confirming the identity and purity of the protein	SDS-PAGE, Western blotting, or mass spectrometry

purity and yield of the protein and may require optimization and modification depending on the properties of the protein being purified.

Proteins consist of long chains of amino acids (*see* Fig. 1) that are linked to each other by peptide bonds between their carboxyl group (COOH) and the amino group (NH_2) of the adjacent amino acid. At the beginning of the chain, or polypeptide, the first amino acid's amino group is unbound by another amino acid, and at the end of the chain, the final amino acid's carboxyl group remains unbound. The start and end of a given polypeptide are, therefore, referred to as its N-terminus (NH_2) and C-terminus (COOH), respectively. Each of the natural 20 amino acids that are found in proteins are distinguished from one another by their variable "R group," or side chain, which largely determines the physicochemical properties of that amino acid (*see* Table 2). The sequence of amino acids determines the conformation of the protein and, consequently, its overall function. In their native states, proteins are complex-folded molecules whose functions are linked to their three-dimensional structure [1]. The folding of polypeptide chains is highly complex and is the end result of multiple intra- and inter-polypeptide chain interactions. Hydrogen bond formation between amino acids yields different repeating patterns along a polypeptide chain, such as α-helices and β-strands, which are structures that form in order to decrease the energy states of the primary polypeptide sequence. During protein folding, amino acids with hydrophobic side chains can fold inward away from contact with water. When

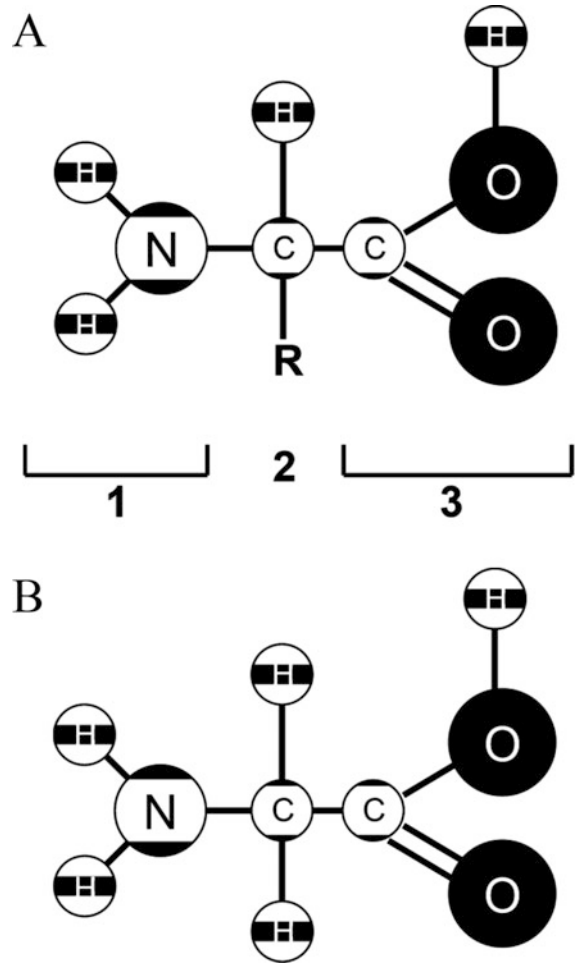

Fig. 1 Amino acid structure (**a**) with the amino group [1] on the left; the amino acid side chain R [2] and the carboxyl group [3] on the right (the acidic group). The amino acid Glycine, shown in (**b**), with only a hydrogen atom as its side chain, is the smallest of the 20 amino acids

these hydrophobic amino acids have retreated inward, the rest of the polypeptide chain is free to take up its final conformation, known as its tertiary structure, which is held together by weak noncovalent bonds, including van der Waal's interactions, hydrophobic packing, hydrogen bonds, and salt bridges [2–4]. Some proteins are composed of more than one polypeptide, or more than one copy of the same polypeptide, that adapt a stable organized superstructural arrangement known as a quaternary structure. Following translation, the last step in the biosynthesis of many proteins involves post-translational modification (PTM). PTMs play critical roles in regulating protein structure, function, localization, and stability. Such modifications can include: (a) the addition of functional groups including carbohydrates

Table 2
Amino acid abbreviations and polarity

Amino acid name	Three-letter code	One-letter abbreviation	Polarity
Alanine	Ala	A	Nonpolar, hydrophobic
Arginine	Arg	R	Polar, charged, hydrophilic
Asparagine	Asn	N	Polar, uncharged, hydrophilic
Aspartic acid	Asp	D	Polar, charged, hydrophilic
Cysteine	Cys	C	Polar, uncharged, hydrophilic
Glutamic acid	Glu	E	Polar, charged, hydrophilic
Glutamine	Gln	Q	Polar, uncharged, hydrophilic
Glycine	Gly	G	Nonpolar, hydrophobic
Histidine	His	H	Polar, charged, hydrophilic
Isoleucine	Ile	I	Nonpolar, hydrophobic
Leucine	Leu	L	Nonpolar, hydrophobic
Lysine	Lys	K	Polar, charged, hydrophilic
Methionine	Met	M	Nonpolar, hydrophobic
Phenylalanine	Phe	F	Nonpolar, hydrophobic
Proline	Pro	P	Nonpolar, hydrophobic
Serine	Ser	S	Polar, uncharged, hydrophilic
Threonine	Thr	T	Polar, uncharged, hydrophilic
Tryptophan	Trp	W	Nonpolar, hydrophobic
Tyrosine	Tyr	Y	Polar, uncharged, hydrophilic
Valine	Val	V	Nonpolar, hydrophobic

(glycosylation), acetate (acylation), phosphates (phosphorylation), and lipids; (b) structural changes including proteolytic cleavage of the protein, disulphide bond formation between inter- or intra-chain cysteine residues, or racemization of proline residues; (c) covalent linkage to other polypeptides such as SUMO protein (SUMOylation), ubiquitin (ubiquitination), or ISG15 (ISGylation); and (d) chemical modification of individual amino acids by deamination, deamidation, or eliminylation. Proteins are therefore complex, fragile, and highly interconnected polymers.

In order to determine the structure and function of any protein, it is first necessary to purify it. The history of protein purification dates back over 200 years to 1789 when Antoine Fourcroy reported on attempts to isolate substances from plants that had similar properties to egg albumen. One hundred years later, Franz Hofmeister obtained the first crystals of a protein, namely,

ovalbumin, and now more than 50 years have elapsed since Max Perutz and John Kendrew used X-ray crystallography to decipher the first protein molecular structures, namely, those of hemoglobin and myoglobin, for which they consequently received the Nobel prize in Chemistry [5]. Large-scale protein production for commercial purposes first appeared during World War II, driven by an acute need for blood proteins. Thus, the Cohn fractionation procedure, developed by American biochemist Edwin J. Cohn, for the purification of albumin and other serum proteins was first described in 1946 [6] and was still in recent use, although not as widely as it was in the past. Enzymes, in particular, became targets for purification and crystallization, with large-scale applications being developed in the food, detergent, and cosmetic industries among others. In many cases, these enzyme preparations, which were usually obtained from microbial sources and included amylases, lipases, and proteases, were pure enough in terms of activity as was necessary for their intended purpose. The purification processes involved were minimal, and such "process enzymes" were in fact not very pure at all. Most of the methodologies for purifying proteins from native sources were conceived in the 1960s and 1970s enabling small quantities of highly purified proteins to be obtained from large quantities of biological materials such as animal and plant tissues. Such were the quantities of materials needed that in one case, and by extrapolation, it would have necessitated starting with 500,000 sheep brains in order to produce 5 mg of sheep growth hormone. Since that time, the rapid progress made in molecular biology led to the point where theoretically any protein could be produced in recombinant form and in unlimited quantities from heterologous hosts such as bacteria, fungi, cell cultures of insect, plant and animal origin, and even whole animals and plants. In 1988, yeast-derived recombinant bovine chymosin, a new substitute for neonatal calf rennet in cheese making, was the first recombinant enzyme to gain approval from regulatory authorities for use in food. The revolution in recombinant DNA technology brought additional improvements to almost every stage of recombinant protein production and was accelerated even further with the introduction of the polymerase chain reaction (PCR) and associated methodologies. Rational approaches to "protein engineering" by site-directed mutagenesis of the corresponding recombinant gene, gene fusion methods, gene codon content optimization, improvements to expression vectors, and tailoring of host-cell genotypes all ensured that many problems with issues such as protein activity, stability, ease of purification, yields, post-translational modifications, folding, and downstream processing could be addressed. Recombinant products, such as protein hormones and their receptors, hematology-associated proteins including clotting factors and clot-busting molecules, and proteins with immunomodulatory functions including antibodies and vaccine

components, are now big business. The biopharmaceutical manufacturing industry uses genetic information to discover, develop, manufacture, and commercialize recombinant biotherapeutics that address significant medical needs.

The physicochemical properties of proteins, such as size/shape, charge, hydrophobicity, and solubility, have been exploited over the years leading to the development of techniques that enabled individual proteins to be purified from a crude source. *Protein chromatography* is a collective term applied to a set of techniques in which individual protein components of a starting mixture of proteins (the "mobile phase") are differentially retained as they pass through a solid medium or "stationary phase." A stationary phase typically consists of beads of varying diameter and internal surface area, packed into a column. The mobile phase, which can be aqueous or organic, then moves the sample through the column, and the separation efficiency and speed of elution of the analytes depend on their relative affinities for both phases. Such differing partition coefficients between protein components in the mixture leads them to move at different rates, thus achieving separation. Size/shape differences between proteins can be exploited by methods such as size exclusion chromatography and ultracentrifugation. Unlike other modes of chromatography, such as affinity or ion-exchange chromatography, molecules do not bind to the porous bead resin in size exclusion, which makes it relatively "mild" in comparison and also means buffer conditions can be tailored to suit the type of sample or the downstream requirements.

The overall charge of a protein is the result of all of the individual charges of its constituent amino acids, and this can vary at different pH values. At its isoelectric point (p*I*), the overall charge of a given protein is neutral, as all of the positive charges balance all of the negative charges. At lower pH values, a protein takes on a more positive charge, and at higher pH values a protein adopts a more negative overall charge. The charge of a protein can be exploited for the purpose of purification by methods such as ion-exchange chromatography, isoelectric focusing, and chromatofocusing. Ion-exchange chromatography uses resins of varying charge as the stationary phase. Buffers of different ionic strength are then used to competitively interact with the stationary phase to elute bound proteins. In the case of isoelectric focusing, the analyst uses agarose, polyacrylamide, or starch gels in which an immobilized pH gradient has been established.

An electric field is then applied, and individual protein species are resolved according to their p*I* values. Chromatofocusing recapitulates isoelectric focusing chromatography using a fast protein liquid chromatography (FPLC) instrument whereby buffers of different pH are used to generate a gradient for eluting proteins.

The hydrophobicity of a protein is determined by the quantity, nature, and distribution of hydrophobic amino acids throughout

the polypeptide chain. Although tending to be internalized within the folded protein, external hydrophobic patches or pockets often exist and can aid in the purification of a protein by methods such as hydrophobic interaction chromatography (HIC). HIC works in an analogous way to conventional chromatography in that the difference in affinity between analytes (proteins) and the stationary phase is due to hydrophobic regions on protein surfaces interacting with the hydrophobic stationary phase in the column. This binding is promoted at higher salt concentrations and suppressed in low salt concentrations.

Indeed, it is worth noting that newer commercially available mixed-mode resins have gained some popularity where a common strategy for purification is to use stationary phases that facilitate adsorption of proteins through a combination of both electrostatic and hydrophobic interactions. Subsequent protein elution can then be achieved by electrostatic charge repulsion strategies (*see* Chapter 2).

Protein solubility in water is the result of interactions between water molecules and hydrophilic amino acids on the polypeptide chain. The solubility of a protein, for instance, can be altered by pH, temperature, and ionic strength. This fact can be exploited by methods such as ammonium sulfate precipitation, in which the strength of that salt in the mixture is progressively increased to a point at which the protein of choice starts to precipitate or is said to "salt out" of solution. The biological affinity of a protein for a particular immobilized ligand on the stationary phase can also be utilized for the purposes of purification. Thus, enzyme–substrate, ligand–receptor, and antibody–antigen interactions can be exploited for affinity chromatography by first binding the interacting molecule to a solid support and using it to trap the target partner protein molecule. Antibody-based protein chromatography protocols can serve to purify either the target protein (antigen) with which the antibody interacts or the antibody itself. Thus, antigen- or antibody-bound stationary phases (e.g., agarose beads) incorporate one of these molecules, and the protein of interest is retained due to the antibody–antigen interaction.

Small-scale protein isolations, with polypeptide sequence determination as the end point objective, demand the recovery of a highly purified product, and acceptable sacrifices can be made in terms of protein losses during purification steps. High recovery and economy of processing would be primary considerations, however, during the commercial production of large amounts of protein on a continual basis. Protocols for the purification of proteins using many of the methods mentioned above are detailed throughout this volume as are methods for protein quantitation and analysis of purity (*see* Chapter 16). In most cases, homologous proteins differ to a rather small degree between most of the higher mammals. Thus, a protocol designed for the purification of a given native

protein from a pig tissue may not need much adjustment for application to the isolation of its human counterpart from the corresponding human tissue. Whether the objective is to generate purified native protein or its equivalent recombinant form, the starting source material should ideally express significant quantities of that protein [and therefore its corresponding messenger RNA (mRNA) in the case of a molecular cloning project]. The investigator should do their homework, review the literature, and be mindful of issues including tissue-specific expression, possible temporal expression, and the existence of mRNA splice variants leading to multiple protein isoforms. The choice between yield and purity of final product will obviously depend on the intended purpose. If small amounts of highly pure protein are required for peptide sequencing analysis, ultimately leading on to DNA coding sequence identification, then sacrificing yield for the sake of purity may be logical. The advent of major genome sequencing programs and associated bioinformatics software packages now means that many genes for both known proteins and proteins of unknown function are becoming available and are being directly cloned and expressed in recombinant form from heterologous host cells. The cloning procedure usually ensures that when expressed, these molecules are "tagged" with some sort of additional "affinity handle," a short peptide or small protein domain that serves to facilitate purification of its fusion partner. These tags are the equivalent of molecular Velcro™, and this approach obviates the need to develop purification strategies that exploit the physicochemical properties of the target protein during purification from its native or heterologous host species (*see* Chapter 7 for a detailed discussion on such tags and their uses). The production of recombinant proteins has greatly facilitated structural studies using Nuclear Magnetic Resonance and X-ray crystallography, both of which require milligram quantities of pure protein. Researchers can now avail of off-the-shelf products for every step of the protein purification process—from cell disruption, to purification resins and reagents for concentrating and desalting the final product. Suppliers of certain reagents and equipment are noted where pertinent throughout this volume and a general, although not exhaustive, list of suppliers of protein purification-associated products and technologies is given here (*see* Table 3).

The term "Proteome" was first introduced by Marc Wilkins and Keith Williams in 1994 to describe the entirety of all proteins encoded in a single genome expressed under distinct conditions. The development of Matrix-Assisted Laser Desorption/Ionization (MALDI) and Electron-Spray Ionization (ESI) techniques, in conjunction with mass spectrometric analysis of large and fragile biomolecules, was a major breakthrough in protein analysis that has ultimately enabled rapid high-throughput protein identification on a global scale [7]. In general, protein identification is done in either

Table 3
Major conglomerate suppliers that provide many of the products and technologies required for protein purification, and an indication of the types of products that they offer

Supplier	Products/services/technologies	Website
ThermoFisher	Elisa kits for the quantification of protein yields Various protein extraction/purification kits including cell surface protein isolation kit, tissue extraction kit (total protein extraction), and kits for glycoprotein isolation An assortment of columns, resins, and immobilized phases Ligands and target molecules such as antibodies and protein A, which are well established in the field of protein purification Magnetic beads for IMAC	www.thermofisher.com
Merck Millipore	Purification & Detection Products FLAG® System Magnetic Agarose Agarose Beads for Epitope Tag Protein Purifications HIS-Select® Technology EZview™ High Visibility Resin Recombinant Protein Expression MAT-Tag™ System An assortment of buffers and other chemicals and equipment required by an analyst	www.sigmaaldrich.com
Cytiva	Size exclusion chromatography columns—Sepharose and Sephadex. Affinity chromatography columns (Histidine tagged- Metal chelate and Protein A tagged) Various media resins for ion chromatography Lab scale chromatography systems and software Autosamplers, filters, gaskets and sealings, and a large assortment of other essential chromatography accessories	https://www.cytivalifesciences.com/

of two ways, "top down" or "bottom up." Top down involves the analysis of the full protein structure by way of ESI or MALDI techniques typically using Time of Flight Mass Spectrometry (TOF MS). Bottom up involves digestion of the protein prior to analysis followed by subsequent MS analysis of the peptide fragments [8]. Focusing on protein targets that are relevant to disease processes requires first determining where and to what extent a given protein is expressed. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or liquid chromatography followed by mass spectrometric analysis of individual proteins is a popular method for comparative proteome-wide screening studies. Prior selective removal of the most abundant proteins—the albumins and immunoglobulins in the case of sera or plasma for example—significantly increases the chances of finding proteins that are rarer and probably more interesting. In Chapter 15, the power of LC-MS for biomarker discovery in clinical proteomic studies is

outlined with two protocol methods to analyze clinical patient samples using One-Dimensional Reverse Phase Chromatography (RPC) or High pH Reversed-Phase Peptide Fractionation.

The ultimate goal of any proteomics study is to determine the roles of all proteins encoded by a given genome. This entails elucidating the structures, functions, and protein–protein interactions of many proteins in parallel. One widely used approach for the simultaneous production of hundreds of proteins starts with their expression in a heterologous host such as *Escherichia coli*. The purification of recombinant proteins expressed from complementary DNAs in such heterologous systems typically involves a host-cell lysis step followed by the affinity purification of the tagged target protein from the lysate using an affinity resin. Simultaneously dealing with large numbers of samples poses a particular challenge, however, one that has fostered fruitful academia-industry collaborations. Automation-friendly lysis and affinity-capture reagents are now available for high-throughput protein purification procedures using multi-well platform formats, and many have been validated with a range of robotic liquid handlers. The principal advantages to using automated systems over manual pipetting include the former's greater reproducibility and consistency. The expression, capture, and assay of several hundred tagged proteins can now be performed in just a few hours. There are frequently issues with recombinant protein insolubility, structural integrity, and degradation when using cell-based expression systems, however, and the expression of cDNAs by transcription and translation in a "cell-free" in vitro format is one way around this problem [8, 9]. Thus, protein arrays are being developed whereby proteins, either tagged or untagged, are produced from cDNAs that have been immobilized on an inert surface.

High-throughput approaches have benefitted much from the advances made in the development of bioinformatics tools for protein identification, characterization, structure prediction, and analysis. For example, a typical two-dimensional liquid chromatography coupled to tandem mass spectrometry (2D–LC–MS/MS) generates in the order of tens of thousands of tandem MS spectra per day making impossible the efficient manual analysis of such data. Data processing using powerful bioinformatics software is necessary to get the most from such sequence information and to save time. Bioinformatics tools for proteome data set browsing and analysis are available both as propriety software and open-source software tools [7] (*see* Table 4). Pivotal to peptide and protein identification is comparisons of properties observed following separation (including mass, p*I*, amino acid composition, and fragmentation patterns) with predictions that are generated in silico. Thus, Internet resources are available for identifying peptide sequences based on their observed masses following protein fragmentation, and online prediction tools are available to help identify proteins

Table 4
Bioinformatics and web-based tools for protein identification, characterization, structure prediction, and analysis

Hosting site	Description	Link
Expasy: Bioinformatics resource portal of the SIB Swiss Institute of Bioinformatics	A site containing descriptions and links to a wide range of bioinformatics tools made available through the expasy server	https://www.expasy.org/
US Food and drug administration (FDA).	The National Center for Toxicological Research conducts research in both bioinformatics and chemoinformatics with the goal of developing methods for the analysis and integration of complex genomic, transcriptomic, proteomic, and metabolomic datasets	http://www.fda.gov/ScienceResearch/BioinformaticsTools/default.htm
Online Analysis Tools—Protein Chemistry	Additional Bioinformatics tools that are not available from the other links above	molbiol-tools.ca
RCSB Protein Data Bank	RCSB PDB is the US data center for the global Protein Data Bank (PDB) archive of 3D structure data for large biological molecules (proteins, DNA, and RNA)	RCSB.org
Tools for data analysis		
BLAST	Finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance	https://blast.ncbi.nlm.nih.gov/Blast.cgi
ProtParam	Allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL or for a user-entered protein sequence	https://web.expasy.org/protparam/
SIEVE	Thermo Scientific SIEVE software is an automated solution for the label-free, semiquantitative differential expression analysis of proteins, peptides and metabolites, and liquid chromatography-mass spectrometry (LC-MS) data sets	www.thermofisher.com
MASCOT daemon	Mascot Daemon is a Microsoft Windows client application that automates the submission of data files to Mascot server	www.matrixscience.com

(continued)

Table 4
(continued)

Hosting site	Description	Link
MaxQuant	MaxQuant is a quantitative proteomics software package designed for analyzing large mass-spectrometric data sets. It is specifically aimed at high-resolution MS data	www.MaxQuant.Org
The Trans-Proteomic Pipeline	Software tools for the analysis of MS/MS datasheets	www.proteomecenter.org
Skyline	Skyline is a freely available, open-source Windows client application for building a variety of quantitative methods and analyzing the resulting mass spectrometric data.	skyline.gs.washington.edu
Proteome discoverer	Proteome Discoverer software simplifies a wide range of proteomics workflows, from protein and peptide identification to post-translational modification (PTM) analysis to isobaric mass tagging for quantitation. Supports multiple database search algorithms (SEQUEST, Z-Core, Mascot, etc.) and multiple dissociation techniques (CID, HCD, ETD)	www.thermofisher.com
Uniprot	Provides a comprehensive, high-quality, and freely accessible resource of protein sequence and functional information	www.uniprot.org
Cytoscape	An open-source software platform for visualizing complex networks and integrating these with any type of attribute data	www.cytoscape.org
Ingenuity	Provides intuitive web-based applications for quickly analyzing and accurately interpreting the biological meaning in acquired genomics data	https://digitalinsights.qiagen.com/
GOEAST	A web-based software toolkit providing easy to use, visualizable, comprehensive, and unbiased Gene Ontology (GO) analysis for high-throughput experimental results	omicslab.genetics.ac.cn/GOEAST/
KEGG	KEGG is a database resource for understanding high-level functions and utilities of biological systems from molecular-level information, especially large-scale molecular datasets generated by high-throughput experimental technologies	www.genome.jp/kegg

(continued)

Table 4
(continued)

Hosting site	Description	Link
DAVID	The Database for Annotation, Visualization, and Integrated Discovery (DAVID) provides a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large list of genes	david.ncifcrf.gov
Phyre2	View human sequences and structures and map genetic variants, used to predict the structure of proteins that have not yet been experimentally determined	http://www.sbg.bio.ic.ac.uk/phyre2
Databases		
Peptide atlas	PeptideAtlas is a multi-organism, publicly accessible compendium of peptides identified in a large set of tandem mass spectrometry proteomics experiments	www.peptideatlas.org
Pride	The PRIDE PRoteomics IDEntifications (PRIDE) database is a centralized, standards compliant, public data repository for proteomics data, including protein and peptide identifications, post-translational modifications and supporting spectral evidence	www.ebi.ac.uk
Human Proteinpedia	Human Proteinpedia is a community portal for sharing and integration of human protein data. It allows research laboratories to contribute and maintain protein annotations	www.humanproteinpedia.org
GPMDB	Large database of detailed information about the evidence supporting the observation of proteins, peptides, PTMs, and SAVs using modern tandem mass spectrometry-based proteomics	https://www.thegpm.org/
NeXtprot	A knowledge platform on human proteins. A comprehensive resource that provides a variety of types of information on human proteins	www.nextprot.org
Human Protein Atlas	A site dedicated to summarizing current knowledge on proteome families	www.proteinatlas.org

based on their p*I* and amino acid composition. There are also software programs for discovering potential protease cleavage sites on a protein, for determining the theoretical isotopic distributions of peptides/proteins and for assisting the analyst with predicting possible oligosaccharide structures that occur on proteins based on their experimentally determined mass (*see* Table 4).

In this compendium, the reader will find detailed overviews of protein purification methodologies coupled with user-friendly, step-by-step protocols for both selected classical techniques and methods for isolating recombinant proteins.

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Chapter 2

Mixed-Mode Chromatography and Its Role in Monoclonal Antibody Purification

John Joseph Milne

Abstract

As the biopharmaceutical industry matures and embraces process intensification methodologies allied to the emergence of newer personalized medicines, a key constant is the regulatory need to purify products that satisfy the criteria of safety, quality, and efficacy in each batch of released product destined for clinical use. Downstream processing operations and in particular chromatographic separations continue to play a key role in manufacturing strategies with the industry being well served by commercially available resins that provide different options to purify a particular target molecule of interest. In recent years, mixed-mode chromatography, a technique based on multimode interactions between ligands and proteins, had attracted much attention. This short review will discuss the concept and benefit of mixed-mode chromatography in purification strategies and specifically look at its application in the purification of IgG subtype monoclonal antibodies, a key product class in today's industry.

Key words Protein purification, Downstream processing, Mixed-mode chromatography

1 Introduction

The biopharmaceutical industry worldwide continues to perform very strongly with annual sales predicted to grow steadily with the emergence of new treatment modalities such as cellular and gene therapy products now attracting great interest, with the first products now gaining approval from regulatory agencies. However, recombinant proteins, in particular, monoclonal antibodies (mAbs), still dominate the market and represent the strongest driver of growth in terms of the number of products in clinical development and gaining ongoing approval [1, 2]. Biopharmaceutical products are typically complex products that are manufactured using complex manufacturing strategies to produce the target product of interest.

The process for recombinant protein manufacture typically commences with the generation of a mammalian cell line that is

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genetically engineered to express the molecule of interest most commonly in large-scale cell culture operations. Chinese hamster ovary (CHO) cells are most often used to manufacture proteins as they have a proven regulatory track record in producing therapeutics with human-like post-translational modifications necessary for desirable safety and efficacy profiles for patients [3, 4]. Product titers generated from upstream processes have continued to improve over recent years due to improved technologies for optimum cell clone screening under process-relevant conditions along with improved cell culture media formulations and better understood and controlled cell culture configurations [5–7]. The trend toward greater process intensification is currently a key driver within the biopharmaceutical industry. Process intensification will lead to smaller process footprints, increased utilization of newer single-use technologies, and greater flexibility for multiple product manufacture that can contribute to a decrease in manufacturing costs and speed to market [8, 9]. Advances in upstream processing have moved the process bottleneck toward downstream processing, and to address the increase in product titers, there is a developing interest among manufacturers in moving to an entirely continuous integrated downstream process that potentially could bring many benefits in terms of increased flexibility and quality [10, 11]. While more work still needs to be done to determine critical attributes of the target drug during a continuous manufacturing strategy, it is suggested that a fully continuous, commercial, and end-to-end process may be realized within the next 5 years [12].

Following the manufacture of the target protein in the production bioreactor, cells are separated from the conditioned media using a harvesting/clarification strategy that typically utilizes centrifugation and/or depth filtration operations. The resultant supernatant solution containing the "impure" product of interest is then subjected to a series of downstream operations that have the effect of removing process and product contaminants to provide a drug substance with an acceptable purity and safety profile. The drug substance is finally formulated with excipients to control tonicity, osmolality, buffer conditions, surfactant concentration, and, in the case of lyophilized powders, the concentration of a specific lyoprotectant. The formulated drug product is finally aseptically filled under highly classified conditions into the final mode of delivery to await final product release for parenteral administration to the patient [13].

Robust and reproducible downstream processing is crucial to successful biopharmaceutical manufacture. Downstream processing typically involves a series of individual steps operating in batch mode where each unit operation is completed before moving to the next operation in the sequence (Fig. 1). Within all downstream processes, chromatography continues to play the major role

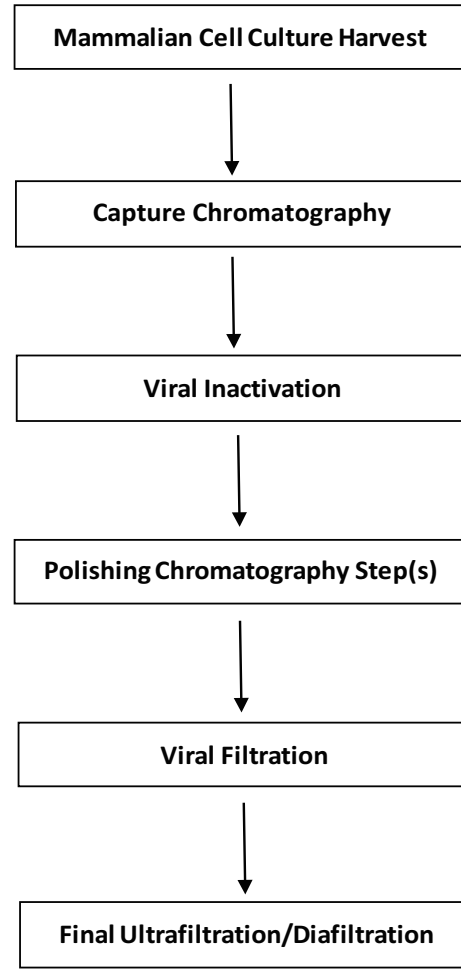

Fig. 1 General downstream process used in recombinant protein manufacture

in protein purification, and with the inclusion of additional filtration and viral clearance steps, products of requisite quality fit for their intended use can be produced. In recent years, technical suppliers have begun to provide manufacturers with newer chromatography resins with ligands that can exploit more than one property of a protein to promote purification. In this regard, "mixed (multi)-mode" resins that can provide unique purification capability through a specific combination of discrete chemistries are becoming more popular. These resins can provide benefit in terms of streamlining and reducing complexity in protein purification processes. This chapter will provide a general introduction to the concept of mixed-mode chromatography (MMC) and an example of its application in the polishing phase of monoclonal antibody manufacture.

1.1 The Role of Chromatography in the Purification of Monoclonal Antibodies

Purification design, optimization, and scale-up are crucial phases in the context of commercially producing proteins that will demonstrate the required attributes of safety, quality, and efficacy, as mandated by regulatory agencies. Within such workflows, liquid chromatography still plays the central role with respect to protein purification and achieving mandated critical quality attributes in the final product. Liquid chromatography essentially describes a technique where compounds dissolved in a specific mobile phase can be partitioned by application of the sample mixture to a solid matrix appropriately packed within a chromatography column shell. When looking at strategies to purify proteins, it is the fundamental properties of the target protein itself that will determine the type and sequence of chromatographic steps used in its purification. A solid matrix and associated ligand will be selected for use based on its properties to facilitate the separation of sample components based on their molecular properties. The characteristics and functional properties of any protein result directly from the amino acid composition of that protein. As proteins will be comprised of different numbers and sequences of amino acids, it is this difference that can be exploited in chromatographic purification. In general terms, a mixture of proteins when applied to a chromatography matrix will interact differently with ligands immobilized on that matrix surface with the specific attributes of the proteins being exploited for separation. Based on the most commonly exploited surface chemistries in the purification of recombinant proteins and monoclonal antibodies for therapeutic use, chromatography can be broadly classified into affinity chromatography [14], ion-exchange chromatography, [15], and hydrophobic interaction chromatography [16], respectively.

Chromatographic purification exploits differences in the properties of the target protein and properties of contaminants with separation being mediated using a specific polymeric material packed into a traditional chromatography column shell. Differences in properties of size, charge, hydrophobicity, and affinity can all be exploited with separation being based essentially on one discrete attribute. The classic chromatographic strategy to purify monoclonal antibodies of the IgG subtype is to capture the target antibody generated in cell culture on a Protein A affinity ligand immobilized on the matrix beads and to operate the step in bind-and-elute mode [10]. As binding to Protein A is not mediated by ionic strength and the pH of cell culture harvest material generally facilitates binding to this ligand, the clarified feedstock can be applied directly to the Protein A column without the need for any sample preconditioning. Robust and reproducible removal of host cell protein and DNA removal can be realized, and hence, Protein A chromatography has now become the capture chromatography step of choice for monoclonal antibodies. Protein A resins have undergone much development with vendors now offering modern resins with high dynamic

binding capacities and associated alkali stability to facilitate resin reuse strategies in commercial manufacturing applications [14, 17].

In IgG purification following the capture chromatography step, the target antibody is subsequently polished historically using two orthogonal ion-exchange steps, namely cation and anion-exchange chromatography. The cation-exchange step is typically operated in bind-and-elute mode, whereas the anion-exchange step typically operates in flow-through mode, and after some prior sample conditioning of the Protein A eluate, either ion-exchange step can be selected to follow as the second chromatography step in the sequence. Both ion-exchange steps operate by exploiting the normally high isoelectric points ($pI > 8$) exhibited by IgG subtype antibodies. It is most common currently for a three-step chromatography strategy to be employed in IgG purification with additional and deliberate viral clearance steps also incorporated in the downstream process in order to meet final product specification and regulatory mandate. Alternative polishing strategies can be used, and both hydrophobic interaction and hydroxyapatite chromatography have been used in IgG purification to replace the bind-and-elute cation-exchange step. The flow-through anion-exchange step where IgG transits through the column while contaminants bind to the column is traditionally included as one of the selected polishing steps in IgG manufacture. The chromatography resins typically used in IgG purification operate based on one main type of interaction between the target protein and the selected resin ligand. In recent years, technical providers of chromatography resins to the industry have looked to bring new innovations to the market, so manufacturers can now avail of resins that contain immobilized ligands that utilize more than one mode of orthogonal interaction with protein components. These so-called mixed (multi)-mode resins and their use specifically in the purification of monoclonal antibodies of the IgG subtype will be explored in subsequent sections.

2 Mixed-Mode Chromatography (MMC): An Introduction

Mixed-mode chromatography (MMC) is a separation technology that utilizes more than one binding mechanism to mediate the interaction between proteins and ligands immobilized on a stationary phase. Historically, mixed-mode phenomena in chromatography were considered to be only "secondary interactions" that were thought to be the main cause of the peak tailing often observed in chromatographic separations. It was realized subsequently that such interactions could possibly be exploited to increase the effectiveness of protein separations. The concept was essentially developed through MMC with modern commercially available MMC resins now offering a diverse range of separation options that can be

used in a variety of biopharmaceutical purification applications. In MMC, the properties of the target protein and the mobile phase conditions, respectively, will dictate the separation mechanism that predominates, and hence, MMC can provide a distinct and novel selectivity for analytes. By careful selection of the conditions of the mobile phases that moderate interactions in the binding and elution steps, the interactions of analytes with stationary phases can be purposely and often strategically controlled.

MMC has gained popularity in recent years, and a commonly used strategy is to use ligands that facilitate adsorption of proteins through a combination of electrostatic and hydrophobic interactions with subsequent elution being achieved by electrostatic charge repulsion. Studies have shown that such MMC steps can have a comparable or better separation performance in ion-exchange and hydrophobic interaction modes when compared with two individual weak-cation exchange and hydrophobic interaction chromatography columns operating in sequence [18]. As two or more types of interaction contribute significantly to the retention of solutes, MMC can offer many advantages in terms of achieving high resolution, high selectivity, high sample loading, when compared with conventional chromatography [19, 20]. High selectivity and resolution are key attributes in developing a robust purification strategy, and hence, the use of MMC resins that combine electrostatic and hydrophobic interactions can offer a more discrete purification when compared with using individual chromatography steps that utilize charge or hydrophobicity separation alone. The associated benefit of achieving such purification performance in one MMC step should also not be underestimated. With the current industry interest in achieving greater process intensification and process flexibility, the ability to reduce the number of chromatography steps by replacing two conventional, single-mode chromatography steps with a MMC step incorporating both corresponding modes of separation is a powerful option. When one also considers the necessity for additional sample preconditioning prior to any chromatography step, the ability to utilize one chromatography step instead of two steps is of interest to chromatographers assuming equivalent purification factors and yields can be achieved. It is also not uncommon for mixed-mode resins to be able to tolerate higher conductivities in the sample load, which also provides more flexibility in the purification process as direct capture of the target protein from cell culture feedstock is possible [21, 22]. Removing the necessity for pretreatment of cell culture feedstock produced after cell culture harvest/clarification operations has clear benefits in terms of cost and time savings, which are also not an insignificant consideration particularly at large scale. In general terms, the ability to reduce the number of discrete operations in a downstream process has an immediate pay back in terms of reducing effort and potential for equipment or

human error. In facilities engaged in the manufacture of multiple batches of a product per year or indeed in multiproduct facilities engaged in campaign manufacturing for a series of products, reducing downstream processing time through the incorporation of MMC presents clear operational advantages.

MMC resins can provide a unique selectivity and can be used to separate proteins from closely related contaminants that may co-purify when using individual ion-exchange or hydrophobic interaction chromatography steps, respectively. Mixed-mode resins also offer good flexibility in that they can be used in the capture, intermediate purification, or polishing phases of chromatography, albeit that it is in the latter two stages where the greatest deployment is observed particularly in the purification of monoclonal antibodies. MMC resins have been used in the purification of a large variety of targets including monoclonal antibodies, recombinant proteins, fusion proteins, and viruses, which clearly demonstrates the versatility of this separation technique [23].

With respect to their mode of preparation MMC stationary phases can be distinguished as being produced by "physical" or "chemical" methods. In the case of physical preparation, the stationary phase is formed from two or more types of packing materials. There are different methodologies that can be used in the physical method that are relatively simple to operate [19]. Columns with different stationary phases may be connected in series [24]; two stationary phases can be packed on top of each other in the same column [25] or can be premixed together, and the resulting "hybrid" stationary phase can then be packed into the same column [26]. In the case of chemical preparation, more than one functional group is immobilized on ligands attached to the same stationary phase. Chemical methods of stationary phase production in MMC all require an assessment of the effective combinations of different types of functional groups that can be immobilized on the surface of the selected stationary phase as determined by experimental design [19]. The specific selection and design of differing combinations of ligands used for MMC has been the subject of review previously [27].

2.1 Commercially Available Mixed-Mode Resins

As MMC has evolved to be a desirable option for recombinant protein purification, the portfolio of commercially available MMC resins from leading technical providers and vendors has increased. Vendor companies provide a range of resins and will provide technical documentation to support the application and proper use of their respective chromatography resins. Such information will describe best practices for effective use with respect to packing protocols, optimum resin performance, appropriate sanitization, and regeneration regimes to ensure reproducible performance on repeated use. Table 1 provides a sample list of some commercially available MMC resins from a variety of vendors that references the

Table 1
Some commercially available mixed-mode chromatography resins used in protein purification applications

Medium	Structure	Functionalities	Vendor
Capto™ adhere	Ligand: N-benzyl methyl ethanolamine Base matrix: Capto™	Electrostatic interaction, hydrogen bonding, and hydrophobic interaction	Cytiva
Capto™ adhere ImpRes	Ligand: N-benzyl methyl ethanolamine Base matrix: Capto™ ImpRes	Electrostatic interaction, hydrogen bonding, and hydrophobic interaction	Cytiva
Capto™ Core 700	Ligand: octylamine Base matrix: high-flow agarose	GF, AIEX, and HIC Core bead technology with ligand-activated core and nonfunctionalized shell allows capture of contaminants while target molecules are collected in flow through	Cytiva
Capto™ MMC	Ligand: N-benzoyl-homocysteine Base matrix: Capto™	Thiophilic interaction, hydrophobic interaction, hydrogen bonding, and electrostatic interaction	Cytiva
Capto™ MMC ImpRes	Ligand: N-benzoyl-homocysteine Base matrix: Capto™ ImpRes	Thiophilic interaction, hydrophobic interaction, hydrogen bonding, and electrostatic interaction	Cytiva
PPA Hypercel™	Ligand: aromatic (PPA—phenylpropylamine) amine Base matrix: Hypercel™ rigid cellulose	Electrostatic interaction and hydrophobic interaction	Sartorius
HEA Hypercel™	Ligand: aliphatic (HEA—hexylamine) amine Base matrix: Hypercel™ rigid cellulose	Electrostatic interaction and hydrophobic interaction	Sartorius
MEP Hypercel™	Ligand: 4-Mercapto-Ethyl-Pyridine (4-MEP) Base matrix: Hypercel™ rigid cellulose	Electrostatic interaction and hydrophobic interaction	Sartorius
CMM Hypercel™	Ligand: contains both a primary amine and a carboxyl group Base matrix: Hypercel™ rigid cellulose	Electrostatic interaction and hydrophobic interaction	Sartorius
Eshmuno™ HCX	Couples Eshmuno™ tentacle structure with a hydrophilic polyvinyl ether base matrix	Electrostatic interaction and hydrophobic interaction	Merck Millipore

(continued)

Table 1
(continued)

Medium	Structure	Functionalities	Vendor
Toyopearl™ MX-Trp-650M	Ligand: the amino acid tryptophan that has weak carboxyl cation exchange and indole hydrophobic functional groups Base matrix: hydroxylated polymethacrylic polymer	Electrostatic interaction and hydrophobic interaction	TOSOH Biosciences
Nuvia™ cPrime	Ligand: hydrophobic weak-cation exchange Base matrix: macroporous highly cross-linked polymer	Hydrophobic interaction, hydrogen bonding, and electrostatic interaction	Bio-Rad Laboratories

resins' respective base matrix, ligands, and general functionalities. For further information and technical details on specific resins and their mode of use, the reader is referred to the corresponding websites of the vendors mentioned in Table 1.

2.2 Purification of IgG Antibodies Using Mixed-Mode Resins at Commercial Scale

It is becoming increasingly common in addressing the polishing of IgG antibodies to incorporate a mixed-mode chromatography step with several options being provided by technical providers. Mixed-mode resins can be operated in bind-and-elute mode where the target molecule is first bound and subsequently eluted from the column in a separate mobile phase or in flow-through mode where impurities are bound to the column matrix in the binding step, and the target molecule will concomitantly pass through the column. As with all large-scale chromatography processes, a suitable step design and optimization prior to process scale-up is crucial to address the variability in feedstock inherent in all biological processes. Mixed-mode resins that incorporate both electrostatic and hydrophobic interactions can provide a wider window of operations when compared with conventional ion-exchange resins. As the binding mechanisms in such mixed-mode resins are more complex and typically stronger than in traditional ion-exchange resins, the specific elution conditions of pH and ionic strength have both to be established to determine the optimal set of conditions to maximize both recovery and purity. Due to the difficulty in predicting binding and elution conditions of mixed-mode resins, specific screening studies are typically now accomplished using high-throughput platforms such as mini-columns or microtiter plates using a design of experiments methodology. One early study used a 96-well microplate method combined with SELDI-MS to determine optimal binding and elution conditions for processing a recombinant allergen using two commercially available resins, hexylamine (HEA)

HyperCel™ or phenylpropylamine (PPA) HyperCel™ [28]. As there is a choice when using MMC between operating in classic bind-and-elute and flow-through mode, careful consideration needs to be given to designing robust and reproducible processing conditions. The number and complexity of interactions exploited in MMC is a direct result of the process conditions. Optimized design of process conditions can also lead to advantageous outcomes by providing chromatographers with a wider operational window that in turn can increase selectivity for the molecule of interest from a panel of closely related contaminants.

The purification phase of IgG manufacture begins typically with the first step in the downstream process: capture of the antibody directly from cell culture harvest. It is most common for manufacturers to use an immobilized Protein A ligand that has a direct affinity for IgG antibodies. Protein A has a high affinity for the Fc region of immunoglobulin isotypes and in nature is found as a binding receptor in the cell wall of the Gram-positive bacterium *Staphylococcus aureus* [29]. To capture the target IgG antibody from the clarified cell culture supernatant, the feed is applied directly to the column at neutral or near neutral pH values. Following a washing step to remove nonspecifically bound material, the bound IgG is removed from the column by application of an elution buffer with a low pH typically in the range 3.0 ± 0.5. Protein A has found wide acceptance in IgG purification as a capture step given the resultant high recoveries and high yields achieved that can be achieved in a single chromatography step [30].

There are some challenges in the use of Protein A linked primarily to the high cost of the resin with the resultant high cost of use particularly in large-scale manufacturing campaigns. However, as many commercially available process resins are now compatible with strong alkali that facilitates cleaning and hence multiuse regimes, the cost issue may not be as concerning. The requirement to incorporate a low-pH elution step to remove bound IgG antibody can also be problematic as antibodies have variable stability to such low-pH environments, and in general terms, proteins have a propensity to aggregate at lower pH values. One opportunity to address this issue would be to use a capture resin that does not require the very low-pH elution conditions necessary in Protein A chromatography. MMC and in particular MEP Hypercel™ can serve as an alternative to Protein A in the capture step for IgG due to its particular ligand (4-mercapto-Ethyl-Pyridine) and functionality (*see* Table 1). In the case of MEP Hypercel™, its ligand utilizes hydrophobic interaction through an aromatic residue and thiophilic interaction through a sulfur atom to facilitate binding of immunoglobulins [31]. MEP Hypercel™ is immunoglobulin selective and can be used in capture and intermediate purification applications for enhanced host cell protein and aggregate removal. This MMC resin also permits direct immunoglobulin

capture from cell culture feedstocks at neutral pH and is essentially independent of subclass or species, which is an advantage over Protein A resins, the latter having variable affinities for immunoglobulins. A key advantage of using MEP Hypercel™ is the milder elution conditions necessary to remove bound IgG with pH values in the range 4.0–5.5 being typically used. These milder elution conditions can contribute to minimizing formation of antibody aggregates which are problematic as they can cause side effects, reduce efficacy and induce immune responses in patients [20]. Obviously, when selecting any resin for use in a biopharmaceutical manufacturing process, it is the totality of benefits that the resin offers that is most important factor to consider. With MMC, increased functionality of resins, increased flexibility and window of operations, robustness and reproducibility are all important in the context of maximizing yield and purification factors.

Once the capture step has been completed given the high purity (>95%) of IgG that results, the remaining chromatography steps utilized in the purification process can essentially be defined as "polishing" steps designed to remove remaining contaminants to acceptable levels (*see* Table 1). As outlined previously, there are several options to polish an IgG fraction to ensure that it meets final specification and it is usual to incorporate two additional chromatography steps to complement the earlier capture step noting that Protein A is still the most widely used resin for capture applications. Chromatographers have commonly used traditional ion-exchange and hydrophobic interaction chromatography to complete the polishing phase. With the advent of MMC chromatography, further options now exist, and increased deployment of MMC is likely to continue with respect to IgG purification [32]. MMC resins can operate in polishing applications in bind-and-elute or flow-through mode, which provides a degree of flexibility in process design. Careful process optimization is required to define optimum loading conditions for IgG on MMC resins as conditions suitable in traditional single-mode chromatography are often suboptimal when transferred directly in MMC. Optimization studies typically include a full factorial DoE with respect to pH, ionic strength and protein load. Subsequent monitoring of outputs such as IgG antibody yield, removal of dimer and aggregated IgG (D/A), host cell protein removal, and removal of leached Protein A if it was used in the capture step can all be used as surrogates of step performance [23]. One such DoE study was executed to determine optimal loading conditions for purification of an IgG antibody Protein A capture in flow-through mode on Capto™ adhere [33]. Capto™ adhere is a primarily marketed as a multimodal ion exchanger designed for intermediate purification and polishing of monoclonal antibodies after a capture step on Protein A medium. In the referenced study using a DoE approach, loading conditions were successfully developed as a compromise between overall yield

and contaminant clearance [33]. Following optimization, the MMC step operating in flow-through mode could polish a Protein A eluate (starting conditions: 200 mg/mL, pH 7.0, conductivity 8.5 mS/cm) and produce a fraction after MMC polishing with yield >90%, leached Protein A below the limit of detection (decreased from initial 36 ppm), host cell protein at 15 ppm (decreased from initial 210 ppm), and dimer/aggregate ratio of 0.5% (decreased from initial 3.3%) [23].

Alternatively, similar polishing of monoclonal antibodies can be achieved using Capto™ adhere ImpRes and Capto™ MMC ImpRes, respectively (*see* Table 1). The multimodal anion-exchange ligand in Capto™ adhere ImpRes exhibits several functionalities that can interact with proteins most notably ionic/hydrophobic interactions and hydrogen bonding and the resin can operate in either bind-and-elute and flow-through mode. The multimodal cation-exchange ligand in Capto™ MMC ImpRes, N-benzoyl-homocysteine interacts with proteins primarily through hydrophobic interactions, hydrogen bonding, and thiophilic interactions, respectively. Studies have shown that following optimization of the elution step, both these resins can provide antibody fractions in high yield, with good clearance of aggregates, host cell proteins, and leached Protein A [34, 35].

HEA and PPA HyperCel resins (Table 1) have also found use in the polishing of IgG antibodies. These resins were designed to complement MEP Hypercel, and their mode of action is based on a combination of electrostatic and hydrophobic interactions of target proteins with the respective HEA (hexylamine) or PPA (phenylpropylamine) ligand. At neutral pH where protein binding is usually accomplished, hydrophobic interaction predominates. Binding to these ligands is typically carried out in a low ionic strength binding buffer that removes the need to add salt to the system. This provides an advantage over traditional hydrophobic interaction chromatography where the addition of large quantities of salt is required to induce optimum interaction between target protein and ligand. With these MMC resins, elution is driven essentially by electrostatic charge repulsion, and unlike traditional hydrophobic interaction chromatography, protein is eluted in dilute buffer, which removes the need for diafiltration that in turn has a payback in terms of increased process efficiency. Studies have shown that a target IgG molecule could be successfully separated from plasma impurities on HEA, PPA, and MEP Hypercel™ resins with HEA Hypercel™ providing the best results [36].

It is now clear that recently available MMC resins examples, of which have been provided in this review, can provide novel solutions to manufacturers who are interested in developing efficient, intensified, and robust purification processes for recombinant proteins and monoclonal antibodies. MMC resins can operate in different modes and provide unique selectivity. To maximize results,

diligent chromatography design and optimization is necessary as the mode of interaction in MMC is more complex when compared with single-mode chromatographic techniques. The ability to shorten a purification process by removing a chromatography step and perhaps also a necessary associated feedstock conditioning step can contribute to overall increases in efficiency and decreases in associated manufacturing costs.

3 Summary

As the biopharmaceutical industry looks at newer innovations to address the need for greater process intensification in biologics manufacturing, alternative purification strategies are an obvious area of focus. Chromatography remains the focal point in protein purification, and newer chromatography resins that can provide better windows of operation are most desirable. MMC can provide unique selectivity, and as these resins are based on multiple interactions, it affords the chromatographer greater options to purify certain analytes.

In the case of IgG purification, MMC resins can be included as an ideal polishing step following capture on Protein A chromatography. MMC resins can process high salt-containing feedstocks, and neutralized Protein A eluate may be processed without any adjustment in conductivity. MMC resins incorporating ligands operating through a combination of electrostatic and hydrophobic interaction can successfully replace two single-mode steps, ion-exchange and hydrophobic interaction, which has the effect of reducing purification time, complexity, and cost.

Although optimizing the operating conditions in an MMC step is more complex and challenging given the complexity of interactions between ligands and proteins, as more MMC ligands become available commercially, greater deployment of the technique in purification processes will occur in the future.

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Chapter 3

Continuous Countercurrent Chromatography in Protein Purification

Thomas Müller-Späth

Abstract

Continuous countercurrent chromatography can be applied for both capture and polishing steps in the downstream processing of biopharmaceuticals. This chapter explains the concept of countercurrent operation, focusing on twin-column processes and how it can be used to alleviate the trade-offs of traditional batch chromatography with respect to resin utilization/productivity and yield/purity. CaptureSMB and MCSGP, the main twin-column continuous countercurrent chromatography processes, are explained, and the metrics by which they are compared to single-column chromatography are identified. Practical hints for process design and application examples are provided. Finally, regulatory aspects, scale-up, and UV-based process control are covered.

Key words Continuous chromatography, Countercurrent chromatography, Twin-column chromatography, Yield-purity trade-off, CaptureSMB, MCSGP, Cost savings

1 Introduction

1.1 Continuous Countercurrent Chromatography for Protein Purification

Chromatography is an integral part in the manufacturing of biologics due to its capability to purify target compounds with high yields, high purity, and under conditions that preserve their structure and activity. Most modern downstream purification processes for biopharmaceuticals include at least two single-column chromatography steps. The first chromatography step is generally referred to as the capture step, and its main purpose is the removal of process-related impurities (such as host cell protein and DNA) and up-concentration of the target compound. Further downstream processing steps are referred to as polishing steps, and they serve to remove product-related impurities (such as aggregates and fragments of the target compound).

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In this chapter, the terms "continuous chromatography" and "continuous countercurrent chromatography" are used to describe processes that use solid stationary phases packed into columns. Filtration or extraction processes are not within its scope.

In the literature, several definitions of the term "continuous chromatography" can be found. An orthodox definition of "continuous chromatography" implies that the inlet and the outlet streams of the process are not interrupted over time and that the feed is operated at a uniform flow rate [1]. Less strict definitions allow short interruptions of the streams for valve switching. Even less strict definitions require only the entering or the feed stream to be uninterrupted (or interrupted only for valve switching) while the product elution can occur discontinuously. It is worth mentioning that volume reduction, which is specified as one of the major aims of the capture step, can be achieved formidably using a discontinuous elution (in product peaks). In this chapter, the terms "continuous chromatography" and "continuous countercurrent chromatography" are used more generally to describe chromatography processes that are designed to run in a cyclic manner and include a start-up phase, a cyclic steady-state phase, and a shutdown phase. In the cyclic steady-state phase, product eluate concentration and quality are identical from cycle to cycle. Reaching a steady state implies that the process uses recycling or transfer of part of the target compound. These steps can be well achieved by directing the material from a first column to a second column of the same type. Therefore, continuous chromatography processes generally use two or more columns. A decisive additional motivation to use two or more columns is the enabling of countercurrent principles. In countercurrent chromatography, the mobile phase (buffer) and the stationary phase (resin) move in opposite directions. In practice, the movement of the stationary phase is simulated by inlet and outlet port switching (Simulated Moving Bed chromatography). Through the countercurrent movement, the compounds to be separated/adsorbed "see" the stationary phase more often (one can imagine this as walking against the sense of movement of an escalator as in contrast to walking in the same movement direction ... one will see many more steps in the case of the former, the countercurrent of movement), allowing better separation (polishing applications) or higher resin saturation (capture applications). Countercurrent chromatography requires continuous operation in a cyclic manner, whereas the opposite is not necessary. For example, alternating batch chromatography ("flip-flop" chromatography) is continuous but not countercurrent and, therefore, does not enjoy the advantages of countercurrent chromatography.

In downstream processing, the main purpose of the capture step is increasing the concentration of the product (bind/elute mode) and the removal of a large part of the process-related

impurities. Polishing steps are generally used for removal of product-related impurities and are run either in bind/elute mode or in flow-through mode.

Continuous countercurrent chromatography can address these challenges and provide improvements over traditional batch chromatography. Today, continuous countercurrent chromatography is used by industry mainly for capture applications and predominantly used in the purification of monoclonal antibodies (mAbs) from clarified cell culture harvest; however, its application in oligonucleotide and peptide purifications is rapidly growing.

1.2 Trade-Offs in Batch Chromatography

Despite its great separation power, chromatography in general does have intrinsic disadvantages. These include mainly high buffer consumption (usually several liters of buffer/solvent are required to purify 1 g of pure product), low productivity (typically only a few grams of product can be produced per liter of packed bed per hour), and high stationary phase costs (typically several thousand US$ per liter of stationary phase). Stationary phase costs are driven up further by underutilization of the resin in terms of capacity or lifetime. In single-column (batch) chromatography, these shortcomings are amplified by trade-offs between process performance targets that cannot be optimized concomitantly.

In capture chromatography, there is a trade-off between capacity utilization and productivity (throughput). In capture in batch mode, the column is loaded with feed until a certain value in relation to the dynamic capacity is reached. Exceeding the dynamic capacity leads to breakthrough of the product and at the column outlet and to yield losses. The dynamic capacity is dictated by the sharpness of the breakthrough curve (caused by mass transfer effects). Decreased loading flow rates (increased residence times) lead to sharper breakthrough curves and improved resin capacity utilization but entail decreased productivities. On the other hand, increasing loading flow rates increase productivity; however, they lead to shallower breakthrough curves, lowering the dynamic capacity and the capacity utilization.

Due to their overloading capability, continuous countercurrent capture processes show a better load (capacity utilization)-productivity curve than batch processes (*see* Fig. 1).

In polishing chromatography in batch mode, there is a trade-off between yield and purity in the case of difficult separations where impurities overlap with the product in the front or in the tail of the product compound peak or on both sides. This means that the product pool has to be narrowed to exclude impurities overlapping with the product to ensure that purity specifications are met. Broadening the product pool fraction increases the yield but lowers the purity. By means of continuous countercurrent chromatography, due to internal recycling, the yield–purity trade-off can be alleviated.

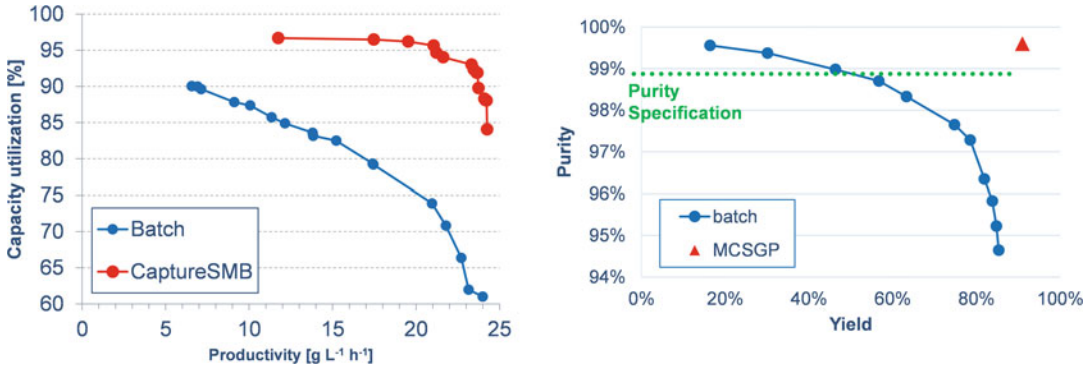

Fig. 1 Trade-offs of capture (left) and polishing chromatography

Thus summarizing, continuous countercurrent processes are beneficial in the capture step in case of a diffuse breakthrough curve and in polishing steps when an overlap between the product and the impurities is present, which includes many separation challenges in biologics downstream processing. In capture cases where steep breakthrough curves are present at high productivities, and in polishing applications where impurities and product are well separated, continuous countercurrent chromatography can only offer marginal benefits in terms of performance. Yet there may be advantages in terms of scheduling and manufacturing cadence. The more the respective trade-off "hurts" (capacity utilization vs. productivity in capture chromatography, yield vs. purity in polishing chromatography), the stronger is incentive to pursue implementation of continuous countercurrent chromatography.

In the following section, continuous countercurrent capture and polishing processes are presented in greater detail.

1.3 Process Performance Parameters

The process performance of chromatography processes is determined with respect to the parameters listed in Table 1. While purity includes several critical quality attributes (CQAs), the other process parameters are considered process attributes that are relevant to process economics. Continuous countercurrent capture processes generally improve over batch processes with regards to the process attributes load, productivity, capacity utilization, buffer consumption, and product concentration. In contrast, continuous countercurrent polishing processes mainly improve on yield.

2 Capture Applications

2.1 Introduction and Process Principle

Due to its high specificity, affinity chromatography is perfectly suited for removal of nonproduct-related impurities and the reduction of the volume to be processed in subsequent downstream steps. However, affinity stationary phases are rather expensive,

Table 1
Process parameters in chromatography

Process parameter	Unit	Definition
Purity	[%], [ppm]	Purity = mass of impurity in product pool / mass of product in product pool
Yield	[%]	Yield = mass of product in product pool / mass or product loaded
Load	[g/L]	Load = mass or product loaded / total column volume
Productivity	[g/L/h]	Productivity = mass or product in product pool / total column volume / total processing time
Capacity Utilization	[%]	Capacity Utilization = Load / SBC
Buffer Consumption	[L/g]	Buffer Consumption = volume of Buffer used / mass or product in product pool
Pool concentration	[g/L]	Capacity Utilization = mass or product in product pool / pool volume

and typically, their capacity is only partially utilized in the capture step due to mass transfer effects. These effects lead to shallow product breakthrough curves, that is, to a breakthrough of product at the column outlet before the column is fully saturated. A typical column load is a feed volume corresponding to 90% of the volume that would correspond to 1% DBC (dynamic breakthrough capacity) [2]. The 1% DBC concentration corresponds to 1% of the feed concentration and is determined for a fresh column. The safety factor of 90% accounts for titer variations and column capacity decline that occurs a as result of repetitive fouling and cleaning. With this, the typical capacity utilization of single-column capture processes is only 40–60% of the static binding capacity (SBC) of the stationary phase. Capacity utilization can be increased by lowering the flow rate/increasing residence time; however, this comes at the cost of productivity (throughput) [3].

Continuous countercurrent capture chromatography processes were introduced around 2005. They use a loading zone of two interconnected columns whereby the first column is loaded beyond its dynamic breakthrough capacity (DBC), typically up to 60–80% DBC. With these loads, the SBC utilization of the first column reaches 90% and more. The breakthrough product is not lost but captured on the second column. This concept is shown in Fig. 2. The SBC corresponds to the area above the breakthrough curve (A + B + C), and the DBC corresponds to area A. CaptureSMB increases capacity utilization from CU(Batch) = A/(A + B + C) to CU(CaptureSMB) = (A + B)/(A + B + C). Thus, based on a single-column breakthrough curve, the process parameters and

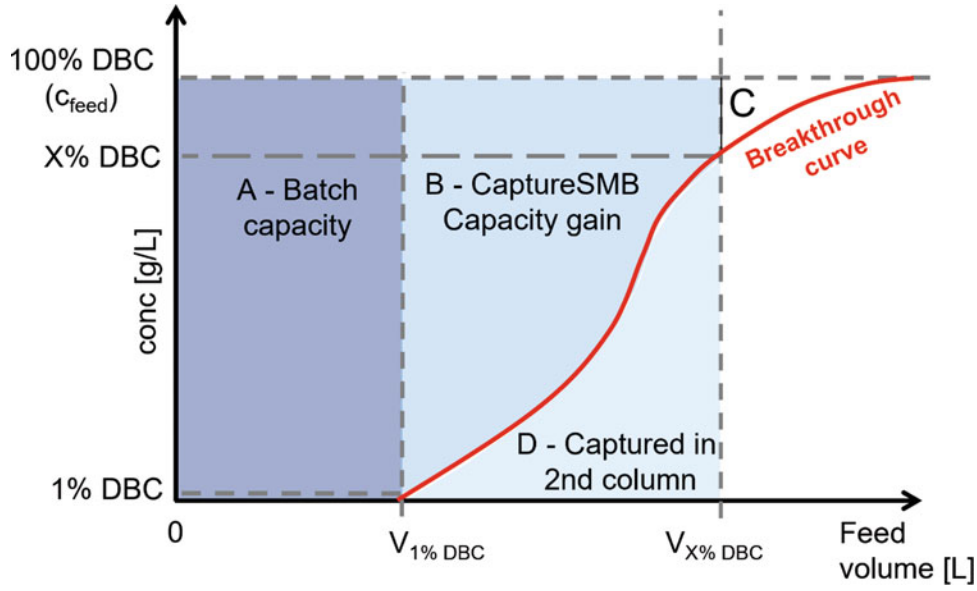

Fig. 2 Schematic illustration of the capacity gain obtained through interconnected loading (e.g., CaptureSMB process). $V_{1\%\ DBC1}$, $V_{X\ \%\ DBC1}$: Load volumes of feed corresponding to 1% Feed concentration (1%DBC), and X % feed concentration, respectively (X% DBC). The area A corresponds to the resin capacity utilized in batch chromatography, when loading to 1% DBC without safety margin. B corresponds to the additional capacity that is utilized when the column is loaded to X% DBC. Area D corresponds to the material breaking through from the first column (and captured on the second column during interconnected loading). Area C corresponds to the unused capacity of the first column when the column is loaded up to X% DBC

improvements of CaptureSMB related to the load can be predicted without actually running a CaptureSMB experiment (Fig. 2).

In the twin-column capture process CaptureSMB, the interconnected loading step is the first process step. In the next step, the first column is washed in series, whereby unbound product is transferred from the voids in between the resin particles of the first column onto the second column that still has available capacity to adsorb this product. After the first column has been washed, the columns are disconnected and the first column is continued to be washed and eluted while the second column is continued to be loaded with feed. The feed flow rate in this phase may be adjusted to synchronize with the first column that is washing, eluting, and cleaning. Once the first column is eluted, cleaned, and re-equilibrated, the second column that has been partially loaded until now is connected again to the first column, despite upstream of the first column. The loading is resumed on the upstream column, overloading again to 60–80% DBC as in the first step. The process continues as before, just with the columns in exchanged order. The process schematic for this two-column process (CaptureSMB) is depicted in Fig. 3. Because of the increased load in CaptureSMB, the capacity utilization, the productivity, the

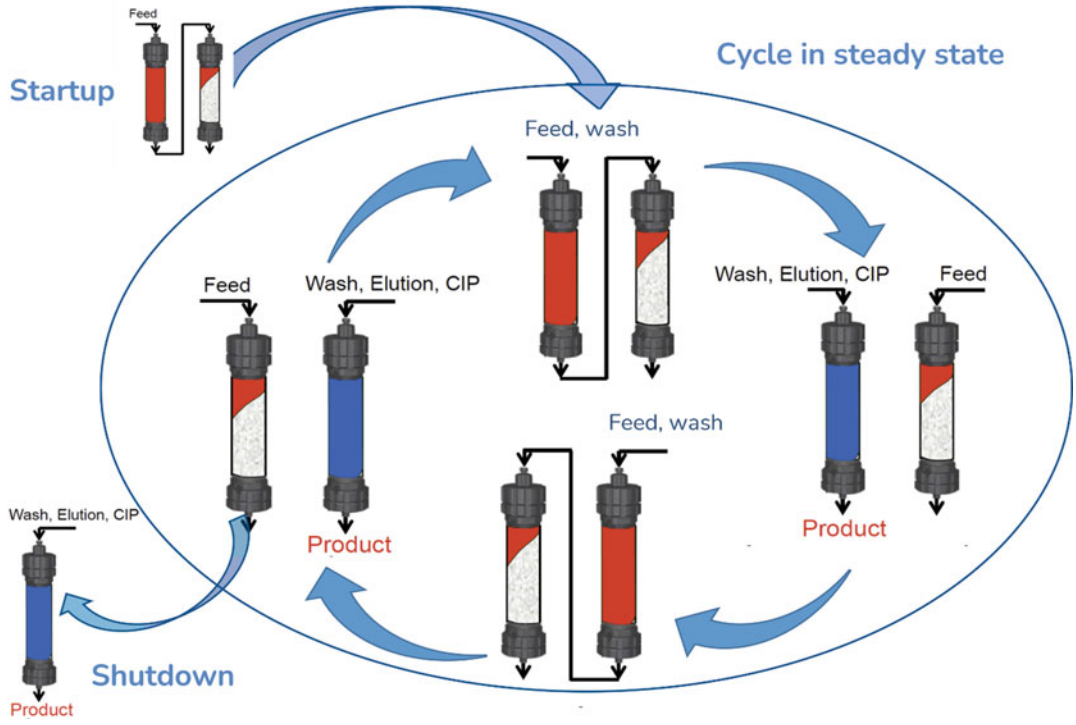

Fig. 3 Schematic of the CaptureSMB process including start start-up step, cyclic phase, and shutdown step

buffer consumption, and the product concentration are increased. Consequently, resin costs and buffer costs are reduced. Resin cost savings are particularly relevant in the case of capture with expensive affinity stationary phases that are used only for a few cycles, either because of chemical stability or because they are run in clinical manufacturing.

The process can operate multiple cycles until all starting material is consumed (downstream of fed-batch fermentation) or in conjunction with continuous fermentation. Start-up and shutdown methods allow entering and exiting the cyclic phase with minimal time consumption.

Several multicolumn countercurrent capture setups have been presented that differ in the number of columns used. Apart from CaptureSMB that uses two columns, a number of processes have been presented using three columns (3C-PCC and derivatives) [2, 4, 5] and more columns (4C-PCC, BioSC, SMCC, BioSMB). These processes carry out the same tasks as the two-column process, but more activities are parallelized with increasing number of columns. When parallelizing tasks, the column carrying out the most time-consuming step becomes rate limiting, forcing the tasks of other columns to be slowed down to synchronize column switching. The processes can operate with a uniform feed flow rate,

whereas the product elution takes place discontinuously, that is, in discrete product peaks. This is by no means a disadvantage as discontinuous elution entails that the product is eluted at a higher concentration and volume reduction is one of the main objectives of capture chromatography.

2.2 Practical Hints for CaptureSMB Process Design

The following points are helpful:

- For Capture Process Design, recording of the breakthrough curve is a must. The breakthrough curve helps determine the process improvement potential through use of CaptureSMB as described above (*see* Fig. 2). Moreover, the breakthrough curve is used for CaptureSMB process design.
- The protocols in terms of buffers, buffer volumes, and residence times can be transferred from single to continuous countercurrent capture without any or with minor adaptation. The elution buffer and wash buffers may need to be slightly adapted in response to the higher eluate concentration/higher load.
- The method works also with membrane adsorbers, as the housing of membrane adsorbers leads to a shallow breakthrough curve, although mass transfer is fast.
- The method works with both fed batch and continuous bioreactors (coupling occurs to the surge tank after the bioreactor).
- Model-based numerical optimizations of protein A-based countercurrent capture processes show that these processes can be run at higher throughput when short bed heights are selected and the improvement over batch chromatography becomes larger. Optimal column bed heights are 5–10 cm. However, the availability of columns with these bed heights for the required scale should be considered (clinical manufacturing, commercial scale).

2.3 Application Examples of Continuous Countercurrent Chromatography for Capture

To compare countercurrent capture processes for mAb capture using protein A resins with two, three, four, and more columns, a simulation study for 2.5 and 5.0 g/L feed titer was carried out. The processes were compared in terms of capacity utilization and productivity [6].

The application of continuous countercurrent capture processes for monoclonal antibody (mAb) has been evaluated by Genentech and Genzyme/Sanofi. In both cases, a three-column setup (3C-PCC) was used (with a two-column interconnected loading zone) [2, 5]. Genzyme/Sanofi have also evaluated a four-column setup for the capture of enzymes using hydrophobic interaction (HIC) and pseudo-affinity stationary phases [4]. Godawat et al. showed a comparison between batch and multicolumn processes, leading to column size reduction (factor 23–35), resin capacity utilization increase (1.3–3.3 fold), and buffer savings (20–70%)

when using the multicolumn process [4]. This analysis included process integration of continuous countercurrent chromatography with continuous fermentation instead of batch fermentation, which contributed to a large extent to the savings.

Mahajan et al. have evaluated the improvements of continuous countercurrent capture too [2]. They found that the countercurrent chromatography process can save approximately 40% on resin costs, buffer demand, and processing time.

CaptureSMB was evaluated by Angarita et al. for mAb capture using protein A affinity chromatography, and similar improvements over single-column capture were found in terms of resin capacity utilization and productivity (up to 30–40%) [7]. An investigation of the impact of feed flow rate showed that improvements over batch chromatography grow with increasing flow rate, which is expected as the DBC of the stationary phase decreases with increasing flow rate. This affects the batch chromatography to a much greater extent than the CaptureSMB, which is loaded to a much higher DBC value than the batch process.

The capture of mAb fragments using CaptureSMB was shown by Ulmer et al. [8] indicating resin cost savings of 40% in comparison to single-column batch chromatography ($2 million for 100 kg annual production).

A detailed cost modeling analysis for a 3C-PCC process was carried out by Pollock et al. [9] showing savings of 30% in the proof-of-concept scenario, while savings in phase III and commercial manufacturing were lower. This was attributed to full lifetime utilization of the stationary phase in commercial scale, while in the proof-of-concept scenario the resin was discarded after a few cycles, leading to high relative resin costs [$ resin cost/g product] so that resin cost savings by continuous countercurrent chromatography processes have a larger impact. However, in terms of absolute numbers, according to Pollock et al. [9], with an estimated cost savings per 10 kg batch of 30–45 kUS$, the annual costs savings through continuous countercurrent processes in commercial scale could exceed US$ 1 million.

3 Continuous Countercurrent Chromatography for Polishing Applications

3.1 Introduction

Although most applications of continuous countercurrent chromatography in protein purification have been reported for the capture step, and most of them involving protein A for mAb capture, continuous countercurrent chromatography is applicable to polishing steps too. It is most useful in cases where the product needs to be separated from a large number of product-related impurities, such as aggregates, fragments, isoforms, or undesired conjugates. In these cases, a yield–purity trade-off exists in preparative single-column chromatography as described above.

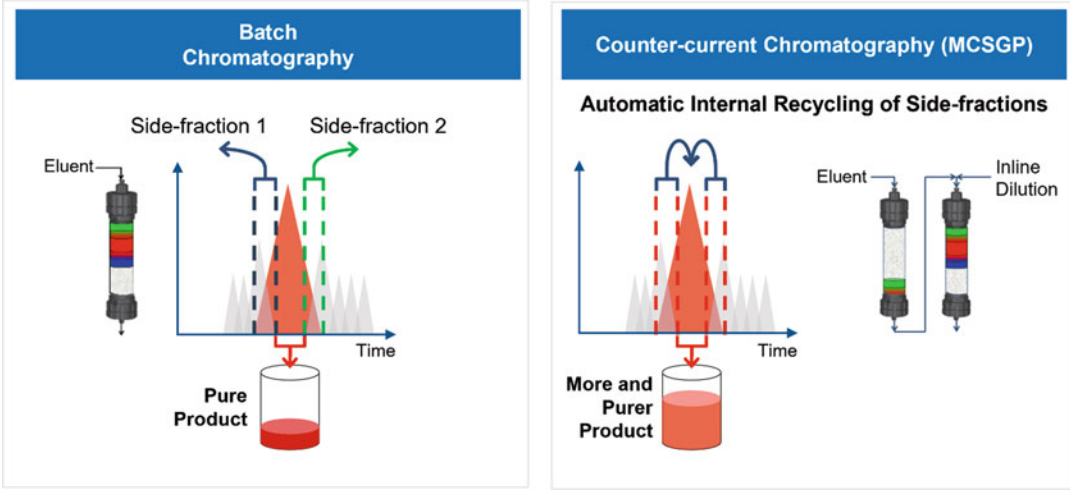

Fig. 4 Different side-fraction treatment in traditional single-column (batch) chromatography (left) and continuous countercurrent chromatography (MCSGP, right)

The MCSGP process (Multicolumn Countercurrent Solvent Gradient Purification) is a continuous countercurrent chromatography process capable of resolving the yield–purity trade-off. This is achieved by automatic internal recycling of impure side fractions from a first column to a second column. Thereby, the impure side fraction stream is diluted inline to ensure re-adsorption of the product on the second column. While the impure side fractions are re-purified inside the process, new feed is added and product is withdrawn repetitively. The concept of MCSGP is shown in Fig. 4, and the process principle is described in detail in the literature.

The MCSGP process parameters can be derived directly from a preparative single-column batch program that uses a linear gradient.

3.2 Practical Hints for MCSGP (Multicolumn Countercurrent Solvent Gradient Purification) Process Design

The following points are intended as guide to help determining if MCSGP is applicable and could be beneficial:

- MCSGP process is most beneficial if there is significant target compound yield improvement potential in single-column chromatography. In a typical case, if 60% yield can be obtained in single-column chromatography at target purity, 90% yield can be achieved in MCSGP.
- MCSGP productivity is improved when utilizing gradients that start with a high percentage of buffer B (the modifier-containing buffer). This is due to the lower level of inline dilution required during the internal recycling steps for impure side fractions.
- The MCSGP process parameters are determined from a single-column "design chromatogram" that is divided into different sections. The distribution of product, weakly and strongly

adsorbing impurities should be known, for instance, by offline analysis. Preferably the "design" chromatogram, from which the MCSGP operating parameters are derived, should fulfill the following specifications:

  - The linear gradient should be operated between min. 10% B and max. 70% B. Gradients starting with a higher percentage of buffer B are preferred. Shallower gradients are preferred. Isocratic operation is possible if re-adsorption of impure side fractions on the second column can be achieved through inline dilution.
  - Product elution should occur in the center of the gradient, and the gradient should not be excessively long, for example, elution in between min. 2 and max. 15 column volumes (CV) after elution start, whereby product elution should be completed at least 0.5 CV before wash/CIP step starts.
  - At least part of the product (20–50%) isolated in the fractions of the batch run fulfills the purity specifications.

- For process characterization, a series of MCSGP runs can be carried out automatically without intermediate shutdown and start-up methods. This saves time and buffer. New MCSGP conditions should be operated for at least five cycles to confirm steady state.
- It is recommended to collect the two eluates from each cycle together (each column will deliver one eluate per cycle). By pooling the two eluates and analyzing the pool, an average product concentration and purity is obtained, which is representative of the process performance.
- The MCSGP process parameters are determined from a single-column "design chromatogram" that is divided into different sections. The distribution of product, weakly, and strongly adsorbing impurities should be known from offline analytics.

If one of the above conditions is not met, a new "design" batch run with improved conditions should be recorded. The operating parameter determination procedure for MCSGP design has been automated in a software tool ("MCSGP wizard", ChromIQ software, ChromaCon AG, Switzerland).

The design procedure leads to an operating point with improved product yield at approximately the same productivity as the underlying "design" batch chromatography run. Productivity can be optimized by increasing the load and/or gradient steepness.

3.3 MCSGP Applications

MCSGP is generally applicable to ternary purifications that utilize linear gradients. MCSGP has been successfully applied to protein, peptide, and oligonucleotide purifications using ion exchange, hydrophobic interaction, and reverse phase chromatography.

Application examples include the purification of mAbs, of mAb isoforms, bispecific antibodies, antibody conjugates, fusion proteins, PEGylated proteins, and peptides and oligonucleotides from chemical synthesis.

The purification of mAb isoforms was shown by Müller-Späth et al. [10, 11]. Apart from removing unwanted isoforms, antibody isoform separation could be potentially interesting for production of "biobetters" or production of more homogeneous starting material for ADCs. MCSGP can also be applied to straighten out product isoform patterns resulting from variations in upstream fermentation. This was shown for the three cases of Trastuzumab (Herceptin®), Bevacizumab (Avastin®), and Cetuximab (Erbitux®) [11].

The application of MCSGP to PEGylated protein purification has been presented in the literature [12]. In summary, mono-PEGylated α-Lactalbumin was to be purified from a mixture of higher PEGylated species. It was shown that the same product quality (93% purity) could be obtained using batch and MCSGP chromatography. However, the yield of the batch run was only 56%, whereas the yield for MCSGP was 83%. Furthermore, the buffer consumption was reduced 50% using MCSGP.

In addition, the purification of common light-chain bispecific antibodies using cation-exchange MCSGP was shown by Müller-Späth et al. [13]. The purification of bispecific antibodies requires a center-cut purification to separate the target bispecific antibody AB from the parental AA and BB monoclonal antibodies and their charged isoforms that are co-expressed by the host cells. Also, in this case, MCSGP alleviated the yield/purity trade-off by increasing the yield from 37% (single-column batch chromatography) to 87% (MCSGP).

In another case study, MCSGP was used to isolate a fraction of an antibody–drug conjugate (ADC) model system with a narrow drug–antibody ratio (DAR) distribution [14]. A fluorescent dye was coupled to Trastuzumab through the antibodies' lysine residues leading to a broad DAR profile due to the high number of available Lysine residues. Using CEX MCSGP, antibody conjugates with DAR = 2, DAR = 3, DAR = 4, and DAR = 5 were produced. In all cases, yield improvements were substantial (e.g., from 22% to 100% for DAR = 3). MCSGP could be interesting for producers of first-generation ADCs that use a nonspecific coupling chemistry to produce high-purity ADCs. Another advantage of MCSGP is that it can also produce ADCs with uneven DARs, such as DAR = 3.

An MCSGP scale-up cost evaluation has been carried out by Takizawa in collaboration with Sandoz for a biosimilar manufacturing process showing significant cost savings of the continuous countercurrent chromatography process and a net present value of several million US$ for a large biopharmaceutical product [15].

3.4 Scale-Up of Multicolumn Countercurrent Chromatography Processes

The scale-up of continuous countercurrent chromatography processes to clinical scale and beyond has been achieved in multiple cases (2021). As in single-column chromatography, the scale-up is usually done by increasing the column diameter, keeping the bed height constant. Although the minimum bed height considered "packable" in large scale is 10 cm, in clinical scale shorter bed heights (5–10 cm) should be considered, as continuous countercurrent chromatography processes can offer further productivity advantages over batch chromatography when combined with shorter bed heights. Scale-up equipment for continuous countercurrent chromatography for manufacturing under GMP is available from various vendors. A twin-column skid is available from YMC Process Technologies. Scale-up equipment for continuous countercurrent chromatography relies on the same proven pump, valve, and detector technology as batch chromatography. It is worth mentioning that several continuous countercurrent chromatography skids offer the flexibility to operate also in parallel batch mode (without interconnected loading phases) or in integrated batch mode with inline dilution between two columns of different modalities (e.g., CEX and AEX polishing).

For sequential loading processes (CaptureSMB, 3C-PCC, 4C-PCC, SMCC), resin costs savings and buffer volume savings are in the range of 40–60% compared to batch affinity chromatography. This is due to the increased load that can be achieved in continuous countercurrent chromatography. In clinical manufacturing, resins are generally not used to the end of their lifetime before product-changeover, so relative resin costs are very high. In commercial manufacturing scale, where resins are used to the end of their lifetime, stationary phase costs become less important than at clinical scale. Particularly for very large-scale processes where bioreactors produce dozens of kgs of mAb in a single batch, buffer savings through continuous countercurrent chromatography correspond to 10,000s of liters of buffer per batch.

Angelo et al. have shown the scale-up of CaptureSMB from lab scale to pilot scale. They confirmed a productivity improvement of 2–3× and a reduction of the buffer demand by 50%. Good consistency between lab and pilot scale was shown. No difference in quality attributes (Aggregates, Fragments, HCP, DNA) was observed [16].

While for batch capture processes several multicolumn alternatives exist (CaptureSMB, 3C-PCC, 4C-PCC, SMCC, etc.), the MCSGP process is the only multicolumn chromatography option for bind/elute polishing of biopharmaceuticals. The state-of-the-art MCSGP process uses in a twin-column configuration.

3.5 Regulatory Aspects of Continuous Chromatography

Regulatory authorities support the introduction of continuous manufacturing in pharmaceuticals production as they promise to lead to drug quality advantages [17] and lowering of costs of therapeutics for patients. Continuous manufacturing is in line with FDA quality initiatives (QbD).

No specific FDA guidance exists about continuous manufacturing apart from the definition of a "lot." Nothing in FDA guidance prohibits the use of continuous manufacturing.

Both definitions of "batch" and "lot" are applicable to continuous manufacturing (21 CFR 210.3), providing great flexibility in terms of batch and lot definition allowing mass-based, time-based, volume-based, or raw materials-based definitions.

Based on the definitions in FDA guidance "Q7A Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients," the interconnected loading steps (CaptureSMB) and internal recycling steps (MCSGP) are to be considered as parts of the normal process and not considered reprocessing or reworking.

Additional questions regarding use of continuous countercurrent chromatography in production have been addressed: for CaptureSMB, a process characterization and validation concept has been presented [18] that shows compatibility of the process with traditional risk-based process validation approaches.

Moreover, virus studies for continuous countercurrent chromatography have been carried out using representative model viruses (MVM, X-MuLV), examining virus clearance, virus carryover, and the effect of resin aging on virus clearance in the CaptureSMB process [19, 20]. None of these studies have raised concerns about the feasibility of continuous countercurrent chromatography for protein capture.

3.6 Control of Multicolumn Countercurrent Processes

In continuous countercurrent chromatography processes, online information on product quality can be obtained more regularly and over longer periods of time compared to batch processes. Cyclically continuous processes produce a steady output of product elutions that lend themselves to state-of-the-art process analytical tools (PAT). One major advantage of continuous countercurrent chromatography is the possibility of "looking inside the process" by means of the detectors that are situated at the column outlet. During the interconnected steps (loading in CaptureSMB, internal recycling in MCSGP), the fluid path is such that the detectors are located in between two columns, that is, the material exiting the first column is guided through the detector onto the second column for re-adsorption. As opposed to single-column chromatography, material that has passed the detector is not lost (or collected) but transferred to the next column during the interconnected step, and the target compound does not leave the system. This opens great opportunities for either:

1. Direct control based on reaching certain threshold values (e.g., "start collect if UV > 300 mAU), like in single-column chromatography. For capture processes, the UV-based control concepts for AutomAb (for CaptureSMB) and DeltaUV (for 3C/4C-PCC) are based on this principle. For polishing processes (MCSGP), the Autopeak control uses the direct control.
2. PAT (process analytical tools) based on cycle-to-cycle monitoring, comparison, and feedback control within the same continuous chromatography run (e.g., elution peak area growing from cycle to cycle could be indicative of column overloading). Cyclic steady state of the process can be confirmed by comparing and overlaying the UV signals from cycle to cycle. In cyclic steady state, an exact overlay of the UV profiles is expected, leading to a specific "fingerprint" with constant product concentration and quality from cycle to cycle.
3. PAT (process analytical tools) based on cycle-to-cycle at-line analysis using an external analytical device (such as an HPLC) that allows extraction of concentration and even purity information, giving a maximum of process insights. Additional equipment complexity and validation efforts are the downside of this control solution.

Cycle-to-cycle control can be considered less responsive than direct control as it requires at least two elution peaks for comparison, yet it can deliver much more information in terms of process trends and in case of at-line monitoring, even of product quality.

So far, mainly UV traces are being used for controlling multicolumn processes; however, other modes of detection are also possible (e.g., conductivity to monitor gradient reproducibility, or backpressure to monitor column fouling). Careful detector calibration is important in all cases.

UV-based feedback control concepts have been demonstrated for both capture and polishing processes [21]. In the following, the direct UV-based control concept for capture applications, AutomAb, will be explained in greater detail (Fig. 5).

In the interconnected loading step of CaptureSMB, the UV monitor located in between the two columns initially "sees" the UV signal corresponding to the impurity baseline as mAb is adsorbed on the column and the impurities flow through. As the column saturation progresses mAb starts to break through from the first column, and the signal at the detector starts to rise above the impurity signal. The AutomAb controller continuously integrates the UV signal, subtracting the impurity baseline, thereby determining the mass of mAb that has broken through and is re-adsorbed on the second column. Once a defined mass threshold is reached, the interconnected loading step is stopped, and the process continues.

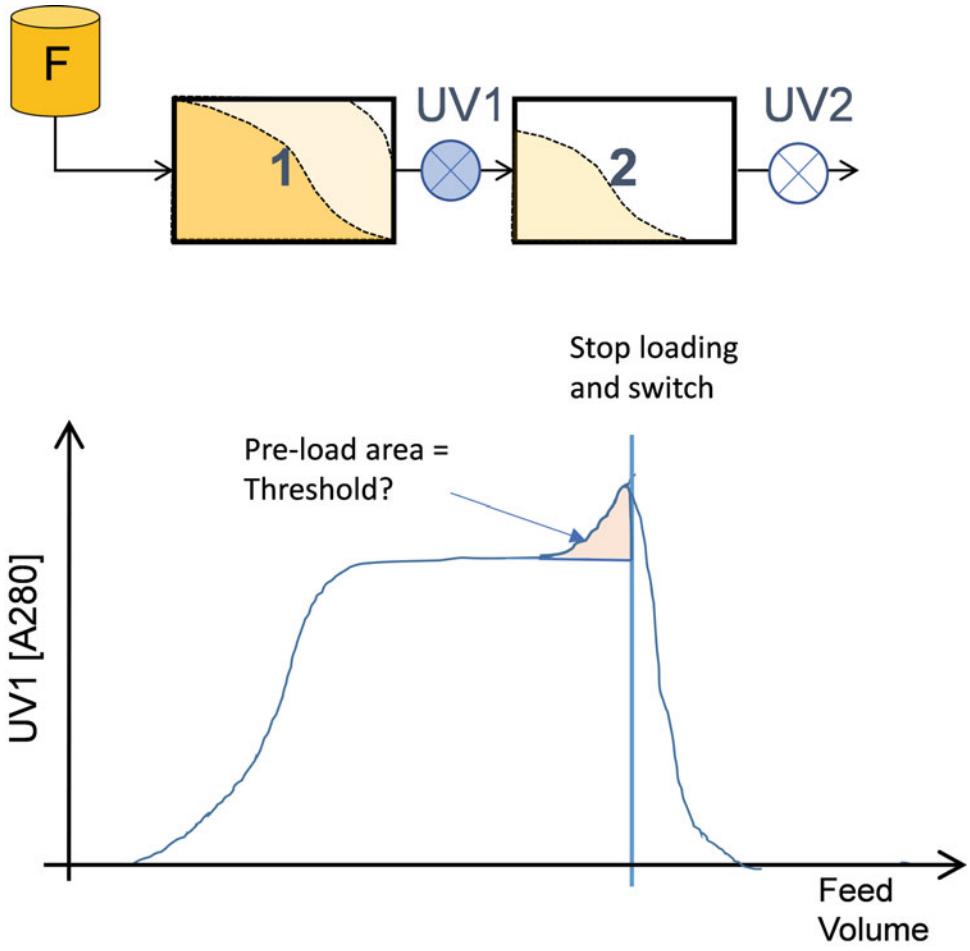

Fig. 5 UV-based dynamic process control principle (AutomAb). The controller monitors the breakthrough signal of the target protein and continuously integrates the area below the breakthrough curve that is proportional to the mass of target that has left column 1 and is captured by column 2. Once the area reaches a defined threshold, the loading is stopped, and the next process step is initiated (interconnected washing)

By using the integral of the UV signal instead of an absolute value threshold, the slope of the breakthrough curve or the 100% breakthrough value (in terms of absolute value) are not important, which enables AutomAb to cope with changing feed titers and column aging.

As the CaptureSMB process uses only two columns, the interconnected loading may be stopped whenever the threshold is reached, as there are no other columns operating in parallel that need to be considered, which could severely reduce process productivity by introducing waiting times.

Due to this high flexibility, CaptureSMB is ideally suited to be run in conjunction with continuous upstream that is operated over several weeks with changing feed titers and column degradation effects.

A difficulty can arise from low mAb titers and high impurity signals as the mAb breakthrough is not clearly visible in the UV signal. In such cases, screening for a different wavelength with a better mAb versus impurity signal ratio may help. Finally, if the signal is still not clear, it is always possible to revert to operation for CaptureSMB with fixed loading times, adding safety margins, such that the expected titer range is fully encompassed.

In continuous countercurrent polishing processes (MCSGP), direct control concepts are acting on the interconnected loading step too, by triggering the change from interconnected to parallel phases based on UV thresholds (Autopeak). Although less important for proteins, these control concepts are very relevant in peptide purification using reverse phase chromatography, where temperature and counter-ion effects play an important role [22].

3.7 Continuous Countercurrent Chromatography in Product Development

Continuous countercurrent chromatography provides a very efficient way to produce analytical impurity standards for further characterization with high purities and concentrations. By means of the N-Rich process, selected regions of a chromatogram are amplified by internal recycling, whereas nontarget compounds are depleted. Using this process, purities that would otherwise require HPLC chromatography, when operated in single-column mode, can be obtained using semi-preparative columns. With this automated process, the amount of time and number of samples generated to provide impurity standards is reduced by approximately one order of magnitude [23].

4 Summary

Multicolumn countercurrent processes can be used as replacement for any batch chromatography step to improve productivity and process economics. Due to the elevated load, that is typically 40–60% increased over batch chromatography, continuous countercurrent capture processes (such as protein A affinity capture of mAbs) deliver a 40–60% reduction in resin costs, 40–60% reduction of buffer consumption, and 40–60% increase in product concentration at the same productivity as single-column batch chromatography. In terms of productivity, optimizing the process with regards to flow rates and bed heights allows for further improvement over

batch chromatography. As opposed to single-column batch chromatography, continuous countercurrent capture processes allow running under conditions that lead to shallow breakthrough curves (such as elevated flow rates leading to higher productivity), because the breakthrough material is captured on the second columns. In batch chromatography, shallow breakthrough curves lead to early product breakthrough and to losses in yield.

An elevated productivity leads to the following production options:

1. More product can be produced within the same timeframe and the same bed volume (relevant to very large-scale manufacturing scenarios).
2. The same amount of product can be produced within the same timeframe using smaller columns (relevant in clinical scenarios).
3. The same amount of product can be produced using the same bed volume within a shorter time frame (relevant for CMOs and multipurpose plants to increase project turnover).

When replacing batch steps by continuous countercurrent chromatography processes, the economic advantages of multicolumn countercurrent processes are cumulative and improve with the number of batch steps replaced. Also, hybrid processes are possible. For instance, a multicolumn countercurrent capture step can be used instead of a batch capture step, while the polishing steps remain in (integrated) batch mode. With this setup, a downstream process with three chromatography steps that includes continuous countercurrent capture can be realized with just two chromatography skids. A downside of continuous countercurrent chromatography is the elevated equipment complexity that increases with the number of columns used. Continuous countercurrent chromatography is available through all scales (*see* Fig. 6), and scale-up has been demonstrated in several cases.

In polishing chromatography, the increased yield of continuous countercurrent chromatography helps improve the economics of the chromatography step and induces savings on the upstream side. If less product is lost downstream, upstream can be downsized or abbreviated (fewer bioreactor runs).

Fig. 6 Bench-top development and GMP scale-up systems side by side (Contichrom CUBE and Contichrom TWIN)

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Chapter 4

Chromatographic Purification of Viral Vectors for Gene Therapy Applications

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Abstract

Chromatography has been a mainstay in the downstream processing and purification of biopharmaceutical medicines. Until now, this has largely involved the purification of protein products such as recombinant enzymes and monoclonal antibodies using large-scale column chromatography methods. The development of advanced therapeutic medicinal products (ATMP) is heralding in a new era of therapeutics for a range of indications. These new therapeutics use diverse substances ranging from live stem cell preparations to fragments of nucleic acid enclosed in a viral delivery system. With these new technologies come new challenges in their purification. In this chapter, the challenges faced in producing and purifying viral vectors capable of delivering life-altering gene therapy to the patient will be discussed. Current methods of chromatography capable of adaptation to meet these new challenges and advancements that may be needed to increase the purification capabilities for these new products will also be discussed.

Key words Viral vector, Gene therapy, Adenovirus, Adeno-associated virus, Lentivirus

1 Introduction

Gene therapies are treatments for disease, which involve inserting a recombinant gene into the body. Gene therapies are being developed to treat genetic disease, cancer, and other chronic conditions [1]. At its simplest level, gene therapy represents a step away from traditional treatment plans for lifelong conditions, which have focused on regular doses of enzyme replacement therapy or moderating biochemical interactions in the body via monoclonal antibody therapy. These established types of treatment, although extremely successful, often rely on the patient receiving treatment on an ongoing basis. Gene therapy represents a new frontier in patient treatment and focuses on treating the underlying cause of disease. As an example, inherited retinal dystrophy is a rare genetic disorder, which causes vision loss due to mutations in the RPE65 gene responsible for producing all-trans retinyl isomerase, an

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enzyme required for the functioning of retinal cells in the eye. Luxturna is a gene therapy designed to treat retinal dystrophy. Luxturna® uses an adeno-associated virus to deliver working copies of the RPE65 gene to the patient's retinal cells, enabling the patient to produce functional copies of the enzyme [2]. In the case of Luxturna®, the therapeutic gene is delivered directly into the patient's body known as in vivo gene therapy. In other instances, gene therapy may be used ex vivo to alter a sample of cells outside of the body before their reintroduction into the patient.

Chimeric antigen receptor T cell therapy (CAR-T) is an example of an ex vivo gene therapy. The treatment works by collecting and then modifying a patient's immune cells to treat cancer. Tecartus™ is a CAR-T therapy designed to treat mantle cell lymphoma (MCL), an aggressive type of non-Hodgkin lymphoma. The patient's immune cells (T cells) are isolated from a blood sample and genetically engineered by delivering new genes to the T cell to express a chimeric antigen receptor that increases their ability to recognize and kill lymphoma cells. These modified immune cells are infused into the patient, increasing their ability to fight cancer [3].

For both in vivo and ex vivo gene therapy, an effective delivery system is required to transport the genetic material into the cell and encourage the cell to accept and express the new gene. Serendipitously, evolution has developed entities capable of entering a specific cell type and delivering or incorporating genetic material into that cell—viruses. In the case of gene therapy, viruses are adapted and used as a shuttle (called a vector) to carry therapeutic DNA into the body. The use of viral vectors presents us with a novel production challenge: growing and isolating viruses, which, instead of containing the genetic information to replicate and create viral copies, now contain the DNA payload to be delivered to the patient. Moreover, we need to develop mechanisms to effectively purify and isolate full vectors from those that have not successfully taken up the DNA payload or those that are of insufficient quality to be administered to a patient. Traditional chromatographic purification technology provides an opportunity to address these challenges.

1.1 Viruses Used in Gene Therapy Applications

A selection of viruses are popular choices for gene therapy techniques including adeno-associated viral vectors, adenoviral vectors, and lentiviral vectors. Each of these virus types has their own production and purification challenges.

Adeno-associated virus (AAV) vectors are popular choices for in vitro and in vivo gene therapy applications. The viral particles have low immunogenicity, are replication defective, and are not overwhelmed by the presence of neutralizing antibodies in the patient [4]. Multiple serotypes of AAV are already being used in authorized commercial gene therapy products. AAV1 is used in

Glybera® (designed to treat lipoprotein lipase deficiency but later withdrawn from the market), AAV2 is used in Luxturna® (designed to treat children and adults with retinal dystrophy), and AAV9 is used in Zolgensma® (designed to treat spinal muscular atrophy) [5].

AAV is a member of the *Parvoviridae* family of small (typically 20–26 nm in diameter), non-enveloped, single-stranded DNA viruses. Wild-type AAV can integrate into the human DNA genome using a specific site on chromosome 19 (and occasionally via random integration) [6–8]. However, for therapeutic applications, AAV can be engineered to enter the host cell nucleus and form episomal concatemers, head to tail circular DNA, which remains intact in cells but is not transferred during mitosis and does not interfere with the host genome. In this way, the therapeutic virus may transmit genes, which can be expressed for several months [9]. Early mechanisms of engineering AAV for gene therapy involved the use of a helper virus to grow AAV in culture. These helper viruses represent a safety concern for patients as they can be highly immunogenic and, therefore, represent a process-related impurity that must be removed effectively during downstream purification. More advanced AAV production techniques have aimed to eliminate this problem by replacing the need for helper virus in upstream production with the introduction of a three-plasmid system. In this system, necessary genes for viral replication are transfected separately into the host cell, allowing for the safe production of replication deficient viral vectors [10]. AAV is a relatively small, stable choice of vector. It is relatively stable to heat, nonionic detergents and proteolytic enzymes, making it well suited to purification by a range of chromatographic techniques [11].

Adenoviruses (Ad) are another class of viruses with applications in gene therapy. They are a group of medium-sized, non-enveloped, double-stranded DNA viruses. They are larger than AAV, typically 70–90 nm in diameter [12]. Adenovirus is typically more immunogenic than other gene therapy vectors but can induce transgene expression in multiple cell/tissue types. Ad vectors are being explored for various cancer therapies [13]. Structural similarities between Ad and AAV have allowed for parallel development in purification techniques for these two viruses. However, differences in separation strategies exist, as well as differences in the chromatographic media used for their purification [11].

Lentiviruses (LV) are a subclass of retroviruses. They are structurally distinct from AAV and Ad. They are large viruses (90–120 nm in diameter), containing an RNA genome and surrounded by a lipid envelope [14]. Perhaps the most well known of the lentiviruses is Human Immunodeficiency Virus (HIV). These viruses are well-known for their long incubation times, their name deriving from the Latin word “lente” meaning slow. Vectors based on HIV-1 have been developed, which are capable of delivering

genes to a wide variety of cells, both in vivo and ex vivo. Lentiviral vectors are already being used in licensed gene therapies, notably in ex vivo CAR-T cell therapy. LV can stably integrate into the host genome, which allows for long-term transgene expression. Like the other viruses selected for gene therapy applications, LV have low immunogenicity [15–17]. In addition, the virus is engineered to be replication deficient to ensure patient safety. In the early stages of development, this was achieved by removing all non-necessary genes from the HIV-1 genome except those required for particle production, infection, and integration. Current systems take this concept a step further, by separating the required genes into four separate plasmids to be co-transfected into the host cell used to produce the viral particles, ensuring that the viral particle which meets the patient is replication defective [18]. The use of LV has some disadvantages. Enveloped viruses are typically more fragile and prone to inactivation, and the viral envelope can exhibit poor stability during purification. Furthermore, as an RNA virus, the vector relies on an active reverse transcriptase enzyme, another element whose stability must be carefully controlled during production. The virus can be affected by fluctuations in multiple factors including variations in cholesterol levels, temperature, and osmolarity [19–23].

2 Purification of Viral Vectors

In general terms, viruses can be challenging to purify. They are both larger and more fragile than the protein products, which have been the mainstay of the biopharmaceutical industry thus far. As well as these inherent characteristics of the product, the mechanism of production for gene therapy can introduce novel product and process-related impurities not previously encountered by biomanufacturers. A challenge specific to viral vector purification is the separation of full viral capsid from empty viral capsid to ensure that the patient receives a full, active, dose of the viral therapy [11]. For current downstream purification processes, viral yields are low, and high purity is difficult to achieve. In the case of AAV, yields of between 10^{12} and 10^{14} recombinant AAV vector particles may be necessary to achieve a clinical dose. To meet these demands, we must develop purification strategies capable of delivering high-quality, safe, and efficacious product to the patient [4].

Viral vectors can be purified at laboratory scale by centrifugation using a buoyant density gradient. Early versions of this technique employed a gradient of cesium chloride, which immediately renders the technique unsuitable for therapeutic applications due to the toxic nature of cesium chloride. More recent protocols employ a mixed approach of precipitation, aqueous two-phase extraction, and a centrifugal gradient of iodixanol. This could be

considered an improvement on the gradient centrifugation technique as it reduces the toxicity of process-related impurities. Iodixanol gradient centrifugation may also help to prevent aggregation of viral particles and is less prone to inactivating the virus [24–27]. However, these lab-scale protocols for the purification of virus do not scale up efficiently and would be impractical, costly, and time-consuming at large scale. For production of gene therapy products, a range of chromatography techniques may be applied to purify viral vectors for patients.

2.1 Purification of Viral Vectors Using Chromatography

For capture chromatography, several affinity-based options are commercially available. Some AAV and LV serotypes have affinity for heparin, an anionic polysaccharide essential for many cellular processes. Affinity chromatography based on a heparin ligand or pseudo-affinity chromatography variations using sulfated resins such as Cellufine® sulfate are useful for the purification of a subset of LV and AAV serotypes, for example, AAV2 and AAV6. However, not all serotypes will interact with heparin, for example, AAV1, AAV4, and AAV5, and therefore, they cannot be purified using this ligand [28–30]. Importantly, a subset of cellular proteins also have affinity for heparin, so this form of chromatography must be followed by subsequent purification steps to remove these undesirable host cell impurities [31].

Other options for affinity capture of viral particles include the use of antibody capture. Resins using affinity ligands based on camelid antibodies are available. Camelid antibodies are smaller than other monoclonal antibodies making them well suited for the application [32]. Affinity chromatography for AAV using 14 kDa camelid antibodies has been shown to capture AAV particles with yields of 50–92%. A range of affinity resins based on this technique are available each with specificity for a subset of AAV serotypes [32]. More recently developed stationary phases have aimed to provide versatility with affinity for multiple serotypes, for example, POROS™ CaptureSelect™ AAVX resin shows good affinity for AAV1-AAV9 as well as some chimeric and recombinant capsid variants [33].

In the case of Ad purification, immobilized metal affinity chromatography is a possible choice for virus capture. The virus binds to charged divalent zinc ions covalently linked to the resin via a chelating ligand such as iminodiacetic acid. The bound virus may be eluted from the column by changing the pH of the mobile phase or by the addition of a competing ligand such as imidazole or glycine [34].

Although a powerful chromatography step, affinity chromatography has a number of drawbacks and limitations for gene therapy applications. For example, buffer conditions required for elution from affinity-based stationary phases can be harsh and lead to product loss. Affinity resins may not be completely selective for a

particular virus as is the case for heparin affinity, which may interact with a number of cellular proteins. The technique relies on affinity for the viral capsid and is, therefore, not able to distinguish full capsid from nontherapeutic empty capsid. Furthermore, affinity resins are typically expensive and commonly subject to ligand leaching over time, on repeated use.

Ion-exchange chromatography is an effective mechanism of purifying virus. The interaction between these large particles and the stationary phase is mediated by charged proteins on the viral capsid. In the case of Ad, the viral particles have an overall negative charge at neutral pH and will interact with anion-exchange resins [34, 35]. This interaction is mediated by the net charge of the proteins on the viral capsid [36]. Due to its strong net negative charge, Ad can bind to anion-exchange resin under high salt conditions, precluding the interaction of most proteins with the resin and aiding their separation. In contrast, DNA has a higher negative charge density than Ad and can be separated by its stronger affinity for the stationary phase. Ion exchange is a highly successful chromatographic technique for the purification of virus with high yields and good separation achieved [37–39]. Importantly, the p*I* for full viral capsids is different to that of empty capsids—providing a mechanism for separating full capsid, which has taken up the therapeutic gene of interest from empty viral capsid which is a particularly challenging product-related impurity [40].

Size exclusion chromatography (SEC) has traditionally been a less popular choice of purification protocol for large sale of production of biopharmaceuticals due to the time-consuming nature of the technique. SEC has been invaluable to the industry in analytical testing regimes that are required for final batch release. Going forward, SEC could find favor in the purification of large and fragile virus molecules. The technique applies little stress to the molecule in terms of shear or changes in pH or conductivity. In addition, SEC generally results in high product yields. SEC chromatography protocols can be designed so that the large virus is completely excluded from the pores in the resin and will elute in the void volume of the column. For large viral particles, the vector may become trapped in the pores of certain chromatography resins representing a source of viral loss. SEC can be optimized to exclude virus from the pores completely, both reducing sheer stress and viral loss by entrapment in resin matrices [41]. SEC employs an isocratic elution profile and can insulate the virus from changes in mobile phase conditions, which could cause damage. In the case of Ad, rapid changes in ionic strength, as well as exposure to a pH greater than 8.0, can result in a conformational change associated with loss of activity, aggregation, or disruption of viral particles [11]. However, this technique has a number of disadvantages. First, any large proteins or super-aggregates will elute in the void volume with the viral particles. Second, the technique has

limitations with respect to the volume of sample that may be loaded as a percentage of total column volume. This invariably means that the feedstock containing virus has to be concentrated before being applied to the column, a step that can also lead to product losses [11].

Another technique that may be applied to the purification of viral particles is hydrophobic interaction chromatography. This form of separation is based on interactions between hydrophobic moieties on viral capsid proteins and a hydrophobic stationary phase. A significant disadvantage of the technique is the necessity of using a high salt loading buffer to encourage binding to the stationary phase. Viral products may have limited tolerance for the changes in conductivity and osmolarity required for the technique, which can result in viral degradation or inactivation [11, 24].

Many traditional bead-based chromatography techniques have limitations when it comes to purifying viral vectors. The fragile nature of virus particles limits the mobile phase conditions that can be used, and more fundamentally, the large viral particles are poorly suited to traveling through the small pores on stationary phase resins affecting both yield and infectivity [38]. In short, it may be that the established biopharmaceutical chromatographic techniques based on porous stationary phase in large stainless-steel columns might not represent the best strategy for purification of these new products. Alternative stationary phases, previously less popular for production of protein-based biologics, may now find favor in the purification of gene therapies.

Macroporous stationary phase supports such as those provided by membrane chromatography or stationary phase monoliths offer some advantages for the purification of large biomolecules such as virus [42]. The columns aim to combine the separating power of traditional stationary phase resins with convective mass transport by providing a stationary phase with highly interconnected flow-through pores. This allows for reduced sheer stress on viral particles under significantly higher flow rates (10×) [43, 44]. Membrane or monolith-based chromatography systems have not been widely adopted in the purification of protein products in part due to the difficulty in adapting these technologies for certain stationary phase chemistries (for example, protein A resin, currently a mainstay of the monoclonal antibody production process) [45]. These systems are not best suited to the extremely high volumes necessary for production of a blockbuster biologic medicine [46]. These limitations in stationary phase chemistry and scale do not have as significant an impact on their application in the purification of gene therapies, which, as we have seen, can be purified very successfully using relatively straightforward chemistries such as ion-exchange chromatography. Furthermore, the patient cohort for gene therapy is still typically small, meaning batch sizes for gene therapy products are significantly smaller than for other types of biopharmaceuticals

allowing for the use of lower-capacity columns. Small batch sizes lead to another larger consideration for the production of gene therapy—the facility in which the product is produced. These therapies could be made more affordable for the patient if the need for large capital investment in a dedicated manufacturing facility could be reduced. Transitioning toward a more flexible multiproduct approach whereby a facility could be operated to make batches of several different products to treat different patient cohorts would provide clear benefits. Membrane and monolith columns are significantly less expensive than their resin-based counterparts. This means that these columns may be more easily adapted into a disposable or even single-use platform. The column is used for the duration of its useful lifespan, and when a facility is used to manufacture a batch of a different product, the column can be removed and/or replaced. This eliminates the risk of cross contamination between products, reduces time spent designing and validating a cleaning program, and allows for greater flexibility within the manufacturing facility [47]. This should result in shorter time to market, reduced production costs and capital investment for the company, and makes drugs designed for small patient cohorts more affordable.

3 Conclusion

As advancements in novel medicines and advanced therapies are made, new challenges will emerge in their production and purification. Adaptations to and advancements in standard chromatography workflows must keep pace to ensure successful and economic purification of these novel therapies, which will in turn lead to positive outcomes for future patients.

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Chapter 5

Scale-Up of Protein Purification: Downstream Processing Issues

John Joseph Milne

Abstract

Large-scale chromatography operations continue to occupy the central position in the overall strategy for downstream processing and purification of therapeutic protein products for human use. As the biopharmaceutical industry looks forward to embracing new therapeutic modalities such as viral vector-mediated gene therapy, it is becoming evident that chromatographic separations will be also be crucial for success in that discipline. The current industry focus on cell culture intensification strategies that can result in increased process efficiency and lower cost of goods is presenting challenges to the robustness and economics of chromatography processes. To ensure robust and reproducible commercial manufacturing strategies, there is always a mandate to increase the scale of chromatography unit operations that are typically developed and optimized in small-scale development trials. This chapter discusses the key factors in typical chromatography operations that need to be carefully considered and modeled during the process scale-up phase in order to maintain the purity, yield, and quality of a product purified at smaller scales.

Key words Protein purification, Downstream processing, Chromatography, Scale-up

1 Introduction

When compared to traditional pharmaceutical products, biopharmaceuticals are complex high molecular weight products derived from living organisms, cells, animals, or plants. Development pipelines are increasingly being populated by new biopharmaceutical products, and most pharmaceutical companies are now embracing biopharmaceuticals as they look to build successful pipelines into the future. Innovations and ongoing developments in molecular biology have led to progress in scientific disciplines including genomics, metabolomics, and proteomics that in turn have facilitated the development of new classes of therapeutic products. The current market for biopharmaceutical products represents an increasingly significant proportion of total pharmaceutical annual sales resulting mainly from the continuing interest in monoclonal antibodies as therapeutic agents [1, 2]. It is clear as we look to the

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future that therapeutic drug approvals will continue to be dominated by monoclonal antibody-based products, and those products will be expressed in mammalian cell expression systems and administered by means of conventional parenteral delivery. It is also evident that new therapies based on cellular and gene therapies are beginning to gain momentum, while continuing interest in biosimilar products will have a major impact worldwide on brand monopolies for monoclonal antibodies and recombinant proteins that have or are due to come off patent [3]. While vaccines will continue to be an important class of therapy against infectious disease, the recent positive deployment of mRNA vaccines against SARS-CoV-2 has also opened up the possibility of mRNA providing an alternative to the use of conventional vaccines.

The system of choice for the expression of complex recombinant proteins still remains, the mammalian cell with expression in Chinese Hamster Ovary (CHO) cells grown in suspension fed-batch culture being the most popular choice to date for manufacturers. CHO cell cultures offer manufacturers the ability to produce complex protein products with human-like post-translational modifications that are necessary for product quality and efficacy [4, 5]. Due to continuing advances in cell line engineering, improvements in cell culture media formulations, and improved control within the bioreactor environment, product titers have increased with titers of >5 g/L now being routinely achieved in conventional stirred tank bioreactors. Currently, many developments taking place in the area of mammalian cell culture processing are focused on approaches that can lead to greater upstream process intensification [6]. Such intensification strategies are utilizing perfusion and other technologies that present opportunities for more cost-effectiveness and flexibility in manufacturing. One approach aimed at increasing volumetric productivity by converting fed-batch cell culture processes to more continuous cell culture processes is attracting considerable interest within the industry due to the prospective advantages that such an approach may bring [7].

Production of therapeutic products involves several core disciplines with downstream processing occupying the central position in the overall process of ensuring the ultimate safety, quality, and efficacy of the target product. Downstream processing can be defined as a series of separation and purification activities that together can produce a protein product fit for its intended use with attributes of robustness and reproducibility being key for overall success. Downstream operations are typically carried out at a scale that is dependent on the amount of product required, and in order to avoid increased capital investment and optimize facility usage, incremental and disruptive innovation continues to be required in bioprocessing [8]. Protein purification using preparative chromatography is currently the central enabling technology in

downstream processing for recombinant protein manufacture [9]. Large-scale process chromatography has played a key role in the purification of therapeutic proteins and although alternative techniques such as the use of membrane adsorption are currently receiving attention in certain limited circumstances, preparative chromatography is likely to continue to remain the primary purification platform utilized by the biopharmaceutical industry. However, due to the steadily increasing number of monoclonal antibody approvals [2], the increased volumes observed in fed-batch cell culture harvests (>10,000 L), and the associated high levels of protein expression that are now realizable, increased productivity by means of integrated and continuous production strategies will most probably emerge in the future to address the currently problematic bottleneck in downstream processing [7, 10, 11]. Indeed, the combination of high-density perfusion cell culture with a directly coupled continuous capture chromatography step (*see* Chapter 3) is receiving much attention in this regard and may provide a viable alternative t operate in batch mode [12].

1.1 Protein Purification Using Chromatography

Following expression of the target product in large-scale cell culture and subsequent harvesting operations, the product is subjected to the downstream phase of manufacture. Downstream processing proceeds through a series of sequential unit operations to achieve solid–liquid fractionation. Currently, the key operation in the downstream process is liquid chromatography in which a sample dissolved in a mobile phase is passed through a stationary phase held in a column shell. In preparative chromatography, the stationary phase is selected on the basis of its ability to purify the target molecule from a wide variety of process contaminants. In the case of non-affinity based chromatography processes, product concentration using ultrafiltration and diafiltration or a suitable sample load adjustment step are used to appropriately condition the feedstock prior to application of the feedstock on to the capture chromatography column. Chromatography strategies that rely on specific affinity-based interactions generally have the advantage that the feedstock can often be applied directly on to the stationery phase without the need for any prior sample conditioning. The downstream phase of protein manufacture is designed to specifically take the target molecule from the upstream stage to the final drug substance stage achieving the required level of purity and recovery and to do so safely, reproducibly, and economically. In order to achieve product approval, regulatory agencies require that process characterization, risk mitigation, and validation are carried out prior to market authorization. Each of these disciplines presents distinct challenges and requires a thorough knowledge and understanding of the manufacturing process in order to produce products that are fit for their intended use [13].

Within industry, the primary method of protein purification currently used is process chromatography, which is typically performed on a large scale. Such chromatography must be shown to be robust and reproducible to be able to address the variations inherent in the use of cell-based protein expression systems. When chromatography is used as a method of purification, process development will typically involve distinct phases of bench scale development, optimization of chromatographic parameters, and scale-up to the required final scale. Scale-up of chromatography presents unique challenges in terms of both the media selected and the equipment hardware used and is a critical issue for the successful commercialization of biopharmaceuticals [14].

In order to design a purification process that is robust and reproducible to deliver a product of acceptable quality, the basis of each constituent chromatography step in the overall scheme is assessed in small-scale experiments. Purification schemes for currently approved protein products typically involve at least three orthogonal chromatography steps, each offering a different selectivity and exploiting alternative attributes of the target product and/or contaminants in order to maximize the overall purity of the product [15]. The advent in recent years of mixed-mode chromatography (*see* Chapter 2), a separation technology that utilizes more than one binding mechanism to mediate the interaction between proteins and ligands immobilized on a stationary phase has provided advantages in process design and optimization. Mixed-mode media can be divided into those that augment anion exchange with hydrogen bonding, metal coordination with electrostatic interactions, and hydrophobic interactions with other chemistries [16, 17]. An example of how mixed-mode resins can facilitate process design is the case of Capto™ adhere, a strong multimodal anion exchanger developed by Cytiva. When used in combination with a Protein A-based affinity capture chromatography step, monoclonal IgG_1 can be successfully purified with acceptable levels of key impurities in a two-step process, thus reducing the conventional IgG_1 purification strategy by one column step, which is not an insignificant development [18]. It is important that as a purification process is being developed at small scale, reference is made to the predicted final scale of the process, so that specific issues relating to the scale-up can be fully appraised. This is particularly important if the protein is to be purified according to the guidelines of current good manufacturing practice as defined by regulatory authorities [19].

Within a typical downstream process, chromatography is generally regarded as being composed of three distinct phases, namely capture, intermediate purification, and final polishing. Each of these phases requires a different focus, and different challenges will be presented as the purification process is scaled up. The initial capture step involves protein isolation from crude feedstock and

thus requires a chromatography medium with a high dynamic binding capacity and high operating flow rate. The binding capacity of the medium for the target protein in the presence of many impurities, rather than resolution, is the most critical factor when capturing the target protein. As current process volumes generated in cell culture can be large, fast flow resins possessing a large bead size range are used to reduce pressure issues, improve overall processing times, and thus stabilize the protein product quickly. Although there is a developing interest in applying single-use production strategies for recombinant proteins, even including the use prepacked affinity chromatography columns [20, 21], economic constraints generally require the capture resin to be recycled for a validated number of uses when large-scale capture applications are involved in which the volume of medium is large. Due to the crude nature of the starting material, the ability to clean and sanitize media effectively to permit reuse of chromatography media is also a very important issue to be considered. Resolution of the target protein from host-cell contaminants becomes more important in intermediate purification applications. To ensure productivity and process economics, the binding capacity of the medium for the target protein is also important. Since bead size correlates with resolution, smaller bead sizes are often more appropriate at this stage of purification. Speed is usually less critical in intermediate purification applications since impurities affecting stability of the target will have been removed in a successfully optimized capture step. In polishing chromatography, the main issue is resolution, to ensure removal of trace contaminants and also structural variants of the target, which can lead to immunogenicity concerns following therapeutic administration [22]. High-efficiency media with small bead sizes are typically deployed in chromatography focused on final polishing applications. As the use of a smaller bead size will result in increased pressure, lower volumetric flow rates are used with increased process times in result. In overall terms, the performance criteria to be considered in developing an optimized purification process are speed, recovery, capacity, and resolution. The priority given to these attributes will be dependent on the positioning of a chromatographic step in the process and specifically whether the purification step is operating in capture, intermediate, or polishing mode.

1.2 Scale-Up of Protein Chromatography

Following the initial phase of small-scale scouting experiments, typically a variety of chromatography media will have been screened in order to select the most appropriate medium that can provide a basis for separation of the target protein from impurities in the sample feed material. Once a medium has been defined, the next stage in the process is to design specific chromatographic procedures that can optimize the dual requirements of product recovery and purity, by defining the buffer solutions used during sample

Table 1
Parameters to be defined during the optimization phase of development

Process parameters	Sample preconditioning required Sample concentration (load/mL resin) Sample volume (load/mL resin) Product concentration (load/mL resin) Resin bed height (cm) Linear flow rates (cm/h) Process volumes Pressure drop Packed column qualification Buffer conditions (pH, conductivity) Fractionation scheme if used Stability of resin to cleaning and sanitization agents
Product parameters	Stability Solubility in buffers Storage conditions Storage time

conditioning, media equilibration, and product elution. Typically, at this stage, the purification process will be capable of producing milligram quantities of protein. The main result of these process optimization activities is that product information and process parameters will have been considered in detail and where possible defined to ensure a smooth technical transfer when the process is ultimately scaled up (*see* Table 1). When the running conditions and chromatography parameters have been determined for any column purification step, the final issue to resolve is how the chromatographic process can be scaled appropriately to a level required for required large-scale production [23]. Scaling from laboratory to pilot plant can involve scale-up factors of 50- to 100-fold, while further increasing from pilot plant to commercial production scale will involve another 10- to 50-fold scale-up [24]. Scale-up of chromatography should involve a detailed consideration of chromatographic, non-chromatographic, and equipment-related factors, and the role these factors collectively can play in successful process scale-up.

An increase in the scale of chromatographic purification is most typically achieved by increasing the column diameter and volumetric flow rate, while at the same time ensuring that the media bed height and linear flow rate remain constant [25] (*see* Table 2). Increasing the column diameter during scale-up while ensuring that the column cross-sectional area is increased in proportion to the process volume, with the bed height being kept constant, should equate with successful scale-up. This is due to the fact that the overall residence time of the target molecule from small-scale development through to large-scale columns remains constant. In

Table 2
Status of various process parameters during chromatography scale-up

Remain constant	Column bed height Linear flow rates (cm/h) at all stages Sample attributes (concentration and conditioning) Ratio of sample volume to media volume Ratio of gradient volume to media volume Buffer specifications
Increased	Column diameter Volumetric flow rate (mL/min) Sample volume proportionally Gradient volume proportionally Buffer volumes proportionally
Issues to be addressed	Decreased wall support as column diameter increases Quality of packing/bed instability Increased pressure drop Uneven flow distribution Zone broadening

theory, the respective separations should be very similar for both column diameters and, therefore, provide the basis for successful scale-up. However, as the column scale increases, other factors such as choice of buffers and their preparation, media packing, column engineering, and process hygiene can present limitations and often present problems particularly at the scale of manufacturing required in commercial biopharmaceutical manufacturing [26]. Thus, a proper scale-up strategy is a crucial consideration in process design involving the optimization of many factors in order to ensure robust and reproducible purification of a protein therapeutic over the lifetime of the product license. The remainder of this review will consider some of the main issues in chromatography scale-up and the approaches taken by industry to provide acceptable solutions.

2 Downstream Processing Issues

Preserving the quality of resolution and the level of purification determined at laboratory scale is the key to successful chromatography scale-up [27]. Whenever a requirement exists to increase the scale of a chromatography process from small-scale through pilot-scale and on to full commercial manufacturing while adhering to the conditions of good manufacturing practice (GMP), a series of key issues need to be considered to determine the effect on the overall scale-up process. Such issues can be categorized into those relating to the choice of medium and the chromatographic separation process. In commercial manufacture, equipment and hardware issues can often present challenges due to the need to use large columns and automated process skid systems.

2.1 Chromatography Media

In protein purification applications where chromatography is utilized, it is the physical and chemical properties of the medium used in the process that are the main route to achieving the required purity. Chromatographers now have an increasing portfolio of commercially available resins from which to choose with vendors offering media with improved performance, mode of action, and reusability. The rational selection of chromatography media prior to screening experiments is critical for successful downstream processing for industrial chromatography applications [27]. Modern processes require media that are not only consistent and selective but that also offer high dynamic capacities and low cycle times to be compatible with the increasing product titers resulting from intensified cell culture processes [11].

The main considerations when choosing a medium for use in a production process for therapeutic products are a route to scalable and robust processing while at the same time ensuring that all procedures can be sufficiently validated. As the volume of medium required increases, the available commercial supply of media with demonstrated lot-to-lot consistency and full regulatory support documentation is mandatory. Such issues should be noted prior to early small-scale trials and if not resolved might indicate that a different media should be considered for use. As mentioned, there are an increasing number of suppliers who offer a wide portfolio of media that are compatible with the current process requirements of industry and quality requirements of regulators, so exploring all available options is advisable.

Media and capital equipment costs at large scale should not be taken for granted, and cost analysis is frequently an important component when considering scale-up to commercial manufacture. Affinity-based separations while offering clear advantages for overall effectiveness can often be very expensive particularly as column volumes are increased. Protein A, a protein naturally found in the cell wall of *Staphylococcus aureus*, has the ability to selectively interact with the fraction-crystallizable (Fc) domain of immunoglobulins [28]. A regularly cited limitation with the use of Protein A media for the purification of monoclonal antibodies is the cost of the media itself, which is an order of magnitude more expensive when compared with media using non-proteinaceous ligands [29]. The particle size distribution of chromatography media impacts directly on scalability due to its effect on column pressure profiles. The particle size chosen for any purification step will depend on the nature of the feed sample and on the degree of resolution required. The desired resolution will depend on whether the chromatography is operating in capture, intermediate, or final polishing mode.

The inherent stability of the medium chosen is an important attribute in large-scale manufacture, which can be overlooked during small-scale development studies. An ideal medium for large-

scale applications should exhibit good chemical and physical stability profiles. The chemical instability of packed column beds can be caused by leakage of the ligand following repeated purification cycles and from the deterioration in the quality of the media following treatment with harsh cleaning agents necessarily deployed in resin reuse strategies. In large-scale manufacturing operations, resins are recycled due to economic necessity for a validated number of uses, often >100 times. A medium with low-ligand leakage that can withstand cleaning-in-place/regeneration protocols will lower overall production costs, which is always highly desirable. At the same time, assurance is required that adventitious contamination arising from the medium itself or from carryover from the feed material of a previous batch will be minimized in the product stream.

In order to satisfy regulatory scrutiny concerning the important issue of process validation, full documentation and technical support is required for media used in a GMP process. Column media should be considered a critical raw material, and new batches must be tested before being introduced into a production cycle. Currently, for therapeutic products, the use of media prepared using animal-free components and validated manufacturing technologies are mandated by regulatory agencies. A guarantee of the supply of the chosen media along with full and proper vendor certification should also be ensured prior to specifying a medium for use in commercial production campaigns.

2.2 Column Packing at Large Scale

Ensuring reproducibility of media packing as the diameter of the column increases is often regarded as the most problematic aspect in scale-up. It is not surprising that small-scale columns are easier to pack reproducibly. One limitation in the approach to scale-up by increasing the column diameter while maintaining packed bed height is that commercial manufacturers typically supply column diameters of a set-defined size. From a practical viewpoint, the packing of large-diameter columns becomes more difficult, and the stability of the resultant packed bed can also be reduced. Instability in the media bed at large scale is due in the main to the decreased physical support offered by the wall of the column as the diameter increases. This can result in high pressures caused by compression of the medium via drag forces exerted by fluid flow through the bed. In general terms, decreased stability may result in unpredictable flow distribution during processing leading to hysteresis, edge effects, and media compression [30], all of which are undesirable as they will affect process reproducibility.

The packing procedure itself can become quite challenging when large-diameter columns are used. To satisfy the needs of larger production processes, biopharmaceutical manufacturers will often utilize chromatography columns with diameters in the range 1–2 m for the initial capture step following harvesting/clarification

operations. The size of column required will be dependent on the binding capacity of the column media for the protein of interest and the stage of purification itself. In large-scale cell culture processes that generate large amounts of target product, it may not be possible or even reasonable to capture the target product in one cycle due to the limitations in column availability and the resultant cost implications of media particularly if expensive affinity-based capture chromatography is being utilized. Thus, it is more common for the total volume of initial feed solution to be processed through a smaller diameter column and to utilize a series of discrete cycles to achieve the required volumetric throughput.

Insufficient quality in column packing can cause channeling in the bed, which will lead to broadening or splitting of peaks and an obvious decrease in the resultant resolution. Manual packing of chromatography columns can be used within industry and is often appropriate if the column diameter is relatively small (<0.3 m). This type of packing is performed by pouring the required amount of prepared medium slurry into the column shell and pumping an appropriate buffer through the column adaptor, thus inducing the medium bed to pack. This operation is obviously quite manual and at stages is open to the surrounding environment. Thus, media packed using this method is usually sanitized with a caustic solution prior to equilibration and processing to remove any potential bio-burden that could have been introduced. Automated pack-in-place technologies can be utilized, which allow for more consistent and efficient packing operations when large-diameter columns are used [31]. Such systems also allow for packing operations to be carried out in a more closed and controlled manner and are composed of diaphragm pumps and associated valving that serves to direct the flow of buffers or resin slurries to appropriate valves on the column or slurry vessels, respectively [32].

All chromatography media will require different conditions to achieve optimum packing, and trials should take place in order to develop robust and optimal packing procedures prior to scale-up [26]. When a column is packed, the integrity of that packing is usually qualified by estimating parameters such as the asymmetry factor (As) and the height equivalent to the theoretical plate (HETP). Both of these factors are determined from an analysis of the chromatogram that is generated when a small volume (<0.25 column volumes) of a low molecular weight tracer compound (NaCl or acetone) is applied and eluted from the packed column [31, 33]. Throughout the lifetime of a packed chromatography media, the continued monitoring of asymmetry and HETP is useful and may be used as a first indication of the deterioration of column integrity as the number of processing cycles is increased, which, in turn, would lead to a decision to replace the media in order to ensure process reproducibility and product quality.

2.3 Cleaning of Chromatography Media

The maintenance of proper sanitary conditions is crucial to current biopharmaceutical processing operations. Whatever media and column systems are selected for a particular application at large-scale, the issue of cleaning and sanitization is, therefore, of high importance. If a chromatography medium is to be reused, which is typical in commercial operations, assessment of the cleaning regime employed between cycles will form a major part of the required resin lifetime study [34]. The potential for carryover of impurities from one column run to the next must be minimized, and any cleaning regime must be capable of removing residual contamination while at the same time not compromising the performance of the media. Indeed, a validated cleaning strategy for packed chromatography columns and associated equipment is a major component in the overall process validation requirements requested by regulatory agencies in their guidance documents.

Cleaning and sanitization protocols are most usually performed after each batch of material is processed on the column. These activities are designed to mitigate the risk by minimizing exposure of the medium to contaminating bioburden, thus prolonging the lifetime of the medium and reducing downtime due to the need to repack with fresh medium. Notwithstanding the time involved, repacking of columns can necessitate the use of large volumes of buffers, and a requalification of the packed bed quality should also be performed following repacking to ensure reproducibility.

Cleaning-in-place (CIP) strategies, which are often performed after every cycle, must be designed having regard to the stability of the medium used, the properties of the feed material applied, and the position of the step in the purification sequence. The important decision to reuse a medium in a process can often depend on the ability to clean the medium satisfactorily. Media used in the first capture step can be more difficult to clean due to the nature of the feedstock applied. However, at the capture stage, media volumes are generally large and reuse presents the only option, particularly given that the medium may be very expensive. During polishing operations, feed samples should be much cleaner and hence column volumes will generally be smaller. However, at this late stage in manufacturing, carryover and any potential ligand leaching could significantly compromise product quality, and a decision should be made as to the merits of a reuse strategy in this case [35]. Sanitization cycles typically use alkali (0.5–1 M NaOH) to reduce the build-up of endotoxins and bioburden. If the CIP protocol is carefully designed, it is possible to reuse a medium many times without negative effects. In one study, a Protein A medium was used for 150 cycles with cleaning-in-place being performed after every cycle. It was demonstrated in this study that the yield and purity were

consistent after each cycle. In addition, there was no detectable carryover, and the binding capacity for the target IgG_1 antibody remained at a high level (>85%) when compared with the initial cycle [36].

2.4 Production Equipment

While in theory at least chromatographic processes can be scaled efficiently by taking note of key chromatography parameters and scaling accordingly, one aspect of large-scale downstream processing that will clearly be an issue is the nature of the equipment used. Glass and acrylic columns are used as scale is increased, but for very large-diameter columns, stainless steel is usually the material of construction. Column adaptors at large scale (0.5–2.0 m) will be very heavy, and contingency must exist within a facility for packing and maintenance operations. This will invariably involve the use of hydraulic lifting equipment to protect column hardware and to ensure operator safety. When a column is specified for inclusion in a GMP process, the manufacture of all components of the column and the materials of construction therein must be fully traceable to minimize the risk associated with leaching, particularly given the harsh cleaning solutions that will be used during processing.

Large-scale chromatography systems are generally automated systems that use a series of pipings, inlet and fraction valves, flow meters, air detectors, air-traps, pressure sensors, and buffer mixing capability for application requiring gradient formation. Such equipment may give rise to increased dead volumes, leading to dilution, higher pressure drops, and peak broadening, which will cause extra dilution of the product fraction or even loss of resolution if the application is sensitive to variations in plate number in the system used. Chromatography systems are typically constructed from inert piping and are configured to minimize hold-up volumes and to ensure that the fluid path can be properly cleaned and sanitized to minimize contamination. Proper qualification of chromatography equipment can identify problems prior to undertaking production activities [14]. It is generally necessary to further refine previously optimized parameters (e.g., flow rates) upon scale-up as a direct result of differing equipment design.

2.5 Non-chromatographic Factors Applicable at Large Scale

Several factors not directly related to the chromatography process per se become an issue when the downstream process increases to large scale. These factors can affect the purity and yield achieved from a purification cycle when the resultant product fraction is characterized and compared with the corresponding purification step performed at small scale. As the size of the cell culture bioreactor increases, both the concentration of the target molecule and the distribution of the associated panel of contaminants present will change due to the natural variation that will take place during cell culture. In general, during scale-up design, culture feedstock should be supplied from reactor runs performed close to the

predicted final scale so that the effect of bioreactor conditions on product quality can be determined. When a chromatography column step itself is upscaled, there will be an obvious increase in the amount of buffer solutions required to achieve purification. As volumes increase, the storage space taken up by such solutions in a facility can be problematic. Reproducibility in buffer preparation at large scale becomes very important, and realistic specifications for pH and conductivity must be set in order to ensure that buffer manufacturers can effectively prepare solutions that will perform robustly during processing. The use of expensive organic solvents is often required to achieve the desired level of purification in reverse phase chromatography applications. In addition, processing suites where such activities take place within a facility will have to be designed carefully to ensure that the room is properly vented and risk of explosion is removed. This will ensure that operators and personnel entering the area can be protected from the effects of large volumes of concentrated solvents. Such requirements can add considerable expense to a downstream process and will require that facilities must be dedicated to such activities, which will reduce overall flexibility.

If a purification step has been carefully optimized, the volumes of buffers required at each stage of the chromatography should have been minimized. If scale-up is planned, it is more appropriate to have the target product molecule elute in one to two-column volumes rather than in five to ten-column volumes. This feature will decrease the volume of buffers required, reduce the resultant storage required, and lower accompanying material costs. A model study has shown the effect of increasing elution volume per cycle as column volume is increased for the affinity purification of a monoclonal antibody [26]. Furthermore, if the volume of a bioreactor is increased and the associated capture chromatography step has to be scaled up by increasing the number of individual cycles rather than the diameter of the column, the additional volumes of buffer required will necessitate higher costs. Membrane chromatography is now emerging as a cost-effective alternative to conventional chromatography for certain applications. The lower consumption of buffer volumes that results from using membrane chromatography coupled to the cost savings on hardware when compared with traditional chromatography has attracted much attention from manufacturers [30, 37].

3 Summary

Continued developments in cell culture technology and the clear industry focus on greater process intensification are placing an increasing demand on manufacturers to develop robust and reproducible downstream processes to address the increased product

titers that are now achievable. Due to the crucial role that chromatography plays in downstream processing, satisfactory scaling-up of chromatography operations continues to be a key factor in the successful commercial manufacture of therapeutic proteins. Similar challenges will remain in the future as the industry diversifies the panel of therapeutic modalities that will be produced for patients. A considered and rational design of a chromatography process must take place to ensure effective scale-up of that process can be achieved. Such design must have regard to all factors that can affect scale-up from media properties, packing operations, column hardware, ancillary equipment, and facility issues. This review has described some of the key issues that will present themselves whenever a decision is made to increase the scale of a chromatographic-based purification step. Due to the current exciting pipeline of high-dose protein products in development and the emergence of new classes of antibody products, the impetus to continue to find new innovations in large-scale downstream processing will remain crucial for overall success.

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Chapter 6

Approaches to Avoid Proteolysis During Protein Expression and Purification

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Abstract

All cells contain proteases, which hydrolyze the peptide bonds between amino acids of a protein backbone. Typically, proteases are prevented from nonspecific proteolysis by regulation and by their physical separation into different subcellular compartments; however, this segregation is not retained during cell lysis, which is the initial step in any protein isolation procedure. Prevention of proteolysis during protein purification often takes the form of a two-pronged approach: first, inhibition of proteolysis in situ, followed by the early separation of the protease from the protein of interest via chromatographic purification. Protease inhibitors are routinely used to limit the effect of the proteases before they are physically separated from the protein of interest via column chromatography. In this chapter, commonly used approaches to reducing or avoiding proteolysis during protein expression and purification are reviewed.

Key words Protease, Protease inhibitor, Proteolysis, Protein expression and purification, Bioinformatics

1 Introduction

Protein stability can be defined as "the persistence of molecular integrity or biological function despite adverse influences or conditions, such as heat or other deleterious conditions" [1]. One of the key deleterious conditions during protein purification is the presence of proteolytic enzymes, referred to as proteases. Proteolysis is the directed degradation of proteins by specific proteases and occurs ubiquitously in nature where homeostatic levels of proteins in cells are governed by a fine balance between their rates of synthesis and their rates of degradation (*see* Fig. 1). Proteases have been referred to as "Nature's Swiss Army knife" due to their diverse roles in protein cleavage [2].

Proteases are employed by all living cells to maintain a particular rate of protein turnover achieved by continuous degradation and synthesis of proteins. Catabolism of proteins provides a ready

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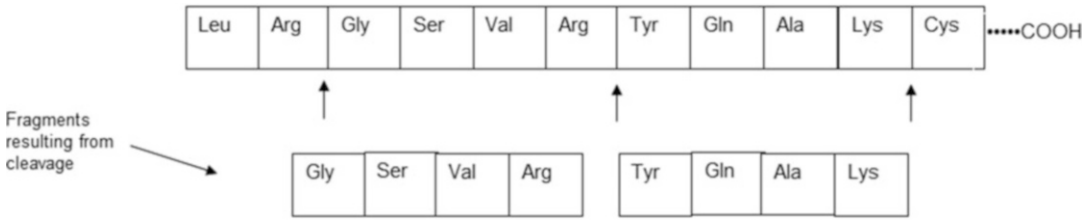

Fig. 1 The schematic depicts a peptide chain fragment, indicating where the chain will be cleaved by trypsin. Trypsin cleaves at the carboxyl side of Arginine (Arg) and Lysine (Lys) amino acids. The cleavage points are indicated by arrows. The amino acid fragments resulting from the cleavage are indicated

pool of amino acids that can be reused as precursors for protein synthesis. Intracellular proteases participate in governing protein turnover for the cell; for example, in *Escherichia coli*, the adenosine triphosphate (ATP) dependent protease La, the *lon* gene product, is responsible for hydrolysis of abnormal proteins [3]. The turnover of intracellular proteins in eukaryotes is also affected by a pathway involving ATP-dependent proteases [4].

Issues with proteases are less acute when purification is from a recombinant host such as *E. coli* since such hosts have been engineered to minimize proteolysis. Nonetheless, issues can arise that may be avoided by judicious choice of expression host. Proteins purified directly from "native" tissues are a different matter and present a far greater challenge. In the latter case, the lysis of subcellular organelles may cause the release of a number of ill-defined, damaging proteases. In this review, the term "protein of interest" will be used to denote the target protein, recombinant or otherwise, to be purified.

Originally, proteases were thought to be involved solely in the degradation of unwanted proteins, but they are now known to take part in a wide range of important signaling and physiological processes such as angiogenesis, apoptosis, and blood clotting [5]. The ability of many proteases to digest proteins at specific cleavage sites (*see* Fig. 1) has given rise to a host of biotechnological applications for these enzymes [6]. Research in this area now employs bioinformatics [7] and systems biology [8] among other tools to investigate the complex interlinked proteome. Based on this research, some proteases are recognized as biomarkers for disease states and have become the target of therapeutic intervention in an attempt to modulate a variety of signaling pathways [9].

2 Protease Classification

Proteases belong to the hydrolase class of enzymes (Enzyme Classification 3.4), which catalyze the hydrolysis of various bonds with the participation of a water molecule. The proteolytic process

Table 1
Broad classification of proteases based on their site of action along a protein chain

Peptidase type	Mode of action	Common examples
Exopeptidase	Hydrolyzes terminal amino acids from a protein chain. They may act on the amino, or carboxyl, terminal of the peptide chain	Aminopeptidases and Carboxypeptidase
Endopeptidase	Hydrolyzes internal peptide bonds of a protein	Trypsin, Chymotrypsin, Pepsin, and Papain

Table 2
Broad classification of proteases based on active site amino acid or metal ion [44]

Protease type	Active site amino acid or metal ion	Common examples
Serine protease	Serine	Subtilisin (EC 3.4.21.62, an endopeptidase sourced from *Bacillus subtilis*, [45])
Cysteine (thiol) protease	Nucleophilic cysteine thiol	Papain (EC 3.4.22.2, an endopeptidase sourced from *Carica papaya,* [46])
Aspartate protease	Aspartic Acid (often, two highly conserved aspartate residues)	Plasmepsin (EC 3.4.23.39, an endopeptidase produced by the *Plasmodium* parasite, [47])
Glutamate protease	Glutamate and glutamine dyad	Glutamate carboxypeptidase 2 (EC 3.4.17.21, an exopeptidase sourced from *Homo sapiens*, [48])
Threonine protease	Threonine (commonly activated by a histidine)	TSP50 peptidase, an endopeptidase sourced from *Homo sapiens*, [49])
Asparagine protease	Asparagine (commonly as a dyad with an acidic residue)	MeTr peptidase, an endopeptidase sourced from *Homo sapiens*, [50])
Metalloproteases	Catalysis requires an active site metal ion (e.g., zinc or cobalt)	Adamalysin (EC 3.4.24.46, an endopeptidase from the rattlesnake *Crotalus adamanteus,* [51])

involves the cleavage of peptide bonds that link amino acids together in the polypeptide chain. Proteases are defined as either exopeptidases or endopeptidases depending on their site of action (*see* Table 1). Proteases are also categorized into four major groups according to their active site and mode of action (*see* Table 2). Another classification divides proteases based on their pH optimum. Thus, acid proteases are optimally active at acidic pH values: these were originally identified in the mammalian stomach as components of digestive juices (e.g., Chymotrypsin). Neutral proteases and alkaline proteases are optimally active at neutral and alkaline pH, respectively.

3 Proteolysis

3.1 Proteolysis: Prevention Is Better Than Cure

Proteases are essential enzymes in all life forms, and in normal circumstances, they are typically packaged into specialized organelles to minimize the chance of unwanted proteolytic activity. Within these organelles, there are specific regulators associated with each protease, controlling their action. Prior to an initial chromatography step, it may be necessary to lyse or disrupt cells to liberate the protein of interest in soluble form. It is at this stage that a protein is most vulnerable to proteolysis. When cells are disrupted prior to chromatography, proteases that are normally located in different subcellular compartments become separated from their regulator molecules and exposed to the protein of interest, thus increasing the likelihood of undesired proteolysis [10]. Realistically, it is impossible to remove all proteases present in a cell lysate; however, careful selection of a host cell (if the protein of choice is recombinant) or a cell type (if the protein of choice is native) in conjunction with specific sample preparation protocols can reduce unwanted proteolysis during purification [11]. Approaches to reduce proteolysis during heterologous protein expression and native protein extraction for chromatography purposes will be discussed here by way of examples.

3.2 Reducing Proteolysis During Heterologous Protein Expression

During the production of recombinant proteins, the protein of interest may be exposed to a host cell protease to which it is particularly susceptible. There are many design strategies that can be employed to reduce such proteolysis. The gram-negative bacterium *E. coli* is a widely used host for heterologous protein expression in both research and industry. The approaches described below refer to expression in this host system but will also apply to other organisms in many cases.

3.2.1 Use of Alternative Expression Strains

Simply altering the host strain may reduce proteolysis of recombinant proteins. There are many commercially available protease-deficient strains for heterologous protein expression; for example, *E. coli* BL21 is deficient in two proteases encoded by the *lon* (cytoplasmic protease) and *ompT* (periplasmic protease) genes, while other strains lack *Prc* and *DnaJ* protease genes ([12], *see* Table 3).

3.2.2 Subcellular Targeting of Expressed Proteins

Proteins may be expressed in a subcellular compartment where they are less likely to encounter a protease. Targeting to the periplasmic space of *E. coli* by use of signaling motifs such as PelB or DsbA may avoid degradation by cytoplasmic proteases during expression. The appropriate signal peptide must be deduced by trial and error (*see* Ref. [13] *for an example*). This approach, combined with selective cell lysis, will reduce the likelihood of the expressed protein coming

Table 3
Some commercially available protease-deficient *E. coli* strains that are used to express recombinant proteins

Strain name	Protease deficiency	Supplier
UT5600	Deficient in *OmpT* (an outer membrane protease that cleaves between sequential basic amino acids)	New England Biolabs Inc.
CAG626	Deficient in Lon (a protease that degrades abnormal/misfolded proteins)	New England Biolabs Inc.
CAG597	Stress-induced proteases at high temperature	New England Biolabs Inc.
CAG629	Stress-induced proteases at high temperature and Lon protease	New England Biolabs Inc.
PR1031	Deficient in DnaJ—a chaperone that can promote protein degradation	New England Biolabs Inc.
KS1000	Deficient in Prc (Tsp), a periplasmic protease	New England Biolabs Inc.
Rosetta	Deficient in Lon and OmpT	Novagen
Rosetta-gami B	Deficient in Lon and OmpT	Novagen
Origami B	Deficient in Lon and OmpT	Novagen
BL21 Star™(DE3) pLysS	Deficient in Lon and OmpT	Invitrogen
BL21 Star™(DE3)	Deficient in Lon and OmpT	Invitrogen
BL21-AI	Deficient in Lon and OmpT	Invitrogen

in contact with cellular proteases. Extracellular expression, where the protein of interest is secreted into the culture medium, is challenging in *E. coli* since the protein must cross two cell membranes. However, such systems have been developed and do not require cell lysis. This strategy may provide a means of avoiding proteases altogether (*see* Ref. [14] *for an example*).

3.2.3 Reducing Protein Misfolding

Protein degradation by proteolysis occurs naturally when misfolded proteins are produced in the cell. Misfolded proteins are commonly produced during heterologous protein expression and can occur due to a difference in codon usage between *E. coli* and the expressed protein's native environment. The codon bias may be reduced by using specific cell lines (e.g., *Oragami*™ or *pRARE*™ from *Novagen*), lowering growth temperature (*see* [15] for an in-depth review) or by altering the growth medium (e.g., polyol inclusion [16]).

3.2.4 Use of Fusion Proteins

Another approach to protect recombinant proteins from unwanted proteolysis is to fuse them to a protein tag. These tags can improve protein folding and solubility and also act as a convenient handle for purification. Examples here include proteins fused to Glutathione-S-Transferase (GST) or Maltose Binding Protein (MBP). Vectors incorporating such tags are commercially available; GST (pGEX plasmid system from *Cytiva*) and MBP (pMAL plasmid system from *New England Biolabs*).

3.2.5 Alternative Expression Hosts

If the above approaches fail, it may be necessary to consider expression in another heterologous host (e.g., another prokaryote, yeast, or mammalian cell line). Several commercially available expression systems have been developed in recent years [17–19].

3.3 Proteolysis Reduction During Native Protein Purification

The purification of proteins from cells (plant or animal) other than specialized prokaryotic expression hosts can be more problematic. In the case of eukaryotic cells, for example, cell lysis may cause the release of lysosomal proteases that may attack a protein of interest. In this case, a number of basic procedures to minimize protease activity can be considered.

3.3.1 Source of Native Protein

Native protein sources, such as mammalian tissues, often exhibit differing protease levels (e.g., liver and kidney samples contain a much higher concentration of proteolytic enzymes than skeletal or cardiac muscle, [20]). Careful selection of protein source may sufficiently reduce protease activity during extraction and purification.

3.3.2 Low-Temperature Lysis

Cell lysis is normally carried out on ice to keep degradative processes to a minimum. The extract buffer should also be chilled to 4 °C before use.

3.3.3 Work Quickly

It is important to prepare a clarified cell extract for purification as quickly as possible to minimize contact between the protease and the protein of interest. A clarified extract is normally achieved by centrifugation using a refrigerated centrifuge. It is important to proceed to the first purification step as quickly as possible after cell lysis and clarification. The initial purification step should be designed to separate the protein of interest from proteases.

3.3.4 Control of pH

Typically, cell lysis is carried out at neutral or slightly alkaline pH. This will minimize the activity of acid proteases; however, it will not affect neutral or alkaline proteases.

3.3.5 Lysis Buffer Additives

A range of compounds may be added, sequentially or simultaneously, to the lysis buffer to reduce protease action. Salts may be added to alter the osmotic concentration of buffer, whereas glycerol (5–15% v/v) and sucrose (2.5 mM) may be added to stabilize

Table 4
Protease inhibitors: stock solutions and storage conditions

Inhibitor activity	Inhibitor	Solvent	Molarity	Storage
Serine	PMSF[a]	Dry methanol or propanol	200 mM	−20 °C
Serine	3,4-DCL	Dimethylsulfoxide	10 mM	−20 °C
Serine	Benzamidine	Water	100 mM	−20 °C
Cysteine	Iodoacetic acid	Water	200 mM	Prepare fresh
Cysteine	E64-c	Water	5 mM	−20 °C
Thiol (serine & cysteine)	Leupeptin	Water	10 mM	−20 °C
Metallo	1,10 Phenanthroline	Methanol	100 mM	RT[c] or 4 °C
Metallo	EDTA[b]	Water	0.5 M	RT[c] or 4 °C
Acid Proteases	Pepstatin	DMSO	10 mM	−20 °C
Aminopeptidase	Bestatin	Water	5 mM	−20 °C
Threonine	Leupeptin	Water	10 mM	−20 °C

[a]PMSF is toxic. Weigh this compound in a fume hood and wear appropriate personal protective equipment
[b]Does not inhibit pancreatic elastase
[c]*RT* Room temperature

Table 5
General protease inhibitor mix

Stock Inhibitor	Volume (μL)
PMSF (100 mM) or 3,4-DCI (10 mM) or Benzamidine (5 mM)	200
Iodoacetate (200 mM) or E64-c (5 mM)	200
1,10 phenanthroline (100 mM) or EDTA (500 mM) or Leupeptin (10 mM)	100
Pepstatin (10 mM)	100
Double-distilled water	400
Final volume	1000

proteins. In addition to these compounds, there are a range of specific protease inhibitors that may be added to lysis buffers to stabilize proteins (*see* Table 4). Many of these agents are commercially available as inhibitor cocktails, or they can be prepared in-house (*see* Table 5 and Subheading 4.3).

3.3.6 Alternative Approaches

In some cases, it may be possible to heat shock an extract to temperatures up to 70 °C or more; however, this approach will *only* work if the protein of interest is heat stable. The heat shock inactivates degradative enzymes, such as proteases, while maintaining the activity of the protein of interest. Another approach is to use

salting-out to precipitate and stabilize the protein of interest. In this process, ammonium sulfate is added (slowly, with stirring) up to 70% w/v saturation to render proteins insoluble. The precipitated proteins may be collected by centrifugation. Many proteins are surprisingly stable as precipitates and can be stored in this form for extended periods before being resolubilized by a simple resuspension and dialysis step.

Once the source of the protein of interest has been optimized, a commonly used approach toward prevention of further unwanted proteolysis during protein isolation and purification is to include protease inhibitors during sample preparation, purification, and characterization.

4 Protease Inhibition

4.1 Protease Inhibitor Selection and Preparation

Proteolysis avoidance, or reduction, is preferable to dealing with a protease after it has begun to act. However, if proteolysis is unavoidable, understanding the protease you are dealing with will help in choosing alternative protein isolation and purification strategies or, in the worst case, selecting a suitable inhibitor. One should also consider whether the protease activity is a problem all the time or only during certain conditions (e.g., induction, isolation from cancer cell lines, *see* [21]). Judicious inhibitor choice will depend on the correct empirical identification of the protease involved.

The identity of a protease can be determined in several ways; however, the simplest method is to incubate the sample of choice with a single inhibitor from the group of inhibitors (Serine, Cysteine, Thiol, Metallo-, etc.) listed in Table 2. The degree of proteolysis can be simply identified from Sodium Dodecyl–Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE) analysis of the protein sample post-inhibitor treatment; increased protein band smearing on the gel or a change in the expected protein size will indicate potential proteolysis. Proteolysis inhibition, indicated by a maintenance of correct protein size with no protein band smearing after incubation with inhibitor, will permit the identification of a suitable agent.

Predictive Tools to Identify Potential Proteases Responsible for Degradation

There is a great demand for bioinformatics tools that can predict protein cleavage events with high accuracy by utilizing both sequence and structural information [22]. With specific proteolytic cleavage sites identified, the protease(s) responsible for degrading the protein of interest can be predicted using peptide characterization software (*see* Table 6). Subsequently, the proteolytic activity can be inhibited by addition of protease-targeted inhibitors. Use of

Table 6
Bioinformatics tools to predict protease activity and guide selection of appropriate protease inhibitors

Name	Description	Webserver	Reference
Cascleave2.0	Prediction of caspase substrate cleavage sites	http://www.structbioinfor.org/cascleave2/	[52]
CaMPDB and Calpacchopper	Calpain cleavage prediction	http://calpain.org/	[53]
DeepCleave	A deep learning predictor for caspase and matrix metalloprotease substrates and cleavage sites	https://deepcleave.erc.monash.edu/	[54]
GPS-CCD	Prediction of calpain cleavage sites	http://ccd.biocuckoo.org/	[55]
iProt-Sub	Mapping and predicting protease-specific substrates and cleavage sites	iprot-sub.erc.monash.edu.au	[56]
LabCaS	Prediction of calpain cleavage sites	http://www.csbio.sjtu.edu.cn/bioinf/LabCaS/	[57]
PCSS	Peptide classification using sequence and structure	https://modbase.compbio.ucsf.edu/peptide/	[58]
PeptideCutter	Predicts potential cleavage sites cleaved by proteases	https://www.expasy.org/resources/peptidecutter	[59]
PoPS	A computational tool for modeling and predicting protease specificity	http://pops.csse.monash.edu.au/	[60]
Procleave	A bioinformatics approach to predicting protease-specific substrates and specific cleavage sites by considering both their sequence and 3D structural information	http://procleave.erc.monash.edu/	[61]
PROSPER	Predicting protease substrate cleavage sites	https://prosper.erc.monash.edu.au/	[62]
PROSPERous	High-throughput prediction of substrate cleavage sites for 90 proteases	http://prosperous.erc.monash.edu/	[63]
ScreenCap3	Prediction of caspase-3 cleavage sites using experimentally verified noncleavage sites	http://scap.cbrc.jp/ScreenCap3/	[64]
SitePrediction	Predicts the cleavage site of a protease	https://www.dmbr.ugent.be/prx/bioit2-public/SitePrediction/	[65]

the MEROPS online protease database (https://www.ebi.ac.uk/merops/) can assist in the search for inhibitors of identified enzymes.

Protease Activity in Extracts

There are alternative experimental strategies for the specific identification of proteases including fluorescence labeling [23], substrate tagging [24], zymography [25], and activity-based probes [26]; however, these techniques are generally more expensive and labor intensive. The type of proteolysis encountered in a given tissue sample can be divided into two broad categories:

Minor hydrolysis: An exopeptidase that cleaves off one, or more, terminal amino acids may cause little disruption to the integrity or function of a protein. This degree of degradation may go unnoticed, and catalytic activity, for example, may be unchanged. In some cases, significant cleavage of an enzyme by peptidases may occur without loss of activity or function. Such proteolysis may only be detected by electrophoretic heterogeneity or by mass spectrometry. A consequence of this hydrolysis where protein activity or function is partially lost may be extremely difficult to detect.

Catastrophic hydrolysis: In this case, hydrolysis renders a protein devoid of activity or function. The protein may not be detectable by traditional techniques such as activity assay or using antibody probes. In this case, it may be useful to monitor lysates for protease activity using an appropriate screening assay.

Once the type of protease has been identified, individual inhibitors can be chosen from Table 4, or a typical *general-use* protease inhibitor mix can be prepared immediately before use from the stock concentrations outlined in Table 5. Protease inhibitor solutions must be correctly stored after they have been prepared. An aliquot a stock solution of inhibitor should be prepared and stored at the correct temperature (*see* Table 4) to maintain the properties of the inhibitor. Single-use aliquots should be prepared to reduce the risk of stock contamination. Ensure that the protease inhibitor/inhibitor mix is combined with the cell sample *immediately prior* to cell disruption. If the individual protease inhibitor/inhibitor mix is to be prepared fresh, then it must be used within 1 h of preparation.

It should be noted that the generic protease inhibitor cocktail outlined here is not guaranteed to work in all circumstances. The success of any mix will depend on the correct empirical identification of the protease involved.

4.2 Commercially Available Universal Protease Inhibitor Mixes

There are several types of commercially available "Universal Protease Inhibitors" that may also be used (e.g., *Complete Protease Inhibitor Cocktail Tablets*, Roche). Additionally, many companies offer inhibitor panels, such as the *Protease Inhibitor Panel* (Merck Sigma Aldrich), which is a cost-effective method for personalized protease cocktail inhibitor generation [27].

4.3 Supplementary Inhibitor Components

Additional Inhibitors

If a particular protease is thought to be dominant within a sample preparation, the cocktail mix may be supplemented with additional specific protease inhibitors [20, 28–35]. Commonly used specific individual protease inhibitor components are listed in Table 7.

Table 7
Additional inhibitors that can be used to supplement protease inhibitor mixes

Inhibitor	Solvent	Molarity	Storage
Serine Protease Inhibitors			
Aprotinin (Does not inhibit thrombin or factor Xa)	Water	300 mM	−20 °C (at pH 7)
Chymostatin (Inhibits chymotrypsin-like serine proteases such as chymase, cathepsins A, B, D, and G. It also inhibits some cysteine proteases such as papain)	DMSO	10 mM	−20 °C
Antithrombin III (Inhibits thrombin, kallikreins, plasmin, trypsin and factors Ixa, Xa, and Xia)	Water	10 Units/mL	−20 °C (at pH 7)
TLCK (Inhibits chymotrypsin-like serine proteases)	1 mM HCl	100 μM	Prepare fresh
TPCK (Inhibits chymotrypsin-like serine proteases)	Ethanol	10 mM	4 °C
DIFP (Highly toxic cholinesterase inhibitor. Broad spectrum serine protease inhibitor. Hydrolyzes rapidly in aqueous solutions and must be added immediately before use)	Anhydrous isopropanol	200 mM	−20 °C
Antipain (Inhibits serine proteases such as plasmin, thrombin, and trypsin. Also inhibits some cysteine proteases such as calpain and papain)	Water	10 mM	−20 °C
A2-Macroglobulin (Broad spectrum protease inhibitor)	Water	100 mM	−20 °C
Cysteine Protease Inhibitors			
N-Ethylmaleimide	Water	100 mM	Prepare fresh
Metalloprotease Inhibitors			
Phosphoramidon (Strong inhibitor of metalloendoproteases, thermolysin, and elastases, but a week inhibitor of collagenase)	Water	1 mM	−20 °C

Table 8
Commonly used phosphatase inhibitors

Name	Typical working concentration	Stock molarity	Typical inhibitory targets
p-Bromotetramisole	0.1–1.5 mM	100 mM	Alkaline phosphatases [66, 67]
Cantharidin	20–250 μM	2.5 mM	Protein phosphatase 2-A [66, 68]
Microcystin LR	20–250 nM	2.5 μM	Protein phosphatase 1 and 2-A [66, 69]
Imidazole	50–200 mM	1 M	Alkaline phosphatases [70, 71]
Sodium molybdate	50–125 mM	1 M	Acid phosphatases and phosphoprotein phosphatases [68, 71]
Sodium orthovanadate	50–100 mM	1 M	ATPase inhibition, protein tyrosine phosphatases, phosphate-transferring enzymes [71, 72]
Sodium tartrate	50–100 mM	1 M	Acid phosphatases [69, 71].

Phosphatase inhibitors may also be required since many enzymes are activated by phosphorylation; hence, dephosphorylation must be inhibited if enzyme activity is to be maintained. Again, an empirical approach is required to identify whether a phosphatase inhibitor is required (*see* Subheading 4.1 and Table 8). Protein phosphatases can be divided into two main groups: protein tyrosine phosphatases and protein serine/threonine phosphatases, which remove phosphate from proteins (or peptides) containing phosphotyrosine or phosphoserine/phosphothreonine, respectively [36]. Inhibitors commonly used include p-Bromotetramisole, Cantharidin, Microcystin LR (Ser/Thr Protein Phosphatases and Alkaline Phosphatase L-Isozymes), Imidazole, Sodium molybdate, Sodium orthovanadate, and Sodium tartrate (Tyr Protein Phosphatases and Acid and Alkaline Phosphatases, *see* Table 8). There are also a number of commercially available Phosphatase Inhibitor Mastermixes (e.g., PhosphataseArrest™ Phosphatase Inhibitor Cocktail, G-Biosciences). These are often supplied in convenient, ready to use 100X solutions that are simply added to the protein extraction buffer or individual samples. These mixes can be sourced as either broad spectrum phosphatase inhibitor cocktails or phosphatase inhibitors for targeting a particular set of phosphatases.

4.4 Supplementary Inhibitors

The addition of supplementary chemical components to disrupt protease activity should be carefully assessed on a small scale since such components may alter the function/stability of the protein of interest (*see* Table 9). Moreover, additional protease inhibitors should be introduced to the sample with caution since protein modifications, such as alteration of protein charge, may occur. These alterations may interfere with further protein characterization studies. For example, 2-mercaptoethanol will reduce cysteine

Table 9
Supplemental chemical/enzyme additions to protease inhibitor buffer [73]

Item and typical working concentration	Advantages	Disadvantages	Uses/typical protease targets
2-mercaptoethanol (1 mM)	Reduction cysteine protease activity	Unfolding of target proteins containing disulfide bridges	Cysteine proteases
Dithiothreitol (2 mM)	Reduction cysteine proteinase activity. Low odor	Unfolding of target proteins containing disulfide bridges	Cysteine proteases
EDTA (5 mM)	Removal of metal ions involved in proteolysis impeding proteolysis	The chelating effect of EDTA will affect the structure of metalloproteins and dramatically reduce the purification of polyhistidine-tagged proteins	Non-His-tagged protein targets or non-metalloprotein targets
Thiourea (2 M)	Proteolysis inhibitory effects, in conjunction with improved protein solubilization	Thiourea is considered a possible human carcinogen and mutagen	General purpose protease inhibitor
Detergents (e.g., SDS or deoxycholate; 2% v/v)	Useful in solubilizing membrane proteins	May activate some proteases	Serine proteases
Sucrose (2.5 mM) /Glycerol (5–15% v/v)	Stabilizes proteins	May need to be removed by dialysis	General stabilization of lysosomal membranes to prevent protease leakage
DN*ase* (100 U/mL)	Reduction in the crude lysate viscosity	Requires incubation step of 10 min at 4 °C in the presence of 10 mM $MgCl_2$	Can be included in the cell lysis buffer for optimal efficiency

protease activity but may also disrupt target proteins containing disulfide bridges. Ethylenediaminetetraacetic acid (EDTA) is a metal ion chelating agent included in protease inhibitor buffers because certain proteases employ metal ions during catalysis, thus, sequestering divalent metal ions will impede proteolysis. However, if one is purifying polyhistidine-tagged proteins or metalloproteins, then the chelating effect of EDTA will dramatically reduce their activity. For His-tag chromatography, EDTA should be removed by dialysis or by using a buffer exchange resin prior to chromatography. Inclusion of 2 M thiourea may also prevent proteolysis: Castellanos-Serra and Paz-Lago [37] noted the protease inhibitory

effects of thiourea addition in conjunction with its efficiency in solubilizing proteins. DN*ase* (100 U/mL), although not a protease inhibitor, can be included in the cell lysis buffer, as this will serve to reduce the viscosity of the crude lysate. The reaction is allowed to proceed for 10 min at 4 °C in the presence of 10 mM $MgCl_2$.

4.5 Protease Inhibition During Chromatography

The introduction of contaminating proteases from skin, nonsterile water, and so forth can be avoided by sterilizing all plasticware and by wearing appropriate personal protective equipment. All buffers should be filter sterilized (0.2 μm) into autoclaved bottles (sterile filtering will not remove contaminating proteases but will remove protease-secreting microorganisms). Additionally, sterile filtration of the protein eluate, once purification is complete, is recommended.

Cell disruption, as with all other parts of the purification procedure, should take place at 2–8 °C. This temperature will not only reduce the activity of proteases but will also aid in stabilizing the target protein (reduction in thermal denaturation). Kulakowska-Bodzon and colleagues [38] provide an excellent review on protein preparation from various cell types for proteomic work. In general, all buffers and materials should be pre-chilled to 2–8 °C. Rapid purification at this lower temperature will reduce the risk of unwanted proteolysis. It is advisable not to store such samples at 2–8 °C for more than 1 day between purification steps, rather store them at −20 °C.

Gel filtration (size exclusion) chromatography is often used as the final step in protein purification where it is used to desalt and buffer exchange the target protein (thus eliminating the need for dialysis). Contaminating proteases can also be separated from the protein of choice if there is significant separation between elution peaks for the protease and the protein of choice. This is the case only where there is a considerable difference between the size of the protease and the size of the protein of interest. If a multistep purification strategy is being used, a purification strategy that delivers the optimal separation between protease and the protein of interest from the beginning is best. This, however, may not always be feasible, as other factors must be considered in designing a purification strategy (e.g., physicochemical properties of the target protein, cost, and time).

5 Proteases in Chromatography

5.1 Use of Proteases for Purification and to Remove Purification Tags

Some purification protocols require the addition of specific proteases. Common examples here include the use of *enterokinase* (recognition site D-D-D-K) or *TEV protease* (recognition site E-N-L-Y-F-Q-G) to remove polypeptide and protein purification tags from recombinant proteins. More recently, designed and non-specific proteolysis during preparative chromatography has been

used to assist in glycoprotein characterization [39, 40], lipid transfer protein purification [41], and antibody profiling [42]. In all cases, it is critical to ensure that any buffer containing protease inhibitor is exchanged, by dialysis or a suitable buffer exchange resin, prior to the addition of the desired protease.

5.2 Post-Chromatographic Analysis

Protease inhibition can be either reversible or irreversible. Most of the serine and cysteine protease inhibitors are irreversible, whereas the aspartic and metalloprotease inhibitors are reversible. Even when the inhibitors are added at an early stage, they may be lost during purification and subsequent handling steps, resulting in proteolysis post-chromatography. The reapplication of protease inhibitors may, therefore, be necessary as purification progresses.

Even with increased numbers of purification steps, very few protocols will remove all proteases from a sample preparation; however, one can hope to achieve an adequate reduction in the level of these unwanted activities. Each purification protocol will have a unique definition of "adequate protease reduction" based on a number of variables including the activity of the remaining proteases, further downstream applications of the protein of choice, and the cost of further protease removal. Additional purification steps often result in a reduced final yield; therefore, a trade-off between protease reduction and yield must be expected.

An apparently pure protein that gives a single band on a Coomassie Blue-stained SDS–PAGE gel should be reanalyzed over time to ensure protease activity is absent from the purified sample. This may be carried out by simply storing an aliquot of the purified protein solution at room temperature and analyzing samples of this by SDS–PAGE at regular intervals. If the protein is degraded (indicated by a smear or a reduced "band size" of the protein of choice), protease contamination is present, and an additional purification step (or supplemental inhibitor addition step) is required.

Care must be taken to rule out the possible loss of enzyme activity due to other destabilizing factors during protein purification. These other factors include, but are not limited to, thermal denaturation, oxidative damage, protein deamidation, and surface absorption. Thermal denaturation of proteins is the decreased stability of a protein caused by extremes of temperature. Thermal denaturation can be reduced if the purification procedure is carried out at 2–8 °C. All buffers and chromatography columns/resins should be pre-chilled to 2–8 °C and the purified protein stored at the correct temperature.

Oxidative damage to proteins can be divided into a number of categories; however, improper disulfide formation is the most pertinent here. Thiol oxidation may be crucial for correct protein folding. The formation of incorrect intra- or intermolecular disulfides is a detrimental process that can often result in loss of activity and/or protein aggregation. Oxidative damage can be avoided by

not exposing the protein of interest to thiol-reducing compounds (e.g., β-mercaptoethanol) during purification thus maintaining the correct folded state of the protein.

Deamidation of glutamine and asparagine residues in a protein during storage is a nonenzymatic degradative process that leads to the introduction of a negative charge and can affect structure and activity. Software that can predict deamidation sites has also been developed [43].

Surface adsorption is caused by the binding of the protein of interest to a surface such as the purification column and/or the column resin material by virtue of its physicochemical properties (e.g., surface charge or hydrophobicity). Nonspecific protein adherence can cause sheer stress damage to the protein during purification; however, this can be circumvented by careful selection of the purification column (type/grade of glass or plastic) and purification resin.

6 Conclusion

The presence of proteolytic enzymes can result in target protein degradation during protein purification. Careful selection of source organism/tissue, along with judicious use of protease inhibitors, can reduce these degrading effects. Commonly used inhibitors are listed here (*see* Tables 4 and 5), along with supplemental compounds (*see* Tables 7 and 8) for easy selection. Protease inhibitors can be added individually or as part of a mix; however, optimal inhibitor selection is an empirical process.

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Chapter 7

Tagging Recombinant Proteins to Enhance Solubility and Aid Purification

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Abstract

Protein fusion technology has had a major impact on the efficient production and purification of individual recombinant proteins. The use of genetically engineered affinity and solubility-enhancing polypeptide "tags" has a long history, and there is a considerable repertoire of these that can be used to address issues related to the expression, stability, solubility, folding, and purification of their fusion partner. In the case of large-scale proteomic studies, the development of purification procedures tailored to individual proteins is not practicable, and affinity tags have become indispensable tools for structural and functional proteomic initiatives that involve the expression of many proteins in parallel. In this chapter, the rationale and applications of a range of established and more recently developed solubility-enhancing and affinity tags is described.

Key words Protein, Tag, Solubility, Affinity chromatography

1 Introduction

The biotechnology industry demands rapid and efficient procedures for expressing and purifying authentic proteins. The advent of genetic engineering brought with it the ability to design, express, and manipulate any DNA sequence and produce the encoded protein in recombinant form using a heterologous host such as *Escherichia coli*. In this chapter, techniques for synthetic DNA synthesis and assembly are replacing in vitro gene fusion and site-directed mutagenesis methods, all of which permit the optimization of transcription and translation regulatory signals including codon content. The result is that virtually any polypeptide can be produced in recombinant form in bacterial, fungal, or eukaryotic host cells or using in vitro "cell-free" translation systems. Major post-expression stumbling blocks remain, however, including protein insolubility, incorrect folding, host cell toxicity and protein instability. Sometimes the result of enforced hyperexpression of

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the desired protein, these impediments can often be overcome by expressing the protein of interest as a fusion with certain partner "tag" peptides or proteins. Thus, numerous solubility-enhancing or affinity tags of either natural origin or of artificial design have been exploited to enhance protein solubility and yield and facilitate purification. Unlike purification strategies that start with native sources of the desired protein, the chromatography of recombinant proteins is much simpler thanks to the range of tag-associated affinity-based recovery systems that has been developed. Recombinant proteins that are joined to a tag with moderate affinity and high specificity for a particular ligand can thus often be isolated from crude host cell lysates in a single step following selective binding to matrices on which the ligand has been immobilized. Tag-fusion expression vectors are commercially available for the purposes of enhancing solubility, enabling one-step affinity-based purification and facilitating the detection of the recombinant protein of interest. In structural and functional proteomic studies, high-throughput protein purification can only be achieved with the assistance of affinity tags as it is clearly not practicable to tailor purification procedures to so many individual proteins.

Escherichia coli remains an important prokaryotic host cell for recombinant protein expression because of its easy manipulation, high biomass-to-cost ratio, and the commercial availability of strains that have been optimized for this purpose. Some heterologous proteins that are produced in *E. coli* form aggregates of insoluble folding intermediates known as inclusion bodies, which are often the result of insufficient time for correct folding being available under conditions of hyperexpression. Protein denaturation and refolding in vitro is expensive, time consuming, and can lead to losses in both yield and bioactivity. Inclusion body formation can often be prevented by optimization of host culture conditions or by fusion of the desired protein to some of the larger affinity tags that are available. Affinity tags include enzymes, protein domains, or small polypeptides most of which bind with high specificity to a range of substrates such as carbohydrates, small biomolecules, metal chelates, antibodies, or antibody-binding molecules. Other tags that only possess solubility-enhancing properties are frequently used in tandem with an affinity tag to aid purification.

In order to generate a tagged protein, the DNA sequence that encodes the protein of interest is joined to the expression vector sequence in such a way as to generate an in-frame fusion with the polypeptide tag-coding sequence. Alternatively, the entire DNA sequence encoding the desired fusion protein may be procured commercially from a synthetic DNA vendor. The fusion tag may be placed at either the N-terminus or the C-terminus of its partner depending on the strategy used, and optimal positioning will depend on the individual protein and be influenced for instance

by factors such as the location of the protein's active site. Commercially available expression vectors are usually designed so that a short flexible hydrophilic "linker" or "spacer" peptide is located between the tag and its fusion partner. This may serve two purposes: (i) to ensure sufficient spatial separation between tag and recombinant protein, so as to maximize tag availability during chromatography but minimize any potential interactions with its partner protein, and (ii) to facilitate the inclusion of an endoprotease cleavage site that can be subsequently exploited for removal of the tag. The choice as to which tag might work best with a given target protein must usually be determined experimentally. Here, we report on the principal solubility and affinity tags that are currently available for use.

2 Solubility-Enhancing Fusion Partners

2.1 Maltose-Binding Protein

Maltose-binding protein (MBP) was one of the first fusion partners to be used for the purposes of alleviating problems associated with the expression and purification of recombinant proteins. MBP is a part of an *E. coli* pathway that is responsible for the uptake and efficient catabolism of maltodextrins [1]. MBP binds strongly to amylose resin [2], and one-step affinity chromatography of MBP-tagged proteins using amylose-agarose columns can typically lead to a fusion protein of 70–90% purity [3]. A monoclonal antibody (B48) also binds MBP and has shown potential as a purification tool for MBP-fusion proteins [4]. In addition to acting as an affinity tag, the 42 kDa MBP can enhance the expression and folding of fusion proteins in which it is the N-terminal [5–7] or C-terminal partner [8]. The *MBP* sequence provides an optimum context for translation initiation, and the expressed protein can be located in the cytoplasm or secreted to the less reducing environment of the periplasm, depending on whether the secreted or nonsecreted form of MBP is used as partner [9]. There is much evidence of MBP-associated enhancement of the folding/solubility of otherwise insoluble fusion partner [8, 10–15]. This enhancement is passive and not completely understood [16], and there is some evidence to suggest that it may be the result of MBP-recruited chaperones being located in the vicinity of the fusion partner [17]. Expression vectors for generating proteins fused to MBP are available from several sources (e.g., the pMAL series from New England Biolabs; the Addgene repository [18]). Following purification, the MBP tag can be excised by specific protease cleavage in the polypeptide linker region between the fusion partners. The MBP system is widely used in combination with a small affinity tag and vectors for generating combinatorially tagged polyhistidine-MBP fusion proteins have also been described, enabling the user to derive additional benefit from the

improved specificity of purification using Immobilized Metal Affinity Chromatography (IMAC; *see* Subheading 3.1) [19–21] and to address the observation that not all MBP fusions can be purified on amylose [22].

2.2 Glutathione S-Transferase

Recombinant glutathione S-transferase (GST), a 26 kDa protein whose corresponding gene was originally obtained from the parasitic helminth *Schistosoma japonicum*, is another well-established solubility/affinity tag [23, 24]. Fusion proteins are affinity purified from crude lysate preparations on immobilized Sepharose glutathione matrices and recovered under mild nondenaturing elution conditions in the presence of reduced glutathione. Although inexpensive, large-scale production of GST fusions is a lengthy process however, a result of the slow binding kinetics of GST to glutathione–sepharose resins [25]. Fusion to the GST partner ensures that translation of the recombinant protein initiates efficiently and affords some protection against intracellular protease cleavage. The GST-tag can be fused at the N- or C-terminus and has successfully been used in bacteria, yeast, mammalian cells and baculovirus-infected insect cells. In many cases, fusion proteins are soluble and form dimers in aqueous solution, but overall GST is a poor solubility enhancer. It is a homodimer, an unwelcome complication making it an unsuitable partner for the isolation of many oligomeric proteins and has exposed cysteine residues that can promote oxidative aggregation [26]. Some insoluble GST fusions can still be purified by affinity chromatography following solubilization with mild detergents [27]. Typically, expression vectors for GST fusion proteins (such as the pGEX series from Merck) encode specific endopeptidase cleavage sites that have been engineered between the tag and the partner proteins. GST moieties can, therefore, be enzymatically cut off and removed by affinity chromatography on a glutathione-Sepharose matrix, and the desired fusion partner is then purified to homogeneity by other chromatographic methods such as ion exchange or gel filtration. Fusing proteins to GST is a popular molecular biology technique for studying biomolecular interactions ("GST pulldowns"; [28–30]). In high-throughput proteomics, GST fusion proteins have been directionally immobilized onto protein microarrays [31, 32]. Successful structure-function studies involving protein–protein and DNA–protein interactions also have been described [28]. Affinity chromatography, followed by removal of the GST-tag, has been used to purify numerous proteins prior to structural analyses and crystallography, and crystal structures have also been described for fusion proteins that retain the GST-tag [33–35]. The GST-tag is also widely used in tandem with smaller affinity tags such as polyhistidine, thus, permitting additional benefit to be had from using IMAC purification systems (*see* Subheading 3.1).

2.3 Small Ubiquitin-Related Modifier

The addition of Small Ubiquitin-Related Modifier (SUMO) protein is a reversible post-translational modification that has been shown to occur in eukaryotic cells. SUMOylation however, unlike ubiquitination, does not lead to degradation of the target protein at the 26S proteosome but appears to regulate protein transport to different intracellular compartments, such as the nucleus, and to play a role in transcriptional regulation [36, 37]. The SUMO conjugation/deconjugation pathway is highly conserved in eukaryotes but absent in prokaryotes. Yeast has one SUMO gene (SMT3), while three genes have been identified in vertebrates (SUMO-1, -2, and -3) [38–40]. Although the overall sequence identity between SUMOs and Ub is low, they share a common three-dimensional structure [41]. The SUMO pathway is similar to that already described for ubiquitin, in that it is an adenosine triphosphate (ATP) dependent ligase cascade (ligases E1, E2, and E3), which serves to couple SUMO through an isopeptide bond to the e-NH2-group of lysine residues of the acceptor protein [42, 43]. SUMO itself is a 100 amino acid protein that has been shown to modulate protein structure and function by covalent modification of target proteins [40, 44, 45]. SUMO specific proteases remove and recycle SUMO from target proteins. The SUMO proteases, such as yeast Ulp1, recognize a Gly-Gly-containing motif found at the C-terminus of SUMO, with a strict requirement for tertiary structure elements only present on correctly folded SUMO. Consequently, such cleavage of proteins that are joined to SUMO is restricted to the junction between SUMO and its protein partner. The advantage to this is that erroneous cleavage within the target protein is much less likely to occur [46–48].

In general, recombinant fusion of yeast SUMO (Smt3) to the N-terminus of target proteins for expression in bacteria has been shown to work well, leading to the enhancement of both protein solubility and expression [47, 49, 50] and in particular with difficult to express proteins [47–49, 51, 52]. The SUMO Tag can then be removed by digestion with Ulp1, thus, regenerating the native N-terminus of the recombinant fusion partner [47]. SUMO fusion systems that facilitate efficient expression of recombinant proteins in *E. coli* have been described [47, 49, 53, 54]. Lee and Kim (2009) used a sticky-end polymerase chain reaction (PCR)-based strategy to design a SUMO fusion protein expression vector that allows directional cloning of any target gene [55]. Their approach involved joining the protein of interest to hexahistidine (His6)-tagged Smt3 (Smt3 is the yeast SUMO protein), thus making it possible to carry out both fusion protein purification and SUMO protease cleavage using a Ni^{2+}-resin column. This kind of one-column strategy is useful in high-throughput platforms for the purification of recombinant proteins. Comparative studies have demonstrated that SUMO can outperform other commonly

used fusion tags in enhancing expression and solubility of several fusion partners [48, 56].

The *E. coli* protein ElaD is a ubiquitin (Ub) protease that has been shown to specifically cleave Ub fusion proteins, but not SUMO fusion proteins [57], implying that SUMO is a better choice of fusion partner when *E. coli* is used as host. The SUMO tag can be cleaved from the fusion protein using Ulp1 protease, which specifically recognizes the SUMO tertiary structure, and the resultant native protein does not retain any redundant amino acids [58]. In eukaryotic protein expression systems, premature processing by endogenous desumoylases can limit the utility of SUMO as a fusion partner. The "split SUMO" approach, was designed to overcome this problem and involves first fusing the N-terminus of the target protein to the C-terminal half of SUMO (CTHS), which is not cleaved by endogenous desumoylases but does retain the capacity to enhance expression of its fusion partner. Following protein purification, the full SUMO structure is then reconstituted by incubation with the N-terminal half of SUMO (NTHS), which interacts strongly with the CTHS, thus generating a substrate for SUMO protease [51]. In another attempt to address the same issue, a double mutant of the yeast Smt3 protease (termed SUMOstar) was developed, which is not recognized by native desumoylases. When used as fusion partner, SUMOstar was shown to lead to the enhanced expression of a range of test proteins in several eukaryotic cell lines and a baculovirus system [59–61]. Elsewhere, protease-resistant SUMO mutants were developed that are resistant to endogenous cleavage and serve as stable fusion tags in yeast and eukaryotic systems [62].

2.4 Other Solubility-Enhancing Fusion Partners

Thioredoxins are universal oxido-reductases that facilitate the reduction of other proteins by cysteine thio-disulfide exchange. *E. coli trxA* is a small protein (109aa, 11,675 kDa) that demonstrates high solubility in the *E. coli* cytoplasm. It also has an inherent thermal stability and is located on the cytoplasmic side of the adhesion zones between the inner and the outer bacterial cell membrane. Though not itself an affinity tag, these two properties of *trxA* may be conferred on *trxA*-fusion partners and can therefore sometimes be exploited to enable the rapid purification of *trxA*-fusion proteins. Release from the *E. coli* cytoplasm is achieved by osmotic shock or freeze/thaw treatments. Thioredoxin can be fused to the amino- or carboxyl-terminus of the protein of interest, but typically, the *trxA* coding sequence is placed at the 5′ end as it promotes efficient translation initiation of the recombinant gene [63, 64]. A variety of expression vectors for this purpose are commercially available, and derivatives of *trxA* have been engineered to possess affinity for immobilized metal ions (e.g., His-Patch Thio-Fusion system from Thermo Fisher Scientific) or avidin/streptavidin [65]. Various cytokines, growth factors, and fluorescent

proteins have been shown to retain remarkable solubility in the *E. coli* cytoplasm when expressed as c-terminal fusion proteins with *trxA* [66–69]. Subsequent examples, in which the solubility of archaeal proteins and bioactive viscotoxins were considerably enhanced, serve to demonstrate the utility of *trxA* as a versatile fusion partner [70, 71]. Protein structural studies are frequently hindered by the difficulty in obtaining diffracting crystals of the target protein, and a fusion partner may sometimes aid the crystallization of difficult targets [72]. *TrxA* itself crystallizes in several different forms, and one screen of a variety of fusion partners demonstrated significant benefits when *trxA* was joined via the tetrapeptide linker sequence GSAM to proteins that are difficult to crystallize [73].

N-utilization substance A (NusA) is a well-conserved essential transcription elongation and anti-termination factor [74]. Fusion with the highly soluble *E. coli* NusA has been shown to enhance the expression and solubility of proteins to which it is joined at the N-terminus in particular [75]. One comprehensive study compared the solubility-enhancing capabilities of NusA and MBP using a diverse set of aggregation-prone partner proteins [16]. It was concluded that both tags, although very different in terms of their biological functions and physiochemical properties, performed very similarly overall as solubility promoters and played passive roles in the folding of their fusion partners. Another hydrophilic fusion partner is the *E. coli* protein disulfide isomerase I (DsbA), which can increase the cytoplasmic and periplasmic solubility of target proteins [76]. Vectors enabling the use of NusA, MBP, and GST in combination with a hexahistidine tag are available permitting IMAC purification of fusions proteins that benefit from the solubility-promoting properties of the larger tag partner [77, 78]. One interesting tag is Fh8, a highly soluble and thermal stable protein when produced recombinantly in *E. coli* (maintaining secondary structure integrity up to 74 °C) [79]. Fh8 has a calcium-binding moiety, and in the presence of calcium, such proteins expose a large hydrophobic surface that can absorb to hydrophobic matrices such as phenyl sepharose. Hydrophobic Interaction Chromatography (HIC) has, therefore, been used to successfully purify Fh8 fusions with elution being achieved by removal of bound calcium through the use of chelating agents like ethylenediaminetetraacetic acid (EDTA) [80]. Elsewhere, IMAC was used to purify an Fh8-hexahistidine tagged protein [81]. HaloTag, which is based on a genetically engineered haloalkane dehalogenase protein that can bind to several synthetic ligands, has been shown to improve solubility of its fusion partners [82]. Other reported solubility/affinity tags include derivatives of the *Staphylococcus aureus* protein A (Z-tag and ZZ-tag; [83, 84]), a mutated derivative of the *Streptococcus* protein G β1 domain (GB1; [85–87]), truncated derivatives of the highly secreted and rapidly folding β-FFase from

Arthrobacter arilaitensis (Ffu fusion tags) [88]; solubility controlling peptide tags (SCP tags) [89]; Solubility Enhancing Peptide tag (SEP tag) [90]; NT* tag [91]; polyionic tags [92]; Phage T7 protein kinase (T7PK; [93]); the Seventeen kilodalton protein (Skp; [93]); and the small polypeptide Solubility Enhancing Tag (SET; [94]).

3 Affinity Tags

3.1 Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography (IMAC), a now ubiquitous form of affinity chromatography, was first introduced in 1975 [95] and is currently the most widely used technique for purifying recombinant proteins. IMAC is a selective tool for the separation of metal-binding peptides and is based on the interaction of certain amino acid residues (e.g., accessible His, Ser, Cys, Glu, and Asp residues [96–98]) on the surface of peptides and the metal ions within an immobilized metal chelate [99]. The reversible nature of this interaction means that it can be first exploited for peptide adsorption and then subsequently disrupted under various elution conditions, involving making alterations to pH or salt concentration. The differential affinity of proteins for immobilized metal ions stems from the coordination bonds formed between the metal ions involved and the electron donor groups present in some amino acid residues (e.g., His, Cys, Trp, and Arg). In IMAC, these electron donor groups form complexes with transition metal ions like Cu^{2+}, Co^{2+}, Zn^{2+}, or Ni^{2+}, which are typically immobilized on polymeric supports with chelating pendant groups such as such as iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA). The retention of proteins on IMAC supports is influenced by a number of variables, including the nature of chelating groups, the specific metal ion [99, 100], and the surrounding milieu (salt concentration and pH). Each chelating group exhibits its own selectivity and adsorption capacity toward a specific protein.

Histidine exhibits the strongest interaction with immobilized metal-ion chelates, and as a result the oligohistidine tag, His_6, is one of the most frequently used fusion tags for protein recovery. Ni (II)-nitrilotriacetic acid (Ni-NTA) exhibits a high affinity for adjacent histidine residues and is one of the most commonly used IMAC matrices for target protein capture by metal-ligand covalent bonding. In general, the order for the adsorption capacity of proteins with accessible histidines and chelating ligands is IDA (tridentate) > NTA (nitrilotriacetic acid, tetradentate) ≥ CM-Asp (carboxymethylated aspartic acid, tetradentate) > TED (pentadentate) [101]. Controlled release of captured proteins can be achieved by increasing the concentration of imidazole in the purification buffer, by changing the pH or stripping the metals off the resins with strong metal chelators such as EDTA.

The popularity of histidine (His)-tag affinity purification is due in part to its high affinity with Ni-NTA, a relatively inexpensive matrix that is able to withstand multiple regeneration cycles under stringent conditions, but also due to the ease of controlled release using mild (e.g., nondenaturing) conditions. Additionally, as the tertiary structure of the His-tag is not important for purification, an insoluble His-tagged recombinant protein can be purified by IMAC under denaturing conditions and subsequently refolded (for a review *see* [102]). IMAC binding of a polyhistidine-tagged fusion protein allows the fusion partner to remain free in solution and thus to be able to fold unhindered by any constraints due to the fact that it is bound to a solid support [103]. A gradual decrease in denaturant concentration induces protein refolding, and elution is achieved by increasing the imidazole concentration or by using a decreasing pH gradient [104, 105]. Elsewhere, an artificial chaperone-assisted IMAC (AC-IMAC) procedure for protein refolding and purification from inclusion bodies was developed [106]. In this work, the authors first overexpressed His-tagged enhanced green fluorescent protein (EGFP) and then refolded and purified it from solubilized inclusion bodies by AC-IMAC. In a systematic comparison of refolding by AC-IMAC versus refolding by IMAC or in bulk solutions, it was shown that in addition to the high purification advantage associated with IMAC, the AC-IMAC method was superior to either IMAC or AC in its high refolding efficiency.

Several further key advantages have established His-tag IMAC as the most widely used method for purifying recombinant proteins for biochemical and especially structural studies. The His-tag combines the advantages of being inert, of low-immunogenicity, and of small size (0.84 kDa) [101, 107]. In most cases, its small size means the His-tag does not interfere with the biochemical activities of the partner protein [108–114] or with most downstream applications [101]. IMAC can be performed in the presence or absence of chaotropic agents and is compatible with strong denaturing reagents such as urea and guanidinium–HCl, as well as a large number of nonionic detergents, making it extremely useful in the initial steps of protein purification immediately after the extraction/isolation procedure [115, 116]. His-tagged proteins can be readily detected by Western blotting using anti-His antibodies. His-tag IMAC purification processes have been adapted for high-throughput protein screening, and such approaches have potential in areas such as target discovery, therapeutic reagents identification, and the discovery of novel protein functions [117–120].

There are reports of His-tags altering the binding characteristics or structure of their partner protein when compared to the corresponding native protein [121, 122]. Hang et al. showed that although His-tagged subunits of the terminase enzyme from bacteriophage-γ formed holoenzymes with wild-type catalytic activity,

one of the subunits displayed an altered capacity to interact with DNA that was not seen in its native counterpart [121]. The length, composition, and location of the His-tag can, therefore, require optimization depending on the sequences of the native protein [122–124]. The use of His-tags is not recommended for purifying proteins that contain metal ions. Likewise, the presence of cysteine and naturally occurring histidine-rich regions in host proteins may result in unwanted protein binding during IMAC [125].

Despite the universal application of IMAC, there are always difficult-tagged proteins that show weak binding to the metal chelating resin. This can be caused by concealment of the His-tag and may be alleviated by switching its position to the other terminus of the protein [126] or by introducing a linker to separate the His-tag from the partner protein [127]. These difficulties can also be overcome by increasing the length of the His-tag to eight or ten histidines [128, 129], and a vector has been described that allows the parallel cloning of target genes with different His-tag lengths using a single insert [55].

IMAC is very sensitive to the presence of metal chelators [101]. In *E. coli* expression systems, the cell lysate contains many unspecific weak chelators such as dicarboxylic acids from the citric acid cycle. Under stress conditions, *E. coli* can also produce highly specific metal chelators known as metallophores [107]. One report linked the failure to purify low-abundance His-tagged proteins from *E. coli* lysates to metal-ion leakage from purification columns [130]. In that study, the authors used His-tagged GFP (His_6-GFP) to examine the effect of *E. coli* lysate on the protein-binding capacity of IMAC columns and concluded that low-molecular-weight components of the lysate, associated with the periplasm, severely reduced the binding capacity of the column. By removing the periplasmic material before cell lysis, the authors observed a tenfold increase in the yield of His_6-GFP when it was diluted with *E. coli* lysate before purification to simulate a low-abundance protein. The separation of contaminant proteins from the target protein is often difficult due to similarities in physicochemical properties. One interesting report described the design a host cell tailored for highly efficient protein purification [131]. The authors outlined a highly efficient strategy, based on proteomic analysis and elution chromatography, in which a protein of interest may be isolated from co-purifying contaminants. Strains of *E. coli* were first engineered to be deficient in three prevalent host proteins that were found in a strategic fraction of an elution profile of Ni(II)-IMAC. Recombinant GFP was then used as a model protein in the expression system, and its elution was directed to the optimized fraction with a His_6-tag, thereby easing its recovery. His-tag/IMAC-based strategies remain dominant in the area of recombinant protein

purification, and examples of novel applications continue to emerge. The purification by IMAC of whole viral particles engineered to express polyhistidine-tagged surface proteins is another application of interest [132–135].

3.2 Other Affinity Tags

Strep-tag is an eight amino acid peptide that binds to the biotin-binding pocket of Streptavidin. Optimization of both partners has led to the development of Strep-tag II peptide and Strep-tactin, the latter being an engineered derivative of Streptavidin [136]. The Strep-tag II polypeptide binds Strep-tactin with an affinity that is two orders of magnitude greater than its affinity for Streptavidin. The tag itself is short, proteolytically stable and biologically inert, and the Strep-tag II-Strep-tactin affinity system is, therefore, widely used for the one-step isolation of bioactive and highly pure Strep-tag II-tagged proteins under physiological conditions [137, 138] and has even been used to purify intracellular organelles [139]. The larger 38 amino acid Streptavidin-binding peptide (SBP) was selected as a robust high-affinity streptavidin-binding aptamer [140]. Tagging recombinant proteins with SBP is of particular interest when intermediate amounts of protein (up to 0.5 mg) need to be produced and purified in a high-throughput manner. An additional advantage here is that a wide variety of streptavidin-derivatized materials is commercially available.

Short oligomers of arginine (Arg-tags) have been used in the past as tags to facilitate protein purification by ion-exchange chromatography. These cationic peptides, such as the R_9 tag which consists of nine arginines, are versatile in that they can also promote cellular internalization and surface immobilization of recombinant proteins to which they are fused [141].

Calmodulin-binding peptide (CBP), derived from human skeletal muscle myosin light chain kinase, binds calmodulin with high affinity [142]. Stringent washing conditions coupled with the lack of calmodulin-binding proteins in *E. coli* means that CBP-tagged fusion proteins can be recovered in good yield with high specificity. The tag is not suitable for use in eukaryotic cells as it interacts with endogenous proteins and can interfere with calcium signaling pathways.

Cellulose-binding domains (CBDs) are non-catalytic domains that have been identified in many proteins, and their size, relative locations, and affinity for their natural substrate varies considerably [143]. Cellulose is an attractive matrix for the affinity purification or immobilization of CBD-tagged proteins. It is inert, has low nonspecific affinity, is available in many forms, and has received approval for many pharmaceutical uses. CBDs have been used for applications involving the immobilization of enzymes, cytokines, or other ligands [144]. Some cellulose-CBD interactions are very strong indeed and often require the use of strong chaotropic agents to promote release of the CBD-tagged protein, thus necessitating

protein refolding in vitro. Others can be eluted under milder conditions using ethylene glycol. A method for purifying CBD-tagged molecules has been reported whereby they are induced to self-aggregate leading to their selective pull-down [145]. Elsewhere, CBD tags have been used to immobilize antibodies and cells onto regenerated cellulose hollow fiber membranes [146, 147].

Chitin-binding domains have also been exploited as tags for the affinity purification of recombinant proteins. The IMPACT™ system (Intein-Mediated Purification with an Affinity Chitin-binding Tag; NEB) is based on fusing the target protein to a chitin-binding domain in tandem with an intein protease. Following affinity selection of the fusion protein on a chitin matrix, the intein undergoes specific cleavage by a thiol reagent or pH and temperature shift, which releases the target protein from the chitin-bound tag [148–150].

FLAG-tag is an eight amino acid peptide that was designed for immunoaffinity chromatography [151]. FLAG-tagged proteins may be recovered from crude lysates in a rapid one-step procedure and detected in easy immunoassay formats. Although highly selective and very frequently used in research, its binding capacity is low making scale-up a costly undertaking, and being immunogenic, the tag must be removed in the case of proteins intended for therapeutic use. The HA, c-Myc, and T7 epitope tags are often used to tag recombinant proteins [93, 152, 153], but such antibody-based affinity systems are not routinely used for purification purposes. Softag1 and Softag3 were developed as small peptides that are recognized by polyol-binding mAb. Elution can be achieved under mild conditions, and such "gentle immunoaffinity chromatography" is useful for studying protein interactions within multi-subunit protein complexes [154]. S-tag, a 15 amino acid polypeptide derived from the N-terminal helix of RNAse A, complements the fold of immobilized truncated RNAse A with very high affinity, thereby providing a system for the efficient purification of S-tag-fused proteins [155].

Elastin-like polypeptides (ELPs) have the property of becoming reversibly insoluble at relatively low transition temperatures and have been used successfully as affinity tags [156–160]. "Inverse Transition Cycling" (ITC) describes the technique in which an ELP tag is used to reversibly precipitate and purify a genetically fused target protein [161, 162]. The method is highly scalable and eliminates the need for expensive affinity resins and apparatus, and when coupled with a self-cleaving intein tag, the requirement for tag removal using an additional protease step is removed [163].

3.3 TAP Tagging

Tandem Affinity Purification (TAP) tagging is a method first described using yeast cells that enables the rapid in situ purification of protein complexes as a result of their co-purification with a "TAP-tagged" recombinant protein. The original TAP tag

consisted of two IgG-binding domains of *Staphylococcus aureus* protein A and a calmodulin-binding peptide (CBP) separated by a protease cleavage site [164, 165]. TAP tagging, which involves two sequential purification steps, has been widely used to identify protein complexes in yeast, insect, human, and plant cells [166–173]. The S3S-TAP-tag (a cleavable S-tag combined with Strep-tag II; [174]) is a universally applicable TAP-tag system suitable for the isolation of mammalian protein complexes. Strep/FLAG–TAP (SF-TAP) tag is a small tag that combines tandem Strep-tag II and FLAG tags and eliminates the need for an intermediary proteolytic cleavage step [175]. Another tandem tag is FF-ZZ, which consists of two FLAG tags (FF) followed by two protein-A IgG-binding domains (ZZ). Replacing CBP with FLAG resulted in higher recovery during purification [176]. Additional tag combinations have been described whereby the CBP moiety has been replaced with other tags in an effort to address various issues including the yield of TAP-tagged protein and background due to nonspecific protein interactions (SF-ZZ, GS-tag, PTP-tag [177–179]; *see* Table 1). "CHiC tag" was constructed based on a combination of His-tag and a choline-binding domain followed by a TEV protease cleavage site [180]. CHiC-tagged proteins were purified by diethylaminoethyl-cellulose affinity chromatography and Ni^{2+}-IMAC with the CHiC tag also functioning as a solubilization partner. TAP tagging is frequently to be found as a key part of strategies designed to study protein interaction networks [172, 173, 181–183].

3.4 Tag Cleavage and Removal

Expression vectors are often designed to facilitate inserting a short flexible hydrophilic linker or spacer peptide between the tag and its fusion partner. Here, an endoprotease cleavage site is invariably included to facilitate removal of the tag following purification. This is important due to the potential impact that the tag may have on the function or structure of the recombinant protein. It is of particular relevance both in the case of therapeutic proteins, where protein authenticity is paramount, and for structural studies where the protein's native conformation may be lost due to the presence of the tag. A limited number of proteases are exploited for the purpose of tag removal (*see* Table 2), and these are reviewed in detail elsewhere along with detailed protocols for their use [150, 151]. The need for enzyme removal, the expense involved, and the frequent failure to cleave has spurred attempts to develop other effective tag-removal strategies. Intein-derived protein segments have been developed as self-cleaving tags [160, 184–186]. Polypeptide sequence-dependent hydrolysis by Nickel (Ni) ions has also been demonstrated [187]. In one interesting study, a Ni-assisted cleavage tag (SNAC-tag) was developed and shown to often work effectively in cases where enzymatic cleavage failed [188].

Table 1
Selected tags for solubility enhancement and the affinity purification of recombinant proteins

Tag name	Tag type	Purification	Comments
Thioredoxin (TX)	Solubility enhancement	Phenylarsine oxide matrices	Often poor yield and quality of product. Tandem tagging with polyhistidine enables purification by IMAC (e.g., His-patch ThioFusion system, https://www.thermofisher.com). Other vector sources include http://www.addgene.org, https://www.merckmillipore.com.
Glutathione S-transferase (GST)	Solubility enhancement and affinity	Glutathione-sepharose matrices	Elution under mild conditions with free glutathione or IMAC when used with Hexa-histidine tag, for example, vector series (pGEX) available from https://www.sigmaaldrich.com. Other vector sources include http://www.addgene.org, https://promega.com, https://www.origene.com, https://www.atum.bio, https://www.merckmillipore.com, and https://www.thermofisher.com.
Maltose-binding protein (MBP)	Solubility enhancement and affinity	Cross-linked amylose resins	Elution under mild conditions with free maltose or IMAC when used with Hexahistidine tag. pMAL and pIVEX vectors available at https://international.neb.com and http://www.addgene.org, respectively. Other vector sources include https://www.sigmaaldrich.com, https://www.atum.bio, and http://www.abvector.com.
Small Ubiquitin-Related Modifier (SUMO)	Solubility enhancement	None when used alone	Enhanced solubility and expression in *Escherichia coli* and eukaryotic expression systems. SUMO-His_6 fusions purified by IMAC. Vectors sources include https://www.lucigen.com, https://www.thermofisher.com, and http://www.addgene.org.
NusA	Solubility enhancement	None when used alone	IMAC when used with Hexahistidine tag, for example, pET-44 vectors available from https://www.merckmillipore.com.
Fh8	Solubility enhancement and affinity	Hydrophobic Interaction Chromatography (Calcium-dependent interaction)	Derived from *Fasciola hepatica* 8 kDa antigen (69aa). See https://www.atum.bio.

HaloTag	Solubility enhancement and affinity	Chlorokane	Covalent binding and release of target by proteolysis. See https://promega.com.
Z-tag, ZZ-tag, and GB1 tag	Solubility enhancement and affinity	Protein A-sepharose and IgG resins	Mostly tagged in tandem with polyhistidine, enabling IMAC purification to be used. Vector sources include https://www.cytivalifesciences.com and http://www.addgene.org.
Arg-tag	Affinity	Cation-exchange resins	Eluted with a NaCl linear gradient at alkaline pH. Resins include SP-Sephadex (https://www.sigmaaldrich.com), HiTrap SP-sepharose FF columns (https://www.cytivalifesciences.com).
FLAG-tag	Affinity	Monoclonal antibodies/affinity gels for detection and purification (https://www.sigmaaldrich.com)	Competitive elution by FLAG octapeptide when using anti-FLAG M2 antibody. Calcium/EDTA-dependent affinity procedure in the case of M1 antibody. Tag is removable by enterokinase cleavage. Vector sources include http://www.addgene.org, https://www.origene.com, https://www.atum.bio, https://www.merckmillipore.com, and https://www.thermofisher.com.
Streptavidin-binding peptide (SBP)	Affinity	Immobilized Streptavidin	Eluted with Biotin (2 mM). Vector sources include https://www.agilent.com and https://www.sigmaaldrich.com.
Strep-tag II	Affinity	A modified streptavidin (Strep-Tactin; multiple commercial sources).	Can be advantageous when purifying metalloproteins and when NMR is to be used. Eluted with biotin and derivatives, in particular desthiobiotin. Strep-tag vector sources include https://www.iba-lifesciences.com.
C-myc-tag	Affinity	Monoclonal antibody-based detection (multiple commercial sources)	Mab9E10 (Multiple suppliers). Physiological conditions followed by elution at low pH.
Softag1, 3	Affinity	Monoclonal antibody-based detection	Mild elution conditions (https://www.lucigen.com).
S-tag	Affinity	S-fragment of RNaseA (https://www.merckmillipore.com)	Elution conditions are harsh: 3 M guanidine thiocyanate, 0.2 M citrate pH 2, 3 M magnesium chloride. Tag removal by enzymatic cleavage is recommended to obtain functional proteins.

(continued)

Table 1
(continued)

Tag name	Tag type	Purification	Comments
Calmodulin-binding peptide	Affinity	Immobilized calmodulin (Sources include https://www.iba-lifesciences.com)	Binds Calmodulin in the presence of 0.2 mM $CaCl_2$. Eluted in 2 mM EGTA without or with 1 M NaCl. Not to be used for expression in eukaryotic cells. Vectors available from https://www.agilent.com (pCAL series).
Cellulose-binding domain	Affinity	Cellulose matrices	Tag size varies between 4 and 20 kDa. Eluted with either urea (4 M) and guanidine HCl (Family I CBDs) or ethylene glycol (Family II/III CBDs). Vector sources include http://www.addgene.org and https://www.agilent.com (pCAL-n).
Chitin-binding domain	Affinity	Chitin matrices/beads (https://international.neb.com). No need for additional protease step to remove tag	Used in tandem with an intein. Intein self-cleavage is then induced with dithiothreitol (30–50 mM), β-mercaptoethanol or cysteine. Vectors available at https://international.neb.com.
Biotin Carboxyl carrier Protein (BCCP)	Affinity	Biotinylated proteins purified on avidin/streptavidin resins	PinPointXa series of vectors and SoftLink avidin resin (https://promega.com).
Elastin-like peptides (ELP)	Affinity	Temperature-induced aggregation, separation, and resolubilization	Target protein self-association in high-salt buffers at temperatures above 30 °C; intein activity (if present) induced in response to a mild pH shift.
His-tag	Affinity	Ni-NTA agarose/Ni-NTA Superflow (https://www.qiagen.com) Co2+-CMA (https://www.thermofisher.com) Ni Sepharose/Ni Sepharose High Performance (https://www.iba-lifesciences.com) Nickel affinity gel and Cobalt affinity gel (https://www.sigmaaldrich.com)	Imidazole 20–250 mM or decreasing pH gradient. Various commercial sources.
Tandem Affinity Purification (TAP)-tag	Affinity (TAP)	IMAC	Imidazole 20–250 mM or low pH. Various commercial sources.

S3S-TAP-tag	TAP	IgG beads followed by Calmodulin beads (https://www.sigmaaldrich.com, https://www.iba-lifesciences.com, https://www.thermofisher.com)	Calmodulin-binding protein (CBP) fused to two protein-A IgG-(ZZ) binding domains. Contains a TEV cleavage site between CBP and ZZ. There are issues due to large tag size, need for proteolytic removal of tag and CBP interference with calcium signaling in eukaryotic cells.
FF-ZZ TAP tag	TAP	S-tag agarose (https://www.merckmillipore.com), Strep-Tactin beads (Sources include https://www.qiagen.com, https://www.iba-lifesciences.com).	Modified TAP-tag designed for mammalian protein complex isolation. Elution with desthiobiotin. Vector sources include http://www.addgene.org.
Strep-FLAG-TAP (SF-TAP)	TAP	IgG Sepharose resin (https://www.iba-lifesciences.com), Anti-FLAG M2 affinity resin (https://www.sigmaaldrich.com)	Elution either by low pH or with 3xFLAG octapeptide (https://www.sigmaaldrich.com).
GS-tag	TAP	Strep-tactin Superflow resin (Iba-lifesciences.com and https://www.qiagen.com); Anti-FLAG M2 affinity matrix (https://www.sigmaaldrich.com)	Elution with desthiobiotin (https://www.iba-lifesciences.com, https://www.thermofisher.com, https://www.merckmillipore.com) or 3xFLAG octapeptide (https://www.sigmaaldrich.com). Purification under native conditions, no need for enzymatic removal of tag.
PTP-tag	TAP	HPC4 monoclonal antibody (anti-protein C affinity matrix, https://www.sigmaaldrich.com, https://www.thermofisher.com) in presence of calcium	A Strep-II-3xFlag-2xZZ tag. Less background but low yield of affinity co-purified proteins. Eluted with EGTA or ProtC peptide. Substitution of CBP with protein C epitope eliminates CBP-associated issues.

Table 2
Specific proteases for the cleavage of fusion proteins

Protease name	Type of protease	Site of cleavage*
Genenase I	Serine	P-G-A-A-H-Y*
Enterokinase	Serine	D-D-D-D-K*
Factor Xa	Serine	I-E-G-R*
Rhinovirus 3C proteinase	Cysteine	L-E-V-L-F-Q*G-P
Tobacco Etch Virus protease	Cysteine	E-N-L-Y-F-Q*(G/S)
Thrombin	Serine	(G/P)-R*G

4 Tagging Proteins and Structural Studies

Protein samples for structural biology projects are increasingly being generated by recombinant DNA technology. Analytical techniques such as NMR spectroscopy, X-ray crystallography, and cryo-electron microscopy all require considerable quantities of protein, usually in the order of 5–50 mg. IMAC-based strategies using small tags are favored for structural studies as the latter do not substantially increase the size of the target protein, and tag removal may not be required in order to generate good-quality crystals [154]. *E. coli* is the preferred host for recombinant protein expression here, and isotope labeling protocols for NMR spectroscopy and selenomethionine incorporation for X-ray crystallography are now well established. There are obstacles, however, when using *E. coli* as it cannot reproduce eukaryotic post-translational modifications such as glycosylation, which are often essential for the correct folding and bioactivity of proteins, so protein expression in a eukaryotic host may be necessary [155]. Furthermore, some proteins, especially larger proteins and membrane proteins, are either not expressed or insoluble when produced in *E. coli* although the situation is improving as a result of the development of new vectors and host strains alike. A semiautomated large-scale process for the production of polyhistidine-tagged recombinant proteins in the Baculovirus expression system has been described [156].

Hexahistidine tags do not enhance the solubility of their fusion partner, unlike MBP or GST-tags. Crystal growth is impeded as a result of conformational heterogeneity induced by such large tags, however, creating the need for tag removal by following expression. If the tag is to be left in place, however, it is now clear that a rigid short polypeptide spacer, as opposed to a longer more flexible one, is required between tag and partner protein so as to eliminate such conformational heterogeneity and promote the growth of high-quality crystals [27]. MBP remains one of the most commonly

used tags in crystallization studies [189, 190]. Not all proteins rendered soluble by fusion to MBP remain soluble after proteolytic removal of the tag, although circumvention of this problem has been reported using a modified expression vector [157]. In vivo expression systems are increasingly being supplemented by prokaryotic and eukaryotic cell-free protein synthesis systems [158–162]. This approach, although still beset by problems of inefficiency, enables the production of cDNA-encoded proteins that could otherwise interfere with the physiology of their host cell.

5 Concluding Remarks

Protein tagging technology has profoundly impacted on our ability to express and purify recombinant proteins. There is now a considerable repertoire of polypeptide tags, which can be exploited to enhance expression, improve solubility, and greatly simplify the purification and detection of their partner protein. Faced with a requirement for soluble correctly folded protein, the investigator now benefits from custom DNA synthesis, rapid cloning, and gene fusion techniques; the possibility of using of multiple tags simultaneously; the availability of multiple constructs designed to enable direct comparisons to be made between different tags on the same protein; and methods for tag removal following affinity purification. High-throughput protein production, as required by the pharmaceutical industries and structural genomics centers, has additionally benefitted from major advances in robotic instrumentation designed to facilitate the expression and purification of many proteins in parallel. His-tagging has consolidated its position as the method of choice for both pharmaceutical production and as a universal platform for proteomic studies.

It remains unclear as to how the solubility of a given protein may be enhanced by its fusion partner. It has variously been suggested that the tag may act as a nucleation site for the folding of the target protein or that the tag acts as a magnet for chaperone proteins, or is itself a general molecular chaperone ([12] and references therein). Predicting which solubility-enhancing tag will work best with a given protein can be difficult, and it is best to try out a few of these by expressing the target protein from different plasmid vectors. No affinity tag is ideal in every respect, and combinatorial tagging allows the user to simultaneously benefit from the solubility-enhancing property of one tag and the affinity property of another. The European Molecular Biology Laboratory (EMBL) Protein Expression and Purification Core Facility has a growing list of commercial and noncommercial expression vectors suitable for use in a range of expression hosts (*E. coli*, Baculovirus, and *Pichia pastoris*) (https://www.embl.de/pepcore/pepcore_services/cloning/choice_vector/index.html).

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Chapter 8

Magnetic Nanoparticles for Protein Separation and Purification

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Abstract

Proteins are essential for various functions such as brain activity and muscle contraction in humans. Even though food is a source of proteins, the bioavailability of proteins in most foods is usually limited due to matrix interaction with other biomolecules. Thus, it is essential to extract these proteins and provide them as a nutraceutical supplement to maintain protein levels and avoid protein deficiency. Hence, protein purification and extraction from natural sources are highly significant in biomedical applications. Chromatography, crude mechanical disruption, use of extractive chemicals, and electrophoresis are some of the methods applied to isolate specific proteins. Even though these methods possess several advantages, they are unable to extract specific proteins with high purity. A suitable alternative is the use of nanoparticles, which can be beneficial in protein purification and extraction. Notably, magnetic iron and iron-based nanoparticles have been employed in protein extraction processes and can be reused via demagnetization due to their magnetic property, smaller size, morphology, high surface-to-volume ratio, and surface charge-mediated property. This chapter is a summary of various magnetic nanoparticles (MNPs) that can be used for the biomolecular separation of proteins.

Key words Magnetic nanoparticles, Iron oxide nanoparticles, Magnetization, Protein separation, Purification

1 Introduction

Proteins consist of amino acids that are made up of hydrogen, carbon, oxygen, nitrogen, or sulfur [1]. Proteins are essential for the growth of an organism [2], where food serves as a source of proteins [3]. Among humans, proteins are essential for various functions [4], from brain activity [5] to muscle contraction [6]. Even though food serves as a source of proteins, it will be intermixed with other biomolecules, such as polysaccharides [7], and it is tedious to provide the quantity of proteins required for the proper body function for humans per day [8]. Thus, it is essential to extract these proteins and provide them as a nutraceutical

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supplement to maintain protein level and to avoid protein deficiency [9]. Moreover, proteins are also used as a theragnostic agent for the treatment of certain common ailments and diseases, such as cancer [10] and those related to nerves and brain [11]. Hence, the isolation of proteins from natural sources is highly significant in biomedical applications [12]. Chromatography [13], crude mechanical disruption [14], chemical approaches [15], and electrophoresis [16] have been used as extraction methods for the extraction and purification of specific proteins. Even though these methods possess several advantages, it is difficult to extract specific proteins with high purity [17]. Thus, it is necessary to introduce additional or novel approaches for the extraction and purification of proteins.

Nanoparticles of 10–100 nm size (10–1000 nm in certain cases) are gaining significant attention among researchers and scientists to be utilized in biomedical applications, due to their smaller size, morphology, high surface-to-volume ratio, and surface charge-mediated properties [18]. Among several nanoparticles, nanosized magnetic particles have recently emerged in the fields of protein extraction and purification [19]. Iron and iron-based nanoparticles have been employed in the protein extraction processes and can be reused via demagnetization [20]. Nanomaterials have been utilized in various industries and applications, and iron oxide nanoparticles, including magnetite (Fe_3O_4) and magnemite (γFe_2O_3), have shown promising results in protein separation and purification processes [21]. Furthermore, various metal and metal oxides can be transformed into ferro- or superparamagnetic materials, while reducing their size to nanometer scale [21]. Thus, this chapter summarizes various magnetic nanoparticles (MNPs) that are beneficial in biomolecular protein separation.

2 Magnetic Nanoparticles

Nanotechnology breakthroughs in the recent decade have shed light on future diagnostic and therapeutic approaches [22]. During this time, magnetic nanosized particles (MNPs) have received major consideration among researchers, owing to their distinct properties related to their physical, chemical, and mechanical properties. MNPs now have now found application in multiple fields, such as (a) drug delivery, (b) tissue engineering, (c) magnetic resonance image (MRI), (d) cancer therapy via hyperthermia, (e) clinical diagnosis, (f) high-density iron oxide recording, (g) information storage, (h) sensing and imaging of biological samples, (i) tumor therapy, and (j) catalysis [23–25]. Magnetism is a beneficial property of iron oxide nanoparticles along with high yield, purity, and specificity, which makes them suitable for biological applications [26, 27]. Formulation and modification of

the size and other aspects of MNPs are among the most well-known approaches in the current field of health research. Engineered nano-/micro-materials have produced improvements in the detection of biomolecules and biomarkers compared to conventional materials, owing to their stability and size-dependent performance. In particular, magnetic nanoparticles are a fascinating material for researchers due to their magnetic stability and reduced oxidation, with unique biological interaction that can be utilized in scientific, therapeutic, public health, safety, and commercial applications.

3 Methods of Formulation

MNPs can be synthesized using various methods to produce nano- to micro-sized high-quality nanoparticles. Oxides of iron, including γ-Fe_2O_3 and Fe_3O_4, and pure standalone metals (namely iron and cobalt) have all been used to make magnetic nanoparticles. Many recent studies have demonstrated effective synthetic ways to fine-tune the morphology, enhance the stability, and monodispersity of magnetic nanoparticles [28, 29]. Thermal breakdown, coprecipitation, reduction, synthesis of micelles, and hydrothermal procedures are just a few of the effective chemical approaches that are utilized to make high-quality nanosized magnetic particles [30, 31], as shown in Fig. 1.

3.1 Coprecipitation

Coprecipitation is a superficial approach to synthesize iron oxides. This is achieved through the introduction of a base to an aqueous salt of Fe^{2+}/Fe^{3+} at ambient temperature under an inert atmosphere [33]. The nature of salts employed, the ratio of Fe^{2+}/Fe^{3+} ions, the temperature of reaction, the value of pH, and the ionic strength of the media are all crucial factors that are identified to substantially influence the nanosized magnetic particles' composition and morhopology [34–36]. The quality of the magnetite nanoparticles can be engineered to be reproducible upon fixing the synthetic parameters [37]. Under ambient circumstances, magnetite nanoparticles are unstable and readily oxidize to form maghemite or dissolve in acidic media. It is noteworthy that maghemite is a class of ferrimagnet, where oxidation is considered a minor issue. As a result, controlled oxidation was used for the synthesis of maghemite, where magnetite particles are distributed in a specific acidic solution, followed by addition of iron (III) nitrate. The resulting maghemite particles are identified to be stable in both acidic and alkaline conditions [38, 39].

Nevertheless, the primary limitation in Fe_3O_4 synthesis via coprecipitation is controlling the size of the particles to achieve monodispersity, even if the particles of magnetite are transformed into maghemite, after their preliminary production. A broad size distribution of magnetic nanoparticles will lead to an increase in the

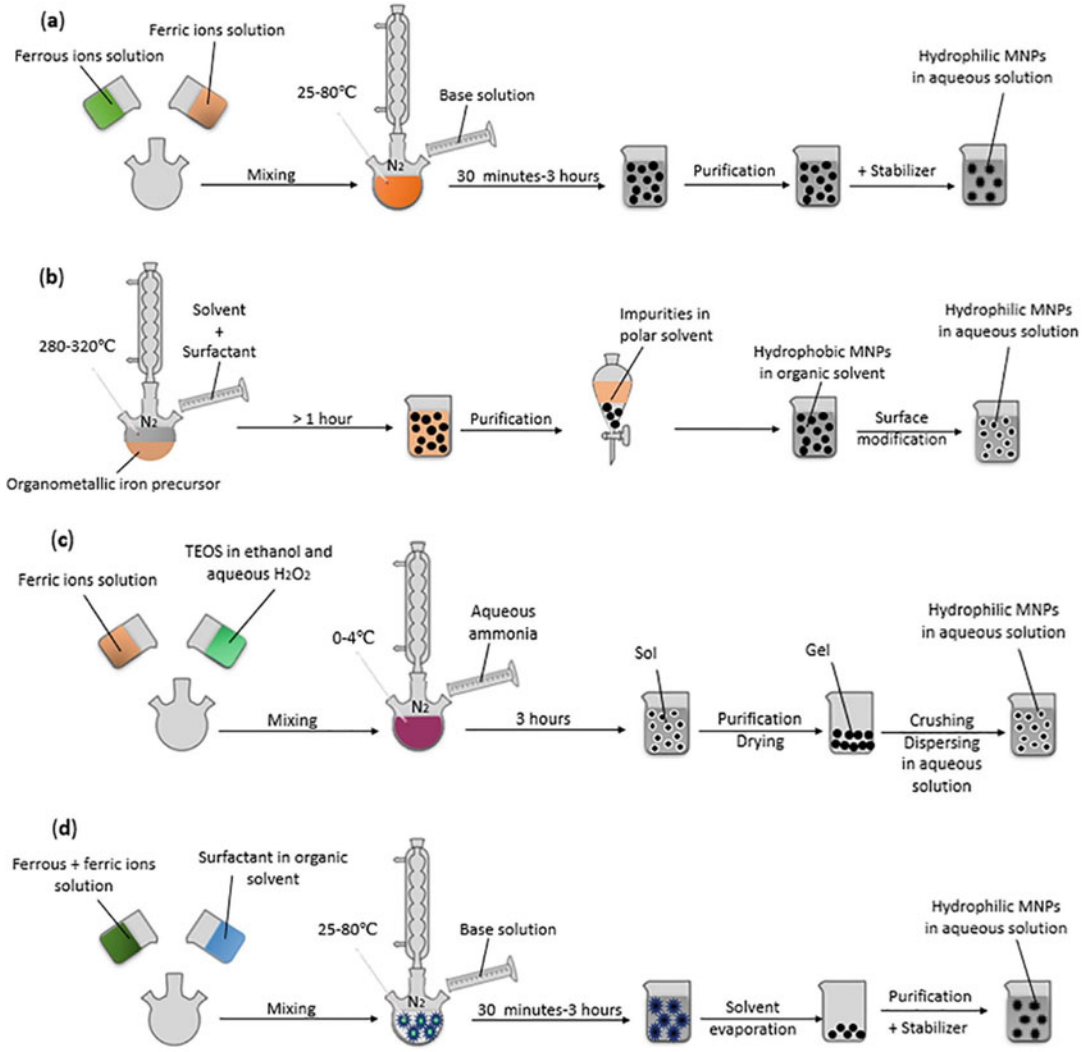

Fig. 1 Distinct chemical synthesis approaches for the formation of magnetite nanoparticles. (**a**) Coprecipitation. (**b**) Thermal decomposition. (**c**) Sol–gel. (**d**) Microemulsion. (Reproduced with permission from [32], © Elsevier, 2021)

blocking temperatures and results in magnetic non-superlative behavior in desired applications, due to their temperature blocking ability which is proportional to the size of the particles [40, 41]. Unfortunately, particles synthesized via coprecipitation are polydisperse in several common aqueous solutions. Thus, it is necessary to control these processes for the manufacture of monodisperse iron oxide magnetic nanoparticles.

The benefits of organic compounds as reducing agents and/or stabilizers has contributed to advancements in the preparation of monodisperse magnetite nanoparticles of various sizes. Nanosized magnetite particles with 4–10 nm diameters, for example, can be

improved in a solution containing 1% polyvinyl alcohol (PVA). Chain-like clusters of magnetite nanoparticles undergo precipitation, when 0.1% of PVA with carboxyl functional group is used as the stabilizing agent [42]. This finding suggests that the selection of the right surfactant is critical to the stabilization of such particles. Numerous organic anions including carboxylate and hydroxy carboxylate ions have been studied extensively for their potential in forming oxyhydroxides or oxides of iron. Moreover, it is crucial to maintain a carboxy group after deprotonation and alpha-hydroxy group after deprotonation for the development of complexes on the surface of magnetic nanosized particles. According to recent studies, oleic acid is the ultimate choice for stabilizing Fe_3O_4. Furthermore, metal ion chelation can impede nucleation and cause the creation of larger sized particles, since the generated nuclei is minimal, while the proliferation of particles is controlled in the system [43].

3.2 Thermal Decomposition

High-boiling conditions can essentially be used to make magnetic monodispersed nanosized crystals with lower sizes via thermal breakdown of organometallic compounds in organic solvents containing surfactants for stabilization. Metal-based acetylacetonates, carbonyls, and metallic cupferronates are the few common precursors of organometals. Surfactants such as oleic acid, fatty acids, and hexadecyl amine are frequently utilized in thermal decomposition [44].

In zerovalent metal precursors, namely, carbonyls, thermal breakdown leads the metal to form initially, although two-step methods can be employed to generate nanosized oxide particles as shown in Fig. 2 [45, 46]. For example, pentacarbonyl of iron disintegrates at 100 °C in oleic acid and octyl ether mixture, followed by the inclusion of trimethylamine oxide $[(CH_3)_3NO]$ as a moderate oxidant at rising temperatures, resulting in monodispersed nanosized γ-Fe_2O_3 crystals with ~13 nm dimensions. Similarly, $[Fe(acac)_3]$ degradation in the presence of 1,2-hexadecanediol, oleic acid, and oleylamine in phenol ether

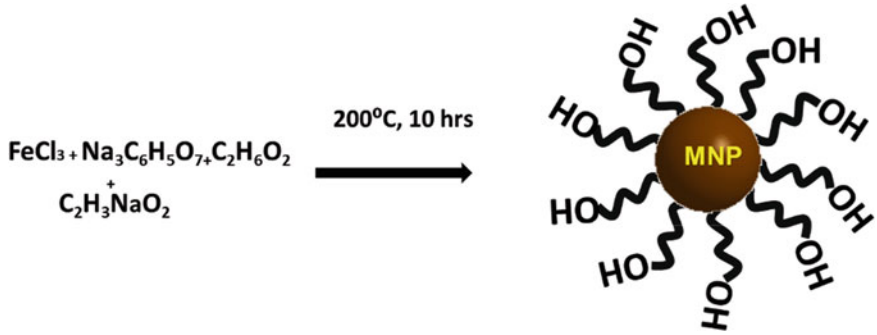

Fig. 2 Chemical synthesis of magnetic nanoparticles (MNP) assisted by thermal approach

was identified to cause the precursor disintegration, especially in the cationic metal centers. This results in the development of oxides, namely, Fe_3O_4. Based on metal-based pyrolysis of fatty acid, a generic decomposition technique for the fabrication of nanosized magnetic oxide crystals with controlled morphology has been developed [47]. It is possible to form nearly monodispersed nanosized Fe_3O_4 crystals with sizes in the adjustable size range of 3–50 nm and with regulated shapes, such as dots and cubes. Rapid formation of nuclei from a supersaturated solution is accompanied by the reduction in the development of particles, resulting in the significant nucleation and growth separation [48]. Nucleation occurs when iron pentacarbonyl is thermally decomposed at a low temperature, whereas growth occurs when the iron oleate complex is thermally decomposed at an elevated temperature.

The nanoparticles discussed above can be dispersed in organic liquids. Water-soluble magnetic nanoparticles, on the other hand, are preferable for biological applications. Nanosized magnetite particles, that are soluble in water, were reported for this purpose. Water-soluble Fe_3O_4 nanocrystals were produced under reflux at 245 °C using $FeCl_3.6H_2O$ as a precursor of iron with a coordinating solvent, 2-pyrrolidone. When the reflux period is 1, 10, or 24 h, the average particle size was regulated (4, 12, or 60 nm), respectively. The particle shape was altered from spherical to cubic morphologies due to an increment in the reflux duration. Using poly (ethylene glycol) terminated with a γω-carboxyl group as a potential capping agent, a one-pot synthesis of nanosized magnetite particles soluble in water was established, which were generated with comparable reaction conditions [49]. These nanoparticles have the capability to be employed as contrast agents in magnetic resonance imaging for cancer diagnosis.

3.3 Microemulsion

A microemulsion is a thermodynamically stable isotropic dispersion of two immiscible liquids in which an interfacial surfactant coating stabilizes the liquid microdomain. The aqueous microemulsion phase of water-in-oil is distributed as microdroplets, which are encased by a surfactant monolayer in the continuous phase of hydrocarbon. The molar water to surfactant ratio establishes the reverse micelle size [46, 47]. By adding a solvent to the microemulsions, such as acetone or ethanol, filtration or centrifugation can be used to extract the precipitate formed during micellar formation.

The microemulsion approach can be used in this way for the fabrication of MNPs [48]. Nanoparticles can be made as spheroids, as well as oblong cross-sections and tubes, using the microemulsion process. Although the microemulsion approach has been used to create a variety of nanosized magnetic particles in a precise manner, particle morphology can often fluctuate over a wide range. Likewise, there is a limited working scope allowing for the production of nanosized magnetic particles synthesized via microemulsions

with poor yield, compared to other approaches, such as thermal breakdown and coprecipitation. To synthesize significant amounts of material, large quantities of solvent are required. As a result, it is not a particularly efficient process, and scaling it up is challenging [50–52].

3.4 Hydrothermal Synthesis

In recent times, a broad spectrum of nanostructured materials have been generated using a hydrothermal approach. By using a liquid–solid solution process, a generic hydrothermal approach for manufacturing a variety of distinct nanocrystals has been developed [53]. Under hydrothermal conditions, the system contains a solid of the metal linoleate, a liquid phase of ethanol and linoleic acid, and a solution of water and ethanol at various operational temperatures throughout the synthesis at the phase boundaries, depending on the phase transfer and separation process. Hydrothermal reduction was used to make hydrophilic and monodispersed microspheres of ferrite using a single-crystalline phase [53–55].

To direct the synthesis, multicomponent mixtures containing sodium acetate, ethylene glycol, and PEG are generally used. Ethylene glycol can be utilized as a reducing agent, as it is stable at a high boiling point. Sodium acetate acts as a stabilizer of particles via electrostatic attraction, which can prevent the formation of agglomerates. PEG surfactant can be used to prevent agglomeration of particles via a polyol process for the formation of monodispersed nanosized metallic particles. The multicomponent strategy appears to be effective in controlling the synthesis of desirable materials. Thermal decomposition appears to be the significant approach developed to date for controlling nanoparticle size and morphology [56, 57]. Furthermore, microemulsions are utilized to form monodisperse nanosized particles with a variety of morphologies as an alternative. However, this approach requires large solvent quantities. Although it facilitates the production of high-quality nanosized particles, the hydrothermal approach is quite understudied for the creation of nanosized magnetic particles. Nanosized magnetic particles synthesized through the coprecipitation method and the thermal breakdown are the most well-characterized to date. The colloidal stability of the magnetic nanoparticles synthesized via aforementioned approaches is attributed to steric or electrostatic repulsion, based on the choice of fatty acids or amine stabilizers and their polarity. Repulsive electrostatic forces are used to stabilize magnetite nanoparticles generated through coprecipitation, due to the positive charge of the particles. Thermally produced nanoparticles, on the other hand, undergo stabilization via fatty acids or surfactants-mediated steric reaction [58–60].

3.5 Sol–Gel Method

The sol–gel process relies on initial hydrolysis and condensation in a solution of colloids to generate a metal oxide network [61]. If the hydrolysis rate is low, smaller particles will be produced. Furthermore, the size of the particles is intrinsically linked to the solvent content, pH, reactive temperature, and other factors. The force of magnetic alignment generated by the magnetic field of the external source is low to withstand the thermal nanosized superparamagnetic iron oxide particles (SPIONs) motion, when the size of magnetic iron oxide nanoparticles (MIONPs) is smaller than a particular value. There will be no residual magnetism when the magnetic field is eliminated as the nanoparticles' magnetization approaches zero [62, 63]. SPIONs are MIONPs and are highly sensitive to external magnetic fields and exhibit magnetism only when they are subjected to an exogenous magnetic field [64–66].

4 Functionalization

Stability is an important aspect to consider when iron oxide particles are used in biological systems, in order to prevent agglomeration. To avoid such issues, various surfactants, organic, inorganic components, hydrophilic molecules, and polymers can be used to alter the surface chemistry of the iron oxide particles in order to make the particle poly-dispersible, reduce oxidation, and act as core materials as shown in Fig. 3. The interaction of other components, with iron oxide nanoparticles can take place by electrostatic ionic attraction, hydrogen bonding, or covalent bonding.

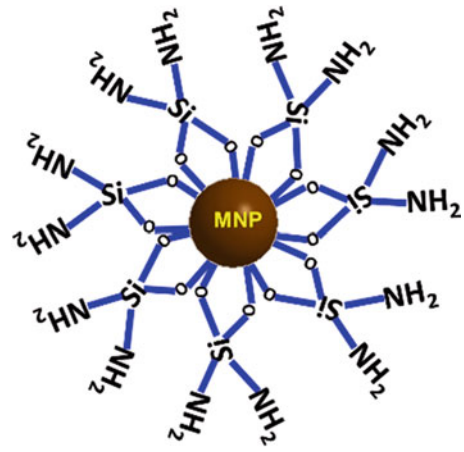

Fig. 3 Schematic representation of magnetic nanoparticles (MNPs) functionalization

4.1 Silica (SiO_2) Functionalized Magnetic Nanoparticles

Silica coating is the best protective material to prevent magnetic nanoparticles from oxidation or interaction with other materials. Through electrostatic interaction with ammonia, the silica or polymer is coated with negatively charged nanosized oxides of iron particles. The shell surface is negatively charged, which helps to modify or adsorb other materials through amine modification. Core-shell nanomaterial is a good biocompatible, nontoxic complete biomaterial used in biomedical applications [67–69]. The core-shell particle facilitates the detection of biological targets. Introduction of organic and inorganic compounds helps to enhance the stability of the magnetic materials. For example, surface modification with an organic silane increases the efficiency of magnetic nanoparticles to form bonds. The coating materials described above are extensively used in clinical diagnosis, protein separation, targeted drug delivery, tissue repair, and biological separations [70, 71].

Magnetic nanoparticles can be loaded on to one-dimensional nano-objects (nanotubes) using a different technique, allowing for the deposition of numerous particles with a large total magnetism on a single nanotube. The inner surfaces of SiO_2 nanotubes were charged with Fe_3O_4 magnetic nanoparticles after they were produced in an alumina template [72–75]. The magnetic nanotubes that resulted were used for bioseparation assisted by magnetic field, biological interaction, and drug administration, with a significant magnetization arising from the presence of multiple magnetic nanoparticles and a large external SiO_2 nanotube surface area.

4.2 Polymer Functionalized Magnetic Nanoparticles

MNPs can be modified using organic polymers such as chitosan (CS), polyethyleneimine (PEI), dextran, and PEG [76, 77]. Surface modification improves MNP stability by inhibiting their agglomeration and oxidation. Surface modification also allows for additional functionalization. MNPs may be coated with dextran, which prevents aggregation and is also biocompatible. MNPs and dextran-coated MNPs have particle sizes of 30 nm and 50–80 nm, respectively. Studies have indicated that the stimulation of cells is inversely proportional to the dextran-coated SPIONs size [78, 79]. Furthermore, macrophages were able to endocytoze ultra-small (30 nm) superparamagnetic nanosized oxides of iron particles coated with dextran (SPIONPs), and no hypersensitivity reactions were observed. These nanoparticles could be employed as contrast agents for magnetic resonance imaging (MRI) applications.

Chitosan (CS) is made up of the monomers glucoside and glucosamine, and is a cationic linear polymer with a standalone amino group [80]. Furthermore, the amino group in the polymer's C2 position is more dynamic, compared to its hydroxyl group. As a result, CS is able to react under mild settings [81]. Nontoxicity, hydrophilicity, biocompatibility, and biodegradability are just a few of the advantages of CS, which are frequently utilized in waste

treatment, health care, and food hygiene applications. The number of amino groups on MIONPs increases after they are changed with CS, allowing them to be linked to bioactive molecules and expanding their prospective applications to form MIONPs with active groups. This significantly improves their ability to attach to physiologically active substances. MIONPs with a CS coating could encapsulate chlorhexidine (CHX) to kill bacteria and planktonic fungal cells, and this method could be used to prevent or cure biofilm-related oral illnesses [82].

Polyethylene glycol is a long-chain hydrophilic and nontoxic polymer. The sol–gel approach, thermal decomposition, and hydrothermal process have all been used to make PEG-coated MNPs [83]. However, these approaches necessitate more stringent experimental conditions or higher temperatures. Thus, a novel synthetic approach for producing PEG-coated MIONPs with high efficiency is required. According to Ling et al. (2019), PEG-terminated hydroxamic acid (HA) and phosphonic acid (PA)-modified MIONPs were stable in the colloidal solution with high dispersibility in aqueous and cell culture medium [84]. This avoids cytotoxicity toward peripheral human primary blood cells, indicating that they could be used in cancer diagnostics and therapy. When there is an excess of iron in the human body, it can lead to reactive oxygen species generation and the development of cancer. After being treated with PEG, the stability in colloidal solution and half-life of MIONPs in blood were improved. PEG-coated MIONPs also did not interfere with cytochrome C (Cyt C's) redox state [84–86].

5 Biomedical Applications

The unique qualities, namely, superparamagnetic behavior, low toxicity, and biocompatibility, of MNPs are especially appealing for applications such as biosensors, antibacterial activity, targeted drug administration, separation of cells, magnetic resonance imaging, and tumor magnetic hyperthermia. Although numerous methods for producing MNPs have been established, significant obstacles remain in the synthesis of MNPs, such as severe aggregation, oxidation, and uneven size. As a result, numerous methodologies for surface modification of MNPs have been devised to improve their properties and generate multifunctional MNPs, which will broaden the use of MNPs.

Biomarkers of clinical disease are most often various forms of proteins at very low concentrations in biological systems. Their quantitative detection is important to diagnose and understand the molecular basis of disease, but it is very difficult in bio-samples. Advancements in nanotechnology in the past decade have provided new insights into future diagnostic approaches.

Compared to their bulk forms, surface-engineered nanomaterials are very attractive due to their relatively enhanced surface-to-volume ratio and unique size-dependent properties, enabling delicate and quantitative biomolecule detection with detection limits of several orders of magnitude lower than conventional techniques. Adsorption capacities for specific proteins such as blood proteins and surface-associated proteins could be confirmed by a proteomics approaches. Functionalized nanoparticles with silica have properties similar to those of super magnetic materials. The amine-modified silica shell also can be coated with active biomolecules and polymers, since it can be easily dispersible in aqueous organic solutions, which is confirmed with binding sites.

5.1 Magnetic Nanoparticles for Protein Separation

In most sectors of biosciences and biotechnology, separation of exclusive molecules, such as DNA [87–89], proteins [90–94], and cells [95, 96] is required to explore cellular mechanisms. Magnetic nanoparticle-based bioseparation is the most well-documented and commonly utilized bioseparation approach due to its distinctive separation via exclusive magnetic properties. The biomolecules are identified via colloidal magnetic nanoparticles and later separated by a magnet, charged via an external electric field source, which can be used for purification of proteins, extraction of RNA/DNA, and precipitation of immune factors, among other things. Because of their tiny size, promising separation capability, and superior dispersibility, nanosized magnetic bead particles have been widely employed for protein purification and separation. Magnetic separation employing antibodies/receptors conjugated with MNPs is one of the current advances in this field since it provides specific antibodies, which bind antigens on the targeted location surface as shown in Fig. 4 [98–103]. For life science applications, it is critical to be able to modify proteins effectively for research and development applications. Purification of native or recombinant proteins is time-consuming, especially when it comes to proteomic applications. The efficient synthesis of MNPs, on the other hand, provides a straightforward and adaptable substrate for protein separation. The target proteins quickly cover the NPs' surface, which reduces the overall unoccupied surface for nonspecific absorption of proteins. As a result, NPs have far greater specificity than microparticles. Additionally, NTA-terminated MNPs can eliminate the requirement for cell lysate pretreatment as a magnet can easily separate the target proteins [104–108].

Separation of proteins via magnets has contributed to the rapid growth of the medical technology sector [109–111]. In general, nanoparticles are advantageous compared to microparticle beads and chromatography resins, due to the lack of any limitation in mass transfer during diffusion of proteins in the separation process. Furthermore, magnetic nanoparticles also possess enhanced protein separation capacity with high purity, low contamination level,

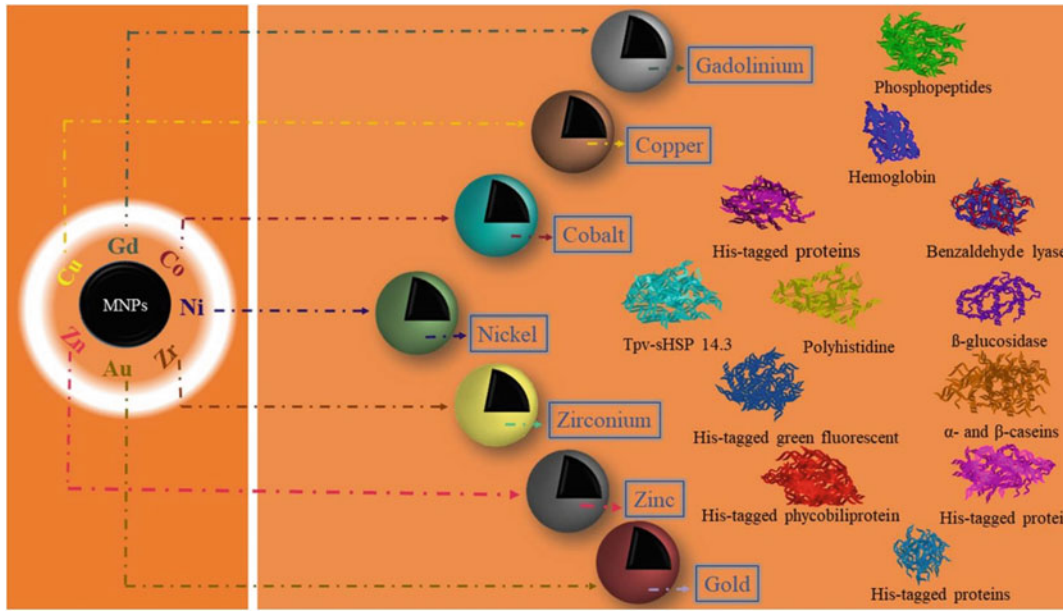

Fig. 4 Functionalized magnetic nanoparticles for the separation and purification of proteins and peptides. (Reproduced with permission from [97])

and reduced processing time. In addition, standalone iron oxide nanoparticles, have been identified to possess significant potential for protein separation applications due to the low cost of separation, lack of high precursor concentration requirement, and the high density of magnetic nanoparticles that can be used without any embedding process [112–114].

5.2 Protein Immobilization

Nanoparticle-based protein immobilization depends on size, composition, and morphology and can be tuned by altering reaction parameters. Particle uniformity can lead to high performance in protein separation without any surfactants or any other substrates [115].

5.2.1 Physical Immobilization

Physical immobilization is a facile method of protein functionalization done via soaking the material in a target biomolecule solution. It requires no extra coupling chemicals, preparaton of surface, or modification of protein. Proteins immobilized via a physical approach on nanosized magnetic iron particles have been created in large numbers [116]. For instance, glucose oxidase was immobilized on the surface of magnetic Fe_3O_4 nanosized particles via a physical approach for deoxygenation of water, resulting in an immobilization of 78% and 640 U/g specific activity [117]. While physical immobilization is facile, it is based on contacts via electrostatic force, van der Waals forces, hydrogen bonds, and interactions of hydrophobic molecules, and the adsorbed species' binding

stability is significantly altered by the microenvironmental conditions [118]. As a result, immobilization of proteins via the physical means can lead to detachment from the support, resulting in activity loss and reaction media contamination, compromising recyclability and robustness. Similarly, direct adsorption of proteins onto surfaces frequently results in protein denaturation and activity losses due to steric interaction-induced conformational changes [119–121].

5.2.2 Covalent Conjugation

Immobilization via covalent bonds is appealing because it can be controlled with appropriate functional groups for the protein attachment [122]. Several covalent binding-based protein immobilization techniques have already been developed and used as shown in Fig. 5. Because functional surface groups can recognize the functional groups of both revised nanosized magnetic particles and proteins, coupling agents are frequently used for cross-linking the proteins and modified nanosized magnetic particles via covalent bonds. Glutaraldehyde cross-linking was used to immobilize glucose oxidase on $CoFe_2O_4/SiO_2$ NPs. However, coupling agents may produce a conformational shift in proteins, causing a reduction in enzyme activity in many circumstances. The immobilized enzymes, for instance, preserved 15–23% of the natural glucose oxidase [124], whereas the glucose oxidase-magnetic nanoparticle bioconjugate lost about 20% of its activity after recycling.

Several enzymes utilized a covalent approach for effective attachment to the surface of magnetic nanoparticles

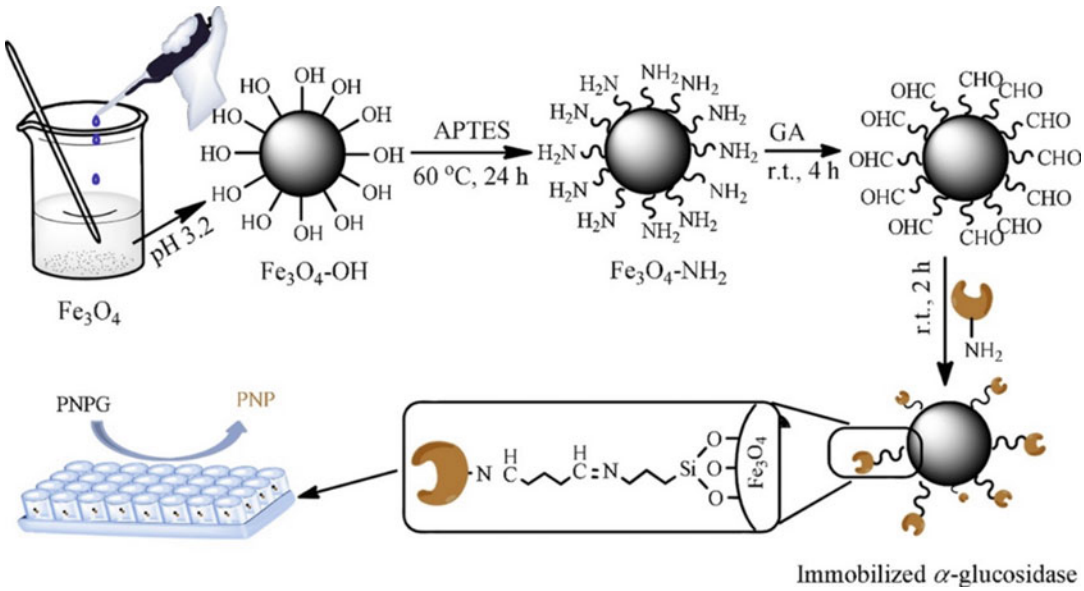

Fig. 5 γ-Glucosidase immobilization on functionalized Fe_3O_4 magnetic nanoparticles for screening of enzyme inhibitors. (Reproduced with permission from [123], © Elsevier, 2019)

[125]. Horseradish peroxidase, lipase, glucose oxidase, laccase, trypsin, pectinase, and triacylglycerol lipase are examples of enzymes that have been separated using MNPs. Nonspecificity is the fundamental issue in the practical covalent immobilization application; therefore, purified proteins are commonly used in immobilization via covalent bonds. When a protein or enzyme degrades, the support must be removed [126, 127].

5.2.3 Biological Affinity

The applications discussed above have been based on immobilization approaches, which are non-site specific, such as attachment via covalent bonding or adsorption. However, their application is severely limited due to their nonspecificity. Site-specific immobilization approaches developed from bioreactions have provided a fresh approach to solving the selectivity challenge, which might be accomplished by forming interactions between active support groups and specific protein residues [128–130]. These attachments are formed in mild conditions due to adequate support modification and protein engineering, which considerably lowers the protein degradation risk. In certain cases, even the selectivity has significantly contributed to the method's popularity as a way of protein immobilization, if a site-selective attachment is sought, the target protein must initially be tagged with biotin. The nanoparticles' surface in complex fluids is defined by the components present in the fluid. The arrangement is dictated by the biomolecule's affinity, which are loosely linked to the surface. Superparamagnetic nanoparticles, in particular, are of tremendous interest because of their magnetic characteristics, enhanced biomolecule separation/transport, and magnet-induced detection [131–135].

Amino acids are bound to nanosized oxides of iron particles (BIONs) primarily via carboxy groups at pH 7 at ambient conditions, with peptides containing residues of glutamate exhibiting high affinity for BIONs. Furthermore, this mechanism might be transferred to larger, more complicated protein structures and employed as an affinity tag. A protein tagged with Glu6 has the similar behavior of adsorption as the standalone homo-hexamer, and both molecules have rapid kinetics of adsorption. Magnetic separation is a fascinating technology for downstream processing since it allows for a reduction in the process steps, which saves time and money.

5.3 Bioseparation

Separation procedures based on adsorption and chromatography are utilized in numerous biological and analytical applications. Adsorption approaches for biomolecule purification and separation have led to the evolution of the biotechnology industry, over the last few decades. The majority of conventional adsorptive processes, via chromatography are carried out in densely packed beds with particle sizes in the tens of microns [136–138]. An efficient separation is influenced by the colloidal stability of nanoparticle

suspensions, surface charge density effects, ionic strength effects, protein charge effects, and protein loading capacities.

The subset of the large pool of MNPs available for biomolecular capture by magnetic forces are now being investigated extensively. They are intriguing due to their enhanced surface-to-volume ratio and biocompatibility [139]. Using high-grade magnetic separation, (a) flow conditions must be optimized to retain all magnetic material; (b) large magnetic field gradients are required to manipulate small particles; and (c) the applied MNPs for the separation process must first be functionalized with the appropriate chemistries. Affinity chromatography is identified to be the most economical technique for significant recombinant protein purification (e.g., His-tagged proteins), due to the effective MNP ions and polyhistidine interaction via affinity tags. For the specific separation of various proteins, several functionalized MNPs have been produced. In most circumstances, the target proteins could be eluted quickly and could be fully utilizing correct elution procedures, such as buffer solutions with varied pH or ion strength. The electric charges in the protein surface and the immobilization platform can alter as the pH changes, reducing the interface between the protein and the nanosized magnetic particles immobilized by ligands [140–142]. Thus, these two factors are considered while selecting a protein elution buffer from nanosized magnetic particles immobilized by ligands. However, utilization of an abnormally high or low (>10 or <4) pH for the elution of protein may have a considerable impact on protein bioactivity.

5.3.1 Separation Strategy

Protein separation using nanomaterials is directly facilitated by the interaction of nanomaterials with protein residues. The interaction between the adsorbent and the biomolecule is based on the affinity and internal bonds between both. Filtration, flocculation, centrifugation, chromatography, and sedimentation procedures are all used in traditional protein isolation processes for medicinal applications [143–146]. The main problem in enhancing downstream processing and opening the path for increased productivity is the development of high-tech or novel solutions. Separation via magnetic particles is a favorable contender for future downstream applications because of several key advantages, such as the following:

1. Target capture and purification in a single step (high affinity and selectivity)
2. Low-energy semicontinuous processing
3. High throughput

In comparison to traditional techniques, magnetic separation can assist in reducing costs while increasing yields and productivity. Low-processing energy costs result from low pressure-mediated semicontinuous or continuous processes. The approach enables a

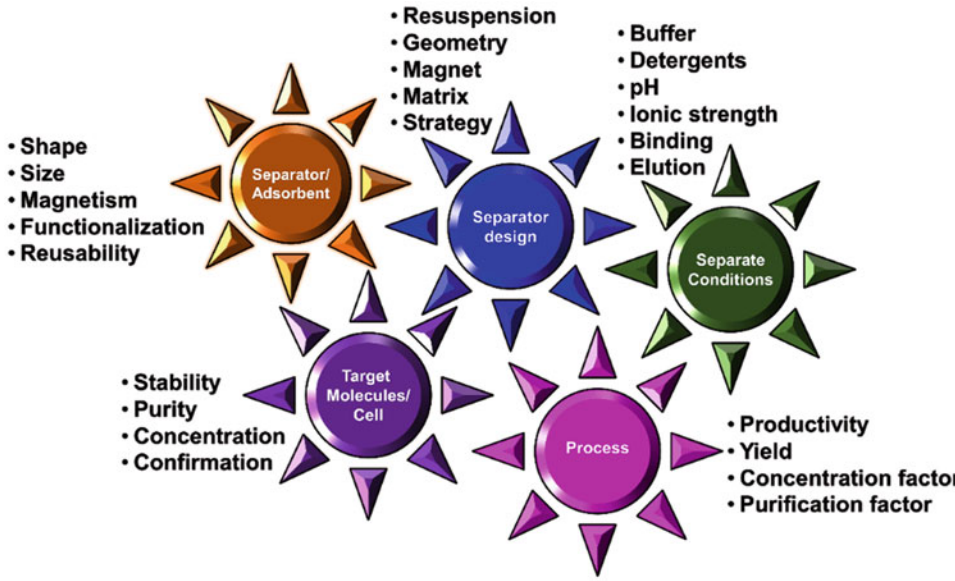

Fig. 6 Role and functions of magnetic nanoparticles (MNP) in protein separation

large number of factors to be adjusted to the needs of each system, which should result in a greater variety of bioproducts that may be used in industry [147].

The first step is to determine which separation approach is most appropriate. This entails considering the system and process, as well as all the relevant variables. The target molecule determines the separation strategy, which includes the separation process via magnetic particles and the magnetic material and target molecule interaction [148]. The parameters for binding with the target and elution are critical for the entire process as shown in Fig. 6, and equilibration durations for binding, particularly for elution, remain a barrier for future refinements.

To isolate molecules in a magnetic field, numerous physical features of magnetically sensitive materials are used. The explicit collection of magnetic particles from nonmagnetic elements is a common application of magnetic separation. To successfully gather all magnetic material, this method employs large magnetic field gradients [97, 149, 150]. Another possibility is to make use of magnetic materials' aggregation and agglomeration effects caused by the creation of magnetic dipoles in magnetic fields. This effect is called magnetic flocculation and can provide rapid separation as the magnetic force is greater compared to other common forces, such as Stoke's drag force and Brownian motion. Additionally, the magnetic force also leads to easier filtration due to larger aggregate size compared to single particles. Flotation can be used to improve the sedimentation and sorting of magnetic particle-mediated protein separation and enhance the utilization of magnetic bead as

adsorbent material for biomolecule separation. Furthermore, magnetic flotation can effectively isolate target molecules from contaminants by collecting or enhancing the flotation action. Nitrogen bubbles are used in the magnetic separation (GAMS) and superparamagnetic extraction (GASE) processes assisted by gas to float nanosized magnetic particles that are bound to target molecules. It can be noted that the magnetic particles can be gathered via a magnet or an extraction phase, allowing for rapid separation. A magnetic centrifuge can help in magnetic sedimentation by elevating the magnetic particles' acceleration forces, allowing for faster separation. Magnetic sorting is a technique for classifying materials with magnetic properties and particles bound to them based on their fluidic and magnetic activities [151–157]. This allows the sorting of nanosized magnetic particles of several morphologies, as well as the sorting of cells. Chemical reactions can take place on the magnetic bead surface, where enzymes and catalysts are immobilized, using magnetically stabilized bed reactors.

Conversely, microbeads with magnetic properties are simpler to utilize in separation operations as they are less prone to aggregation due to magnetic field. In comparison to colloidally stabilized nanoparticles, the huge size allows for a better magnetic and Stokes' drag force ratio, resulting in improved severability in magnetic fields. The process approach has a significant impact on the selection of the optimum magnetic material. Bead selection is also influenced by the target biomaterial and process parameters. Purification methods must use crude low-cost standalone nanosized oxides of iron particles or adsorbents with high specialization, based on the rewards and the target product requirement. Furthermore, the magnetic bead's chemistry and process design, the target protein binding, and the elution parameters also play a crucial role in the protein separation process. Magnetic surface functionalization is a toolkit that may be tailored to the target's attributes and the desired switch circumstances between adsorption and desorption [139, 158–160].

5.4 High-Gradient Magnetic Purification of Protein

Large flux densities of magnetism are present in high-gradient magnetic separators, allowing for local 10^4–10^5 T/m gradients or greater [161]. As a result of the advancement of magnetic technology, several separation procedures for biomolecules, particularly proteins, were developed at the laboratory scale. Despite this, only a few research organizations have focused their efforts on the commercial development of the magnetic protein isolation tool for targeted protein, macromolecules, and cell recovery. This was proposed to be achievable with a high magnetic gradient filter and a unified process with batch-binding stage linkages to handle magnetic adsorbents [162–164]. Magnetic separators have been used to capture pharmaceutically important proteins. Such separators

have purified antibodies from 100 L of supernatant isolated from cell culture via magnetic beads modified with protein A. Similar processing algorithms for the rotor–stator system have recently been enhanced and modified.

The majority of research into improving chromatographic materials and magnetic beads focuses solely on improving binding behavior. As a result, a deeper understanding of the adsorption mechanisms, as well as the desorption steps, is required. While chromatography is widely used in industrial scale-up processes, magnetic isolation must be demonstrated as a viable alternative [165]. The FDA's restrictions on iron oxides as food additives and their biomedical applications will make industrial bioseparation techniques more challenging. Furthermore, device selection for magnetic biomolecule purification is currently challenging. Hence, enhanced effort should be put into developing new equipment via cutting-edge technologies using magnetic nanoparticles for use in protein separation processes. This could be the most difficult obstacle to overcome in the establishment of magnetic particle-mediated protein separation as a viable industrial replacement to traditional approaches for purification in the initial and intermediate downstream processing phases.

5.4.1 Methods of Purification

Almost all fields of biosciences and biotechnologies require isolation of proteins, peptides, and other biomolecules. As a result, separation science is a critical area for further advancements in research and technology. Novel isolation and purification approaches are required to purify low-abundance target molecules in the presence of high impurity concentration in large-scale processes [166–168].

Protein and peptide separation in these sectors is commonly performed via affinity chromatography and other technologies [169]. Affinity ligand methods are presently the exclusive tool available for downstream processing due to their enhanced selectivity and recovery. Numerous applications, particularly at the lab scale, have demonstrated the strength of affinity column chromatography. The limitation of general liquid chromatography procedures is that they are unable to handle samples containing particulate material, making them unsuitable for use in the isolation process during early stages [170–174]. Magnetic affinity, hydrophobic, ion-exchange, or adsorption batch separation methods have proved their utility in this context, as have applications of fluidized beds stabilized with magnets or two-phase systems modified by magnets. Batch magnetic separation works on a fairly fundamental premise as displayed in Fig. 7 [175].

A sample containing the target molecule is combined with carriers of magnets harboring an immobilized ligand with affinity, ion-exchange or hydrophobic groups, or biopolymers with

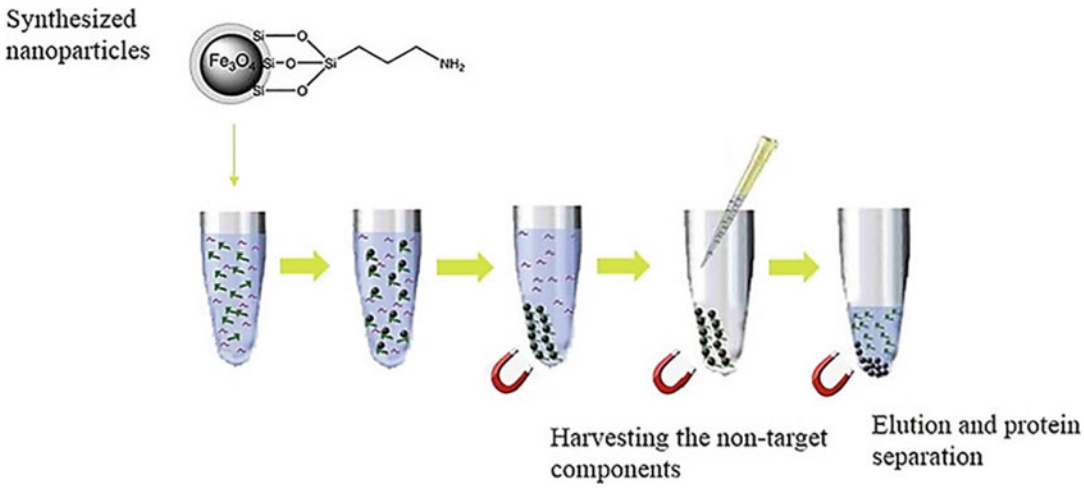

Fig. 7 One-step separation of the recombinant protein by using the amine-functionalized magnetic mesoporous silica nanoparticles: an efficient and facile approach. (Reproduced with permission from [138], © Elsevier, 2019)

magnetic properties possessing isolated structure affinity. After an incubation period, the magnetic particles will bind to the target compounds in the sample, and the entire complex with magnetic property is readily and quickly separated via a magnetic separator. The separated target can be eluted for future research after washing away the impurities. In comparison to traditional separation procedures, magnetic separation techniques offer several benefits [168, 169]. This procedure is typically straightforward, requiring only a few handling steps. All of the decontamination processes can be completed in a single test tube or another receptacle. Expensive systems of liquid chromatography, filters, centrifuges, and other equipment are not essential. In crude samples including materials with suspended solids, the separation process can be carried out directly. In certain circumstances, the disintegration and separation phases might be combined for a reduction in the total separation time.

Magnetic separation techniques are also used in a variety of automated operations for the detection of a wide range of protein analytes. Recently, numerous automated techniques for separation of proteins are available. The target proteins or peptides are normally separated magnetically in a gentle manner. When adopting the very mild magnetic separation approach, even massive protein complexes that are typically broken up by ordinary column chromatography procedures can be preserved. Throughout the isolation process, both the diminished pressures of shear and the enhanced concentration of protein have a definite impact on the process of separation [176, 177]. When target proteins are separated using traditional chromatography techniques, a substantial

volume of diluted protein solution is generally produced. In this situation, instead of ultrafiltration or precipitation, appropriate magnetic particles can be utilized to concentrate them.

5.4.2 Ligand-Based Adsorption

Magnetic affinity adsorption can be done with particles that have affinity ligands immobilized on them. In peptide and protein isolation, streptavidin, protein G and A, and antibodies are frequently utilized for target protein isolation [178, 179]. Particles with magnetic properties along with the abovementioned ligands, that are immobilized, can also be used to immobilize affinity ligands [180]. Other affinity ligands have already been immobilized on commercially available carriers. Standard affinity chromatography processes can be used for additional ligand of interest immobilization to both laboratory-made and commercial particles with magnetic properties. For immobilization, functional groups on the magnetic particle surface, such as $-OH$, $-NH_2$, or $-COOH$ are typically utilized. However, in other situations, particles with magnetic properties are activated, whereas surface modified magnetite particles were prepared in the laboratory via silanization [181]. This process alters the inorganic particles surface with suitable functional groups, allowing affinity ligands to be immobilized easily. In rare situations, enzyme activity can be reduced using magnetic particles of iron oxides. Microspheres encapsulated with a pure polymer outer layer are used in this circumstance.

Biomolecules also act as purification material including β-glucosidase. In comparison to free-glucosidase, immobilized glucosidase has excellent catalytic activity and stability. Furthermore, immobilized glucosidase can be regenerated multiple times while holding over 65% of its primary activity. Such materials have a lot of potential in terms of enzyme purification and immobilization. Furthermore, the purified protein could be confirmed by Western blotting with standard protein molecules.

5.4.3 Antibody-Based Purification

Immunoassays use an immobilized antibody to detect the presence of target molecules. Antibody target identification can be paired with magnetic particles enabling simple separation from the media in immunomagnetic separation. This approach has numerous applications in the sectors of biomedicine, pharmaceuticals, food, and the environment [178]. Some unique and effective solutions to common problems have been identified using nanosized magnetic particles and antibody combinations. Unlike protein purification, exosome extraction, has been difficult, and yields are not as high as those currently possible in protein purification via magnetic particles [182]. Other potential advantages of this approach include the ability to prepare unique architectures that are sensitive to temperature and to detect bacteria via magnetic nanoparticles leading to rapid diagnosis of various ailments. Aside from their enhanced

surface-to-volume ratio, certain MNPs have drawbacks, including limited magnetophoretic mobility, which restricts their separation ability using an external magnet and constrains their usage as scaffolds for exclusive separations [179–181]. Furthermore, multifunctional nanostructures are formed by utilizing magnetic nanowires as a framework, which can be combined with nanoparticles with specific magnetic properties that can be enhanced via biotin functionalization [183]. Strong magnetic MNPs allowed better and rapid separation using streptavidin-biotin conjugated antibodies, which adhered to the surface of nanowires [184]. The antibody-conjugated magnetic (Abs MNws) nanowires cocktail are able to swiftly isolate exosomes from tiny samples with high purity [185, 186].

5.5 Cancer-Cell Separation by Receptor Protein–Ligands Interaction

Circulating cancer cells have attracted attention due to their ability to identify the progression and occurrence of cancer; they can be separated from the preliminary tumor and bloodstream circulation [187–190]. Many reports have described circulating tumor cells (CTC) screening from peripheral blood by various conventional methods. Cell separation is classified into two types as antibody-dependent (labeled method) or antibody-independent method (label free). Antibody-dependent involves the detection of specific tumorigenic cells based on receptor protein binding with antibody [191–193]. The antibody may be conjugated or coated with MNPs or a combination of fluorescence markers with magnets as shown in Fig. 8. The antibody-independent method (label free) is based on the surface functional material of nanoparticles that are attracted to the surface marker of the cell, which is circulating in the whole blood [194–198]. This separation is further divided into two types based on cell type, homogenous and heterogonous separation, depending on the cell morphology, size, existence of biomarkers on the surface, and properties of the cells.

Several methods have been adopted to separate desired cell types from whole blood or cells from a cellular population using MNPs either as direct conjugation or as conjugation with functionalized nanomaterials. The conjugation of biological materials with

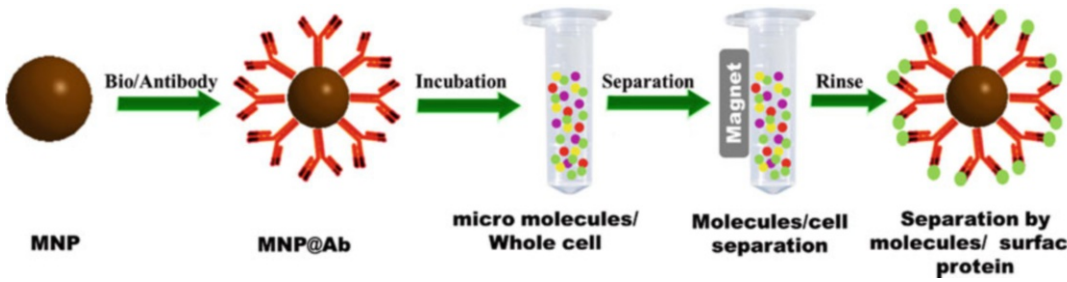

Fig. 8 Separation of a target protein antibody-conjugated magnetic nanoparticles (MNP)

nanomaterials to extract cellular targets is affected by several factors, including the type of biomaterials and the specificity of the marker protein in the cell surface.

Hyaluronic acid (HA) is a biosynthetic natural molecule with a repeating structures of d-glucuronic acid disaccharides and units of N-acetyl d-glucosamine. Because it is biocompatible, biodegradable, and nontoxic, it is also employed as an anticancer agent [199]. The receptor-mediated internalization of HA catabolism molecularly interacts with receptors (CD44) on the surface of cancer cells before being reduced to 50 saccharide units by Hyal-2 on the surface of the cell to produce caveolae which forms endosomes [200]. The endosomes then combine with lysosomes where Hyal-1 breaks them down further into tetrasaccharides. Because HA is present on the surface of nanoparticles, it is feasible to target malignancies [201, 202]. Many tumor cells express CD44, the major location of HA binding on the cell surface. It has a strong link to cancer onset and spread. Around 100 amino acids on CD44 protein recognize repeated disaccharide structure units on HA to create the link module, which is how HA targets CD44. CD44 has proven a useful biomarker for detecting early cancer and distinguishing between tumor and normal cells. HA's value in cancer medication therapy has been established in numerous studies [203].

On another note, folic acids (FA), which are smaller molecules relative to antibodies, have fewer functional groups for bioconjugation and can molecularly interact with folate receptors (FAR) with superior affinity [204]. Due to the small size of FA molecules, immobilizing FA onto nanoparticles causes a reduction in their molecular interaction and capture efficiency with cells [205]. The nanoparticles' enhanced surface-to-volume ratio results in improved adsorption for cell targeting without specificity and reduced purity thereby increasing the turn-around time associated with CTC identification. Processes for enriching CTCs, as well as ways to reduce nonspecific cell adsorption and boost capture efficiency, are critical for lateral identification of CTC. FA coupled on the surface of nanoparticles may lead to more availability of FAs for FAR attachment on the surface of CTCs to potentially improve nanoprobe identification of FAR [206, 207].

Physiological and pathological changes in expression level or state, which correspond with the progression of several disorders such as malignancies, are used as biomarkers including proteins, peptides, DNA, RNA, and other small metabolites as early indicators of diseases. Because of its high viscosity, high cell density, high protein content, and typically complicated composition, magnetic microbeads are generally effective for separating target cells, micro-/macromolecules in whole blood. Magnetic nanoparticles

coupled to antibodies and other biomolecules are an alternative to flow cytometric equipment for protein identification, isolation from blood and detection of surface antigen expression by the specific antibody–antigen response and can be more efficient and less expensive than previous methods [208]. Furthermore, using magnetic nanoparticles as molecular imaging probes allows for noninvasive in vivo analyses of disease antigen expression in a variety of internal organs. Magnetic nanoparticles are promising as therapeutic or diagnostic tools in medicine [209]. They can be immobilized for the detection of biomolecules, separation of cells, affinity purification processes, gene transfer, and in magnetic resonance imaging as contrast agents [29, 210]. The heat they produce in a fluctuating magnetic field can also be utilized as a medication delivery device in targeted therapy and for hyperthermia treatment.

6 Future Perspective

Recently, magnetic nanoparticles have been extensively studied and have been shown to be beneficial for the exclusive and enhanced separation and purification of proteins [211]. However, there are certain limitations among MNPs, which must be eliminated before utilizing them for large-scale applications [212]. For instance, iron oxide or magnetic nanoparticles do not have ability to bind with proteins [213]. These MNPs must be functionalized with biomolecules to enable them to interact with specific proteins [214], to facilitate separation and purification via a magnetization process, and to facilitate reuse via a demagnetization approach [215]. The addition of biomolecules as functionalization agents have been shown to increase their hydrodynamic size, which eventually affects their protein separation efficiency [216]. Thus, in the future, it is essential to analyze their monodispersity, after immobilizing or separating proteins, or utilize capping agents to prevent their agglomeration and maintain stability [217]. In addition, nanosized magnetic oxides of iron particles are blended with other novel metallic or carbon nanoparticles to form nanocomposites, which can be beneficial for one-step protein isolation [218]. Magnetic nanoformulations, such as dendrimers or liposomes can release several nanosized magnetic particles after the exposure of a specific magnetic field to combine with targeted protein and separate them [219, 220]. These types of nanocomposite or nanoformulated magnetic nanoparticles are currently proposed, not only to separate targeted proteins from biological samples but also to separate and purify targeted proteins from protein mixtures in the future.

7 Conclusion

This chapter provides an overview of various magnetic nanoparticles that can be beneficial for protein isolation applications. The magnetic nanoparticles are identified to possess enhanced activity in the isolation of targeted proteins with high efficiency. These nano-sized magnetic particles are also suggested to be valuable in the advanced detection and diagnosis of several diseases, due to their high detection limit. However, the agglomeration in body fluids and increases in their size during large-scale synthesis ultimately reduces their protein separation performance. Thus, it is necessary to introduce nanocomposites or nanoformulations with magnetic nanoparticles to improve their protein separation efficiency in the future.

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Chapter 9

Ion-Exchange Chromatography: Basic Principles and Application

Robert G. Wallace and Keith D. Rochfort

Abstract

Ion-exchange chromatography (IEC) is a fractionation technique that allows for the separation of ionizable molecules on the basis of differences in their electrostatic properties. Its large sample-handling capacity, broad applicability (particularly to proteins and enzymes), moderate cost, powerful resolving ability, ability to perform simultaneous quantitation, and ease of scale-up and automation have led to it becoming one of the most versatile and widely used of all liquid chromatography (LC) techniques. In this chapter, we review the basic principles of IEC, as well as the broader criteria for selecting IEC conditions. By way of further illustration, we outline basic laboratory protocols to partially purify a soluble serine peptidase from bovine whole brain tissue, covering crude tissue extract preparation through to partial purification of the target enzyme using a form of IEC, namely, anion-exchange chromatography. Protocols for assaying total protein and enzyme activity in both pre- and post-IEC fractions are also described.

Key words Liquid chromatography, Anion-exchange, Cation-exchange, DEAE-Sepharose® Fast Flow, Prolyl oligopeptidase, Continuous-bed, Capture, Polish

1 Introduction

Bioseparation involves resolution of the components in complex mixtures encountered in biological and biochemical systems according to differences in their specific properties. This form of chromatography in turn enables scientists to not only determine both the identity and concentration of each component but also, if necessary, the ability to isolate a desired component from other contaminating molecules for further analysis or application. The established approaches for carrying out a bioseparation are typically dominated by liquid chromatography (LC) processes. Resolution of mixtures by LC is based on the principle that, under a given set of conditions, individual solutes dissolved in a *mobile phase* will differentially interact with a chemically modified *stationary phase* as a function of differences in individual solute distribution coefficients

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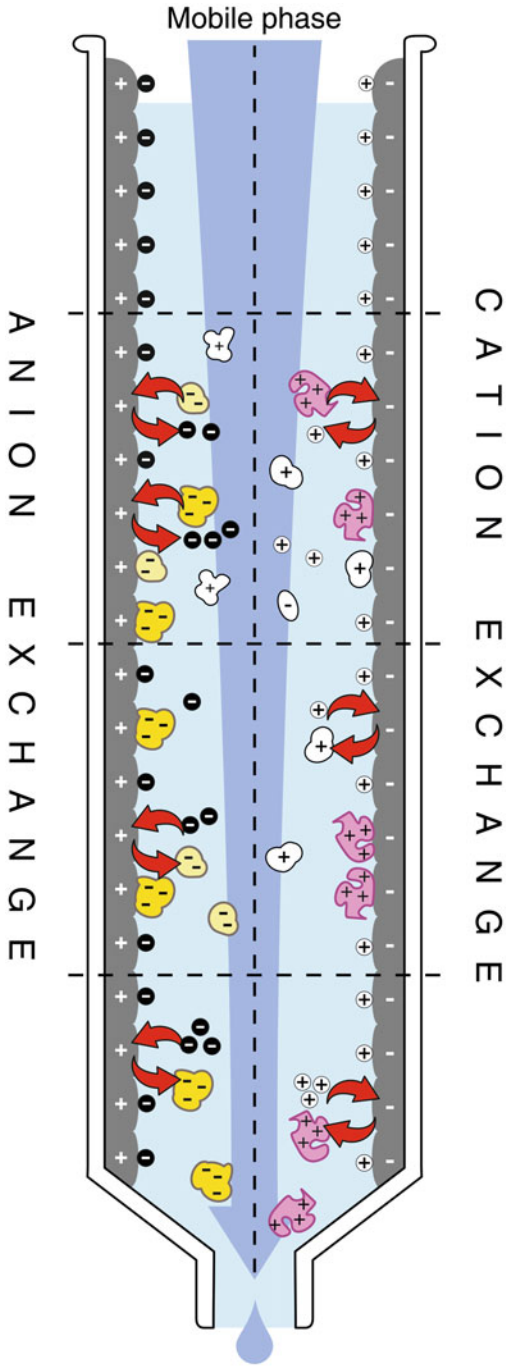
(K). In this way, LC exploits inherent differences between biomolecules (e.g., molecular size, hydrophobicity, ligand specificity, and/or charge) to achieve their separation from one another.

1.1 Basic Principles of Ion-Exchange Chromatography

With its origins dating back to the 1935, ion-exchange chromatography (IEC) was designed specifically for the separation and resolution of charged or ionizable molecules according to differences in their net surface charge [1, 2]. Since then, IEC has proven itself to be one of the premier methods of fractionation of complex mixtures, and its application toward separating and purifying biomolecules from complex biological mixtures has undoubtedly played a significant role in aiding our understanding of specific biomolecules in biological systems and processes. Today, IEC continues to have an influential role in life sciences, and it is one of the most routinely used techniques by chemists and biochemists for the isolation and purification of proteins [3, 4], enzymes [4–6], antibodies [3, 7], peptides [8], amino acids, and nucleic acids [9, 10], as well as simpler carbohydrates [11] and organic compounds [12]. Its large sample-handling capacity, broad applicability (including high performance and high-throughput application formats), moderate cost, powerful resolving ability, ability to perform simultaneous quantitation, and ease of scale-up and automation have led to it becoming one of the most versatile and widely used of all LC techniques. Like other forms of column-based LC (e.g., gel-permeation, affinity, hydrophobic interaction, and size exclusion), this technique comprises both mobile and stationary phases, the former typically an aqueous buffer system into which the mixture to be resolved is introduced and the latter usually an inert organic matrix chemically derivatized to bear bound ionizable functional groups that carry a displaceable oppositely charged counterion. These counterions exist in a state of equilibrium between the mobile and stationary phases, giving rise to two possible IEC formats, namely, anion- and cation-exchange (*see* Fig. 1). Exchangeable matrix counterions may include protons (H^+), hydroxide groups (OH^-), single-charged monoatomic ions (Na^+, K^+, Cl^-), double-charged monoatomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}), as well as organic bases (NR_2H^+) and acids (COO^-).

Molecules can vary considerably in their overall charge and ionization state. The more complex a molecule is, the greater the degree of variation in surface charge distribution, charge density, and overall charge. The pH-dependent ionization of electrolyte groups (weak acids or bases) can impart a net positive or negative surface charge on biomolecules, subsequently enabling their separation from one another via their individual respective interaction with a charged chromatography matrix. In this way, complex mixtures of biomolecules can be efficiently separated and quantitated based on the amounts of each ion inherent to it by IEC. This can be



Column equilibrium

The pH/ionic strength of the column must first be equilibrated using an appropriate buffer solution. Once complete, the mixture containing the protein of interest can be added.

Sample adsorption (binding)

Proteins with a charge opposite to that of the matrix will displace the functional group counterion and preferentially bind to it. Oppositely charged 'contaminant' proteins will not bind to the matrix and will instead pass through the column.

Sample Resolution

By changing either the pH or ionic strength of the mobile phase, protein analytes which have bound to the matrix can be eluted from the column. However, proteins with stronger bonds will remain attached. In this way, fine control of sample resolution may be achieved.

Sample desorption (elution)

Further changing the pH or ionic strength of the mobile phase will enable the elution of the protein of interest from the column to be collected as a purified fraction of the original mixture.

Fig. 1 Ion-exchange chromatography. It is the nature of the counterions displaced from the matrix functional groups which determines the IEC format. Thus, with anion-exchange chromatography (LHS), the stationary phase matrix displays a positively charged functional group with a negative counterion that can be displaced by an anionic sample, thereby enabling matrix adsorption. With cation-exchange chromatography (RHS), the stationary phase matrix displays a negatively charged functional group with a positive counterion that can be displaced by a cationic sample, again enabling matrix adsorption. With either format, sample desorption from the matrix can be achieved by increasing the counterion concentration within the mobile phase

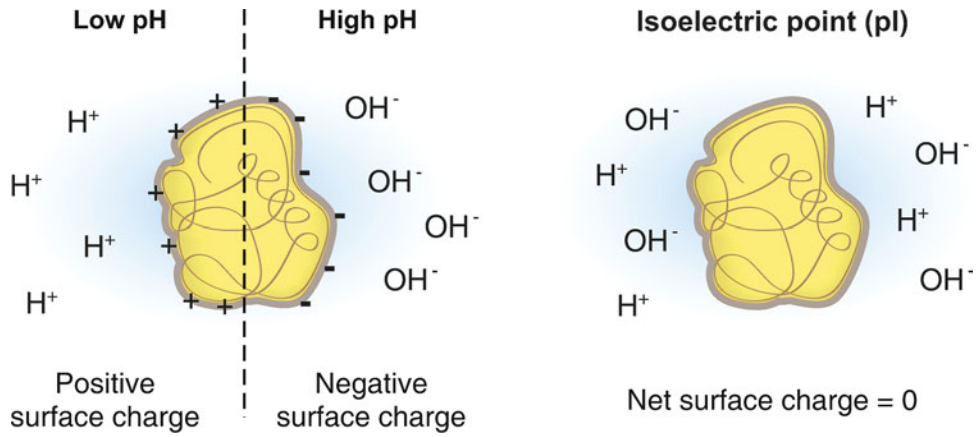


Fig. 2 Molecules can vary in their overall charge, and their ionization state is influenced by their environment. Adjusting the pH of a protein's environment can increase, decrease, or negate the charges present on its surface. However, in a complex mixture, every protein has their own respective pI value, meaning the pH of a mobile phase can influence the net charge on all proteins contained within the sample mixture, in addition to those biomolecules contained within the stationary phase of the column employed. As such, adjustment of the mobile phase, relative to your protein of interest, can thus influence the manner in which a sample is separated by ion-exchange chromatography and enable a level of control over it

explained by taking as an example the separation of a mixture of proteins. During IEC, the pH of the mobile phase will determine the net charge on both the functional group of the matrix and on individual proteins within the sample mixture. The polyampholyte nature of proteins mean they can carry both positive and negative charges; the former largely attributable to the ionization of lysine and arginine side chains and the latter to aspartate and glutamate side chain ionizations. As a general rule, a protein will have a net negative charge above its isoelectric point or pI (i.e., pH at which a protein has zero net charge) and a net positive charge below its isoelectric point (*see* Fig. 2). Naturally, different proteins may have different pI values and therefore a different net charge at any given pH. The pH of the mobile phase can therefore determine its selection such as to ensure that the net charge on a protein of interest within a mixture is the opposite to that of the matrix functional group, ensuring that it will displace the functional group counterion and subsequently bind to the matrix (adsorption). Conversely, oppositely charged "contaminant" proteins will not be retained and in turn will be eluted from the column.

Separation in complex mixtures can be achieved due to the fact that different molecules will have different degrees of interaction with the matrix due to differences in their respective charges. In this way, free, mobile, charged proteins in a solution interact with the solid surface of the matrix, displacing a charged molecule of lesser affinity which was initially residing on the matrix surface, effectively fractionating the mixture based on their respective electrostatic

properties. In this way, ion-exchange chromatography is an incredibly powerful separation technique, in that it can detect minor differences between proteins (e.g., one charged amino acid in difference).

Moreover, this adsorption process is reversible, and there is no permanent change made to the matrix as part of this process. In this way, protein analytes that have strongly bound to the matrix can subsequently be eluted (desorption) by changing either the pH or ionic strength of the mobile phase (*see* Subheading 1.2.3 below). In changing the pH of the mobile phase, one can alter the net charge of the bound protein and thus its matrix-binding ability. More commonly, increasing the concentration of a similarly charged species within the mobile phase to the proteins that have bound creates a competitive environment for adsorption to the matrix. This ultimately leads to the displacement of the captured proteins, which were initially bound to the matrix by the more recently introduced charged competition, allowing them to be eluted from the column and collected for analysis. During anion-exchange chromatography, for example, negatively charged protein analytes can be competitively displaced by the addition of negatively charged chloride ions (e.g., from sodium chloride). By gradually increasing the salt concentration in the mobile phase, the degree of affinity between the salt ions and the functional groups will eventually exceed that which exists between the protein charges and the functional groups, resulting in protein displacement and elution.

1.2 Criteria for Selection of IEC Conditions

In the following subsections (Subheadings 1.2.1, 1.2.2, 1.2.3, and 1.2.4), the broader criteria for selecting IEC conditions are discussed, thereby allowing one to better comprehend and adapt the described protocols [13]. The ensuing materials and methods sections (Subheadings 2 and 3) outline the protocols necessary to partially purify a serine peptidase from bovine whole brain soluble fraction. Specifically, we first describe the preparation of a crude tissue extract for IEC using homogenization, centrifugation, and ammonium sulfate precipitation. This is followed by the method for partial purification of the target protein using anion-exchange LC. Finally, to allow one to accurately monitor purification efficiency, protocols for assaying total protein and enzyme activity in both pre- and post-IEC fractions are also described. Our focus for these protocols is prolyl oligopeptidase (POP, prolyl endopeptidase, EC3.4.21.26), an 80 kDa serine endopeptidase [14, 15].

1.2.1 Selection of Ion-Exchange Matrix

Matrices should be selected relative to their chromatographic properties (e.g., stability, mechanical strength, and flow properties) and behavior toward the biomolecules of interest (e.g., maintenance of biological activity). The choice of a suitable ion-exchange matrix is the single most important aspect of any ion-exchange protocol and is based on several factors:

(a) Purpose of the purification: This may be to concentrate and stabilize a target protein during an initial capture step, to remove bulk impurities during an intermediate step, or to achieve high purity in a final polishing step. In this regard, the size, porosity, and binding capacity of IEC matrix beads should be carefully considered [16, 17]. For example, one would employ a macroporous matrix offering high flow and binding capacity for a primary capture IEC step, while a nonporous (or microporous) matrix offering high resolution would be selected for a polishing step to better remove trace impurities from the sample. Commercially, available IEC matrix beads range in size from 3 to 400 microns. Larger beads are frequently used in initial protein purification stages that require fast elution rates and high capacity but low-to-intermediate resolution, while smaller beads are ideal for final polishing stages requiring higher resolution. Moreover, commercial matrices have binding capacities ranging from less than 2 mg/mL to more than 150 mg/mL.

(b) Desired ion-exchanger charge/strength: As mentioned above, ion-exchange functional groups fall into two charge categories. Positively charged diethylaminoethyl (DEAE) and quaternary ammonium (Q) functional groups, for example, are routinely employed in anion-exchange chromatography, while negatively charged carboxymethyl (CM), sulphomethyl (S), and sulphopropyl (SP) groups are typical cation-exchangers. Both exchanger types can be further categorized as either "strong" or "weak." Strong ion-exchangers are fully ionized over a broad working pH range (i.e., show no loss or gain of charge with varying pH), while weak ion-exchangers are only partially ionized over a narrow pH range (i.e., charge can vary significantly with pH). Consequently, with strong ion-exchangers, individual proteins can adsorb to several exchanger sites, often necessitating more stringent elution conditions (up to 1 M NaCl) that may compromise sample stability and resolution. Strong ion-exchangers are therefore often used for initial development and optimization of purification protocols (and for binding proteins with pI values at the more extreme ends of the pH scale). By contrast, weak ion-exchangers exhibit more flexibility in terms of their selectivity and are therefore a more common choice for the separation of proteins that retain their functionality over the pH 6–9 range, as well as for labile proteins that may require mild elution conditions (Table 1 highlights a range of commercially available ion-exchanger matrices categorized based on charge and strength).

Table 1
Comparison of commercial ion-exchangers

Exchanger type	Ion-exchange group	Functional group	Buffer counterion	Commercial resin	Range
Strong anion	Quaternary ammonium (Q)	$CH_2N^+(CH_3)_3$	Cl^-, $HCOO^-$, CH_3COO^-, SO_4^{2-}	Q Sepharose® Capto® Q Dowex®1X2 Amberlite®/ Amberjet® QAE Sephadex®	pH 2–12
Weak anion	Diethylaminoethyl (DEAE)	$O(CH_2)_2N^+H(C_2H_5)_2$	Cl^-, $HCOO^-$, CH_3COO^-, SO_4^{2-}	DEAE-Sepharose® Capto® DEAE DEAE Cellulose®	pH 2–9
Strong cation	Sulfopropyl (SP)	$(CH_2)_3SO_3^-$	Na^+, H^+, Li^+	Capto® S SP Sepharose® SP Sephadex® TSKgel SP-5PW	pH 4–13
Weak cation	Carboxymethyl (CM)	OCH_2COO^-	Na^+, H^+, Li^+	CM Cellulose CM Sepharose® CM Sephadex® CM Sepharose® CL6B TSKgel CM-5PW	pH 6–10

(c) Sample properties: Selection of the most suitable exchanger functional group for a purification will also be dictated by the target protein biochemical properties such as pI and pH stability. For example, if a protein has a low pI (<5.0) but is more stable at pH values above this, then an anion-exchanger should be chosen and vice versa. The purification of prolyl oligopeptidase by ion-exchange chromatography is a good example. With a pI in the region of 4.8 [18, 19], prolyl oligopeptidase could potentially adsorb to either a strong cation-exchanger or a weak-anion exchanger. However, with a pH optimum from pH 7.4–8.0, coupled with rapid destabilization of the enzyme below pH 5.0, the anion-exchange option is favored. To illustrate this, Fig. 3 (*and* Table 2) demonstrate how anion-exchange chromatography using DEAE-Sepharose® Fast Flow (Cytiva) can be used for the initial-stage purification of prolyl oligopeptidase from bovine whole brain soluble tissue extract.

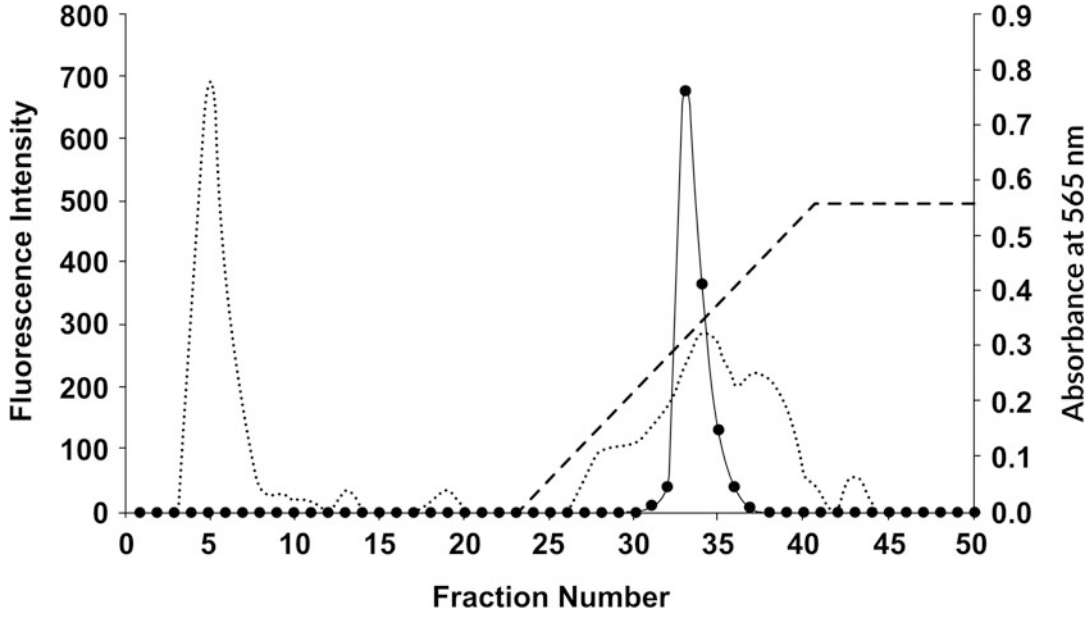


Fig. 3 Partial purification of prolyl oligopeptidase (POP) from bovine whole brain cytosol via DEAE-Sepharose® Fast Flow anion-exchange chromatography. Dialysed post-ammonium sulfate POP fraction (7.4 mL) was applied to a pre-equilibrated 20 mL DEAE-Sepharose® column (50 mM Tris–HCl pH 8.0, 5 mM DTT, 0.5 mM EDTA). Following removal of unbound contaminants, bound POP was eluted with a linear NaCl gradient (- - - -) from 0 to 350 mM. 5 mL fractions were collected and assayed for POP activity (-●-) via fluorimetric assay at excitation and emission wavelengths of 370 and 440 nm, respectively (fluroescence intensity values plotted). Protein (·····) was monitored by BCA microplate assay at 562 nm. Peak POP elution occurred at 180 mM NaCl. DTT: dithiothreitol, EDTA: ethylene diamine tetraacetic acid, NaCl: sodium chloride

Table 2
Partial purification of prolyl oligopeptidase (POP) from bovine whole brain cytosolic fraction

Sample	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	Recovery (%)
Brain cytosol	122.26	1,835,292	15,011	1	100
Ammonium sulfate	24.95	1,578,408	63,263	4.2	86
DEAE anion exchange	10.69	927,209	86,736	5.8	51

Enzyme activity (units) expressed as picomoles of MCA released (i.e., from *Z*-Gly-Pro-MCA) per min at 37 °C. Protein (mg) was determined by BCA microplate assay

1.2.2 Selection of Buffer Conditions

To prevent any variation in matrix and protein net charge, maintenance of a constant mobile phase pH during IEC is essential to avoid pH fluctuations, which can occur when both protein and exchanger counterions (particularly if these are H^+ or OH^- ions) are released into the mobile phase. A number of important factors dictate the choice of the mobile phase buffer:

(a) Buffer charge: Buffering ions that carry a charge opposite to that of the functional groups of the matrix will take part in the ion-exchange process and in turn cause disturbances in the chromatographic environment. In that way, the buffering ion should not interact with the ion-exchanger functional groups (i.e., positively charged buffers should be used in anion-exchange and vice versa). For example, Tris buffers are often used with DEAE exchangers, while phosphate and acetate buffers are frequently used with CM exchangers.

(b) Buffer strength: The minimum buffering strength recommended for ion-exchange is approximately 10 mM within 0.3 pH units of the buffer dissociation constant or pKa (i.e., the pH at which buffering capacity is strongest).

(c) Buffer pH: Finally, many biological macromolecules become denatured and/or lose activity outside of a certain pH range. In that way, a buffer pH should be selected that permits the protein of interest to remain stable, while allowing it to bind reversibly to the matrix. It should also be close enough to the pH at which the protein begins to dissociate from the column to prevent the need to adjust the pH or ionic strength during elution to levels that would destabilize the protein.

1.2.3 Selection of Adsorption and Elution Conditions

The mobile phase can be adjusted to achieve optimal adsorption/elution of target proteins:

(a) pH: Mobile phase pH can be altered to favor either adsorption or elution of proteins. In general, a pH is chosen which will only permit binding of the target protein. This is usually about 1 pH unit above or below the target protein pI. A greater difference in pH would lead to stronger protein binding, the need for stronger elution conditions and decreases in sample resolution, and recovery of target protein activity. A pH change can also be used to induce desorption of the target protein (a pH decrease in the case of anion-exchangers and vice versa).

(b) Ionic strength: Mobile phase ionic strength can also be used to control target protein adsorption and elution. As a general rule, the highest ionic strength that will allow adsorption (e.g., 20–50 mM NaCl) and the lowest ionic strength that will allow elution are recommended.

1.2.4 Selection of Elution Format

In IEC, one can choose to develop the method to target and bind the protein/s of interest and allow the contaminants to pass through the column, or alternatively to target and bind the contaminants and allow the protein/s of interest to pass through the column. These approaches are gradient and isocratic elution, respectively, and each differs in their approach:

(a) Gradient elution: With gradient elution, continuous or discontinuous (stepwise) variations in the ionic strength and/or pH of the eluent are used to promote target protein desorption. While stepwise gradients are technically simpler, continuous gradients generally give better resolution.

(b) Isocratic elution: With isocratic elution, a single buffer is used throughout the entire separation. Sample components (including the target protein) are only loosely/incompletely adsorbed to the column matrix. Since individual proteins will have different distribution coefficients, separation is achieved by their relative speeds of migration over the column. To achieve optimum resolution of sample components, therefore, a small sample volume (1–5% of the bed volume) and a long exchanger column (1:20 diameter/length ratio) are recommended. Although this technique is time-consuming and the desired protein invariably elutes in a large volume, no gradient-forming apparatus is required, and column regeneration is usually unnecessary. More commonly, conditions are selected, which result in the complete adsorption of the desired protein to the column matrix, necessitating an alteration of eluent conditions to achieve its desorption.

2 Materials

Unless otherwise indicated, all chemicals can be purchased from Sigma-Aldrich.

2.1 Preparation of Bovine Whole Brain Cytosolic Extract

1. Buffer A: 100 mM potassium phosphate pH 7.4, 5 mM DTT (dithiothreitol) and 0.5 mM EDTA (ethylenediaminetetraacetic acid) (*see* **Note 1**).
2. Bovine whole brain should be obtained from a freshly slaughtered animal. The brain tissue can be sectioned and frozen at −80 °C for long-term storage.
3. Container with crushed ice.
4. Homogenizer (e.g., Sorvall Omni Mixer, or a standard food blender).
5. Refrigerated centrifuge and rotor (e.g., Beckman XL-80/Type 70.1 Ti Fixed Angle Rotor; 36,000 × *g*).
6. Refrigerated ultracentrifuge (e.g., Beckman L8-M/70Ti rotor; 100,000 × *g*).

2.2 Ammonium Sulfate Precipitation

1. Buffer B: 50 mM Tris–HCl pH 8.0, 5 mM DTT, and 0.5 mM EDTA (*see* **Note 2**).
2. Solid $(NH_4)_2SO_4$ (ammonium sulfate).

3. 1 M NaOH (sodium hydroxide).
4. Container with crushed ice.
5. Dialysis tubing and sealing clips (Sigma-Aldrich).
6. Glass beakers for both "salting out" and dialysis tube preparation steps.
7. Magnetic stirrer and Bunsen Burner.

2.3 Prolyl Oligopeptidase Partial Purification by IEC

1. Buffer B (*see* Subheading 2.2).
2. 350 mM NaCl (sodium chloride) prepared in Buffer B.
3. DEAE-Sepharose® Fast Flow anion-exchange matrix (Cytiva, Sigma-Aldrich), usually supplied in as pre-swollen beads (*see* **Note 3**).
4. Test tubes for the fraction collector.
5. Glass column, low pressure pump (Model EP-1 Econo Pump), fraction collector (Model 2110), silicone tubing and luer-lock fittings (Bio-Rad Laboratories are recommended for all of the aforementioned LC hardware) (*see* **Note 4**).
6. Gradient-forming device with 100–200 mL capacity (Sigma-Aldrich) (*see* **Note 5**).

2.4 Assay of Post-DEAE Fractions

1. Buffer A (*see* Subheading 2.1).
2. Bradford and BCA (bicinchoninic acid) protein assay reagents (Bio-Rad Laboratories and Pierce Protein Research Products).
3. BSA protein assay standard at 1 mg/mL (in ultra-pure water).
4. Both fluorescent standard (1 mM 7-amino-4-methyl-coumarin, MCA) and substrate stock (10 mM *Z*-Gly-Pro-MCA) can be prepared in 100% dimethyl sulfoxide (DMSO) and stored as frozen aliquots at −20 °C (Bachem).
5. 1.5 M Acetic acid.
6. Waterbath at 37 °C.
7. Fluorescence spectrophotometer (e.g., Tecan Infinite® F200).
8. Glass and quartz microcuvettes, the latter for monitoring absorbance at ≤280 nm (method-dependent).

3 Methods

3.1 Preparation of Bovine Whole Brain Cytosolic Extract

The workflow of the steps described below to isolate prolyl oligopeptidase (POP) from whole brain tissue using anion-exchange chromatography is presented in Fig. 4.

All steps to be conducted at 4 °C. Latex gloves should also be worn.

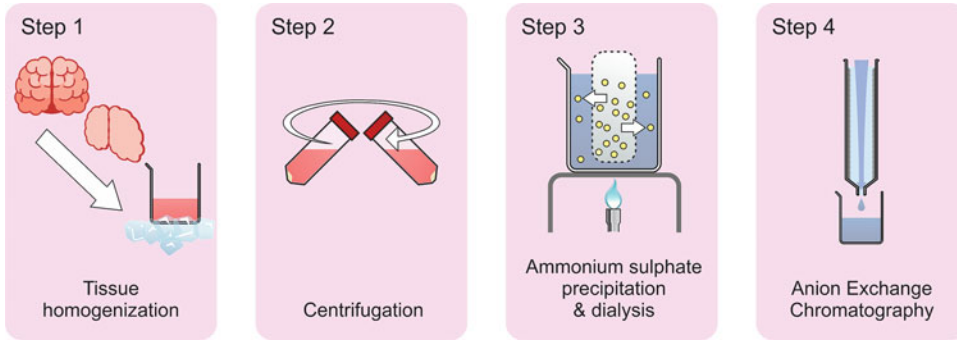


Fig. 4 Workflow of the methodological steps taken to isolate prolyl oligopeptidase (POP) from whole brain tissue using anion-exchange chromatography

1. Extract preparation has been described previously [5]. Briefly, homogenize fully a 50 g whole brain slice in 200 mL of ice-cold Buffer A (*see* Subheading 2.1) (*see* **Note 6**).
2. Centrifuge homogenate for 45 min at 36,000 × *g* to yield a supernatant (S1) and pellet (P1).
3. Resuspend P1 in 100 mL of ice-cold distilled water (osmotic shock step to release occluded POP activity) and centrifuge again as above to yield a second supernatant (S2) and pellet (P2). Discard the P2 pellet.
4. Combine S1 and S2 fractions and ultracentrifuge for 45 min at 100,000 × *g* to yield a whole brain supernatant (S3) for storage as 40 mL aliquots at −20 °C/−80 °C. Discard the pellet (P3).

3.2 Ammonium Sulfate Precipitation

All steps to be conducted at 4 °C. Latex gloves should also be worn:

1. Add solid ammonium sulfate to 40 mL of S3 with constant stirring to give 45% (w/v) saturation (10.67 g at 4 °C) and adjust to pH 7.4 using 1 M NaOH (*see* **Note 7**).
2. Stir for 1 h in a constant gentle manner, and then remove the precipitated ("salted-out") contaminants by refrigerated centrifugation for 45 min at 36,000 × *g*. Following centrifugation, retain the supernatant (S4) and discard the pellet (P4).
3. Add solid ammonium sulfate to S4 with constant stirring to give 75% saturation (9.39 g at 4 °C) and adjust to pH 7.4 using 1 M NaOH.
4. Repeat **step 2** and retain the pellet (P5). Resuspend P5 in 5 mL of Buffer B to create a "post-ammonium sulfate extract."
5. Subject the post-ammonium sulfate extract to dialysis for 12 h against Buffer B (*see* **Note 8**).

3.3 Prolyl Oligopeptidase Partial Purification by IEC

All steps to be conducted at 4 °C. Latex gloves should also be worn. A column flow rate of 1 mL/min and a fraction collection volume of 5 mL should be used throughout:

1. Equilibrate a 20 mL DEAE-Sepharose® Fast Flow column (diameter: 2.5 cm, height: 3.0 cm) with 100 mL of Buffer B.
2. Apply all of the dialyzed post-ammonium sulfate extract (from **step 5** above) to the column and wash through the unbound contaminants with 100 mL of Buffer B.
3. Elute bound POP using a 100 mL linear NaCl gradient (0–350 mM) prepared in Buffer B (*see* **Note 9**).
4. Regenerate the DEAE column with 60 mL of 350 mM NaCl in Buffer B, followed by 100 mL of NaCl-free Buffer B (*see* **Note 10**).
5. Assay (as soon as possible) eluted fractions for total protein and POP activity. Following assay (described below), eluted fractions containing the highest levels of POP activity should be pooled for storage (−20 °C) and further purification.

3.4 Assay of Post-DEAE Fractions

Determination of total protein in pre- and post-IEC fractions can be done by monitoring fraction absorbance at 280 nm (e.g., Tecan Infinite® M200 Pro, NanoDrop® One, WPA Biowave II) or using widely available Bradford or BCA standard assay protocols based on the methods of Bradford [20] and Smith et al. [21], respectively (*see also* Chapter 16). Determination of POP activity is based on a modification of the original method of Yoshimoto et al. using *Z*-Gly-Pro-MCA as substrate [22] and is described below:

1. Prepare the substrate stock (10 mM *Z*-Gly-Pro-MCA) in 100% DMSO. To 200 mL of substrate stock, add 600 mL of DMSO, followed by Buffer A to a final volume of 10 mL. This will yield a final substrate concentration of 200 mM in 8% DMSO (*see* **Note 11**).
2. Add 400 mL of 200 mM substrate to 100 mL of post-DEAE fraction and incubate at 37 °C for 30 min. Fractions should be assayed in triplicate (*see* **Note 12**).
3. Terminate assay reactions after 30 min with 1 mL of 1.5 M acetic acid.
4. For blanking purposes, prepare a negative control by addition of 1 mL of 1.5 M acetic acid to a 100 mL aliquot of Fraction 1 "prior" to addition of substrate (*see* **Note 13**).
5. Monitor liberated MCA by fluorescence spectrophotometry at excitation and emission wavelengths of 360 and 460 nm, respectively (e.g., Tecan Infinite® F200). The fluorimeter gain can be adjusted between 1 and 100 to obtain fluorimetric readings within range of a relevant MCA standard curve (0–10 mM or 0–100 mM) (*see* **Note 14**).

4 Notes

1. Potassium phosphate buffer can be prepared from the 100 mM "acid" (K_2HPO_4) and "base" (KH_2PO_4) forms of potassium phosphate. The base form can then be adjusted to pH 7.4 using the acid form. Moreover, as DTT loses much of its reducing potency within 12–24 h in solution, it should be prepared (in distilled water) as a concentrated stock (100×) and stored in aliquots (−20 °C), only to be thawed and added to buffers immediately prior to use.
2. Tris–HCl buffer can be prepared from 100 mM Trizma® base and adjusted down to the desired pH with concentrated HCl (hydrochloric acid). Moreover, as Tris buffers are temperature sensitive, they should be adjusted to pH 7.8 when being prepared at room temperature (the pH of the buffer will rise to pH 8.0 when the buffer is equilibrated to 4 °C).
3. Various materials ranging from silica and complex polysaccharides (e.g., dextran, agarose and cellulose) to more complex organic polymers (e.g., polyacrylate, polyvinyl, polyether, and polystyrene-divinyl benzene) have been used in the manufacture of ion-exchange matrices for *packed-bed* chromatography formats. IEC matrices are usually porous beads (although fibrous, microgranular, and composite matrices are also available), supplied either as dried preparations or in a pre-swollen state to be used in LC applications ranging from bioanalytical monitoring and research to process-scale protein separations. Vendors also provide ion-exchange matrices in pre-packed IEC columns and microplates for use with standard LC setups, HPLC systems, and high-throughput applications. Also worth noting, *continuous-bed* chromatography formats, which utilize neither bead nor fiber matrices, are now being applied to IEC separations [23, 24]. The IEC matrix is synthesized *within* the column by polymerization of advanced monomers and ionomers to create a continuous porous matrix. Polymer monolithic matrices are formed in situ within the column from a mixture of monomers, a cross-linking agent, a free-radical generator, and a poro-genic solvent, with polymerization initiated by controlled thermal or irradiating conditions. This approach eliminates the requirement for column packing, which may introduce air into the system and in turn impact on process efficiency. Polymer chains coalesce into a homogeneous network of interconnected porous rod structures characterized by mesopores and macropores. The relatively large channels between the porous rod structures allow for the use of a high flow rates, while the high degree of polymer cross-linking provides a large surface area for high binding capacity of ionic functional groups. Continuous bed IEC therefore enable high-resolution biomolecule separations at high flow rates without sacrificing capacity.

4. Column pouring should be performed at 4 °C. Prior to pouring, the column exit valve and tubing should be purged of air with distilled water. A pre-measured volume of the suspended pre-swollen ion-exchange matrix can then be poured into the column in "one" pour (this is essential to avoid "gaps" in the packed column bed, which can reduce column performance and resolution). The buffer volume or "headspace" above the matrix bed should be kept to a minimum (10–20% of bed volume) to ensure accurate delivery of a chosen elution gradient. Moreover, if there is an airspace above the buffer level covering the top of the matrix bed (dependent on column dimensions and bed volume), a small piece of parafilm (~1 cm^2) can be placed floating on top of the buffer over the matrix. This will act as a "shock absorber" to prevent fluid turbulence (which can cause disruption of the matrix bed surface as the buffer is pumped down through the column). Once poured, the column should be washed with several volumes of distilled water to remove preservative.
5. When setting up the gradient maker, ensure that the narrow fluid channel connecting the two gradient compartments is properly purged of air. This can be achieved using a long needle syringe to draw fluid through the channel, or by briefly exerting downward pressure on one of the gradient buffers to force fluid through the channel. Moreover, only gentle magnetic stirring should be used during gradient elution to prevent bubble formation and possible blockage of the gradient fluid channel.
6. Tissue homogenization should be conducted using short, repeated bursts of the homogenizer/blender (i.e., 2–3 s). This will minimize "shearing" and "foaming," both of which can reduce recovery levels of active enzyme.
7. The "salting out" procedure should be conducted on ice in a small glass beaker placed at the bottom of a small polystyrene dry-ice container (the container can subsequently be placed directly onto a magnetic stirrer).
8. Prior to ion-exchange, samples must be dialyzed into the starting buffer (in this case, Buffer B). At least 200 volumes of dialysis buffer are recommended (i.e., relative to the sample volume), with a buffer change after 3–4 h. Dialysis tubing can be prepared by placing a relevant length into a beaker of boiling water containing a large spatula of EDTA (disodium form). This will soften the tubing and remove heavy metal ions. After 5 min in boiling water, the tubing should be rinsed thoroughly in cold distilled water.
9. Linear ionic strength gradients are very reproducible and can be prepared by mixing two buffers of differing ionic strengths in linear volume ratios. Linear pH gradients cannot be

prepared by mixing two buffers of different pH values in linear volume ratios due to differences in the buffering capacities of the two buffers being mixed. In addition, the mixed buffer then must titrate the buffering action of the ion-exchanger and the adsorbed proteins. Consequently, pH gradients are less frequently employed.

10. Avoid leaving high salt buffers on the column for extended periods following regeneration as this may lead to salt crystallization (which necessitates column repouring). Moreover, for medium- to long-term storage, the column should be regenerated in the normal fashion, washed in several volumes of distilled water, followed by several volumes of either 0.02% (v/v) sodium azide or 20% (v/v) ethanol.
11. When diluting the 10 mM *Z*-Gly-Pro-MCA stock, Buffer A should be pre-warmed and added very slowly to the final volume of 10 mL. Moreover, the DMSO should be added in 100 mL increments in parallel with the buffer to prevent the substrate precipitating out of solution. The 200 mM substrate should be prepared from stock only as required.
12. Prior to assay, both fraction triplicates and substrate should be pre-equilibrated to 37 °C. At $t = 0$ min, the substrate should be added to sequentially numbered fractions at exactly 15 s intervals. At $t = 30$ min the acetic acid should be added to fractions at exactly 15 s intervals. In this way, all fractions receive the exact 30 min assay time.
13. When assaying any sample for POP activity, the appropriate negative control should always be: Sample (crude or purified), followed sequentially (at $t = 30$ min) by Stopping Agent (1.5 M acetic acid) + Substrate (200 mM *Z*-Gly-Pro-MCA).
14. The MCA standard curve must be prepared under identical assay conditions and read at the same fluorimeter settings as those used for the assay in order to properly quantitate MCA release for use in the calculation of enzyme activity expression. Units of POP activity are defined as picomoles of MCA released per min at 37 °C.

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Chapter 10

Hydroxyapatite Chromatography (HAC)

Jonathan Cawley

Abstract

Hydroxyapatite (HA) is a mixed-mode media that has been used extensively for the purification of proteins and DNA since the 1950s. Hydroxyapatite possesses a distinctive selectivity that may be applied in the purification of a wide range of biomolecules: immunoglobulins, alkaline proteins, acidic proteins, and DNA. The functional groups of HA can both attract and repel the carboxyl and amino groups on target molecules. This unique selectivity is due to the modalities that can be employed, which are not possible with traditional anion-exchange and cation-exchange chromatography. HA is a powerful chromatography step for reducing host cell-derived impurities and aggregated product, where a 2–4 log reduction in host cell proteins, aggregates, endotoxin, and viruses are routinely achieved. This chapter describes the procedures for: efficiently packing and evaluating a HA column, purifying IgG and acidic proteins respectively using HA chromatography.

Key words Hydroxyapatite chromatography, Ceramic hydroxyapatite chromatography, Mixed-mode chromatography, Antibody purification, Column packing, Column evaluation

1 Introduction

Hydroxyapatite (HA) is a naturally occurring calcium-phosphate mineral ($Ca_5(PO_4)_3OH)_2$. HA chromatography (HAC) is one of the most established chromatography methods for protein separation, dating back to 1956 when first described by Tiselius et al. [1, 2]. Protein separation and purification are usually achieved through a series of sequential chromatography processes until the desired purity and product quality is met. HAC is a mixed-mode separation technique that has been used for the purification of both proteins and DNA. Mixed-mode, or multimodal, chromatography resins primarily retain and repel proteins through hydrophobic, ion-exchange, and hydrogen bonding interactions [3, 4]. HAC is distinct in that it operates based on dual mechanisms of

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Table 1
Commercially available hydroxyapatite media

Media	Supplier	Particle size [μM]	pH stability	Dynamic binding capacity [IgG mg/mL media]	Link
Ceramic Hydroxyapatite (CHT™) Type I	Bio-Rad Laboratories, Inc	Available in three sizes: 20, 40, and 80	6.5–14.0	25–60	https://www.bio-rad.com/en-ie/product/cht-ceramic-hydroxyapatite-type-i-media?ID=7b9e4566-4a07-482a-8082-62dfcb1dd0b1
Ceramic Hydroxyapatite (CHT™) Type II	Bio-Rad Laboratories, Inc	Available in three sizes: 20, 40, and 80	6.5–14.0	15–25	https://www.bio-rad.com/en-ie/product/cht-ceramic-hydroxyapatite-type-ii-media?ID=5ded4ec5-ff5a-47b7-bf10-07253005c83f
Ceramic Hydroxyapatite (CHT™) XT	Bio-Rad Laboratories, Inc	36–44	6.5–14.0	≥60	https://www.bio-rad.com/en-ie/product/cht-ceramic-hydroxyapatite-xt-media?ID=P8W75ISCUMB7
Ca^{++}Pure-HA®	Tosoh Bioscience	39	6.5–14.0	≥50	https://www.separations.eu.tosohbioscience.com/solutions/process-media-products/by-mode/hydroxyapatite

ion-exchange (electrostatic interaction) and metal affinity. Due to its unique properties, ability to remove host cell proteins (HCP), product aggregates, leached protein A, DNA, and viruses from a product stream, HAC is used at all stages in downstream processing, that is, from primary capture to final polishing.

Modern HA media are ceramic, macroporous, and spherical in shape. A variety of commercially available ceramic hydroxyapatite (cHA) media are shown in Table 1. The calcium-phosphate mineral undergoes a sintering process at high temperatures where it is compacted into solid ceramic, non-compressible, beads. This sintering process increases the stability of the media making it more robust and suitable for use in commercial-scale purification processes. Importantly, the separation properties of the hydroxyapatite media are retained post-sintering.

The HA calcium-phosphate media, $(Ca_5(PO_4)_3OH)_2$, contains two types of binding sites, the C-site due to positively charged calcium and the P-site due to negatively charged phosphate groups. These sites are distributed throughout the media, that is, the HA media is both the support structure and ligand—which is not the case in most chromatography media. There are a minimum of three distinct mechanisms of interaction between the molecules in the liquid phase and the HA media solid phase: cation-exchange with the P-sites, metal affinity, and anion-exchange with the C-sites [3]. The factors that will determine which mechanism/s will apply to a given process depends on the operating pH, ionic

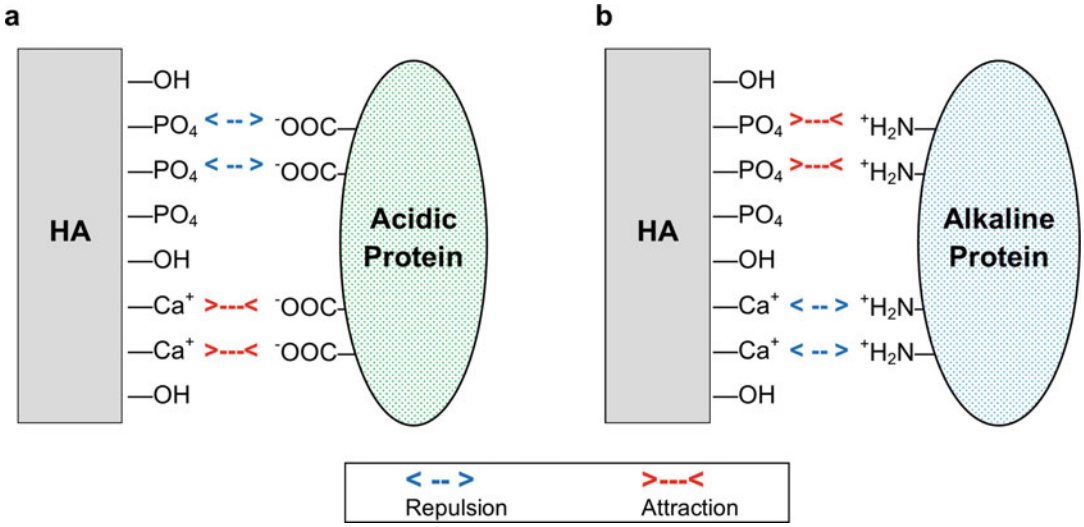

Fig. 1 Schematic representation of protein interaction with HA. (**a**) The carboxyl clusters on acidic proteins are repelled (electrostatically) by PO_4 groups on HA. However, the carboxyl groups form metal coordination complexes with Ca^+ on the HA at acidic and neutral pH. (**b**) Alkaline proteins form ionic bonds between PO_4 and amino groups. However, there is also simultaneous repulsion between Ca^+ and amino groups

strength of the buffers, buffer composition (NaCl and phosphate buffer), and the exposed surface residues on the molecules applied to the column.

Alkaline proteins primarily adsorb to the HA media through cation-exchange of positively charged amino groups with the P-sites. The relative adsorption strength increases as the pH decreases due to the increased charge on these amino groups, (*see* Fig. 1). The elution of alkaline proteins is achieved through an increase in NaCl and phosphate buffer. Eluting alkaline proteins with an increase in NaCl is particularly useful as acidic impurities, for example, DNA, will remain bound to the HA media. Like traditional cation-exchange chromatography (CEX), there is a small correlation between elution and isoelectric point (p*I*). However, in HAC, there is a repulsion between amino groups and the C-sites, which occurs in parallel to the attraction between the amino groups and the P-sites. This dual interaction results in a selectivity that is unique to HAC and distinct from traditional CEX-based separations.

Acidic proteins, that is, proteins that bind strongly to anion exchange media, adsorb to the HA media through metal affinity interaction between carboxyl groups and the C-sites (*see* Fig. 1) [5]. The capacity of the HA media to retain acidic proteins decreases with an increase in pH—this property distinguishes this mechanism from traditional anion-exchange chromatography (AEX) based separations. It is possible to exploit this pH-dependent interaction, between acidic proteins and HA

media, and operate the HAC step in a bind-and-elute mode or flow-through mode. To elute acidic proteins, phosphate buffer is used, which has a strong affinity for C-sites, displacing the bound proteins. An increasing phosphate buffer gradient will elute bound acidic proteins in order of decreasing p*I*.

Phosphoproteins, DNA, and endotoxins interact with the C-sites through their surface phosphate groups. High NaCl solutions will not elute these molecules at a neutral pH. Therefore, phosphate buffer is required to displace the bound molecules from the C-sites. This binding mechanism is utilized for both the removal of cell-derived DNA impurities during the downstream processing of recombinant proteins and for the purification of plasmid DNA [6].

HAC has been used for the purification of IgG since the 1950s and for the purification of IgA and IgM since the 1980s [4, 7, 8]. HAC is not suitable as a primary capture step for IgG due to the composition of the harvested cell culture fluid, that is, the harvested cell culture fluid conductivity and the presence of NaCl and phosphate decrease binding capacity. In addition to this, the presence of chelating agents, which are commonly found in harvested cell culture fluid, may strip calcium from the HA media further decreasing its capacity and stability [4]. A common application of HAC is for the intermediate purification of immunoglobulin proteins following an initial capture with Protein A chromatography. Here, a phosphate buffer or NaCl gradient is used to remove HCPs, DNA, leached Protein A, and endotoxin from IgG monomer. It is possible to achieve two to three log viral clearance across a HAC step where a NaCl gradient is used with a low $NaPO_4$ concentration (*see* Subheading 3.4). A 1000-fold decrease of xMuLV (Murine Leukemia Virus) and a 100-fold decrease of MVM (Minute Virus of Mouse) has been achieved when a human IgG1 (Protein A-purified) was processed on a CHT™ Type I column [9]. HAC has been used for the removal of IgG aggregates, using a phosphate buffer gradient, since the 1990s [4]. A phosphate buffer gradient is also suitable for removing IgA aggregates and for separating IgM monomers from IgM pentamers [10, 11]. However, a phosphate gradient elution is not suitable for all IgGs, so NaCl gradients with a low phosphate concentration are commonly used. This gradient choice enables a significant clearance of aggregated IgG from IgG monomer, and aggregate concentrations up to 60% have been cleared to $<0.1\%$ across a single HAC step [4, 12].

HAC is a flexible chromatography technique that can be applied to a wide variety of purification processes. There are some drawbacks that need to be noted prior to use: the operating pH must remain above pH 6.0, and chelating agents must be avoided in the load material to prevent cHA breakdown. However, the advantages of HAC outweigh these minor limitations. The unique chemistry of HA makes it possible to operate the HAC step in a

mixed-mode state where unique separations that are not possible with traditional individual chromatography methods are achieved. HAC is suited to capture intermediate and polishing chromatography steps, respectively, and is worthy of serious consideration for the purification of biologics within wider downstream processing strategies.

2 Materials

2.1 Column Packing and Evaluation

1. Ceramic HA media: CHT™ Type I, CHT™ Type II and CHT™ XT (Bio-Rad Laboratories, Inc), Ca^{++}Pure-HA® (Tosoh Biosciences).
2. Bench-scale column: Vantage® L Laboratory Column VL 16 × 500 (Merck Millipore) or equivalent.
3. FPLC system: ÄKTA Pure, ÄKTA Avant (Cytiva) or equivalent.
4. Packing buffer: 300 mM $NaPO_4$, pH 7.5 (*see* **Note 1**).
5. Bench-top vortex mixer: Fisherbrand™ Analog Vortex Mixer (Thermo Fisher Scientific Inc.).
6. Column evaluation buffer: 150 mM NaCl in 20 mM phosphate buffer pH 7.3.
7. Column evaluation test solution: 2% Acetone in evaluation buffer, or 1–2 M NaCl.
8. Column storage buffer: 0.1 M NaOH.

2.2 Purification of IgG

1. Pre-equilibration buffer: Reverse osmosis (RO) H_2O.
2. Equilibration/wash buffer: 15 mM $NaPO_4$, 50 mM NaCl, pH 6.7.
3. Elution buffer: 10 mM $NaPO_4$, 0.05–2.00 M NaCl, pH 6.7.
4. Strip buffer: 0.5 M $NaPO_4$, pH 6.7.
5. Pre-sanitization buffer: RO H_2O.
6. Sanitization buffer: 1 M NaOH.
7. Storage buffer: 0.1 M NaOH.

2.3 Purification of Acidic Proteins

1. Pre-equilibration buffer: RO H_2O.
2. Equilibration/wash buffer: 10 mM $NaPO_4$, pH 6.7.
3. Strip buffer: 0.5 M $NaPO_4$, pH 6.7.
4. Pre-sanitization buffer: RO H_2O.
5. Sanitization buffer: 1 M NaOH.
6. Storage buffer: 0.1 M NaOH.

3 Methods

Hydroxyapatite chromatography may be completed at ambient room temperature (18–25 °C). However, the operating temperature may be reduced depending on the stability of the target protein.

3.1 Dry-Packing Method for Bench-Scale Columns

It is possible to pack a small column, diameter 1–4 cm, using the dry-packing method. Here, the resin is added dry to the empty column shell.

1. Determine the target column volume (CV). For example, a 20 cm bed height in a 1.6 cm diameter column shell would have a column volume of 40.2 mL, *see* Eq. 1.

$$\begin{aligned} &\mathrm{CV} = \pi \times \mathrm{radius}^2 \times \mathrm{bed\ height} \\ &\mathrm{CV} = 3.14159 \times 0.8\ \mathrm{cm}^2 \times 20\ \mathrm{cm} \\ &\mathrm{CV} = 40.2\ \mathrm{cm}^3 \mathrm{or\ mL} \end{aligned} \tag{1}$$

2. Determine the quantity of dry resin required. Refer to the product information data sheet for the settled density of the resin. Hydroxyapatite resins have an approximate density of 0.6–0.7 g/mL. For example, a 40.21 mL column would require 26.14 g of dry resin *see* Eq. 2.

$$\begin{aligned} &\mathrm{CV} \times \mathrm{resin\ density} = \mathrm{dry\ resin\ required} \\ &40.21\ \mathrm{mL} \times 0.65\ \mathrm{g/mL} = \mathrm{dry\ resin\ required} \\ &26.14\ \mathrm{g} = \mathrm{dry\ resin\ required} \end{aligned} \tag{2}$$

3. Prepare the column shell, ensuring it is both clean and dry.
4. Prepare the top and bottom column adapters, frits, tubing, and tubing connectors.
5. Mark the target bed height on the column shell with a pen, measuring up from the top of the bottom adapter frit.
6. Prior to opening the dry media, ensure suitable PPE is worn. This includes a laboratory coat, gloves, safety glasses, and a dust mask to prevent inhalation of the dry powder media.
7. Pour the resin into a clean weigh boat and determine its mass using a laboratory balance.
8. Connect the bottom adapter to the column shell.
9. Pour the resin into the column shell (*see* **Note 2**).
10. Place and hold the bottom of the column on a laboratory vortex mixer/shaker for 1–4 min (*see* **Note 3**).

11. Connect the top adapter to the column shell. Move the top adapter down to the top of the settled resin bed. Do not push the top adapter into the resin bed (*see* **Note 4**).
12. Prepare the FPLC system for use, flush the system to remove storage buffer.
13. Hydrate the settled resin bed with packing buffer in reverse flow at 20–60 cm/h for a minimum of 2 CV (*see* **Notes 1** and **5**).
14. Pack the column in forward flow at 500–1000 cm/h for a minimum of 10 CV (*see* **Note 6**).
15. Move the top column adapter down to the top of the column bed.
16. Evaluate the packed column, *see* Subheading 3.3.

3.2 Slurry Packing for Bench to Process-Scale Columns

The slurry packing method is suitable for all column sizes from bench-scale to pilot-scale and process-scale columns.

1. Determine the target CV and quantity of dry media required, *see* Subheading 3.1.
2. Prior to opening the dry media, ensure suitable PPE is worn. This includes a laboratory coat, gloves, safety glasses, and a dust mask to prevent inhalation of the dry powder media.
3. Determine the volume of packing buffer required for a 50% (v/v) media slurry (*see* **Note 1**).
4. Add the phosphate-NaCl packing buffer to a suitable container.
5. Slowly pour in the dry media while mixing gently with a plastic paddle (*see* **Note 7**).
6. Prepare the column and connect the adapters to the FPLC instrument or bioprocess skid.
7. Prime both column adapters to remove air from the tubing, connectors, adapters, and frits.
8. Introduce packing buffer to the column, so the bottom frit is covered with 1–2 cm of packing buffer (*see* **Note 8**).
9. For bench-scale and pilot-scale columns pour the homogeneous slurry into the column shell (*see* **Note 9**).
10. For large process-scale columns, transfer the media slurry to the column shell with a pump and set the column to run in reverse flow at 50–100 cm/h during the slurry transfer (*see* **Note 10**).
11. Connect the top adapter and pack the column by flow with a velocity of 500–1000 cm/h for 10 CV (*see* **Note 11**).

12. Move the top column adapter down to touch the top of the column bed. Do not compress the column bed.
13. Evaluate the packed column, *see* Subheading 3.3.

3.3 Column Evaluation

The packed column must be evaluated prior to use in a chromatography process. There are two key parameters that are calculated to evaluate the packed column [13, 14].

(a) The number of theoretical plates (N), or the height equivalent of a theoretical plate (HETP). HETP allows for columns of various height to be directly compared. A smaller plate height corresponds to increased separation efficiency.

(b) The peak asymmetry factor (As) which is a measure of how symmetrical the peak is (*see* **Note 12**).

$$\mathrm{HETP} = L/N \tag{3}$$

$$N = 5.54 \times \left(V_r{}^2 / W_h{}^2\right) \tag{4}$$

$$\mathrm{As} = b/a \tag{5}$$

$$H = \mathrm{HETP}/d \tag{6}$$

Where L is column length, N is number of theoretical plates, V_r is the retention volume at peak maximum, W_h is the peak width at 50% height, a is the width of the left side of the peak at 10% peak height, b is the width of the right side of the peak at 10% peak height, H is the number of beads per plate, and d is the mean bead diameter (*see* Fig. 2).

1. Prepare the test solution. An acetone solution (1–2% v/v in evaluation buffer) or 1–2 M NaCl may be used.
2. Refer to the resin product information sheet for the recommended evaluation buffer. Typically, a phosphate-NaCl buffer is used.
3. Equilibrate the column with a minimum of 5 CV of evaluation buffer at 50–150 cm/h (*see* **Note 13**).
4. Inject the test solution, 2.5% of CV.
5. Chase the test solution with 3 CV of equilibration buffer at 50–150 cm/h.
6. Store the column in 0.1 M NaOH or recommended storage buffer, minimum of 3 CV at 50–150 cm/h.
7. Determine the *HETP* and *H* (*see* **Note 14**).
8. Determine the As. A value between 0.8 and 2.0 is usually acceptable.

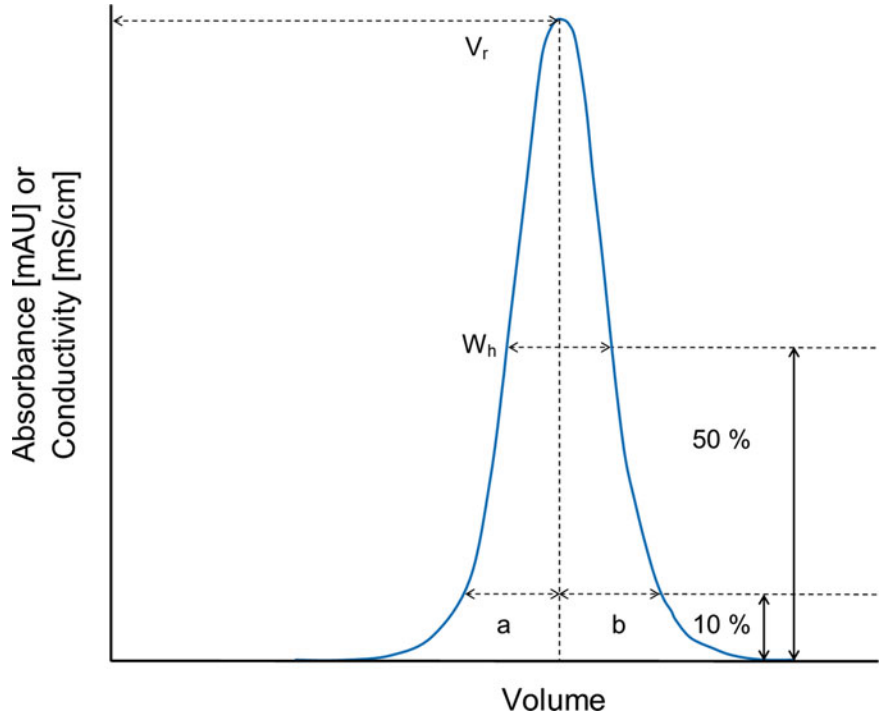

Fig. 2 Schematic chromatogram showing the absorbance or conductivity trace with volume from the column evaluation step

3.4 Purification of IgG

A common application of ceramic HAC is the purification of IgG. HAC is a powerful polishing step to follow directly after Protein A chromatography where aggregates, cell-based impurities (HCP, DNA, and viruses), and process-based impurities (e.g., leached protein A) are removed resulting in an IgG monomer eluate of high purity. The method below describes a generic gradient elution protocol for the purification of IgG. The gradient elution phase is suitable for initial bench-scale screening runs and can easily be adapted to an isocratic elution phase once the most optimal elution conditions are identified for the target protein. This adapted, product-specific method is suitable for both bench-scale and process-scale purifications. A representative chromatogram illustrating the purification of IgG using a bind-and-elute strategy is shown in Fig. 3.

Complete all steps at 150–300 cm/h. The outlet is set to waste for all steps except the elution step.

1. Pre-equilibration rinse: 1 CV of RO H_2O (*see* **Note 15**).
2. Equilibration: 10 CV of equilibration buffer.
3. Sample application: Target 10–40 g/L resin load challenge (*see* **Note 16**) with a 0.2 μM filtered sample adjusted to 10 mM $NaPO_4$, 50 mM NaCl, pH 6.7 (*see* **Note 17**).
4. Wash: 5 CV of equilibration buffer (*see* **Note 18**).

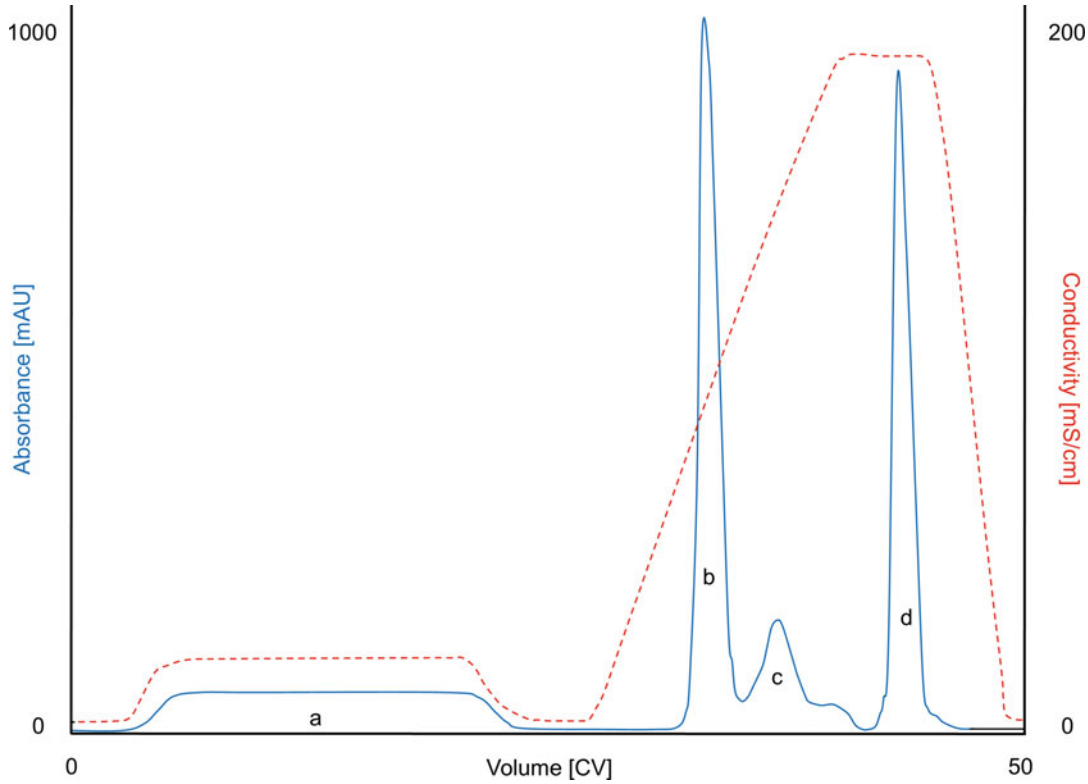

Fig. 3 Schematic chromatogram showing the absorbance and conductivity traces with volume from a bind-and-elute purification of IgG with a NaCl gradient elution. (**a**) The sample application phase where the absorbance (at 280 nm) and conductivity increase and plateau. The protein contributing to the absorbance (at 280 nm) signal here is not retained in the column but flow through (*see* **Note 23**). (**b**) The IgG monomer peak. An increase in NaCl concentration dissociates ionic bonds which results in monomer IgG eluting first. (**c**) The aggregate peak containing dimers and high molecular weight species, generally eluted in order of size. (**d**) The strip peak where non-IgG proteins (HCPs) and DNA are removed by an increase in conductivity and phosphate buffer concentration

5. Elution: 25 CV gradient from equilibration buffer to elution buffer, 10 mM $NaPO_4$, 2.00 M NaCl, pH 6.7. Outlet position: Waste then switch to collection after 0.5 CV (*see* **Note 19**).
6. Clean: 5 CV of cleaning/strip buffer (*see* **Note 20**).
7. Pre-sanitization rinse: 1 CV of RO H_2O (*see* **Note 21**).
8. Sanitization: 4 CV of sanitization buffer (*see* **Note 22**).
9. Storage: 3 CV of storage buffer.

3.5 Purification of Acidic Proteins

HAC is suitable for the purification of acidic proteins in both bind-and-elute mode and flow-through mode. The mode of operation will largely depend on the degree of target protein interaction/repulsion with the HA media when the load material is adjusted to 5–10 mM $NaPO_4$ pH 6.5–7.0. The position of the HA step in the downstream process is to be considered when determining the

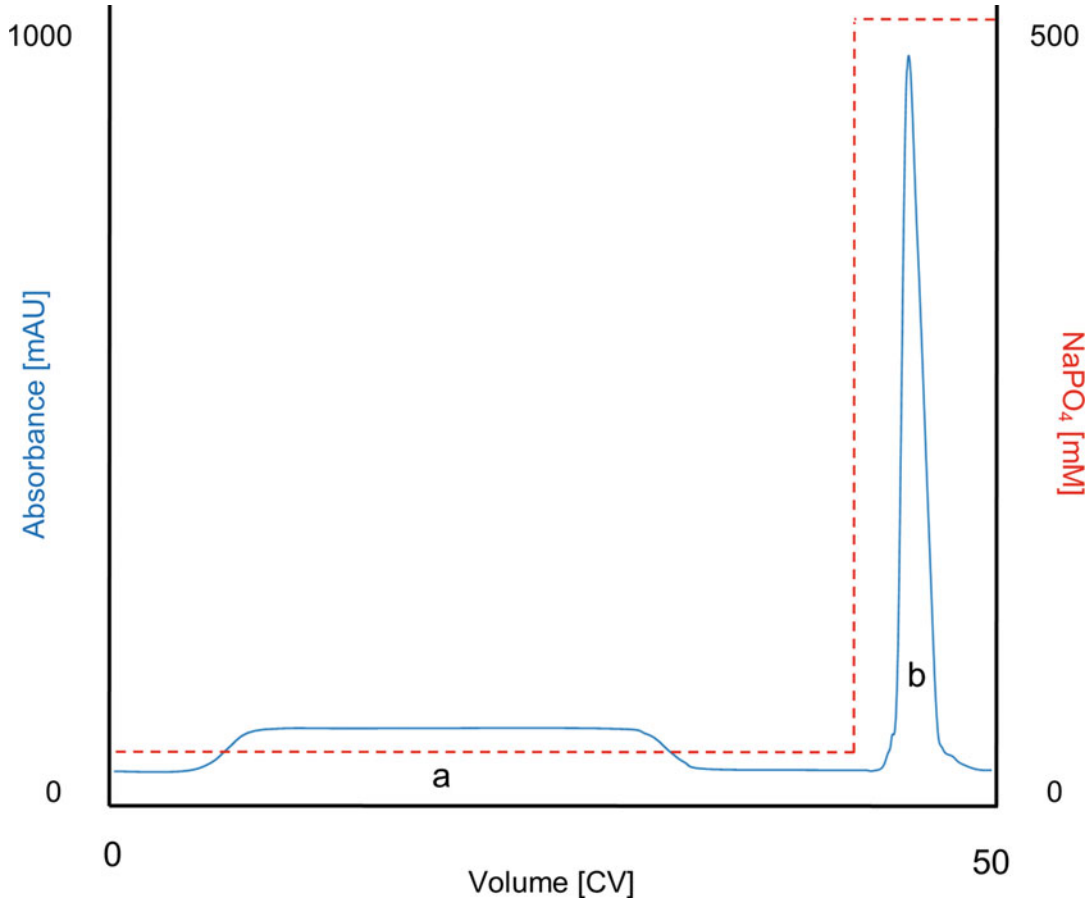

Fig. 4 Schematic chromatogram showing the absorbance and phosphate buffer concentration with volume from a flow-through purification of an acidic protein. (**a**) The sample application and wash phases where the absorbance (at 280 nm) increases, plateaus, and returns to baseline. The protein contributing to the absorbance (at 280 nm) signal here is the target protein which is not retained in the column. (**b**) The strip peak where non-target proteins (HCPs) and DNA are removed by an increase in phosphate buffer concentration

mode of operation for the step, that is, a flow-through mode will dilute the product pool and result in larger volumes further downstream. HAC operated in flow-through mode is comparable to a bind-and-elute mode with respect to impurity reduction. The method described below and illustrated in Fig. 4 is a generic flow-through protocol for the purification of acidic proteins. Scouting runs should be performed to identify optimal phosphate buffer concentration and pH for the target protein to minimize product loss and maximize impurity clearance. This method is suitable for both bench-scale and process-scale purifications. A representative chromatogram illustrating the purification of an acidic protein using a flow-through strategy is shown in Fig. 4. Complete all steps at 150–300 cm/h. The outlet is set to waste for all steps except the elution and wash steps.

1. Pre-equilibration rinse: 1 CV of RO H_2O (*see* **Note 15**).
2. Equilibration: 10 CV of equilibration buffer.
3. Sample application: Target 10–30 g/L resin load challenge (*see* **Note 16**) with a 0.2 μM filtered sample adjusted to 10 mM $NaPO_4$, pH 6.7 (*see* **Note 17**). Outlet position: Waste then switch to collection after 0.5 CV.
4. Wash: 5 CV of equilibration buffer.
   Outlet position: Collect the first 1–5 CV with product (*see* **Note 24**).
5. Clean: 5 CV of cleaning/strip buffer (*see* **Note 20**).
6. Pre-sanitization rinse: 1 CV of RO H_2O (*see* **Note 21**).
7. Sanitization: 4 CV of sanitization buffer (*see* **Note 22**).
8. Storage: 3 CV of storage buffer.

4 Notes

1. Refer to the product information sheet for the most appropriate packing buffer. Typically, a phosphate-NaCl buffer or NaOH solution is used.
2. A small funnel may help minimize resin loss during the transfer of the dry resin to the column shell.
3. Check the column bed height against the pen mark on the column shell. Hold for a further 1–2 min on the vortex mixer/shaker if the resin is not fully settled.
4. Hydroxyapatite resins are rigid and not compressible.
5. Reverse or upward flow must be used for hydrating the resin bed. This pushes air upward and out of the column bed.
6. The packing flowrate should be a minimum of 50% greater than the maximum operating flow rate of the chromatography process.
7. HA media will settle quickly. Mixing is required to keep the media in suspension. However, the mixing must be gentle to prevent the generation of fines. For large process-scale volumes a low-shear impeller is used, set just high enough to prevent media settling.
8. This covers the bottom frit ensuring no air is introduced to the column bed during the media transfer.
9. It is important to ensure the slurry is homogeneous to achieve an efficiently packed column. The slurry should be mixed directly before addition to the column.
10. This reverse flow prevents the media settling during the transfer to the column.

11. The column may also be packed by gravity settling. Here, no flow is applied, and the media is consolidated by gravity settling only. Once finally settled, the top adapter is moved down to touch the top of the settled bed.
12. A normal (Gaussian) distribution would have an *As* value of 1.0.
13. It is important to equilibrate the column prior to the injection of the test solution to remove all traces of packing buffer or storage buffer.
14. Refer to the product information sheet for the acceptable *HETP* and *H* ranges, ≤0.05 cm for HETP and 4–7 for *H* are satisfactory.
15. This rinse step reduces the NaOH concentration in the column from the storage buffer prior to the addition of the equilibration buffer, containing phosphate, which may lead to trisodium phosphate precipitate formation.
16. The target resin load challenge is the amount of target protein loaded (g) per unit volume of the column (L), expressed at g/L. The parameters that will determine what load challenge is feasible include the resin load capacity, the buffer conditions, concentration of non-target protein in the load material and the desired level of purity in the eluate/flow-through collected.
17. The load material is adjusted to match the phosphate buffer and NaCl concentrations in the equilibration buffer, to retain the stability of the hydroxyapatite during the sample application phase.
18. The wash step removes non-bound material from the column.
19. The gradient elution step will result in a series of peaks, which may be fractionated and analyzed individually to identify their composition, that is, IgG monomer, IgG aggregate, and non-IgG protein.
20. The cleaning buffer with a high phosphate concentration elutes remaining proteins (including leached protein A) and DNA from the column that were not eluted during the elution step. This is an important step to ensure the column retains full binding capacity for subsequent purifications.
21. This rinse step reduces the phosphate buffer concentration in the column from the cleaning buffer prior to the addition of the sanitization buffer, containing NaOH, which may lead to trisodium phosphate precipitate formation.
22. The flowrate for the sanitization step may be reduced to achieve a minimum contact time for the sanitization buffer in the column, for example, ≥60 min.

23. A further increase in the absorbance (at 280 nm) from the plateau would indicate that the column is fully saturated, and breakthrough is occurring. During initial screening runs, it is important to assess the capacity of the column, and overloading will result in a lower % product recovery and impact the purity of the eluate.
24. The optimal wash volume for the target protein is determined experimentally. Collecting 1 CV will minimize impurity carryover, while collecting 5 CV will maximize product recovery.

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Chapter 11

Poly-Histidine-Tagged Protein Purification Using Immobilized Metal Affinity Chromatography (IMAC)

Sinéad T. Loughran, Ronan T. Bree, and Dermot Walls

Abstract

His-tagging is the most widespread and versatile strategy used to purify recombinant proteins for biochemical and structural studies. Recombinant DNA methods are first used to engineer the addition of a short tract of poly-histidine tag (His-tag) to the N-terminus or C-terminus of a target protein. The His-tag is then exploited to enable purification of the "tagged" protein by immobilized metal affinity chromatography (IMAC). In this chapter, we describe efficient procedures for the isolation of highly purified His-tagged target proteins from an *Escherichia coli* host using IMAC in a bind-wash-elute strategy that can be performed under both native and denaturing conditions.

Key words IMAC, Protein purification, Polyhistidine, 6xHistidine, Affinity chromatography, Ni-NTA

1 Introduction

Affinity tags are highly efficient tools for purifying recombinant proteins from crude extracts (*see* Chapter 7). The use of genetically engineered affinity tags for improved protein purification is well established, and affinity tags have become indispensable tools for structural and functional proteomics initiatives. The most commonly employed method utilizes immobilized metal affinity chromatography (IMAC) to purify recombinant proteins containing a short affinity tag consisting of polyhistidine (poly His) residues [1]. Histidine residues are infrequently found in globular proteins, amounting to approximately 2% of the amino acid content. At pH 8.0, the tag is small (0.84 kDa) and uncharged and therefore does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell. The popularity of His-tag IMAC is due in part to its high affinity with commercially available Ni-NTA (nickel 2+ ion that has been coupled to nitrilotriacetic acid) resin, which is a relatively inexpensive matrix capable of

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withstanding multiple regeneration cycles under stringent conditions but also due to the ease of controlled release using mild (e.g., non-denaturing) conditions. As the tertiary structure of the His-tag is not important for purification, an insoluble His-tagged recombinant protein can be purified by IMAC under denaturing conditions and subsequently refolded (for reviews, *see* [2] and [3]). The 6xHis affinity tag is technically considered poorly immunogenic (for a review *see* [4]); therefore, it is usually not necessary to remove the tag for the purposes of antibody generation, although consideration should be given to this depending on the application [5]. Additionally, in most cases, the 6xHis tag does not interfere with the structure or function of the purified protein as demonstrated for a wide variety of proteins, including enzymes, transcription factors, and vaccines. An additional advantage of this system is that anti-His antibodies can be used for the detection of tagged recombinant proteins during expression and purification steps [6]. The His-tag can also be directly detected by metal ion-loaded nickel-nitrilotriacetic acid-based chelator heads conjugated to fluorophores, which is a convenient alternative method to immunoblotting [7].

IMAC is based on the interaction between a transition metal ion (in this case, the nickel ion, Ni^{2+}) immobilized on a matrix and specific amino acid side chains (usually 6xHistidine residues). Hochuli et al. (1987) developed a tetradentate chelating adsorbent, nitrilotriacetic acid (NTA), for metal-chelate affinity chromatography [8]. NTA is an aminopolycarboxylic acid with a tripodal tetradentate nature, able to form coordinative interactions with divalent metal ions in a bonding type called chelation [4]. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal [9]. Several groups have developed novel chelating ligands with a number of advantages: increased binding capacity for efficient purification [10], improved selectivity for increased product purity [10], reduced ion leakage [11], and high ion stability that negates the need to recharge the resin after every cycle [12].

IMAC resins have relatively low binding capacities compared to other chromatography resins [13]. In a study to generate a robust IMAC purification process, the binding differences of resin and metal chelator combinations were investigated by generating breakthrough curves with a poly-histidine-tagged bispecific protein [14]. It was found that for protein expressed in CHO cells, Fractogel® EMD Chelate M (Merck Millipore) charged with Zn^{2+} and pre-charged Ni Sepharose® Excel (Cytiva) displayed the highest binding capacities. When the protein was expressed in HEK-293

cells, IMAC Sepharose® 6 Fast Flow (Cytiva) charged with either Co^{2+} or Zn^{2+} bound the greatest amount of protein. Interestingly, this study further identified that the metal binding capacity of the resin lot, the protein capacity to which the resin is loaded, and the ratio of poly-histidine tag residues on the protein all impacted the chromatographic performance and product quality [14].

Strategies to develop more sustainable or "green" approaches to recombinant protein expression and purification are required for the "green economy" transition and to reduce ecological impact (for a summary of the different supports or matrices used in recent years for tag-mediated protein purification and/or immobilization, *see* [15]). One such advance in the search for alternative matrices for His-tagged protein purification has led to the use of bare silica, a low-cost, earth-abundant, and eco-friendly affinity matrix [16]. Adsorption and desorption studies with a purified His6-tagged EGFP showed that binding to bare silica particles of different size and porosity occurred under the conditions tested and that elution could be accomplished with eco-friendly eluants containing L-arginine/L-lysine.

High-level expression of 6xHis-tagged proteins in *Escherichia coli* (*E. coli*) is achieved in this chapter using vectors from the pQE range (QIAgen) (*see* Table 1), which contain a regulatable promoter-operator element consisting of the T5 promoter and two *lac* operator sequences that increase *lac* repressor binding and ensure efficient repression of the powerful T5 promoter (recognized by the *E. coli* RNA polymerase). The *lac* operon promoter system is the most widely used bacterial expression system, encodes

Table 1
Expression systems for His-tagged recombinant protein expression

Plasmid	Source	Description
pQE60/pQE30	QIAgen. www.qiagen.com	High-copy number expression vectors for expression of C- or N-terminal 6xHis-tagged fusion proteins, respectively
pGSLink	*See* [17]	Expression vector for expression of N- or C-terminal 6xHis-tagged fusion proteins linked to protein of interest via a flexible peptide linker [17]
pTrcHis A, B, & C	Thermo Fisher https://www.thermofisher.com/	Expression vectors for C- or N-terminal 6xHis-tagged fusion proteins
pET vector series 14–16 and 19–52	Merck Millipore https://www.merckmillipore.com/	pET vectors permit cloning, detection, and purification of target proteins

proteins that facilitate the uptake and metabolism of β-galactosides, and is subject to both negative regulation (by binding of the *lac* repressor protein to the *lac* operator and preventing transcription) and positive regulation (by binding of an activator to the *lac* promoter and stimulating transcription). IPTG (an allolactose analog; isopropyl-1-thio-β-D-galactopyranoside) is a gratuitous inducer of the *lac* operon, insofar as it competes strongly with the *lac* operator for binding to the *lac* repressor yet is not metabolized in the process and can, thus, be used to induce expression. Recombinant constructs derived from the pQE vectors can be generated such that the encoded 6xHis tag is located at the N-terminus or the C-terminus of the protein of interest (*see* Table 1). The pQE30 vector allows for the incorporation of a N-terminal 6xHis tag, while the pQE60 vector facilitates C-terminal tagging with 6xHis. The C-terminal placement of the 6xHis tag ensures that only full-length proteins are subsequently purified.

Many reports have investigated the positioning of the His-tag, some directly comparing the effect of the position of His-tag in the protein and reporting differential effects [18–20] Song et al. [20] investigated the effect of the polyHis-tagging site on the stability of recombinant alginate lyase from a marine bacterium *Streptomyces* species ALG-5 by the combined use of microchip electrophoresis and an enzymatic depolymerizing activity assay. The authors report that in microchip electrophoresis, C-terminally His-tagged alginate lyase (C-His-AL) was more stable than N-terminally His-tagged alginate lyase (N-His-AL) after the incubation in 50 mM potassium phosphate buffer (pH 7.0) at 37 °C for 14 days. When the enzymatic depolymerizing activity of the same samples was measured, the activity of C-His-AL was not significantly changed for 14 days, whereas N-His-AL showed substantially declined activity after incubation. Elsewhere, Kutyshenko and colleagues studied the effect of the C-terminally attached His-tag and IMAC purification on the activity and structure of the metalloenzyme, L-alanyl-D-glutamate peptidase of bacteriophage T5 (EndoT5), whose zinc binding site and catalytic aspartate are located near the C-terminus. The His-tag by itself did not have a significant effect on either activity or folding of the polypeptide chain, nor on the binding of zinc and calcium ions to the protein. However, the His-tagged EndoT5 samples had low shelf-life, with storage of these samples resulting in an increased propensity for protein self-association and decreased enzymatic activity of EndoT5 [21].

Costa et al. [22] describe the benefits of N-terminus tagging. However, certain features can guide the researcher to either tag at the N- or C-terminus; for example, Cline et al. [23] used N-terminal His-tagging as the C-terminus of their protein of interest was considered to play a role in DNA binding.

Table 2
***E. coli* host strains for recombinant protein expression**

Bacterial strain	Description	Genotype
***E. coli* M15 [pREP4]** (QIAgen)	General expression host; cells contain pREP4 plasmid encoding *lac* repressor in trans, ensuring tightly regulated expression	*NaI*^{S}, *Str*^{S}, *Rif*^{S}, *Thi*$^-$, *Lac*$^-$, *Ara*$^+$, *Gal*$^+$, *Mtl*$^-$, *F*$^-$, *RecA*$^+$, *Uvr*$^+$, *Lon*$^+$ pREP4 (*lacI*^{q}) (Kan^R)
***E. coli* RosettaBlue™**(*MerckMillipore*)	Expression host; provides six rare codon tRNAs	*end*A1, *hsd*R17(r_{K12}^-,m_{K12}^+), *sup*E44, *thi-1*, *rec*A1, *gyr*A96, *rel*A1, *lac*[F′ *pro*A$^+$B$^+$ *lacI*^{q}ZΔM15::Tn10(tet^R)] pRARE(argU, argW, ileX, glyT, leuW, proL) (Cm^R).
***E. coli* Origami™ (DE3)pLysS** (Merck Millipore)	Expression host; two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm	Δ*ara–leu*7697 Δ*lac*X74 Δ*phoA PvuII phoR araD139 ahpC galE galK rpsL F′ [lac*$^+$ *lacI*^{q} *pro]* (DE3) *gor522*::Tn*10 trxB* pLysS (Cam^R, Kan^R, Str^R, Tet^R)
***E. coli* BL21(DE3)** Δ*cyoA*Δ*yfbG*Δ*adhP* [25]	BL21(DE3) mutant knock-out strain deficient in three prevalent host proteins found in a strategic fraction of an elution profile Ni-IMAC	F – *ompT hsdS*$_B$(*r*$_B$– *m*$_B$–) *gal dcm* (DE3) Δ*cyoA*Δ*yfbG*Δ*adhP*
***E. coli* XL10-Gold™** (Agilent)	General expression host, high efficiency transformation	Tet^R, Δ(μχρA)183 Δ(mcrCB-hsdSMR-mrr)173, *end*A1, *sup*E44, *thi-1*, *rec*A1, *gyr*A96, *rel*A1, *lac* Hte[F′ *pro*AB *lacI*^{q}ZΔM15 Tn10(tet^R) Amy Cam^R]

E. coli is an advantageous organism for the production of recombinant protein for several reasons including inexpensive growth media, rapid biomass accumulation, amenability to high cell-density fermentations, and simpler process scale-up [24]. Generally, most recombinant proteins can be cloned and expressed at high levels in *E. coli* (*see* **Note 1**). However, many polypeptide gene products expressed in this host accumulate as insoluble aggregates that lack functional activity. Furthermore, problems with toxicity toward the host cell, protein instability, improper processing, or posttranslational modification and inefficient translation may also be experienced. The use of *E. coli* strain M15 [pREP4] (*see* Table 2; [26]) is advantageous for high-level recombinant protein expression using pQE vectors as the pREP4 plasmid constitutively expresses the Lac repressor protein. *E. coli* strains that harbor the *lacI*^{q} mutation (including RosettaBlue™ (Merck Millipore) and XL

10-Gold™ (Agilent) (*see* Table 2)) also produce enough Lac repressor to efficiently repress the T5 promoter. The formation of stable disulfide bonds is a requirement for proper folding and activity of some recombinant proteins. Without disulfide bonds, these proteins may be degraded or accumulate as inclusion bodies. Bacterial strains with glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) mutations (e.g., Origami™ strains; Merck Millipore (*see* Table 2)) greatly enhance the formation of disulfide bonds in the *E. coli* cytoplasm [27]. Codon usage can be an issue when expressing recombinant genes in a heterologous cell context [28]. Codon use preferences reflect the amounts of corresponding cellular tRNA levels suggesting that recombinant genes containing rare codons may be subject to slower translation due to non-saturating amounts of corresponding tRNAs in the host cell [29, 30]. If the coding sequence contains codons that are infrequently used by *E. coli*, then the protein may not be expressed due to this translational limitation (*see* **Note 2**). Finally, proteomics has been used to design a host cell tailored for highly efficient protein purification in isolation from co-purifying contaminants [25] (*see* Table 2 and **Note 3**). In practice, it is usually worthwhile to test a matrix of several different vector/host combinations to obtain the best possible yield of protein in its desired form (for a review, *see* [31]).

There have been numerous advances in many development areas involving His-tag protein production and purification. Ren et al. [32] described a new purification method expanding on the development of affinity ionic liquids for liquid-liquid extraction and purification of His-tag proteins aimed at reducing costs and working on larger scales. In their study, His-tagged proteins were extracted and purified with a newly synthesized affinity ionic liquid, 1,4,7-triazacyclononane-attached 6,7-dihydro-5H-pyrrolo [1,2-a] imidazolium, in a system that demonstrated a high affinity for His-tagged proteins.

Expanding on the primary goal of purification, many others have reported the use of combinatorial approaches involving His-tags. Mixed-mode chromatography is an expanding field and is discussed elsewhere in this volume (*see* Chapter 2 for a review of the concept and benefit of mixed-mode chromatography for protein purification). For example, Cass et al. [33] described the rapid and efficient purification of a recombinant protein containing two affinity tags at the C-terminus: a His8-tag and a Strep-tag II, purified by sequential steps. Elsewhere, Freitas et al. have shown that His6 and Car9 (a dodecapeptide identified by cell surface display for its ability to bind to the edge of carbonaceous materials [34]) can be used in combination to increase the strength of the binding to an alternative silica matrix, a characteristic that can be explored for immobilization or one-step purification and immobilization processes [16].

To assist with the development of large-scale or high-throughput purification, Liao et al. [35] incorporated the use of silica/boron-coated magnetite nanoparticles to successfully purify both C-terminal and internal 6xHis-tagged proteins successfully within 1 h. Li and colleagues [36] and Yao et al. [37] expanded on this approach further, synthesizing Fe_3O_4/Cu-apatite and Fe_3O_4/hydroxyapatite nanoparticles, respectively, to magnetically separate His-tagged proteins directly from mixtures of lysed cells, with negligible nonspecific adsorption. Elsewhere, Zhang and colleagues describe a method for the efficient purification of His-tagged protein by superparamagnetic Fe_3O_4/Au nanoparticles. Co^{2+} ions in the magnetic nanoparticle shell provided a docking site for histidine, and the nanoparticles exhibited excellent binding capacity (74 μg/mg) and selectivity for the purification of a His-tagged protein from *E. coli* lysate [38]. Fraga Garcia and colleagues [39] developed superparamagnetic core–shell nanocarriers functionalized with a pentadentate chelate affinity ligand and loaded with metal ions (Cu^{2+}, Ni^{2+}, or Zn^{2+}). The charged particles, which can be loaded to a high capacity (250 mg/g) with cell lysate containing protein, were magnetically separated, and protein was eluted at high purity (>98%) and good selectivity.

While the above advances outline developments to allow purifications to be scaled up, the principle of His-tag purification remains the same. More creative applications include those of Ośliźło et al. [40] and Ceglarek et al. [41] who describe the purification of modified bacteriophage T4 from bacterial debris and other contaminating bacteriophages. The process involved incorporating affinity tags, such as His- and Glutathione S-transferase-(GST) tags, into modified T4 capsids via the in vivo phage display technique allowing specific phage to be purified using specific resins.

In a recent development, Lai et al. [42] describe their application of the first, highly specific, small membrane permeable, fluorescent probe (Ni-NTA-AC), capable of binding to intracellular His-tagged proteins in multiple live cell types, even plant cells. The probe utilizes its Ni^{2+}-NTA to target this His-tag and contains ~13-fold fluorescent "turn on" responses. Arylazide is also incorporated to overcome any weak binding between histidines and the Ni^{2+}. While there was minor background staining in certain types of cells (identified to occur when the probe detected endogenously expressed histidine rich proteins), the authors do not believe this will be a problem with overexpressed proteins. These findings build on earlier research such as that described by Murata and colleagues [43] where the authors outlined a "turn on" fluorescent probe system whereby a fluorescent receptor was only turned on when a quencher is replaced by a His-tagged protein, an approach that eliminated the requirement for washing steps to remove unbound fluorescent probes. In a similar application whereby the

presence of a His-tag is used as a binding site, Badar et al. [44] outline the concerns in radiolabeling small proteins. Building on the initial developments of Waibel et al. [45] who used a tricarbonyl method to radiolabel His-tagged proteins with technetium (99mTc), the study describes the expression of a recombinant complement receptor protein (CR2) containing a C-terminal His-tag. However, in addition to facilitating purification, the primary role of the His-tag in this study is to enable site-specific labeling with 99mTc, allowing imaging of activated complement. This radiolabeling approach is one that can easily be used in a wide range of in vitro diagnostic approaches and potentially be extended to include radiotherapy [45].

His-tagging protein targets have also been extended to include oligonucleotide or aptamer technology [46, 47]. Shimada and colleagues [46] report the generation of an oligo-DNA nucleotide aptamer, modified with NTA, which is used in combination with Ni^{2+}, to possess a high affinity for His-tagged recombinant proteins. The authors applied this NTA-modified aptamer technology to ultimately develop a competitive thrombin detection system allowing the concentration of thrombin in a solution to be quantitatively determined.

In 2009, further advances involving aptamer technology and His-tagged proteins were established with a report that a certain isolated RNA aptamer, referred to as shot47, could be used instead of antibodies against His-tags in techniques such as ELISA, immunoprecipitations, and Western blotting [47]. These findings highlight the potential of aptamers to function as cost-effective reagents to detect, purify, and interact with His-tagged targets.

In this chapter, we outline an example of the expression in *E. coli* (RosettaBlue™ host strain) of a His-tagged recombinant protein and describe procedures for His-tagged fusion protein purification using IMAC. Here, we express and purify recombinant human Bfl-1, a member of the intensively researched *bcl-2* gene family that is associated with regulating apoptosis in eukaryotic cells [48].

2 Materials

Use Milli-Q-purified water or equivalent for the preparation of all buffers. Reagents may be acquired from Sigma-Aldrich unless otherwise stated. Use high-quality chemicals. Solutions should be filtered through 0.45 or 0.22 μm filters. Care must be taken to avoid a potential problem when using His-tagged proteins in assays with cells cultured on tissue culture polystyrene. Holmberg and colleagues [49] compared histidine-tagged and native proteins with regard to adsorption properties and observed significantly

increased adsorption of proteins with an incorporated polyHis-tag onto tissue culture polystyrene compared to similar proteins without a His-tag. The authors attributed the effect to electrostatic interactions between negatively charged carboxylate groups on the tissue culture polystyrene surface and positively charged histidine residues in the proteins.

2.1 High-Level Expression of Recombinant His-Tagged Proteins

1. Glycerol stock of transformed *E. coli* (*see* Table 2) cells expressing the His-tagged protein of interest in a suitable vector (*see* Table 1).
2. Luria-Bertani (LB) medium per L: 10 g tryptone, 5 g yeast, 5 g NaCl. Adjust to pH 7.2 with NaOH. Autoclave and store at 4 °C.
3. Ampicillin, stock solution; 100 mg/mL in deionized water, filter sterilize and store at −20 °C. Use at 100 μg/mL within 1 month.
4. Chloramphenicol stock solution; 34 mg/mL in ethanol, store at −20 °C. Use at 34 μg/mL (to maintain pRARE plasmid in RosettaBlue™ host strain).
5. Tetracycline, stock solution; 5 mg/mL in ethanol, store at −20 °C. Use at 12.5 μg/mL (to maintain pRARE plasmid in RosettaBlue™ host strain).
6. Orbital shaker.
7. Spectrophotometer.
8. Cuvettes.
9. Isopropyl-1-thio-β-D-galactopyranoside (IPTG), 100 mM stock; dissolve 2.38 g IPTG in 100 mL deionized water. Filter sterilize and store at −20 °C.
10. Glucose; 20% (w/v) D-glucose solution in deionized H_2O. Autoclave and store sterile solution at room temperature. Add glucose to LB agar with antibiotics to a final concentration of 0.5–1%.
11. Centrifuge.
12. Sonicator.
13. Lysis Buffer A (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 6 M guanidine hydrochloride; 573 g. Adjust pH to 8.0 using NaOH.
14. Lysis Buffer B (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 8 M urea; 480.5 g (MW 60.06 g/mol). Adjust pH to 8.0 using NaOH.

2.1.1 Protein Analysis

1. SDS loading buffer (2×); 100 mM Tris–HCl (pH 7.6), 4% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.2% bromophenol blue. Store at room temperature.

2.1.2 SDS-Polyacrylamide Gel Electrophoresis of Proteins

1. Protein gel electrophoresis system (e.g., ATTO system).

Preparation of SDS-Polyacrylamide Gels

1. Rainbow full range molecular weight marker (Cytiva).
2. Acrylagel, Bis-acrylagel (National Diagnositcs). Acrylagel is toxic, and a known carcinogen. Bis-acrylagel is an irritant. Consult the corresponding material safety data sheet (MSDS) before use.
3. 1.5 M Tris–HCl (pH 8.8).
4. 1 M Tris–HCl (pH 6.8).
5. Deionized water; dH_2O.
6. 10% (v/v) SDS (dissolved in dH_2O).
7. 10% (v/v) Ammonium persulphate (APS) (dissolved in dH_2O). APS is a strong and harmful oxidizing agent. Consult the corresponding MSDS before use.
8. TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine).
9. 10% (v/v) resolving gels and 5% (v/v) stacking polyacrlyamide gels (*see* Table 3).
10. 5 × Tris-glycine running buffer; 15.1 g Tris base, 95.4 g glycine (pH 8.3), 50 mL 10% (w/v) SDS. Make up to 1 L with dH_2O and store at room temperature.
11. 1 × Tris-glycine running buffer; 200 mL 5 × Tris-glycine running buffer, 800 mL dH_2O.

Table 3
SDS-polyacrylamide gel composition

Component	10% Resolving gel (mL)	5% Stacking gel (mL)
Acrylagel	3.33	0.42
Bis-Acrylagel	1.35	0.168
1.5 M Tris–HCl (pH 8.8)	2.5	0
1 M Tris–HCl (pH 6.8)	0	0.312
dH_2O	2.61	1.5475
10% (v/v) SDS	0.10	0.025
10% (v/v) APS	0.10	0.025
TEMED	0.01	0.0025
Total	10 mL	2.5 mL

2.1.3 Coomassie Blue Staining

1. Destain; 450 mL methanol, 450 mL dH_2O, 100 mL glacial acetic acid. Store at room temperature.
2. Coomassie blue stain; 0.25 g Coomassie Brilliant Blue R250, 100 mL destain. Store at room temperature.

2.1.4 Western Blotting

Transfer of Protein to Nitrocellulose Filters

1. Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell.
2. Transfer buffer; 750 mL dH_2O, 2.9 g glycine, 5.8 g Tris base, 3.7 mL 10% (w/v) SDS, 200 mL methanol. Adjust volume to 1 L with dH_2O and store at 4 °C.

Staining of Proteins Immobilized on Nitrocellulose Filters

1. 1× Tris buffered saline (TBS); 6.1 g Tris base, 8.8 g NaCl, 800 mL, dH_2O. Adjust the pH to 7.5 with HCl and the volume to 1 L. Store at room temperature.
2. TBS-T; 1 L 1 × TBS, 1 mL Tween 20. Store at room temperature.
3. Blocking Buffer; 3 g Bovine Serum Albumin, 100 mL TBS-T. Prepare immediately before use.
4. Nitrocellulose blotting membrane.
5. 3MM filter paper.
6. Scalpel blade.
7. Ponceau S.

Immunological Probing

1. Mouse anti-His-HRP; a monoclonal antibody that reacts with polyHistidine residues and is conjugated to horseradish peroxidize (*see* **Note 4**). Dilute to 1/1000 in blocking buffer (Subheading 2.1.4) immediately before use.
2. Substrate (e.g., 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT) or 3,3′,5,5′-tetramethylbenzidine (TMB)). These substrates are light sensitive, protect from light.
3. Stop solution: alkaline phosphatize; 3 M NaOH; Peroxidase; 3 M HCl or 3 M H_2SO_4.
4. Gel documentation system.

2.2 Determination of Protein Solubility

1. Materials from Subheading 2.1.
2. Lysozyme 100 mg/mL. Dissolve 10 mg lysozyme in 1 mL dH_2O.
3. Lysis buffer (1 L): 50 mM NaH_2PO_4; 6.90 g $NaH_2PO_4{\cdot}H_2O$ (MW 137.99 g/mol), 300 mM NaCl; 17.54 g NaCl (MW 58.44 g/mol), 10 mM imidazole; 0.68 g imidazole (MW 68.08 g/mol). Adjust pH to 8.0 using NaOH.

2.3 IMAC Purification of His-Tagged Recombinant Proteins

2.3.1 Denaturing Solutions

Caution: The buffers listed below contain sodium hydroxide, which is an irritant. Lysis buffer A contains guanidine hydrochloride, which is harmful and an irritant. Consult the corresponding material safety data sheet (MSDS) before use.

1. Lysis Buffer A (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 6 M guanidine hydrochloride; 573 g. Adjust pH to 8.0 using NaOH.
2. Lysis Buffer B (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 8 M urea; 480.5 g (MW 60.06 g/mol). Adjust pH to 8.0 using NaOH.
3. Wash Buffer C (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 8 M urea; 480.5 g (MW 60.06 g/mol). Adjust pH to 6.3 using HCl.
4. Elution Buffer D (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 8 M urea; 480.5 g (MW 60.06 g/mol). Adjust pH to 5.9 using HCl.
5. Elution Buffer E (1 L): 100 mM NaH_2PO_4; 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 10 mM Tris–HCl; 1.2 g Tris base (MW 121.1 g/mol), 8 M urea; 480.5 g (MW 60.06 g/mol). Adjust pH to 4.5 using HCl.

2.3.2 Native Solutions

Caution: The buffers listed below contain sodium hydroxide and imidazole, which are irritants. Consult the corresponding material safety data sheets (MSDS) before use.

1. Lysis buffer (1 L): 50 mM NaH_2PO_4; 6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 300 mM NaCl; 17.54 g NaCl (MW 58.44 g/mol), 10 mM imidazole; 0.68 g imidazole (MW 68.08 g/mol). Adjust pH to 8.0 using NaOH. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.
2. Native wash buffer (1 L): 50 mM NaH_2PO_4; 6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 300 mM NaCl; 17.54 g NaCl (MW 58.44 g/mol), 20 mM imidazole; 1.36 g imidazole (MW 68.08 g/mol). Adjust pH to 8.0 using NaOH.
3. Native elution buffer (1 L): 50 mM NaH_2PO_4; 6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 300 mM NaCl; 17.54 g NaCl (MW 58.44 g/mol), 250 mM imidazole; 17.00 g imidazole (MW 68.08 g/mol). Adjust pH to 8.0 using NaOH.
4. Small bench rotary shaker.

2.3.3 Batch Purification

Ni-NTA Agarose is composed of Ni-NTA coupled to Sepharose® CL-6B and the manufacturer's reported binding capacity is 5–10 mg of 6xHis-tagged protein per mL of resin. Caution: nickel-nitrilotriacetic acid is harmful, a sensitizer, and flammable. Consult the corresponding material safety data sheet (MSDS) before use.

1. Ni-NTA resin.
2. Buffers from Subheading 2.3.

2.3.4 Column Purification

1. Ni-NTA spin columns.
2. Buffers from Subheading 2.3.

2.4 Growth for Preparative Purification

1. Materials as described in Subheadings 2.1 and 2.3.

2.5 Buffer Exchange and Concentration of Purified Protein

1. Amicon® Ultrafilter (Merck Millipore).
2. Phosphate Buffered Saline (PBS).

3 Methods

A process strategy for the purification of poly-histidine-tagged proteins from the initial design stage to the final purification steps is outlined in Fig. 1. Initially, an expression screening procedure should be performed under denaturing conditions (Subheading 3.1), which will lead to the isolation of any tagged protein, independent of its location within the cell. Following expression screening, protein solubility can be determined (Subheading 3.2), which will guide the decision to purify under native or denaturing conditions (Subheading 3.3).

3.1 High-Level Expression of Recombinant His-Tagged Proteins

Preliminary analysis of expression levels, cellular localization, and solubility of the target protein should be performed before purification or activity measurements using the methods described in this section. It is useful at this point to predict the physicochemical properties of the fusion protein (*see* **Note 5**) using bioinformatics tools such as the ProtParam tool (*see* web.expasy.org/protparam/). Here, high-level expression of recombinant proteins is achieved by induction with isopropyl thiogalactoside (IPTG).

1. Inoculate 1.5 mL of LB broth supplemented with appropriate antibiotics (pQE vector series—ampicillin at 100 μg/mL; RosettaBlue™ host- Chloramphenicol at 34 μg/mL and tetracycline at 12.5 μg/mL) with single colonies of transformants and grow overnight at 37 °C in an orbital shaker set at 200 rpm (*see* **Notes 6** and **7**).

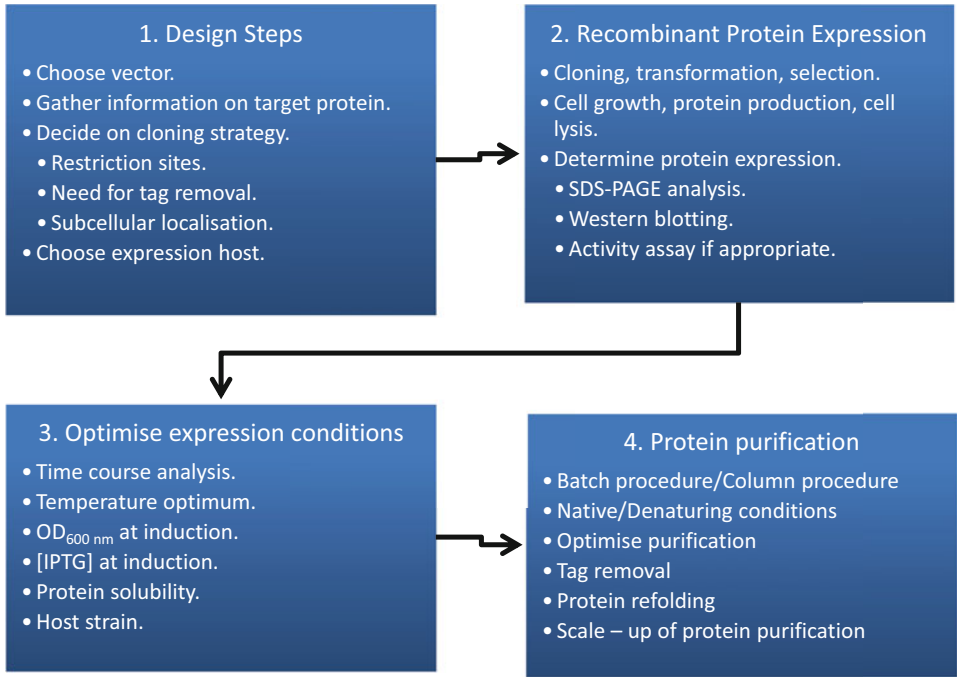

Fig. 1 Strategy for the purification of poly-histidine-tagged proteins. The flow chart shows the various stages in the overall process leading to the purification of a His-tagged recombinant protein

2. Use 500 µL of these cultures to inoculate 10 mL (*see* **Notes 6** and **8**) of supplemented LB broth, and grow as before for 3–4 h until logarithmic phase (Optical Density at 600 nm wavelength ($OD_{600\ nm}$) of 0.5) is reached (*see* **Note 9**). At the same time, set up a control culture, which will serve as an uninduced control in **step 3**.
3. Induce the cultures to express fusion proteins by the addition of IPTG at a final concentration of 1.0 mM followed by growth for a further 5 h as above (*see* **Notes 10** and **11**). At the same time, set up an uninduced control culture.
4. Collect cells by centrifugation at 15,000 × *g* for 5 min. Discard the supernatant and lyse the cell pellets in 400 µL of lysis buffer (Buffer B) (*see* **Notes 12** and **13**). Sonicate on ice for 6 × 10 s with 10 s pauses at 200 W (*see* **Notes 14** and **15**).
5. Remove cell debris by centrifugation at 15,000 × *g* for 15 min (also *see* **Note 16**) and analyze the supernatant (cleared cell lysate) by SDS-PAGE (*see* Subheading 3.1.2) to confirm protein expression at the predicted protein molecular weight.

To optimize the expression of a given recombinant protein, a time-course analysis of the level of expression can be performed by harvesting cells from expression cultures at time zero and at time intervals of 1 h for 5 h and at a final timepoint of 12 h following

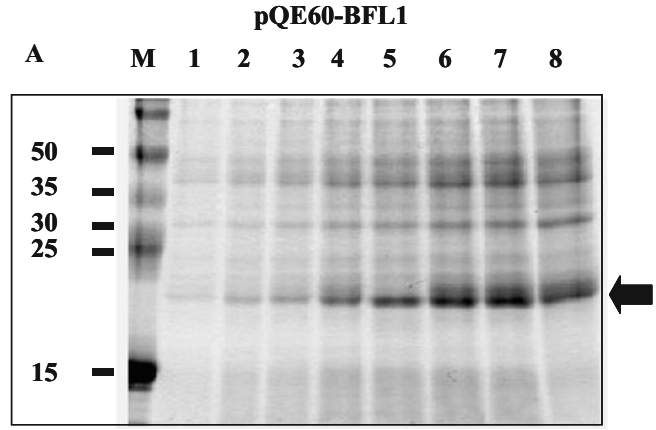

Fig. 2 Variation of growth parameters for optimal expression of pQE60-Bfl-1 in *E. coli* RosettaBlue™. Time-course analysis of recombinant Bfl-1 protein expression. M; Marker, 0–8; 0, 1, 2, 3, 4, 5, and 12 h post induction with 1 mM IPTG. The molecular weight markers are indicated on the left and black arrowheads designate the predicted location of recombinant Bfl-1

induction (*see* **Notes 7**, **10**, and **17**; *see* Fig. 2). The level of recombinant protein expression can be optimized by setting up small-scale expression cultures as above with variations of the following relevant growth parameters; IPTG concentration for induction (varied over the range 0.0025–1.5 mM), optical density measured at 600 nm at the time of induction (varied over the range 0.3–1.0 absorbance units), growth after induction (varied over the range 25–37 °C).

If proteolysis is occurring, take steps to avoid degradation as outlined in **Notes 12**, **18**–**20** (*see* also Chapter 6). Perform Western blotting (as described in Subheading 3.1.4) to verify His-fusion protein expression. Immunoreactivity with the anti-His HRP-conjugated monoclonal antibody will be evident upon chromogenic development following the addition of TMB substrate to the immunoprobed nitrocellulose membrane. The solubility of recombinant proteins can also be determined as described in Subheading 3.2. Finally, if no recombinant protein expression is evident, check that the coding sequence is ligated into the correct reading frame by sequencing. Also, variation of growth conditions as above or a change of expression host may permit recombinant protein expression.

3.1.1 *Protein Analysis*

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with or without subsequent Western blotting with anti-His-tag antibodies is commonly employed to both detect and quantify the tagged protein (*see* **Note 21**). His-tagged proteins can also be visualized directly on such gels following electrophoresis without the need for

Western blotting using a stain specific for proteins fused to an oligohistidine sequence (e.g., InVision™ His-tag In-gel Stain from ThermoFisher Scientific).

Prepare total cellular proteins for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting.

1. To the supernatant prepared in Subheading 3.1, add an equal volume of 2 × SDS gel loading buffer.
2. Immediately heat the sample for 5 min at 85 °C to denature the proteins. Clarify the lysate by centrifugation at 12,000 × *g* for 10 min at room temperature and store at −20 °C until SDS-PAGE analysis.

3.1.2 SDS-Polyacrylamide Gel Electrophoresis of Proteins

Preparation of SDS-Polyacrylamide Gels

Perform SDS-PAGE using 10% (v/v) resolving gels and 5% (v/v) stacking polyacrylamide gels prepared as described in Subheading 2.1.2.

1. Wash glass plates with detergent, then rinse with tap water and then with dH_2O, and finally wipe in one direction with tissue soaked in 100% (v/v) ethanol.
2. Place the gasket around the ridged plate; assemble the plates and secure with clamps.
3. Pour the resolving gel to within 2 cm of the top of the larger plate and overlay with 100% (v/v) ethanol.
4. When set, remove the ethanol and pour the stacking gel. Insert a clean comb that has been wiped in 100% (v/v) ethanol and allow the gel to polymerize for at least 20 min.
5. Fill the electrophoresis tank with 1 × Tris-glycine running buffer to a level of about 5 cm deep.
6. After polymerization, remove the gaskets and clamps and lower the pre-poured gels into the buffer at an angle to exclude air bubbles from the gel-buffer interface. Fill the tank completely with 1× running buffer and remove the comb from the gel. Fix the gel plates firmly in place with the pressure plates. Fill the chamber formed by the inner plates (notched plate facing inward) with 1× running Buffer.
7. Load the samples and attach the electrodes. Perform electrophoresis at a constant current of 30 mA per gel until the blue dye front has reached the bottom of the gel. When complete, separate the plates and place the gel in either Coomassie brilliant blue stain (Subheading 3.1.3) or transfer buffer prior to Western blotting (Subheading 3.1.4).

3.1.3 Coomassie Blue Staining

1. Immerse the gel in Coomassie stain for 30 min with gentle agitation.
2. Remove the gel and immerse in destain solution with constant agitation. Change this solution four or five times at 1 h intervals until all background staining has been removed from the gel.
3. Capture an image of the gel in black and white using a UV trans-illuminator switched to white light only and using a white tray.

3.1.4 Western Blotting

During Western blotting, electrophoretically separated proteins are transferred from the polyacrylamide gel to a solid support, usually a nitrocellulose membrane, and probed with antibodies that react specifically with antigenic epitopes present on the target protein that is attached to the solid support. In this chapter, the bound antibody is detected by a secondary immunological reagent, conjugated to either the alkaline phosphatase or horseradish peroxidase enzyme for detection.

Transfer of Protein to Nitrocellulose Filters

1. Following gel electrophoresis, equilibrate the gel(s) by immersion in transfer buffer for at least 15 min (*see* **Note 22**).
2. Cut the nitrocellulose membrane according to the dimensions of the gel, along with six pieces of 3 MM filter paper required for the gel/membrane sandwich. Immerse nitrocellulose and filter paper in transfer buffer for 15 min.
3. Perform protein transfer as follows (the procedure described is for a Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell): place a pre-soaked sheet of filter paper onto the platinum anode. Use a pipette to roll over the surface of the filter paper to exclude all air bubbles. Repeat this step with two more sheets of filter paper. Then, place the pre-wetted blotting membrane on top of the filter paper and roll all bubbles out. Place the equilibrated gel carefully on top of the nitrocellulose membrane, aligning the gel on the center of the membrane. Roll out any air bubbles. Place another three sheets of pre-wetted filter on top of the gel, with care taken to remove air bubbles. Place the cathode on top of the stack and replace the safety cover on the transfer unit. Transfer proteins for 22 min at 15 V.

Staining of Proteins Immobilized on Nitrocellulose Filters

Ponceau S staining can be used to determine whether uniform transfer of proteins to the nitrocellulose membrane has taken place. Transferred proteins are detected as red bands on a white background. This staining technique is reversible, thus allowing further analysis of blotted proteins by immunological probing. Ponceau S is a negative stain, which binds to positively charged amino acid groups of proteins. It also binds non-covalently to nonpolar regions of proteins.

1. Following electrophoretic transfer, immerse the nitrocellulose membrane in 20 mL of Ponceau S solution and stain for 5 min with constant agitation.
2. Once proteins are visible, verify that transfer has occurred evenly across the membrane. Wash the membrane in several changes of dH_2O until all the stain has been washed away. The membrane can then be used for immunological probing.

Immunological Probing

His-tagged proteins can be readily detected by Western blotting using anti-His antibodies. However, cross-reactivity of the primary antibody with endogenous histidines may be a considerable drawback in mammalian and insect expression systems [50].

1. Following Ponceau S staining, incubate the membrane in blocking buffer for 2 h at room temperature with constant agitation.
2. Remove the blocking buffer and incubate the membrane with the anti-His-HRP antibody (diluted in blocking buffer) at 4 °C overnight with constant agitation.
3. Following overnight incubation, wash the membrane three times in TBS-T for 15 min (*see* **Note 23**).
4. Place the membrane in a clean container and cover with the appropriate substrate; BCIP/NBT for alkaline phosphatase-conjugated antibodies or TMB for horseradish peroxidase-conjugated antibodies. When incubated with alkaline phosphatase or hydrogen peroxidase, bound enzyme catalyzes the production of a colored product that is easily observable.
5. Rinse the membrane in distilled water or add stop solution to stop the reaction. Capture the resultant image using a camera/scanner or gel documentation system.

3.2 Determination of Protein Solubility

Many polypeptide gene products expressed in *E. coli* accumulate as insoluble aggregates (*see* **Note 24**). Eukaryotic proteins expressed intracellularly in *E. coli* are frequently sequestered into insoluble inclusion bodies. Since the interaction between Ni-NTA and the 6xHis tag of the recombinant protein does not depend on the latter's tertiary structure, proteins can be purified either under native or denaturing conditions.

In order to determine if the protein is soluble in the cytoplasm and therefore purifiable under native conditions, the soluble and insoluble fractions of expression lysates are first examined by SDS-PAGE analysis (*see* Fig. 3). If the protein of interest resolves in the soluble fraction, purification under native conditions is possible (proceed to Subheading 3.3.1 for batch purification and Subheading 3.3.2 for column purification). Alternatively, if the protein of interest resolves in the insoluble fraction, the protein can be

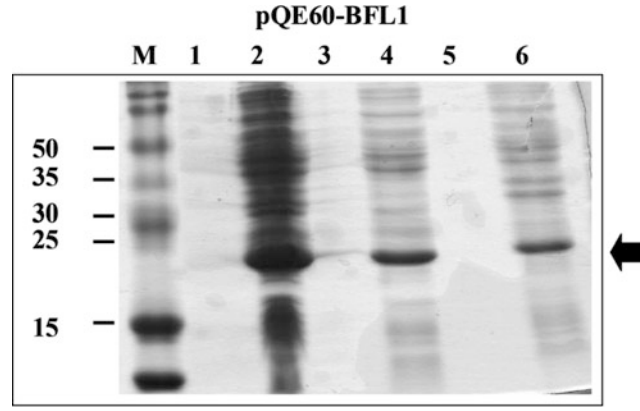

Fig. 3 Determination of protein solubility. Expression cultures harvested at 5 h post induction were lysed in native lysis buffer followed by incubation with lysozyme and sonication. Soluble and insoluble extracts were prepared and analyzed by SDS-PAGE. Lanes: M; Marker, 1 and 2; unrelated experiment, 3 and 5; blank, 4; Extract A, soluble extract, 6; Extract B, insoluble extract. The molecular weight markers are indicated on the left and black arrowheads designate the predicted location of recombinant Bfl-1

purified under denaturing conditions (proceed to Subheadings 3.3.1 and 3.3.2 for batch and column purification procedures, respectively). The Bfl-1-His protein (*see* Fig. 3) was resolved in the soluble fraction as can be seen by comparing the soluble and insoluble extracts in lanes 4 and 6, respectively, indicating that the protein was therefore purifiable under native conditions.

If the tagged protein is insoluble, the expression conditions can be modified in an attempt to enhance solubility. A reduction in growth temperature following induction may lead to an increase in soluble protein. A change of host strain or a reduction in the expression level as induced by IPTG (reduce from 1 mM to 0.005 mM) may permit higher levels of protein expression before inclusion body formation.

1. Inoculate 1.5 mL of LB broth supplemented with appropriate antibiotics (pQE vector series—ampicillin at 100 μg/mL; RosettaBlue™ host- Chloramphenicol at 34 μg/mL and tetracycline at 12.5 μg/mL) with single colonies of transformants and grow overnight at 37 °C in an orbital shaker set at 200 rpm (*see* **Notes 6** and 7).
2. Use 500 μL of these cultures to inoculate 10 mL (*see* **Notes 6** and **8**) of LB broth with antibiotics and grow overnight at 37 °C in an orbital shaker set at 200 rpm.
3. Use 2.5 mL of these cultures to inoculate 50 mL of LB broth with antibiotics and grow as before for 3–4 h until logarithmic phase [$OD_{600\ nm}$ of 0.5] is reached (*see* **Note 9**).

4. Induce the cultures to express fusion proteins by the addition of IPTG at a final concentration of 1.0 mM followed by growth for a further 5 h as above (*see* **Notes 10** and **11**).
5. Harvest the expression cultures by centrifugation at 4000 × *g* for 20 min.
6. Resuspend pelleted cells in 5 mL of native lysis buffer (*see* **Notes 25** and **26**).
7. Add lysozyme (1 mg/mL) (*see* **Notes 16** and **27**) and incubate the sample for 30 min at room temperature (*see* **Note 28**) followed by sonication on ice for 6 × 10 s with 10 s pauses at 200 W (*see* **Notes 14** and **15**).
8. Centrifuge the lysate at 10,000 × *g* at 4 °C for 20 min and aspirate the supernatant and store on ice (soluble extract). Resuspend the remaining pellet in 5 mL of lysis buffer and store on ice (Insoluble matter).
9. Examine the soluble and insoluble extracts for recombinant protein expression by SDS-PAGE analysis (Subheading 3.1.2).

3.3 IMAC Purification of His-Tagged Recombinant Proteins

The purification of His-tagged recombinant proteins can be performed using batch and column procedures under native and denaturing conditions. Native protein purification involves the use of buffers that preserve the native, three-dimensional structure and surface charge characteristics of a selected soluble protein during harvest from an expression host. However, if the tagged protein of interest is insoluble, purification under denaturing conditions may be necessary. Denaturants, such as 8 M urea or 6 M guanidine, can be used to enhance protein solubility (*see* **Note 29**). Incompatible reagents to be avoided during IMAC purification are listed in Table 4 (*see* **Note 30**). Finally, if protein activity/native structure is important (e.g., for biochemical or structural studies), protein refolding following purification under denaturing conditions can be performed (Subheading 3.4).

During the process of purification, collect all eluates and fractions for analysis by SDS-PAGE and Coomassie blue staining. Protein purification steps can also be monitored by UV absorbance at 280 nm (*see* Chapter 16 for details on UV protein absorption assay using the Nanodrop® ND-1000).

3.3.1 Batch Purification

Under Denaturing Conditions

1. Equilibrate 1 mL of 50% Ni-NTA resin by adding of 10 mL of Buffer B.
2. Centrifuge the resin-buffer mixture at 1200 × *g* for 1 min and discard the supernatant.

Table 4
Reagent compatibility with IMAC resin

Reagent	Compatibility	Acceptable concentration
CHAPS	Yes	1% (with caution)
DTE (dithioerythritol)	No	–
DTT (dithiothreitol)	No	–
EDTA	No	–
EGTA	No	–
Ethanol[a]	Yes	30%
Ethylene glycol	Yes	30%
Glycerol	Yes	20%
Guanidine[b]	Yes	6 M
HEPES[c]	Yes	100 mM
Imidazole[d]	Yes	200 mM at pH 7.0–8.0, for elution
KCl	Yes	500 mM
MES	Yes	20 mM
MOPS[E]	Yes	100 mM
NaCl	Yes	1 mM
NP-40	Yes	1%
SDS	Yes	1% with caution
Tris[c,e]	Yes	100 mM
Triton-X 100	Yes	<2%
Tween	Yes	2%
Urea	Yes	8 M
β-Mercaptoethanol[b]	Yes	20 mM (with caution)

[a]Ethanol may precipitate proteins, causing low yields and column clogging
[b]Do not store resin in buffers containing these reagents. Use resin immediately after equilibrating with buffers containing these reagents
[c]Sodium phosphate or phosphate-citrate buffer is recommended
[d]For IMAC column binding, imidazole cannot be used at concentrations higher than 5–10 mM due to competition for binding to the immobilized metal ions
[e]Tris coordinates weakly with metal ions, causing a decrease in capacity

3. Add 4 mL of cleared lysate (from 100 mL culture volumes scaled up by a factor of 10 from Subheading 3.1) (*see* **Notes 30** and **31**) to the equilibrated resin and mix gently for 60 min at room temperature with rotation at 200 rpm on a rotary shaker.
4. Centrifuge the lysate-resin mixture at 1200 × *g* for 1 min and remove the supernatant.

5. Add 4 mL wash buffer (Buffer C) (*see* **Note 32**) to the resin, mix thoroughly and centrifuge at 1200 × *g* for 1 min.
6. Repeat the wash step twice more.
7. Elute by adding four volumes of 500 μL of elution buffer (Buffer D/E) (*see* **Note 33**) to the resin and centrifuge at 1200 × *g* for 1 min. Collect the supernatant/eluate.

Under Native Conditions

1. Prepare expression cultures as in Subheading 3.1 but using 100 mL culture volumes (scaled up by a factor of 10 from Subheading 3.1).
2. Lyse the cells in native lysis buffer.
3. Add lysozyme (1 mg/mL) (*see* **Notes 16** and **27**) and incubate the sample for 30 min at room temperature (*see* **Note 28**) followed by sonication on ice for 6 × 10 s with 10 s pauses at 200 W (*see* **Notes 14** and **15**).
4. Remove cell debris by centrifugation at 15,000 × *g* for 15 min.
5. Equilibrate 1 mL of 50% Ni-NTA resin by adding 10 mL of native lysis buffer, centrifuge at 1200 × *g* for 1 min and remove the supernatant.
6. Add 4 mL of cleared lysate to the equilibrated resin and mix gently for 60 min at room temperature with rotation at 200 rpm on a rotary shaker.
7. Centrifuge the lysate-resin mixture at 1200 × *g* for 1 min and remove the supernatant.
8. Add 4 mL of native wash buffer to the resin (*see* **Note 25** and Table 4), mix thoroughly and centrifuge at 1200 × *g* for 1 min.
9. Repeat the wash step twice more.
10. Elute by adding four volumes of 500 μL of native elution buffer to the resin and centrifuge at 1200 × *g* for 1 min. Collect the supernatant/eluate.

3.3.2 Column Purification

Under Denaturing Conditions

1. Equilibrate a Ni-NTA spin column using 600 μL buffer B. Centrifuge for 5 min at 700 × *g*.
2. Load cell lysates from Subheading 3.1 onto pre-equilibrated Ni-NTA IMAC mini-columns. Centrifuge for 5 min at 700 × *g*. Collect the flow through.
3. Perform two wash steps by adding 600 μL wash buffer (Buffer C) (*see* **Note 32**) followed by centrifugation at 700 × *g* for 5 min. Collect wash fractions.
4. Add 2 × 200 μL elution buffer to the column (Buffer E; pH 4.5). Centrifuge for 5 min at 700 × *g*. Collect the eluates.

Under Native Conditions

1. Equilibrate a Ni-NTA spin column using 600 μL native lysis buffer. Centrifuge for 5 min at 700 × *g*.
2. Add cell lysates (prepared under native conditions as in Subheading 3.3.1) onto pre-equilibrated Ni-NTA IMAC mini-columns. Centrifuge for 5 min at 700 × *g*. Collect the flow through.
3. Perform two wash steps by adding 600 H_2OL native wash buffer (*see* **Note 32**) followed by centrifugation at 700 × *g* for 5 min. Collect wash fractions.
4. Add 2 × 200 μL native elution buffer to the column. Centrifuge for 5 min at 700 × *g*. Collect the eluates.

3.4 Downstream Processes: His-Tag Removal by Proteolytic Cleavage and Protein Refolding

As mentioned by Waugh [37], the Achilles heel of fusion protein strategies has been the removal of purification tags. The addition of a new sequence to engage a new function has been applied here: for example, originally, Tropea et al. [38] and subsequently Zhang et al. [39] introduced a His-tag followed by a tobacco etch virus (TEV) protease site for downstream cleavage following purification. The introduction of this highly specific (yet with a relaxed P1′ specificity) cleavage site ensures no tag/linker residues remain after proteolysis (His-TEV protease digestion for 4 h at 30 °C) and also ensures the protein of interest's native sequence is maintained, allowing for rapid and large-scale purifications of fully functional proteins. Purification may involve cleavage with other enzymes to remove part or all of the fusion tag, using endopeptidases including enterokinase, factor Xa, thrombin, or HRV 3C proteases. The 6xHis tag can be easily and efficiently removed from His-tagged proteins expressed in the pQE30-Xa vector using Factor Xa Protease. Detailed protocols for protein precipitation and differential solubilization following purification are described in Chapter 17.

3.5 Large-Scale Growth for Preparative Purification of Recombinant Protein

Once optimal purification conditions have been determined empirically using small-scale expression and purification experiments, growth for preparative-scale purification can be performed. The procedure below is outlined for native purification and can be modified for large-scale purification under denaturing conditions according to the steps outlined in Subheading 3.3.1. Monitor purification as before by collecting fractions for analysis by SDS-PAGE (*see* Fig. 4).

1. Inoculate 1.5 mL of LB broth (*see* **Note 1**) supplemented with antibiotics (pQE vector series—ampicillin at 100 μg/mL; RosettaBlue™ host- chloramphenicol at 34 μg/mL and tetracycline at 12.5 μg/mL) with single colonies of transformants and grow overnight at 37 °C in an orbital shaker set at 200 rpm (*see* **Notes 6** and 7).

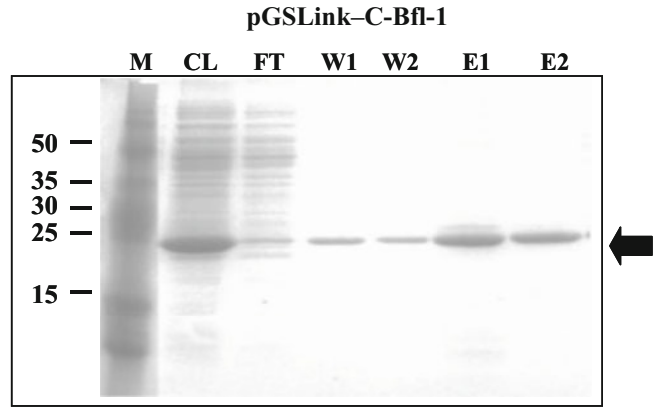

Fig. 4 Optimization of purification of His-Linker-Bfl-1 under native conditions. Recombinant protein purification of His-Linker-Bfl-1 protein under native conditions using Ni-NTA resin. M: Marker, CL: Cleared cell lysate, FT: Flow through, W1 and W2: Wash with native wash buffer, E1–2: Elution with native elution buffer. The molecular weight markers are indicated on the left and black arrowheads designate the location of recombinant Bfl-1

2. Use 500 μL of these cultures to inoculate 20 mL (*see* **Notes 6**–**8**) of supplemented LB broth, and incubate overnight at 37 °C in an orbital shaker set at 200 rpm.
3. Inoculate LB broth (1 L) 1:50 with these cultures (*see* **Note 8**) and grow as above for 3–4 h until logarithmic phase [$OD_{600\ nm}$ of 0.8] is reached (*see* **Note 9**).
4. Induce the expression of tagged protein by adding IPTG at a final concentration of 0.1 mM followed by growth for a further 5 h at 37 °C in an orbital shaker set at 200 rpm.
5. Collect cells by centrifugation at 4000 × *g* for 20 min.
6. Discard the supernatant and lyse the cell pellets in native lysis buffer (5 mL/g wet weight), followed by incubation in lysozyme (1 mg/mL for 30 min at room temperature (*see* **Notes 26**–**28**)) and sonication on ice for six 10 s pulses with 10 s breaks between pulses at an amplitude: 40/output, 200 W (*see* **Notes 14** and **15**).
7. Remove cell debris by centrifugation at 15,000 × *g* for 25 min at 4 °C.
8. Apply the supernatant to 2 mL Ni-NTA matrix that has been packed into a disposable column and pre-equilibrated with native lysis buffer.
9. Collect the flow through and reapply to the resin a further two times.
10. Perform two washes with wash buffer.
11. Elute purified proteins twice using 5 mL volumes of elution buffer.

3.6 Buffer Exchange and Concentration of Purified Protein

1. Pool the eluates from preparative purification and apply to an Amicon ultrafilter.
2. Buffer exchange the 10 mL eluate to PBS (or the appropriate buffer for the desired downstream application) and simultaneously concentrate (by reducing volume from 10 mL to 500 μL three times) by centrifugation at 1252 × *g* and 4 °C.

4 Notes

1. While most recombinant proteins can be cloned and expressed at high levels in *E. coli*, there are exceptions. Some His-tagged proteins show weak binding to the metal chelating resin, which can be due to concealment of the His-tag, and may be alleviated by switching its position to the other terminus of the protein [51] or by introducing a linker to separate the His-tag from the partner protein [17]. Additionally, these difficulties can be overcome by increasing the length of the His-tag to eight or ten histidines [52, 53].
2. *E. coli* RosettaBlue™ (*see* Table 2) and *E. coli* Rosetta™ (Merck Millipore) strains have been engineered to express the tRNAs for rarely expressed codons from a chloramphenicol resistant plasmid, pRARE. The use of *E. coli* RosettaBlue™ or *E. coli* Rosetta™ strains as expression hosts facilitates the expression of proteins that would otherwise be limited by codon bias in *E. coli*.
3. IMAC is very sensitive to the presence of metal chelators [54]. In *E. coli* expression systems, the cell lysate contains many unspecific weak chelators such as dicarboxylic acids from the citric acid cycle. Under stress conditions, *E. coli* can also produce highly specific metal chelators known as metallophores [55]. A recent report linked the failure to purify low-abundance His-tagged proteins from *E. coli* lysates to metal-ion leakage from purification columns [56]. In that study, the authors used His-tagged GFP (His_6-GFP) to examine the effect of *E. coli* lysate on the protein binding capacity of IMAC columns and concluded that low molecular weight components of the lysate, associated with the periplasm, severely reduced the binding capacity of the column. By removing the periplasmic material before cell lysis, the authors observed a tenfold increase in the yield of His_6-GFP when it was diluted with *E. coli* lysate before purification to simulate a low-abundance protein.

4. Sodium azide can be added to each antibody solution to a final concentration of 0.02% (w/v) as a preservative, thus permitting reuse of the antibody. Do not use sodium azide as a preservative when diluting HRP-conjugated antibodies as it is an established peroxidase inhibitor. Also note that the phosphate in PBS can inhibit the alkaline phosphatase reporter enzyme.
5. Predict codon usage, molecular weight, molar extinction coefficient using bioinformatics tools (e.g., such as those available on the bioinformatics resource portal of the SIB Swiss Institute of Bioinformatics *see* expasy.org [57]).
6. In order to ensure good aeration, add medium up to only 20% of the total flask volume. For 1.5 mL culture volumes, use 17 mm × 100 mm sterile snap-cap; for 10 mL culture volumes, use 125 mL Erlenmeyer flasks; for 20 mL culture volumes, use 250 mL Erlenmeyer flasks.
7. If cultures are grown overnight, 0.5–1% glucose may be added to the media in order to reduce target protein expression prior to induction [58].
8. The staging procedure will minimize the shock induced lag phase in growth caused by transfer of a small inoculum to a larger volume of fresh medium and diffusion of vitamins, minerals, and cofactors from the cells [59].
9. Monitor the OD_{600} during growth by removing aliquots aseptically.
10. When directing fusion proteins to the periplasmic space, leakage of the protein to the medium might be enhanced by prolonged inductions (16 h or more).
11. The Overnight Express™ Autoinduction System (Merck Millipore) is designed for high-level protein expression with IPTG-inducible bacterial expression systems [60] without the need to monitor cell growth. The method is based on media components that are metabolized differentially to promote growth to high density and automatically induce protein expression from *lac* promoters.
12. Protease inhibitors can be added at this point as an option (*see* Chapter 6). Serine protease inhibitors should be avoided if the target protein is to be treated with Thrombin, Factor Xa, or Recombinant Enterokinase. Cysteine protease inhibitors should be avoided if the target protein is to be treated with HRV 3C. Although purification may remove active inhibitors, dialysis or gel filtration is recommended before cleavage.
13. Lysing cells in buffer B allows solubilization of most proteins and inclusion bodies and facilitates their direct analysis by SDS-PAGE. To solubilize very hydrophobic receptor or membrane

proteins, Buffer A, containing guanidine hydrochloride (Gu-HCl), can be used, sometimes in combination with detergents.

14. Sonication conditions may vary with the equipment. Alternatively, if a sonicator is not available, pass the sample through a 27-gauge needle several times to reduce the viscosity or use a French press to lyse cells (perform two passes at 20,000 psi using a chilled pressure cell).
15. Excessive sonication can destroy protein functionality.
16. DNase I (5 μg/mL) and RNase A (10 5 μg/mL) can be added at this point (followed by 10–15 min incubation on ice) to reduce the viscosity of the lysate, allowing for more efficient removal of cellular debris.
17. When analyzing the time course of expression, begin with a 100 mL culture in a flask, and take 10 mL samples at each timepoint after induction.
18. In order to reduce proteolytic activity, maintain protein stability and improve yield, perform all manipulations at 4–8 °C. A reducing agent, such as 10 mM β-ME, or a protease inhibitor, such as PMSF, in the equilibration/wash buffer, may improve the structural stability of fragile proteins during sample preparation (*see* Table 4 for reagent compatibility).
19. Additionally, 1 mM EDTA can be added to the equilibration/wash buffer (pH 7.0) to inhibit metalloproteases during the extraction. However, EDTA must be removed before applying the sample to the resin because EDTA complexes with the nickel. A gel filtration column (such as PD-10, Cytiva) equilibrated with the equilibration buffer can be used for this. In some cases, the host cell produces low molecular weight chelators that can also be removed using gel filtration. Alternatively, The "cOmplete™ His-tag purification resin" developed by Roche is compatible with buffers containing EDTA and DTT.
20. Use protease deficient *E. coli*; strains B834, BL21, BLR, Origami™ B, Rosetta™, Rosetta 2, Rosetta-gami™ B, and Tuner™ are deficient in the Lon protease and lack the OmpT outer membrane protease that can degrade proteins during purification [61] (for further detailed procedures on avoiding proteolysis *see* Chapter 6).
21. Considering both of these methods are quite time consuming, Kreisig and colleagues [6] developed a semiquantitative immunoassay based on Förster resonance energy transfer (FRET) that can generate a fluorescent signal dependent on the concentration of the His-tagged protein in *E.coli* cell lysates. Advantages of this development include its ability to work

with both N- or C-terminally tagged proteins, while possessing detection limits similar to other techniques, and importantly, the process can be performed in less than 2 min, including sample preparation.

22. Equilibration facilitates the removal of electrophoresis salts and detergents. If not removed, salts will increase the conductivity of the transfer and the amount of heat generated during transfer.
23. If the primary antibody is not HRP- or AP-conjugated repeat **steps 2** and **3** with an appropriate conjugated secondary antibody, incubating for 2 h at room temperature. Proceed to **step 4**.
24. Depending on the intended application, preferential localization of the recombinant protein to inclusion bodies, secretion to the medium, or the periplasmic space can be advantageous for rapid purification by relatively simple procedures. Vectors are available which can provide a signal sequence (e.g., pelB, DsbA, DsbC) for translocation into the periplasmic space. Thus far, it has not been possible to predict which signal peptide is optimal for the production of a particular recombinant protein in the periplasm. But Karyolaimos et al. present a combined screen involving different signal peptides and varying production rates that enabled the identification of more optimal conditions for periplasmic production of recombinant proteins with disulfide bonds [62].
25. The salt content of this buffer may be increased up to 0.5 M as some proteins may exhibit greater solubility when the cells are lysed in a buffer containing salt. Other proteins, such as those associated with membranes, may partition into the soluble fraction if a zwitterionic detergent (e.g., 10 mM CHAPS) is added to the lysis buffer.
26. An additional option is to freeze the pellet completely at −20 °C or −70 °C, then thaw completely. The freeze/thaw step ruptures the outer membrane allowing lysozyme to access the cell wall.
27. If the bacterial strain contains a plasmid encoding lysozyme (e.g., pLysS or pLysE; Merck Millipore), then additional lysozyme treatment is not necessary.
28. Incubations at room temperature results in elevated proteolytic activities. Alternatively, to avoid proteolysis incubate at 4 °C.
29. Samples containing 6 M guanidine must be dialyzed overnight against buffer containing 8 M urea before loading on an SDS-PAGE gel.
30. A desalting column can be used to adjust the pH of the lysate, to change buffer, remove low molecular weight contaminants and/or to concentrate sample before applying to the column.

31. If the cell lysate is still not clear after centrifugation, filter using a 1 μm filter. Membrane filters that give the least amount of nonspecific binding of proteins are composed of cellulose acetate or PVDF.
32. Wash steps can be monitored for efficient removal of unbound material from the column by analyzing protein content of wash fractions using UV absorbance at 280 nm (*see* Chapter 16 for detailed protocols). Elution can begin when all unbound material has been washed through. This will improve the purity of the eluted target substance.
33. Elution buffer D and/or E may be required for elution. Monomers generally elute in buffer D, while multimers, aggregates, and proteins with two 6xHis tags will generally elute in buffer E.

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Chapter 12

Lectin Affinity Chromatography

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Abstract

Glycosylation is a non-template-driven posttranslational modification during which linked-sugars and glycans are added to the nascent polypeptide. Over 70% of the eukaryotic proteome is thought to be glycosylated. It is now known that correct glycosylation is essential for the correct folding, solubility, stability, and immunogenicity of proteins. In this chapter, we describe the technique of lectin affinity chromatography (LAC), a procedure that has the ability to distinguish different glycans, which are attached to proteins or lipids, termed glycoproteins or glycolipids, respectively. This method utilizes different immobilized lectins that have affinity for specific sugar substrates, to separate a wide range of glycan-attached complexes (Ambrosi et al., Org Biomol Chem 3:1593–1608, 2005). To further enhance the specificity of LAC, a corresponding free sugar may be used to produce a specific elution. In general, the conditions under which lectin affinity chromatography operates are relatively mild resulting in good biological recoveries of the glycoproteins.

Key words Affinity, Lectin, Glycans, Glycoprotein, Con A Sepharose®, Recombinant lectins, Free haptenic sugars, Frontal affinity chromatography

1 Introduction

Lectin affinity chromatography (LAC), utilizing immobilized lectins, is commonly used for isolating and separating glycoproteins, glycoforms, glycolipids, polysaccharides, subcellular particles, and cells and for purifying detergent-solubilized cell membrane components [1–6]. Lectin-affinity-based techniques have the potential to offer a more comprehensive approach to glycan analysis, with glycan analysis becoming increasingly important in the development of glycoproteins as therapeutics. This form of affinity chromatography may be utilized to separate a heterogeneous mixture of oligosaccharides or protein-bound oligosaccharides from complex samples. LAC methodologies are often used as initial steps in mass spectrophotometric (MS)-based glyco-analytical procedures, enabling the preconcentration of glycopeptides or separation of glycoforms of glycoproteins prior to analysis by MS.

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Lectins are proteins capable of specifically recognizing and binding reversibly to specific sugar/glycan structures [7]. They encompass carbohydrate binding proteins from very diverse sources ranging from plants and animals to bacteria and viruses [8]. Lectins were initially studied in plants and were reported to bind their sugar/glycan target with relatively low affinities with dissociation constants in the millimolar or micromolar range. However, recent advances in our understanding of lectin-glycan interactions have demonstrated that interactions of multivalent lectins with complex, branched sugars result in high-avidity binding with nanomolar or even picomolar dissociation constants [9]. The binding of lectins to their corresponding glycans/sugars is specific, non-covalent, and reversible, involving hydrogen bonds, hydrophobic, electrostatic, and van der Waals interactions as well as dipole attraction.

In general, lectins possess biochemical and binding properties that are very useful for their exploitation as ligands for the purification of glycoproteins by affinity chromatography. They do not react catalytically with the glycoproteins or modify them in any way. As noted earlier, immobilized lectins bind glycoproteins non-covalently and reversibly, and therefore, they may be selectively released from an affinity column by competitive elution using a specific/corresponding free sugar or sugar analog (*see* Table 1). Moreover, both lectins and their corresponding glycoproteins are usually stable, and therefore, elution techniques using extreme conditions of pH and/or ionic strength may also be applied to affect a release from a lectin affinity column.

All lectins have a specific binding capacity and dissociation constant for their corresponding sugar. A case study completed in 2003 using surface plasmon resonance (SPR) imaging calculated the solution equilibrium dissociation constant (K_D) and adsorption coefficients (K_{ADS}) for the lectins Concanavalin A (Con A) and Jacalin, which bind to mannose and galactose, respectively. Immobilized thiol-modified carbohydrates were arrayed onto gold films, and SPR imaging was used to determine the K_D for each lectin during elution with increasing concentrations of mannose and galactose. Con A was calculated to have a K_D of 200 μM ± 50 μM, whereas Jacalin had a K_D of 16 μM ± 5 μM. This technique was also employed to work out the adsorption coefficients of both lectins. Con A was calculated to have a K_{ADS} of $5.6 \pm 1.7 \times 10^6$ M^{-1}, whereas Jacalin had a K_{ADS} of $2.2 \pm 0.8 \times 10^7$ M^{-1} [10].

Lectins have been successfully immobilized onto Sepharose® and silica beads (*see* Subheading 3.2 below) producing stable lectin affinity resins where little difference in terms of binding capacity is seen between lectin-Sepharose® and lectin-silica resins. Lectins have also been immobilized onto monolithic supports, miniaturized capillary-based HPLC, and microfluidic chip platforms [11].

Table 1
Lectins and their binding specificities

Lectin	Source	Subunits	Mol. weight (kDa)	Glycan specificity	Eluting sugar/analog	Compatible metal ions
ConA	*Canavalia ensiformis*	Four identical subunits	104	α-Mannose, glucose	200 mM mixture of α-methylmannoside and α-methylglucoside	Ca^{2+}, Mn^{2+}
ECL	*Erytjrina cristagalli*	Two different subunits	54	Galactose, N-acetylgalactosamine, Lactose	200 mM lactose	Ca^{2+}, Mn^{2+}, Zn^{2+}
AAL	*Aleuria aurantia*	Two identical subunits	72	α-1,6-fucose Fucose linked α-1,3 to N-acetyllactosamine	100 mM L-Fucose	None
Jacalin	*Artocarpus heterophylius* (jackfruit)	Four subunits	66	T-antigen, sialyl T-antigen, galactose	800 mM galactose, 100 mM melibiose	None
WGA	*Triticum vulgaris* (wheat)	Two subunits	36	N-acetylglucosamine	500 mM GlcNAc with salt and/or acid	Ca^{2+}
LCA	*Lens culinaris*	Four subunits	50	α-Mannose, glucose	200 mM mixture of α-methylmannoside and α-methylglucoside	Ca^{2+}, Mn^{2+}
PNA	*Arachis hypogaea* (peanuts)	Four subunits	110	Galactose (β3-linked to GalNAc)	200 mM galactose	Ca^{2+}, Mg^{2+}
Mal II	*Maackia amurensis*	Two subunits	130	α-2,3-NeuNAc (sialic acid)	Human glycophorin (glycoprotein)	None
LecB (PA-IIL)	*Pseudomonas aeruginosa*	Four subunits	13	Fucose, mannose Fucose > mannose	200 mM L-Fucose, 200 mM D-mannose	Ca^{2+}
BC2LA	*Burkholderia cenocepacia*	Two subunits	14	Mannose	200 mM D-mannose	Ca^{2+}

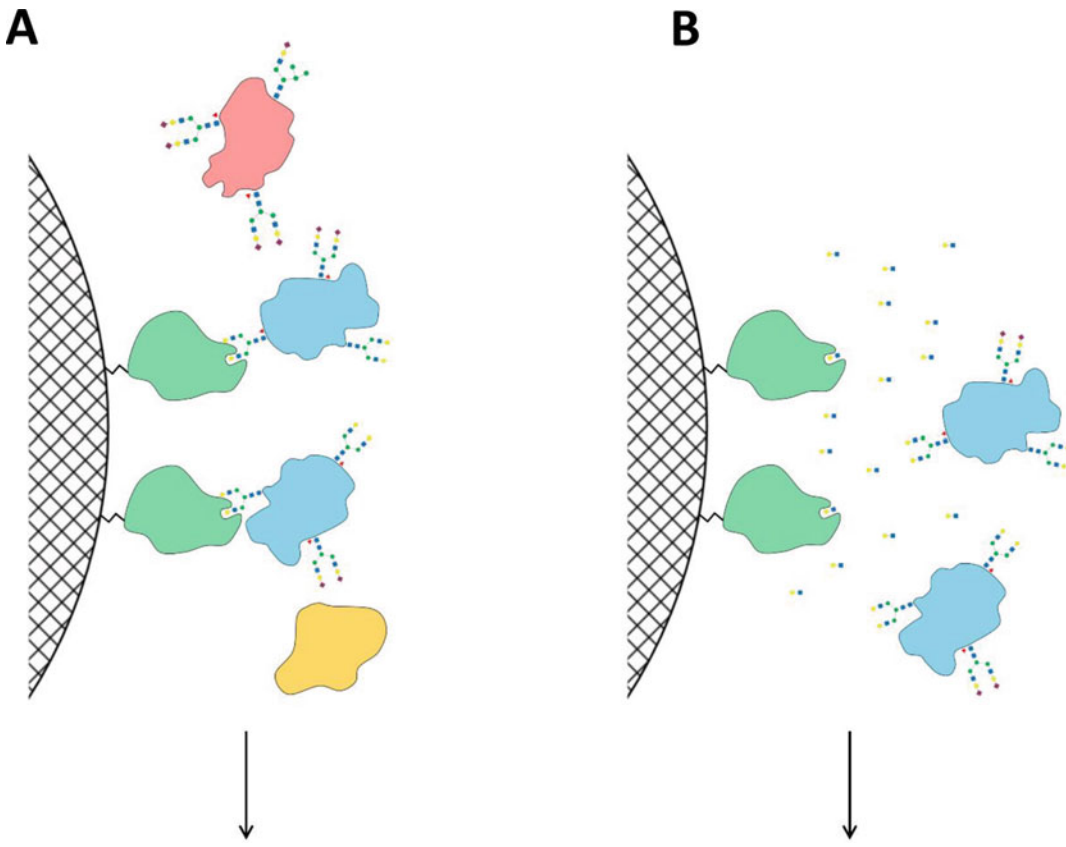

Fig. 1 Schematic diagram showing the principle of lectin affinity chromatography. A lectin (*green*) immobilized on a Sepharose® resin is capable of binding glycoproteins that flow through the column. (**a**) The bound glycoprotein (*blue*) is retained by the lectin, whereas proteins with different glycans (*red*) or no glycans (*yellow*) flow though the resin uncaptured. The unbound proteins are removed from the column with a wash buffer. (**b**) The captured glycoprotein is released with the addition of a specific 0.5 M free sugar solution

Biological samples may be passed over a lectin affinity column, and based on the relative affinity of the lectin, the oligosaccharides or protein-bound oligosaccharides may bind to the immobilized lectin and thus be separated from unbound material (*see* Fig. 1). Further specific separation may be affected by the use of free haptenic sugars in the elution buffer (*see* Fig. 1). This technique may be expanded further, for example, by using multiple lectin columns in conjunction with other analytical methods such as MS and nuclear magnetic resonance (NMR). Separation or sample enrichment/preparation prior to this form of high-end analysis is usually required to achieve the optimal result. Recent significant growth in the biopharmaceutical industry has led to an increased need to be able to separate, purify, enrich, and analyze the glycoforms that are present in any formulation of glycoprotein-based therapeutics. Lectin affinity chromatography has the potential to enrich samples prior to analysis.

LAC techniques are gaining traction in clinical settings where there is a need for the rapid differentiation of glycoproteins. Glycosylation is an essential cellular mechanism required for a variety of cellular processes, such as cell-cell communication, pathogen recognition, and protein function and stability in vivo [12]. Alterations to glycoproteins and glycolipids are commonly found in cancerous and diseased cells [13]. Multi-LAC has been utilized in separating, identifying, and quantifying specific glycoforms [14]. Multi-LAC has been successfully applied to differentiate glycoforms in serum samples from patients with benign prostatic hyperplasia—where atypical glycoproteins were enriched prior to proteomic analysis [5]. The selective binding of lectins to low levels of irregular glycoproteins may be transformative in the clinical space where the early identification of a diseased state is crucial for long-term survival [15].

Some of the earlier discovered lectins like Con A and *Erythrina crista-galli* lectin (ECL) are multimeric glycosylated proteins themselves and are therefore not easily amenable to recombinant production. This has sometimes led to batch-to-batch variation in performance, thus limiting their use for LAC. Recent advances in genome sequencing and bioinformatic analysis have revealed a huge potential reservoir of microbial lectins and lectin-like proteins. Unlike plant lectins, microbial lectins and particularly those from bacterial sources are more amenable to recombinant production in *E. coli* [16]. Furthermore, the addition of affinity tags using recombinant DNA techniques enables consistent one-step purification of these recombinant lectins. Site-directed mutagenesis also allows one to modify the affinities of lectins for their target glycoproteins or different glycoforms of these proteins [16]. This has led to the production of recombinant lectins in highly purified forms, which exhibit more consistent and reproducible specificity and activity (*see* Table 1).

LAC methodologies, such as frontal affinity chromatography (FAC), are also used for glycoprotein characterization since the ability of a glycoprotein to bind to specific lectin affinity matrices is dependent on, and therefore indicative of, its glycosylation [16]. FAC is a highly sensitive, accurate, rapid, and versatile technique for the determination of dissociation constants (K_D) between lectins and fluorescently labeled sugars or glycans [17]. A series of glycan-containing solutions is applied to an immobilized-lectin column, and the elution profile of each glycan (termed the "elution front," V) is compared with that of an appropriate control (V_0) (*see* Fig. 2). In this way, it is possible to establish the relative strengths and binding specificities of a given sugar-binding protein. Here, a general protocol for LAC for the separation of glycoproteins is described using commercially available immobilized lectins. A protocol for immobilizing a lectin of choice onto Sepharose® resin, thus generating a unique lectin affinity resin, is also described.

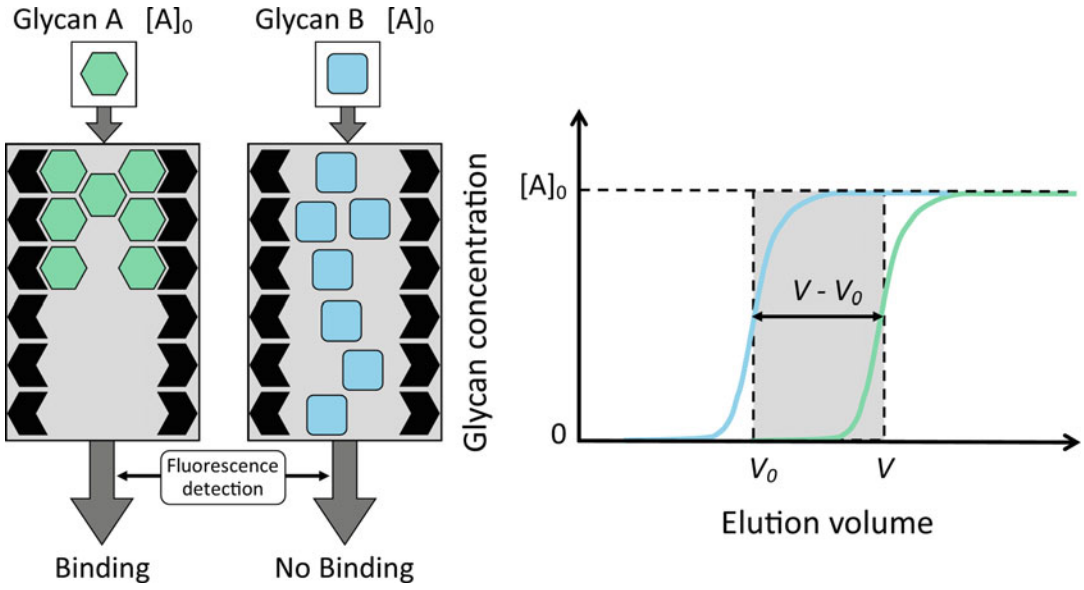

Fig. 2 Glycan capture by a lectin-immobilized column and the subsequent fluorescence detection of the eluate. A glycan solution is applied to a lectin-immobilized column. If a glycan has no affinity with the immobilized lectin, glycan B (*blue*), its elution front (V_0) is detected immediately. However, if the glycan has affinity with the immobilized lectin, glycan A (*green*), its elution front (V) is retarded. The initial glycan concentration, $[A]_0$, is expressed in *M*

2 Materials

2.1 Lectin Affinity Chromatography

1. Con A Sepharose® 4B or Lentil Lectin Sepharose® 4B (Cytiva).
2. Column buffer: 10 mM Tris–HCl, pH 7.5 with 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (*see* **Note 1**). Store indefinitely at room temperature. It is recommended to include sodium azide at 0.02–0.05% (w/v), as a bacteriostatic agent, for long-term column storage.
3. Elution buffer: 10 mM Tris–HCl, pH 7.5 with 150 mM NaCl, 1 mM $CaCl_2$ with 1 mM $MgCl_2$ and the appropriate concentration of corresponding free sugar (*see* Table 1). Store indefinitely at room temperature (*see* **Note 2**).
4. A beaker to prepare the slurry and a standard glass column of appropriate dimensions for the bed volume is required. Degas all buffers prior to use.

2.2 Immobilizing Lectin onto Cyanogen Bromide-Activated Sepharose®

1. Purified lectin protein (ligand) (*see* Table 1).
2. CNBr-Activated Sepharose® 4 Fast Flow (Cytiva).
3. Buffer A: 1 mM HCl.
4. Buffer B: 100 mM Sodium carbonate buffer, pH 8.2 with 500 mM NaCl.

5. Buffer C: 100 mM Tris–HCl buffer pH 8.0.
6. Buffer D: 100 mM Tris–HCl buffer pH 8.0, 500 mM NaCl.
7. Buffer E (Low pH wash buffer): 100 mM Sodium acetate buffer pH 4.0, 500 mM NaCl.
8. Buffer F (Storage buffer): 10 mM PBS buffer pH 7.4, 200 mM NaCl, 10 mM KCl.
9. Dialysis tubing and large beakers.
10. Quartz cuvettes for measuring absorbance at 280 nm.

3 Methods

Lectin affinity chromatography (LAC) may be carried out at room temperature or in a cold room at 4 °C. The final choice of temperature will depend on the stability of the target protein to be purified. In general, it is good practice to pre-chill all solutions to maintain a lower temperature.

3.1 Typical Methodology for Lectin Affinity Chromatography

1. Gently resuspend the appropriate amount of lectin-Sepharose® (*see* Table 1) into the appropriate volume of column buffer in order to make a 75% (v/v) gel slurry. Degas this gel slurry (*see* **Note 3**).
2. Pour the degassed gel slurry into a column of appropriate dimensions (*see* **Note 4**).
3. Continue packing the column until the desired level is reached. Wash the gel with 2–3 column volumes of column buffer to remove any loosely bound or degraded lectin ligand.
4. Wash the packed column with 3–4 column volumes of 0.5 M free sugar in column buffer or the highest concentration of free sugar that will be used (*see* **Note 5**).
5. Wash the column with >5 column volumes of column buffer without free sugar to re-equilibrate (*see* **Note 5**).
6. Slowly load the protein sample onto the column to allow appropriate time for binding without disturbing the gel in the column (*see* **Notes 6** and **7**).
7. Monitor the flow-through and subsequent wash fractions by measuring the A280 until it approaches baseline value (*see* **Note 8**).
8. Using an appropriate specific assay, check the column flow-through and wash fractions for the presence of the protein of interest (e.g., enzyme activity or antibody binding) (*see* **Note 9**).

9. Elute the column with 0.5 M of the appropriate free α sugar or sugar analog in column buffer. Monitor the fractions for absorbance at A_{280} and for activity (using specific assay) and pool peak-activity fractions (*see* **Note 10**).
10. Regenerate the column by washing with 10 volumes of column buffer or until the free sugar concentration is below 20 μg/mL. The column can be reused immediately or stored at 4 °C in column buffer containing 0.02% (w/v) NaN_3.

3.2 Immobilizing a Lectin onto Cyanogen Bromide-Activated Sepharose®

If the lectin that is required is not commercially available as an immobilized resin, then it is possible to prepare a specific lectin affinity resin. Cyanogen bromide (CNBr)-activated Sepharose® is a readily available commercial product. The activation process produces extra reactive hydroxyl groups on the Sepharose®, and most lectins have enough reactive groups on their surfaces to couple to these activated supports. In general, protein ligands chemically couple to activated Sepharose® resin through their primary amines. The immobilization of a lectin ligand to activated Sepharose® beads is carried out under mild reaction conditions, and this, coupled with the general stability of most lectins, means that such lectin immobilizations usually have a good success rate.

1. Obtain purified lectin protein either commercially or through purification of recombinant microbial lectin (*see* **Note 11**).
2. Determine the amount of resin required. Weigh out the appropriate amount of dry CNBr-activated Sepharose® resin and swell it in X volumes of buffer A (*see* **Note 12**).
3. Buffer exchange the lectin protein in buffer B at 4 °C (*see* **Note 13**).
4. Measure the absorbance at 280 nm of the dialyzed lectin preparation in a UV spectrophotometer with a standard quartz cuvette. Calculate the final protein concentration (*see* **Notes 14** and **15**).
5. Add 5 resin volumes of buffer A to the activated Sepharose® resin at 4 °C.
6. Complete the gel activation step by incubating for 4 h at 4 °C with continuous but gentle rotation on a standard bench-top orbital shaker.
7. Centrifuge the resin at 1000 × *g* for 10 min, decant and discard the supernatant.
8. Resuspend the activated resin pellet in 10 resin volumes of buffer B containing the lectin solution and incubate overnight at 4 °C with continuous but gentle rotation (*see* **Note 16**).
9. Centrifuge the resin at 1000 × *g* for 10 min, decant the supernatant and measure its protein concentration (*see* **Note 17**).

10. Add another 5 resin volumes of buffer B to the coupled resin and subject it to gentle but continuous rotation at room temperature for 1 h (*see* **Note 18**).
11. Centrifuge the resin again at 1000 × *g* for 10 min and decant the supernatant.
12. Add 10 resin volumes of the buffer C to the lectin coupled resin and subject it to gentle but continuous rotation at room temperature for 3 h (*see* **Note 19**).
13. Centrifuge the resin again at 1000 × *g* for 10 min and decant the supernatant.
14. Resuspend the lectin coupled resin in 10 resin volumes of buffer D and leave the resuspension for 30 min at 4 °C. Then centrifuge at 1000 × *g* for 10 min and decant the supernatant.
15. Resuspend the lectin resin in 10 resin volumes of the buffer E and leave the resuspension for 15 min at 4 °C. Centrifuge at 1000 × *g* for 10 min and decant the supernatant.
16. Repeat **steps 14** and **15** three more times (*see* **Note 20**).
17. The lectin resin is now ready for use.
18. For storage, resuspend the lectin resin in 5 resin volumes of buffer F and keep at 4 °C (*see* **Notes 21** and **22**).

4 Notes

1. When using Con A lectin columns, the presence of divalent cations (Ca^{2+} and Mg^{2+}) in the column loading buffer significantly improves glycoprotein binding.
2. Prepare $MgCl_2$ within a day or two of use and add it to the buffer only after the pH has been adjusted with 0.5 M HCl. The final concentration of metal ions can be decreased tenfold or more if needed.
3. The volume of lectin beads should be sufficient to bind 100 mg glycoprotein. A 1.0 × 30 cm column will bind 50 mg total glycoprotein. If the amount of glycoprotein in the sample is substantially less than 50–100 mg, decrease the volume of the column accordingly. A 75% (v/v) slurry concentration is where the settled resin volume is 75% of the total volume of the slurry.
4. The ratio of input protein to lectin does not seem to matter as long as the column is not overloaded.
5. It is important to pre-wash the column with the eluting sugar and then to re-equilibrate the column to remove any materials that may have previously bound to the column.

6. A flow rate of 1.0 mL/min, using a 1.0 × 30 cm column, is optimal for most lectin affinity gels and allows for optimal binding of glycoproteins to the immobilized lectin in the column.
7. If detergents are required to solubilize the glyco- or membrane protein, either Triton X-100 or Tween 20 has a negligible effect on binding to lectins. Do not use glucoside-based detergents, for example, octyl glucoside and octyl thioglucoside, as they may interfere with binding.
8. If the A_{280} does not rapidly return to baseline, it may indicate weak interaction of some proteins with the lectin, or overloading of the column.
9. To determine if the column has been overloaded, add a small volume of the flow through (1%) to a small volume of the lectin gel. If glycoprotein is found to be bound to the test column as determined by the appropriate assay, overloading of the original column is indicated. In this case, reapply the flow through with excess glycoprotein to the larger column after the bound proteins of the first run have been eluted with sugar and the column re-equilibrated.
10. Analyze the elution profile obtained from the column. Broad peaks may result during specific elution with the sugar if the glycoprotein dissociates from the immobilized lectin ligand slowly. When this happens, sample recovery may be improved by filling the column with 0.5 M of the corresponding free sugar in the column buffer and allowing the column to stand for at least 4 h. This allows dissociation of the bound material. When the flow is started again, it should give a sharper peak. Other possible remedies include warming a cold column to room temperature in the presence of free sugar, increasing the concentration of the sugar to 1 M, or increasing the NaCl concentration to 1 M. A combination of these approaches may be needed to elute a tightly bound protein.
11. The purified lectin should be prepared in a buffer compatible for coupling, for example, CNBr-activated Sepharose® will react with Tris buffer, and therefore, it is important to remove any traces of Tris from the lectin solution. Recombinant prokaryotic lectins may be sourced from GlycoSeLect Ltd. (https://glycoselect.com).
12. Approximately 2.0 mg of lectin protein may be coupled to every 1.0 mL of swollen activated Sepharose® resin.
13. Buffer exchange may be completed using ultrafiltration centrifuge devices or dialysis tubing or cassettes, for example, dialyze the lectin protein into 500 volumes of buffer B at 4 °C. Replace the buffer after 3 h and continue dialyzing for another 3 h.

14. Concentrate the lectin ligand to a range of 1.0–2.0 mg/mL, which is the optimal range for coupling efficiency.
15. The ratio of lectin ligand to resin may be varied depending on the lectin utilized for immobilization.
16. Performing the coupling reaction with the lectin protein in a buffer other than buffer B will reduce coupling efficiency.
17. This will help determine how much of the lectin has actually been immobilized onto the Sepharose® resin.
18. This step should remove any unbound lectin protein from the resin.
19. This step should ensure that there are no unreacted CNBr sites left on the resin. If left untreated, these sites would cause nonspecific binding to the lectin affinity resin.
20. These steps will remove any remaining uncoupled or unbound lectin protein from the resin.
21. Most lectin affinity resins are stable under these storage conditions for up to 3 years.
22. The coupling efficiency may be calculated by dividing the total amount of lectin protein that appears unbound (as calculated in **step 9**) by the total amount of lectin loaded on the column in **step 4**. Typical coupling efficiencies are in the order of 70%.

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Chapter 13

Single-Step Non-Chromatographic Purification of Recombinant Mammalian Proteins Using a Split Intein ELP Tag System

Sai Vivek Prabhala and David W. Wood

Abstract

Glycoprotein therapeutics are currently used by large patient populations and generate significant revenue for the biopharmaceutical industry. These therapeutic proteins are currently purified at industrial scale using individualized processes involving multiple chromatographic steps. In the absence of a viable affinity platform method, the required chromatographic steps are difficult to develop and inevitably lead to significant yield losses. Further, during preclinical development, there is a need for reliable platform technologies capable of performing high-throughput screening for biologic candidates. Although affinity tags can provide a solution to some of these challenges, they require specific affinity resins, and the tag itself can interfere with the target protein characteristics. Fusion protein systems consisting of elastin-like polypeptide (ELP) and self-cleaving split inteins such as *Npu* DnaE can serve as potential non-chromatographic platform technologies for the single-step purification of tagless glycoproteins expressed in mammalian cells. In this chapter, we demonstrate the use of this technology to obtain highly purified anti-ErbB2 ML39 single-chain variable fragment (scFv) expressed from Expi293F suspension cells.

Key words Intein, Elastin-like polypeptide, Split intein, Recombinant protein purification, Non-chromatographic purification

1 Introduction

Inteins are the protein analogs of self-splicing introns, with the ability to posttranslationally remove themselves from their native host proteins and ligate the flanking peptides via a peptide bond [1]. In nature, contiguous inteins (translated from a single gene transcript) are more commonly encountered than split inteins (translated from different gene transcripts) [2]. Protein chemists have engineered cleaving versions of inteins where the naturally occurring splicing reaction is modified to a cleaving reaction either at the N- or C-terminus under suitable environmental conditions such as pH, temperature, reducing agent, and salt concentration.

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Different contiguous intein systems have been designed for the purification of recombinant proteins of interest (POIs) expressed in *E. coli* and *S. cerevisiae* [3].

Elastin-like polypeptides (ELPs) are polymers composed of repeats of the unit peptide $(\text{Val-Pro-Gly-X-Gly})_n$ where X is any natural amino acid excluding proline and n is the number of repeats (typically ranging from 20 to 3300). ELPs undergo a reversible phase transition phenomena at a particular temperature known as the inverse transition temperature (T_t). Below this temperature, ELPs are highly soluble in aqueous solutions, but when T_t is reached, ELPs become insoluble and form large visible micron size aggregates. T_t can be reduced by insertion of more hydrophobic guest residues and increasing the solution ionic strength, polymer concentration, and length [4].

ELP-POI fusions were first used in the late 1990s to purify recombinant proteins as an alternative to existing affinity chromatography techniques. ELPs are attractive at laboratory scale for purifying recombinant POIs because of the simplicity of the purification protocol, economic scale up to larger expression volumes, and relatively high purity of the final product [5].

Often, protein scientists need pure tagless proteins for performing various sensitive assays and studying the effects of the protein in animal models, this being especially true in the case of therapeutic proteins. This necessitates the removal of the ELP tag from the purified protein. While protease cleavage sites can be incorporated during molecular cloning, the high costs of commonly used proteases can make ELP tag purification uneconomical. Further, proteases can cause nonspecific cleavage events that result in destruction of the target protein, or leave additional amino acids on the POI, which is highly undesirable [6]. To overcome this challenge, both contiguous and split inteins have been incorporated into the ELP-POI fusion protein. Purification systems utilizing contiguous inteins were found to have significant premature cleavage in vivo, resulting in reduced POI yield. ELP tag systems that utilize split inteins do not suffer from premature cleavage, as the intein halves are inactive by themselves and are only capable of cleavage after reassembly [7].

We have previously developed a dual-ELP tagged split intein system utilizing two halves of the engineered ΔI-CM mini-intein and used it to successfully purify four microbially expressed proteins [8]. In this system, the contiguous ΔI-CM intein has been artificially split into two segments, where each is joined to its own ELP tag. The same proteins were also successfully purified using a single-ELP tagged split intein system utilizing the two engineered halves of the *Npu* DnaE mini-intein, which exhibits better pH-sensitivity and tighter control over cleavage kinetics [9]. In this case, the N-terminal segment of the split intein is tagged with ELP, while the C-terminal intein segment is fused to the N-terminus of the POI to allow affinity capture.

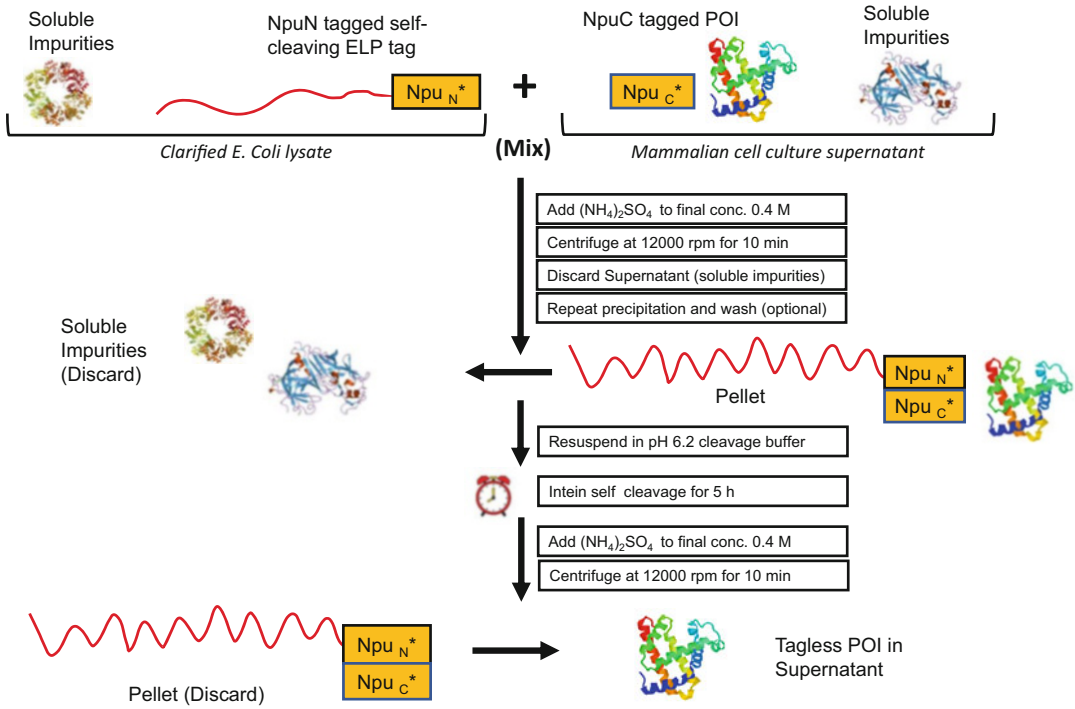

Fig. 1 Schematic of the ELP-tagged *Npu* DnaE split intein method

Here, we extend the application of the ELP tag technology to purify proteins secreted by mammalian cells. In this chapter, we demonstrate the use of the single-ELP tagged *Npu* DnaE split intein system (Fig. 1) to purify the ML39 single-chain antibody (scFv) after secretion from mammalian Expi293F cells. The ML39 scFv targets the extracellular domain of the tumor-associated receptor ErbB2, which is frequently overexpressed in a variety of human cancers [10].

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature unless indicated otherwise. Specific vendors listed below can be substituted in cases where reagents and equipment for general procedures are described (e.g., restriction digests for cloning or gel electrophoresis apparatus).

2.1 Construction of ELP-NpuN and NpuC-POI Fusion Protein

1. *Escherichia coli* cloning strain DH5α (F^- Φ80 *lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1) is from lab stock. The

cells were made competent using the Z-competent cell™ buffer kit (Zymo Research) according to the manufacturer's instructions (*see* **Note 1**).

2. Disposable culture tubes (VWR #47729-584).
3. Ampicillin sodium salt, prepare a stock solution of concentration 100 mg/mL using sterile deionized water.

   Custom primers (Millipore Sigma). To amplify the NpuN fragment from the pET-NpuN plasmid using the *Eco*RI-NpuN-F (5′- GCGGAATTCGGTGACGGTCACGGTGCCTTAAGC TATGAAACGGA-3′) and *Xba*I-NpuN-R primers (5′- GGCT CTAGATTAATTCGGCAAATTATCAACCCG -3′). Plasmids are available on request from our laboratory.
4. 10× Cutsmart® buffer (NEB).
5. *Eco*R1 restriction enzyme (NEB).
6. *Xba*1 restriction enzyme (NEB).
7. Q5 DNA polymerase (NEB#M0491S).
8. 5× Q5 High GC enhancer (NEB).
9. 5× Q5 reaction buffer (NEB #B9027S).
10. 10 mM dNTP stock solution (NEB).
11. Qiagen miniprep kit (Qiagen).
12. Qiagen gel extraction kit (Qiagen).
13. T4 DNA Ligase (NEB).
14. Electrophoresis grade Agarose.
15. *Dpn*1 restriction enzyme (NEB).
16. NEBuilder® 2× HiFi DNA Assembly Master Mix (NEB).
17. PCR machine.
18. 1× Luria Broth (LB) media: Dissolve 10 g NaCl, 5 g yeast extract and 10 g tryptone in 800 mL of deionized water and make up volume to 1 L. Autoclave the media to ensure sterility.
19. LB agar plates supplemented with 100 μg/mL ampicillin. Dissolve 10 g NaCl, 5 g yeast extract, 15 g of agar, and 10 g tryptone in 800 mL of deionized water and make up volume to 1 L. Autoclave the media to ensure sterility. Allow the media to cool and add 1 mL of stock ampicillin solution before pouring on plates. Stores the agar plates in a cold room once solidified.
20. Microbiological incubator for LB agar plates.
21. Temperature-controlled water bath shaker.
22. Temperature-controlled water bath with temperatures ranging from 37 to 80 °C.
23. Gel electrophoresis setup.

2.2 Expression of ELP-Intein Segment

1. *Escherichia coli* expression strain BLR(DE3) (F—*ompT hsd* S_B (r_B^- m_B^-) *gal dcm* (DE3) Δ(srl-recA) 306::Tn10 (Tet^R)) (Millipore Sigma) is from lab stock. The cells were made competent using the Z-competent cell™ buffer kit (Zymo Research) according to the manufacturer's instructions (*see* **Note 1**).
2. Disposable culture tubes (VWR #47729-584).
3. 1× Luria Broth (LB) media: Dissolve 10 g NaCl, 5 g yeast extract and 10 g tryptone in 800 mL of deionized water and make up volume to 1 L. Autoclave the media to ensure sterility.
4. 2× Luria Broth (LB) media: Dissolve 10 g NaCl, 10 g yeast extract and 20 g tryptone in 800 mL of deionized water and make up volume to 1 L. Autoclave the media to ensure sterility.
5. KIMAX® Baffled Culture Flasks (VWR # 89000-988 or equivalent).
6. Incubator orbital shaker.
7. Temperature-controlled water bath shaker.
8. 1.0 M Isopropyl β-D-1-thiogalactopyranoside (IPTG). Prepare a stock solution using sterile deionized water.
9. Refrigerated centrifuge.

2.3 Mammalian Cell Culture

1. Qiagen maxiprep kit (Qiagen).
2. Expi293F cells (Gibco #A14527).
3. Expi293 Expression Medium (Gibco #A1435102).
4. Opti-MEM™ I Reduced Serum Medium (Gibco #31985088).
5. ExpiFectamine™ 293 Transfection Kit (Gibco #A14524).
6. 125 mL Sterile Polycarbonate Erlenmeyer Flask with Vent Cap (Corning #431143).
7. Class II biological safety cabinet.
8. Cell counting slides (Fisher Scientific #0267110 or equivalent) or Countess II cell counter (or equivalent).
9. Trypan Blue Stain (0.4%) (Gibco #15250-061).
10. CO_2 incubator.
11. CO_2 resistant orbital shaker (Thermo Fisher Scientific # 88881101).
12. 15 mL conical tubes.

2.4 Cell Recovery, Lysis, Purification, and Cleavage of Target Protein

1. Refrigerated centrifuge.
2. Ultrasonic cell disruptor (VIRTIS Virsonic100 Cell disruptor ultrasonic converter or equivalent).
3. KIMWIPES™ Delicate Task Wipers (VWR # 21905-026 or equivalent).

4. Vented Safety Wash Bottles (VWR # 16210-923 or equivalent).
5. 50 mL conical tubes.
6. Low salt buffer: 20 mM 2-Amino-2-methyl-1,3-propanediol, 20 mM piperazine-N, N′-bis (2-ethanesulfonic acid), and 1 mM ethylenediaminetetraacetic acid at pH 8.5.
7. 2 M Ammonium sulfate stock solution.
8. Cleaving buffer: 20 mM 2-Amino-2-methyl-1,3-propanediol, 20 mM piperazine-N, N′-bis (2-ethanesulfonic acid) and 1 mM ethylenediaminetetraacetic acid at pH 6.2. Adjust the pH by adding 2 M or higher concentration NaOH.
9. Amicon Ultra-15 Centrifugal Filter Unit (50 KDa MWCO) (Millipore Sigma).
10. 0.2 M Sodium Hydroxide (NaOH).
11. Temperature-controlled water bath/heating block at 98 °C.
12. 2× Laemmli Sample Buffer (Biorad # 1610737). This is also referred to as 2× SDS Loading Dye in the protocol.

2.5 Silver Stain Analysis

1. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific #26616).
2. 12% Polyacrylamide SDS-PAGE gel (0.75 mm thickness is recommended).
3. SDS-PAGE apparatus and tris-glycine running buffers.
4. Fixing solution: Mix 30% v/v absolute ethanol, 10% v/v glacial acetic acid, and 60% v/v deionized water in a graduated cylinder before transferring to a conical flask. Prepare 100 mL for staining one gel.
5. Sensitizing solution: Add about 13 mg of sodium thiosulfate powder to 100 mL of deionized water to prepare the solution.
6. 20% Ethanol solution: Mix 20% v/v absolute ethanol with deionized water to prepare the solution.
7. Staining solution: Add about 204 mg of silver nitrate powder to 100 mL of deionized water to prepare the solution. Wrap the flask with aluminum foil as the solution is photosensitive.
8. Developing solution: Add 3.75 g of potassium carbonate, 15.625 µL of 10% w/v sodium thiosulfate and 31.25 µL of formaldehyde and make up the volume to 125 mL using deionized water.
9. Stopping solution: Add 4 g of Tris base and 2 mL of glacial acetic acid to a conical flask. Make up the volume to 100 mL using deionized water.
10. Clean gel boxes.
11. Gel imaging system.

2.6 Western Blotting Analysis

1. SDS-PAGE apparatus and tris-glycine running buffer.
2. 12% Polyacrylamide SDS-PAGE gel (1.5 mm thickness is recommended).
3. Blotting Grade Blocker nonfat dry milk (Biorad).
4. 1× PBS-T buffer: Add 1 mL of Tween 20 to 500 mL of 1× PBS and make up the volume to 1 L.
5. PageRuler Prestained Protein Ladder (Thermo Fisher Scientific #26616).
6. PVDF transfer membrane (0.45 micron).
7. Blocking Buffer A: Dissolve 2.5 g of blotting Grade Blocker nonfat dry milk in 50 mL of 1× PBS-T buffer.
8. 6×-His tag anti-mouse monoclonal antibody (Thermo Fisher Scientific # MA1-21315).
9. Blocking Buffer B: Dissolve 0.5 g of blotting Grade Blocker nonfat dry milk in 50 mL of 1× PBS-T buffer.
10. Goat anti-mouse secondary antibody conjugated to HRP (Thermo Fisher Scientific #62-6520).
11. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific #34095).
12. Clean gel boxes.
13. LI-COR C-DiGit™ blot scanner.
14. Transfer buffer: Dissolve 28.8 g of glycine, 6.04 g of tris base and 200 mL of methanol in deionized water and make up the volume to 2 L.
15. Transfer apparatus, filter paper strips, sponge pads.

3 Methods

3.1 Construction of ELP-NpuN and NpuC-POI Fusion Protein

3.1.1 Creation of the ELP-Tagged NpuN Construct

1. Set up a 50 μL PCR reaction containing 0.5 μM concentration of each primer (2.5 μL of each 10 μM stock solution), 200 μM dNTP mix (1 μL of 10 mM stock solution), 2–10 ng of template DNA, 10 μL of 5× Q5 reaction buffer, 10 μL of 5× Q5 High GC Enhancer, 0.5 μL of Q5 DNA polymerase and make up the volume with ultrapure water. Start the PCR reaction with a hold at 98 °C for 30 s, followed by 25 cycles of 8 s melting at 98 °C, 20 s annealing (at the lower of the two primer melting temperatures plus 5 °C), and extension at 72 °C for 30 s/kb of DNA sequence. After 25 cycles, hold the reaction at 72 °C for an additional 5 min. This PCR has been set up to amplify the NpuN fragment from the pET-NpuN plasmid using the *Eco*RI-NpuN-F forward and *Xba*I-NpuN-R reverse primers (*see* Subheading 2.1).

2. Digest 1 μg PCR product with *Eco*RI and *Xba*I restriction enzymes by maintaining a 10- to 20-fold excess enzyme factor over the DNA concentration for an hour at 37 °C in 10× Cutsmart® Buffer. Also, digest 1 μg of the previously reported pET-ELP backbone plasmid using the same restriction enzymes [11].
3. Heat the digested vector and PCR products at 80 °C for 20 min to inactivate the enzymes and then resolve via a 1% agarose gel. Excise the vector backbone and PCR product bands and extract the DNA from the agarose gel using Qiagen's gel extraction kit according to the manufacturer's instructions.
4. Ligate the digested PCR and vector backbone products at a molar ratio of 3:1 respectively, according to manufacturer's instructions for the ligase. The molar ratios can be determined by an additional agarose gel, or by absorbance at 280 nm. We strongly recommend a control ligation reaction without the PCR product insert (*see* **Note 2**).
5. Inactivate the ligation product at 65 °C for 15 min (*see* **Note 3**).
6. Transform 5 μL of each ligation product (with and without PCR product insert) into separate vials containing 50 μL each of Z-competent DH5α cells, prepared and transformed according to manufacturer's instructions.
7. Spread the cells on pre-warmed LB agar plates (*see* **Note 4**) supplemented with 100 μg/mL ampicillin and incubate at 37 °C overnight for 12–16 h (*see* **Note 5**).
8. Check the transformation agar plates including the control plate (*see* **Note 6**).
9. Following a successful transformation experiment, prepare multiple overnight cultures using three to five positive colonies from the patch plate in 5 mL 1× LB medium supplemented with 100 μg/mL ampicillin. Incubate each at 37 °C and 220 rpm agitation in a temperature-controlled water bath shaker for 12–16 h.
10. Extract the plasmid from each overnight culture using a Qiagen mini prep kit according to the manufacturer's instructions. The extracted plasmids should then be confirmed by DNA sequencing (*see* **Note 7**).

3.1.2 Creation of NpuC-POI Fusion Protein

1. Design primers for your Gibson Assembly following NEB guidelines on primer design to construct the plasmid encoding signal peptide-NpuC-POI [12]. As part of the Gibson Assembly, create forward and reverse primers to amplify the C-intein (NpuC), signal peptide of your choice (human secreted albumin for scFv-6X His) and your POI with suitable overhangs (*see*

Note 8). Also, create two primers to amplify the pTT vector backbone. In our experience, pTT vectors have resulted in successful transfections in Expi293F cells for different proteins [13]. You can use other vector backbones for Expi293F and for different mammalian cell lines such as CHO or BHK based on your prior experience.

2. Set up four PCR reactions: one for the pTT vector backbone, one for your POI, one for the signal peptide, and the last one for the NpuC fragment. Each 50 μL PCR reaction contains 0.5 μM concentration of each primer (2.5 μL of each 10 μM stock solution), 200 μM dNTP mix (1 μL of 10 mM stock solution), 2–10 ng of template DNA, 10 μL of 5× Q5 reaction buffer, 10 μL of 5× Q5 High GC Enhancer, 0.5 μL of Q5 DNA polymerase with the remaining volume made up by ultrapure water. Start the PCR reaction with a hold at 98 °C for 30 s, followed by 25 cycles of 10 s melting at 98 °C for the pTT vector and 8 s for the other DNA fragments, 20 s annealing (at the lower of the two primer melting temperatures plus 5 °C), and extension at 72 °C for 30 s/kb of DNA sequence. After 25 cycles, hold the reaction at 72 °C for an additional 5 min.
3. Perform a PCR cleanup procedure to remove unreacted reagents.
4. Resolve the PCR products via a 1% agarose gel electrophoresis to verify if the amplicon is of the expected size (*see* **Note 9**).
5. Set up a 20 μL digest comprising of 17 μL of purified pTT vector DNA, 2 μL of 10× Cutsmart® buffer, 0.5 μL of *Dpn*1 enzyme and 0.5 μL of ultrapure water. Perform the digestion reaction for 1 h at 37 °C and then inactivate the enzyme by heating the DNA at 80 °C for 20 min (*see* **Note 10**).
6. Add 5 uL of NEBuilder® 2× HiFi DNA Assembly Master Mix to a PCR tube along with a mixture of the four different amplicons. The vector: insert molar ratio must be between 1:2 and 1:5. The remaining volume can be made up with ultrapure water (*see* **Note 11**). You can refer to NEB resources on how to set up the Gibson Assembly reaction [12].
7. Incubate the assembly reaction at 50 °C for 1 h and cool down the reaction to 4 °C upon completion.
8. Transform 5 μL of the reaction mixture into separate vials of 50 μL each of Z-competent DH5α cells, prepared and transformed according to manufacturer's instructions (*see* **Note 12**).
9. Spread the cells on pre-warmed LB agar plates supplemented with 100 μg/mL ampicillin and incubate at 37 °C overnight for 12–16 h.

10. Following a successful transformation experiment, prepare multiple overnight cultures using three to five positive colonies from the plate in 5 mL 1× LB medium supplemented with 100 μg/mL ampicillin. Incubate each at 37 °C and 220 rpm agitation in a temperature-controlled water bath shaker for 12–16 h.
11. Extract the plasmid from each overnight culture using a Qiagen mini prep kit according to the manufacturer's instructions. The extracted plasmids should then be confirmed by DNA sequencing.

3.2 Expression of ELP-NpuN Fusion Protein

1. Transform the recombinant plasmid which expresses the ELP-NpuN fusion protein (prepared above) into Z-competent BLR (DE3) cells separately according to manufacturer's instructions using a pre-warmed agar plate supplemented with 100 μg/mL ampicillin and incubate at 37 °C for 12–16 h.
2. Pick a single colony from each plate and grow in 5 mL 1× LB medium supplemented with 100 μg/mL ampicillin in a culture tube overnight at 37 °C.
3. Dilute 1 mL of the overnight cultures into multiple 100 mL fresh 2× LB medium in 500 mL baffled flasks and grow at 37 °C with an agitation speed of 220 rpm until the optical density at 600 nm (OD 600) reaches a value between 0.6 and 0.8 (*see* **Note 13**). This normally takes 2.5–3 h.
4. Induce protein expression by addition of 0.5 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) and allow agitation to continue at 16 °C for 18 h.
5. Divide the cells from the flask into 50 mL conical tubes and pellet by centrifugation at 8000 × g for 10 min. The harvested cell pellets can be stored at −80 °C until purification.

3.3 Mammalian Cell Culture

1. Follow the instructions of the Qiagen maxiprep kit to obtain extremely pure pTT-signal peptide-NpuC-POI plasmid DNA (*see* **Note 14**).
2. Count the Expi293F cells using trypan blue solution and cell counting slides (or an automated cell counter) and seed the cells at a density of 400,000–500,000 cells/mL in 30 mL of Expi293 Expression Medium. Sterile polycarbonate Erlenmeyer flasks with vented caps of volume 125 mL are to be used for passaging and transfection.
3. Passage the cells every 3–4 days and incubate in a humidified orbital shaking incubator at 37 °C with shake speed of 125 rpm and 8% CO_2 atmosphere. A maximum cell density of 3–5 × 10^6 cells/mL is recommended, at which point cells should be immediately passaged (*see* **Note 15**).

4. Add 75×10^6 cells into 25 mL (3×10^6 cells/mL) of Expi293 expression medium in a 125 mL Erlenmeyer flask. Perform the transfection according to manufacturer's instructions.
5. Add 1.5 mL of Opti-MEM media to a 15-mL sterile polypropylene conical tube along with 30 μg (1 μg/mL final dilution in the total volume of culture) of plasmid DNA prepared by maxiprep (for SA-NpuC-scFv-6XHis), while in another sterile conical tube, add 80 μL of ExpiFectamine transfection reagent from the kit along with 1.4 mL of Opti-MEM media. Incubate the tubes at room temperature for 5 min.
6. Mix the contents of both 15 mL tubes at room temperature by pipetting and incubate the mixture for 15–20 min. Add the transfection mixture dropwise to the cells slowly, over the course of a few seconds using a micropipette while swirling the cells in a circular pattern.
7. Return the flask to the shaking incubator.
8. At 18 h post-transfection, add 150 μL of ExpiFectamine 293 Transfection Enhancer 1 and 1.5 mL of ExpiFectamine 293 Transfection Enhancer 2 from the kit to the culture and subsequently return the culture to the shaking incubator.
9. At 5–7 days post-transfection, harvest the cell culture supernatant by centrifuging at 3500 × g at 4 °C for 20 min. The cell culture supernatant so obtained is used for the purification of ML39 scFv.

3.4 Cell Lysis, Recovery, Purification, and Cleavage of Target Protein

1. Titrate the cell culture supernatant to a pH of 8.50 using 0.2 M NaOH for ELP purification in 50 mL conical tube(s) (*see* **Note 16**). Alternatively, the cell culture supernatant can also be stored at −80 °C (*see* **Note 17**).
2. Resuspend the cell pellet containing the ELP-NpuN construct in low salt buffer. The resuspension volume corresponds to ten times the mass of the cell pellet. For instance, a pellet weighing 2 g must be resuspended in 20 mL of low salt buffer.
3. Sonicate the resuspended pellet for 10 cycles of 30 s at a power setting of 4–5 Watts, with 30 s on ice between each sonication cycle.
4. Clarify the whole cell lysate by centrifugation at 12,000 × g for 15 min at 4 °C. The ELP-NpuN construct is present in the supernatant so obtained.
5. Mix the ELP-NpuN clarified lysate with NpuC-POI titrated supernatant in a volume ratio of 1:10 (*see* **Note 18**). Mix the 50 mL conical tube(s) by inversion for 10 min (*see* **Note 19**).
6. Add ammonium sulfate to the mixture to a final concentration of 0.4 M (using a 1:5 dilution of a 2 M ammonium sulfate stock solution) and incubate the 50 mL conical tube(s) at 37 °C in a water bath for 15 min (*see* **Note 20**).

7. Centrifuge the sample at 17,000 × g for 10 min at 4 °C. Redissolve the recovered pellet in 4 mL low salt buffer by repeated pipetting.
8. Perform another round of precipitation by adding ammonium sulfate as before to a final concentration of 0.4 M, incubate the conical tube at 37 °C in the water bath for 15 min. Recentrifuge the sample at 17,000 × g for 10 min at 4 °C (*see* **Note 21**).
9. Dissolve the pellet so obtained in 1 mL of cleaving buffer by repeated pipetting.
10. Incubate the sample at 37°C in the water bath for 5 h. Take time point samples for silver stain SDS-PAGE and western blot analysis by mixing 20 μL of 2× SDS loading dye with 20 μL of sample and heating at 98 °C for 5 min (*see* **Note 22**).
11. After 5 h of cleavage, add ammonium sulfate as before at a final concentration of 0.4 M to precipitate the ELP tag. Incubate the sample at 37 °C in a water bath for 15 min and centrifuge at 17,000 × g for 10 min at 4 °C. The cleaved purified product is present in the supernatant.
12. Pipette the supernatant carefully into an Amicon centrifugal tube with a suitable molecular weight cutoff, 50 kDa in this case. Centrifuge at 3500 × g for 10 min to remove any residual ELP tag. Purified scFv can be recovered in the filtrate at the bottom of the tube (*see* **Note 23**). We obtained a final yield of 1.77 ± 0.06 μg of scFv-6X His per mL of cell culture at the end of the complete process as measured by A280 measurements (extinction coefficient at 280 nm = 66,350 M^{-1} cm^{-1}). The course of the purification has been analyzed using silver staining and western blotting (*see* Fig. 2).

3.5 Silver Stain Analysis

1. Run the denatured SDS-PAGE samples on the 12% gel using standard laboratory SDS-PAGE apparatus and Tris-glycine running buffer for 60 min at 180 V using the prestained molecular wight standard. Add 5 μL of sample to each well and 3 μL of the ladder.
2. Carefully remove the gel from the casing and gently rinse it with deionized water in a clean gel box.
3. Add 50 mL of fixing solution to the gel and incubate at room temperature with mild shaking for at least 30 min. Replace the solution with another 50 mL and continue incubation for another 30 min. The gel can also be kept overnight in fixing solution.
4. Rinse the gel with 50 mL of 20% ethanol twice for 10 min each time with mild shaking.
5. Rinse the gel with 50 mL of deionized water twice for 10 min each time with mild shaking.

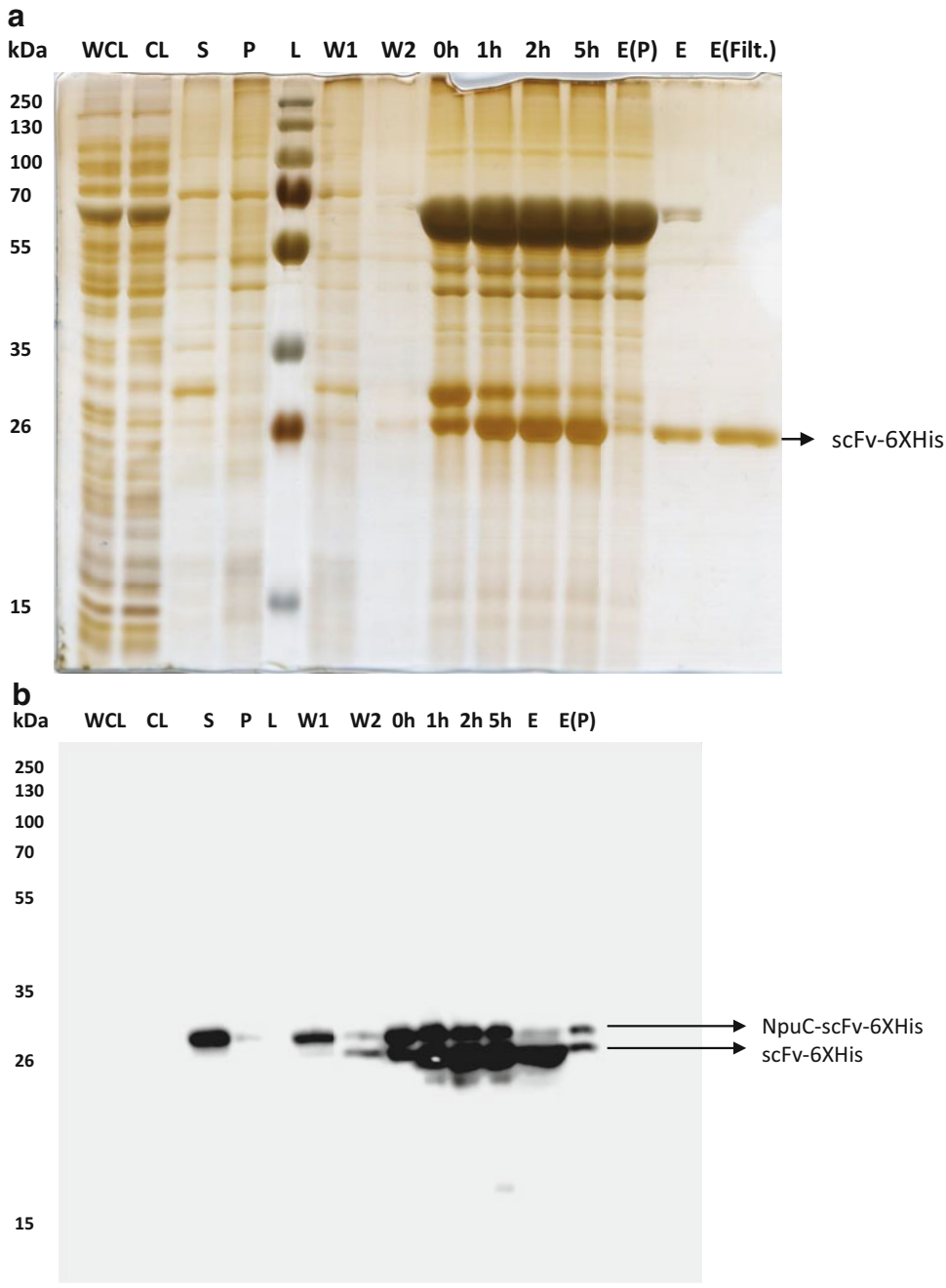

Fig. 2 (**a**) Silver stain gel analysis. (**b**) Anti-His Western blotting analysis. WCL: *E. coli* Whole Cell Lysate, CL: *E. coli* Clarified Lysate, S: cell culture supernatant, P: mammalian cell culture pellet, W1: supernatant of first wash at pH 8.5, W2: supernatant of second wash at pH 8.5, 0–5 h: time point samples indicating extent of cleavage, E: elution sample recovered in the supernatant, E(P): sample from pellet obtained after final centrifugation resuspended in 1× PBS in a volume equivalent to supernatant volume, E(Filt.): elution sample obtained at the bottom of the Amicon tube. Molecular weights: scFv-6XHis = 28.3, NpuC-scFv-6XHis = 32.3 KDa

6. Add 100 mL of sensitizing solution to the gel and incubate at room temperature for 45 min with mild shaking.
7. Rinse the gel twice with 50 mL of deionized water after discarding the sensitizing solution to remove any residual sensitizing solution for 1 min each time with mild shaking.
8. Add 100 mL of staining solution to the gel and incubate with gentle mixing at room temperature for 20 min.
9. Briefly rinse the gel with deionized water for 10 s.
10. Add 125 mL of developing solution to the gel and mix gently. Allow the bands to develop for about 4–6 min.
11. Once the bands are clearly visible, add stopping solution and incubate at room temperature with gentle mixing for 30 min. The gel can then be scanned using a suitable imaging device.

3.6 Western Blotting Analysis

1. Run the denatured SDS-PAGE samples on the 12% gel using standard laboratory SDS-PAGE apparatus and Tris-glycine running buffer for 75 min at 180 V using the prestained molecular weight standard. Add 5 μL of sample to each well and 3 μL of the ladder.
2. Carefully remove the gel from the casing and gently rinse it with deionized water in a clean gel box.
3. Wet the PVDF transfer membrane in 100% methanol for 5 min and immediately transfer it to a clean gel box containing transfer buffer. Additionally, soak filter paper strips in transfer buffer for 5–10 min.
4. Assemble the western blot sandwich by carefully aligning the gel and PVDF transfer membrane in between the filter paper strips and the sponge pads.
5. Perform the transfer operation using the transfer apparatus and transfer buffer by running the setup for 90 min at 300 mA.
6. Carefully pull apart the setup, wash the membrane with 1× PBS-T buffer twice then and place the membrane in 25 mL of blocking buffer A for 2 h.
7. Discard the used blocking buffer A solution and add 20 mL of blocking buffer B solution along with the primary antibody at a 1:10,000 dilution. Incubate the membrane overnight in a cold room.
8. Discard the primary antibody solution the next day and rinse the membrane thrice with 1× PBS-T buffer for 5 min each time.
9. Add 20 mL of blocking buffer B solution along with the secondary antibody at a 1:10,000 dilution. Incubate the membrane at room temperature for 1–2 h.

10. Discard the secondary antibody solution and rinse the membrane thrice with 1× PBS-T buffer for 5 min each time.
11. Transfer the membrane to a fresh gel box and add 500 μL each of the two reagents in the SuperSignal West Femto Maximum Sensitivity Substrate ensuring that the reagents are spread uniformly across the membrane. Incubate the membrane at room temperature for 5 min.
12. Scan the membrane on a LI-COR C-DiGit™ blot scanner or equivalent system.

4 Notes

1. These are the cloning and expression strains we typically use in our laboratory. It is likely that any other cloning and expression strains will also work.
2. The control reaction will give an idea about the number of background colonies which result from incomplete digestion of the vector backbone. This is useful for troubleshooting in cases where one is unable to isolate the correct insert from the transformed colonies.
3. In our experience, in most cases, the inactivation step can increase the transformation efficiency.
4. In our experience, pre-warming the plates at 37 °C for about 30 min before transformation will typically result in higher transformation efficiency.
5. Longer incubation times (greater than 16 h) should be avoided due to possibility of formation of untransformed satellite colonies.
6. Under ideal circumstances, there will not be any colony formation on the control plate (no PCR product insert). The ligation plates will have several hundred colonies. Should similar numbers of colonies appear on both plates, then we recommend repeating the entire procedure with greater care given to the digestion conditions.
7. Typically, one Sanger sequencing reaction can read around 800–900 bp accurately.
8. You will have to perform screening expressions to assess which signal peptide results in significant secretion of the POI. We recommend performing these screening experiments for the NpuC-POI construct instead of just the POI construct. If you do not want a secreted product, you can omit the signal peptide fragment in the assembly mix. You can follow the same procedure used previously for purifying microbial proteins in that case [9].

9. The percentage of gel to be made depends on the size(s) of the amplicon(s).
10. Dpn1 digestion removes Dam methylated DNA (parent DNA in this case), thus ensuring that the subsequent DNA assembly only utilizes PCR amplicons.
11. Optimized cloning efficiency is observed to be 50–100 ng of vector with two- to threefold molar excess of each insert. Make sure to use a fivefold molar excess of any insert less than 200 bp. This will be the case for the amplified NpuC fragment and the signal peptide. Also, the NEB protocol has a total assembly reaction volume of 20 μL. We use 10 μL in our laboratory and have not observed any difference in cloning efficiency.
12. It is a good idea to include controls to gauge the success of the assembly reaction. The negative control includes a reaction mix without the vector backbone. Thus, the negative control plate should not have any colonies as it lacks the ampicillin resistance gene.
13. In our experience, if the same media is used for the large-scale expression, a shorter lag phase is observed.
14. The A260/280 value for DNA prepared by Maxiprep must be between 1.80 and 1.85 for successful transfection.
15. The viability of the cells must be greater than 95% for successful transfection.
16. You can also mix with milder bases such as 0.2 M sodium phosphate or 0.2 M Tris base in case your protein is susceptible to degradation.
17. If you opt to freeze the cell culture supernatant, make sure to avoid repeated freeze/thaw cycles.
18. The volume ratio of mixing is variable from protein to protein and needs to be identified using a scouting experiment involving different ratios. Alternatively, you can work in terms of molar excess if you have pre-purified ELP-NpuN and an assay capable of giving reasonable concentration estimates of your POI in cell culture supernatant, with the ideal ratio being decided by scouting experiments. The volume ratio of mixing has a huge impact on the amount of protein recovered.
19. In our experience, 10 min is sufficient contact time. You may also opt for longer contact times for better POI recovery and yield.
20. We recommend incubating the conical tube in a water bath instead of an incubator because the thermal conductivity of water is higher than that of air. Incubation in the water bath results in the sample reaching a temperature of 37 °C faster. Further, the incubation time may need to be tuned for your

ELP construct. We suggest performing a trial experiment where after addition of 0.4 M ammonium sulfate to ELP-NpuN solution, you can measure the optical density at 600 nm to assess the turbidity of the solution. Avoid large incubation times to minimize product loss as cleavage is not completely absent at pH 8.50. The optimal incubation time will correspond to the highest optical density measurement with minimum product loss.

21. Performing a second round of precipitation results in extremely high purity of the POI.
22. You can allow cleavage to occur for longer times to enhance product recovery.
23. The molecular weight cutoff (MWCO) of your Amicon centrifugal unit tube will vary according to the size of your POI. Other methods such as dialysis can also be used.

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Chapter 14

Clinical Proteomics: Liquid Chromatography-Mass Spectrometry (LC-MS) Purification Systems

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Abstract

Liquid chromatography/mass spectrometry (LC/MS) has become a routine powerful technology in clinical proteomic studies for protein identification, protein characterization, and the discovery of biomarkers. In this chapter, we describe two protocol methods to analyze clinical patient samples using a resin-based depletion column followed by either protein In-Gel enzymatic digestion or protein In-Solution enzymatic digestion using a simple kit-based approach (i.e., using the PreOmics iST sample preparation kit), followed by analysis using one-dimensional reverse-phase chromatography (RPC) or high pH reversed-phase peptide fractionation.

Key words Clinical proteomics, Immunodepletion, Protein separation, Peptide separation, Protein identification, Protein digestion, High-resolution tandem mass spectrometry

1 Introduction

Liquid chromatography-mass spectrometry (LC-MS) is an analytical technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry and is a fundamental tool in clinical proteomics [1]. Monitoring the protein expression pattern in mammalian cells and from clinical patient samples by proteomic technologies offers opportunities to discover potentially new biomarkers for the early detection and diagnosis of diseases including cancer [2]. A biomarker is described as a biological molecule found in blood, other body fluids, or tissues that provide an indication of a normal or abnormal process, or of a condition or of a disease. Substantial advancements in reproducible and rapid sample preparation, along with nano-flow peptide separation, increasingly sensitive and ultra-fast MS-detection, and data analysis have allowed robust and routine quantitation of proteins from complex clinical samples. There are numerous protein and peptide fractionation

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techniques employed including traditional chromatography [3], immunodepletion [4], subcellular fractionation [5], nanoparticle enrichment [6], and ProteoMiner™ technology (a combinatorial library of hexapeptide ligands coupled to beads) [7] for protein expression profiling of clinical samples. Using these techniques, it is possible to carry out relative or absolute LC-MS quantitation between experimental groups (e.g., patient cohorts) from various types of clinical samples. Relative quantitative LC-MS proteomic analysis uses labels to compare protein or peptide abundances between samples. Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) [8] and isobaric tags such as iTRAQ and TMT [9] are commonly used proteomic labeling techniques used in quantitative proteomics. A label-free quantitative LC-MS/MS approach can also be used for differential analysis between patient samples [10, 11]. Absolute quantitation can also be applied to LC-MS with spiked known concentrations of labeled synthetic peptides [12, 13] for selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) MS methods.

These proteomic tools have been used for the differential analysis of various types of clinical biological samples including tissue [10], cell line models [14], primary tumors [14], saliva [15], plasma [16], serum [17], urine [18], and plasma cells from bone marrow [19], to better understand the molecular basis of the pathogenesis of disease and also in the validation and characterization of disease-associated proteins.

There have been many advances in the use of LC-MS in the proteomic analysis of clinical samples, but challenges still remain including the dynamic range of proteins present in biological samples such as serum or plasma where potential biomarkers of interest may be present at very low concentrations, thus requiring the depletion of large serum proteins and also the sensitivity and reproducibility of the LC and MS instrumentation [20, 21]. Albumin and IgG are two major protein components in serum/plasma contributing to approximately 80% of the total protein concentration. Removal of albumin and IgG can allow for the visualization and possible identification of co-migrating proteins on one-dimensional (1D) and/or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). Removal of these abundant proteins will also make possible the detection of low abundant proteins present as the in-spectrum dynamic range of most mass spectrometers is not compatible with the dynamic range of protein concentrations found in plasma/serum samples that have not been depleted [7].

In this chapter, we describe LC-MS-based approaches for analyzing proteins from clinical patient bio-fluid samples that have been immuno-depleted of their high abundance serum proteins

to allow for the detection of medium and low abundant proteins present in the sample. The first approach involves visualizing a depleted serum sample on a Coomassie-blue stained SDS gel and In-gel digestion of the separated proteins using first-dimension reverse-phase liquid chromatography mass spectrometry (1D-RPC LC-MS). The second approach involves the use of high pH reversed-phase peptide fractionation for identification from LC/MS analysis by separating peptides based on their hydrophobicity to provide excellent orthogonality to low-pH reversed-phase LC-MS gradients.

2 Materials

2.1 Immuno-depletion from Serum Sample

1. ProteoPrep® Blue Albumin Depletion Kit (Merck) (*see* **Note 1**).
2. Serum sample from blood prepared and stored using standardized procedures [22].
3. Protein assay, for example, Quick Start™ Bradford Protein Assay (Bio-Rad). Prepare standards using a 2 mg/mL stock of bovine serum albumin standard (BSA; Bio-Rad) at the final concentrations: 1000, 750, 500, 250, 125, and 0 mg/mL, respectively.
4. Microplate spectrophotometer, for example, Multiskan™ GO (Thermo Fisher Scientific).
5. Tube rotator (e.g., SB2, Stuart).

2.2 Protein Precipitation

1. Cold (−20 °C) acetone.
2. Resuspension buffer: 6 M Urea, 2 M thiourea, 10 mM Tris–HCl, pH 8 in LC-MS grade water.
3. Refrigerated high speed centrifuge (e.g., Hettich Mikro 200R).

2.3 Sample Preparation from 1D PAGE Gels (In-Gel Digestion)

1. Gel Stain, for example, Brilliant Blue G–Colloidal (Merck) at 1× concentration (*see* **Note 2**).
2. Gel Destain: 100 µL of methanol:50 mM ammonium bicarbonate (1:1 vol:vol).
3. LC-MS grade water, LC-MS grade methanol, LC-MS grade acetonitrile, LC-MS grade formic acid (*see* **Note 3**).
4. Ammonium bicarbonate solutions: 100 mM and 50 mM. Prepare fresh on the day of use.
5. 10 mM ammonium bicarbonate in 10% (v/v) acetonitrile. Prepare fresh on the day of use.
6. 50 mM Acetic acid solution.

7. Trypsin solution 12.5 ng/μL trypsin in 10 mM ammonium bicarbonate containing 10% (v/v) acetonitrile. Resuspend the trypsin at 1 μg/mL in 50 mM acetic acid (100 μg vial resuspended in 100 μL of 50 mM acetic acid buffer). Remove the required amount of this 80× trypsin stock needed and refreeze the unused portion in 10 μL aliquots and store at −20 °C. Make the 1× trypsin shortly before use by diluting in 10 mM ammonium bicarbonate containing 10% (v/v) acetonitrile to a concentration of 12.5 ng/μL (1:80 (v/v)). Use sequence grade trypsin (*see* **Note 4).**
8. ProteaseMAX™ Surfactant Trypsin Enhancer solution: Resuspend a 100 μg vial of ProteaseMAX™ (Promega) with 100 μL of 50 mM ammonium bicarbonate to give a 1% solution (*see* **Note 5**). Freeze 10 μL aliquots and store at −20 °C.
9. 25 mM DTT in 50 mM ammonium bicarbonate. Prepare shortly before use.
10. 55 mM iodoacetamide in 50 mM ammonium bicarbonate. Prepare shortly before use.
11. TFA Trypsin stopping solution: 50% Trifluoroacetic acid (TFA) solution. Prepare this solution under a fume hood.
12. C18–10 μL ZipTips® (Millipore).
13. Wetting solution: 100% acetonitrile.
14. Equilibration buffer: 2% acetonitrile containing 0.1% TFA.
15. Elution buffer: 50% acetonitrile containing 0.1% TFA.
16. Vacuum centrifuge (e.g., SpeedVac™).

2.4 One-Dimensional Reverse-Phase Chromatography

1. Solvent A: 2% acetonitrile in LC-MS grade water containing 0.1% formic acid. Prepare 100 mL. Prepare this solution in a fume hood.
2. Solvent B: 80% acetonitrile, 20% LC-MS grade water, 0.8% formic acid. Prepare 100 mL. Prepare this solution in a fume hood.
3. Sample loading buffer 1: 2% acetonitrile in LC-MS grade water containing 0.1% trifluoroacetic acid (TFA). Prepare 1000 mL. Prepare this solution under a fume hood.
4. NanoLC system, for example, Ultimate® 3000 RSLCnanoLC system (Thermo Fisher Scientific) or a similar system.
5. Mass Spectrometer with an Orbitrap mass analyzer, for example, Orbitrap Fusion Tribrid (Thermo Fisher Scientific).
6. Column: Acclaim®PepMap100 75 μm × 50 cm, nanoViper C18, 3 μm, 100 Å (Thermo Fisher Scientific).
7. Trap column: PepMap C18 trap cartridge (300 μm × 5 mm) (Thermo Fisher Scientific).

2.5 Sample Preparation for Protein In-solution Using PreOmics iST Sample Kits

1. iST Sample Preparation Kit (PreOmics): This kit contains all chemicals needed to denature, reduce, and alkylate proteins and the enzymes needed to digest proteins and carry out peptide clean up. The enzyme provided is a Trypsin/LysC mix.

2.6 High pH Reversed-Phase Peptide Fractionation

1. High pH fractionation kit (Thermo Fisher Scientific) contains a high pH solution (0.1% triethylamine) and spin columns containing pH resistant, reversed-phase resin.
2. Prepare 10 mL of 0.1% trifluoracetic acid (TFA) solution by adding 10 μL of TFA to 10 mL of LC-MS grade water.
3. Prepare the elution solutions as follows:

Fraction number	Acetonitrile (%)	Acetonitrile (μL)	Triethylamine (0.1%)
1	5	50	950
2	7.5	75	925
3	10.0	100	900
4	12.5	125	875
5	15.0	150	850
6	17.5	175	825
7	20.0	200	800
8	50	500	500

3 Methods

3.1 Immuno-depletion of Serum Sample

1. Transfer 0.4 mL of the ProteoPrep Blue albumin and IgG depletion resin per sample to a spin column and centrifuge at 8000 × *g* for 10 s.
2. Wash the resin with 0.4 mL of equilibration buffer and centrifuge at 8000 × *g* for 10 s.
3. Add 50 μL of serum to the resin and incubate at room temperature rotating for 10 min using a tube rotator.
4. Reapply the eluate back onto the resin for a further 5 min rotating at room temperature before centrifuging again at 8000 × *g* for 10 s. Transfer the supernatant to a fresh tube.
5. Wash the resin with 100 μL of equilibration buffer, centrifuge at 8000 × *g* for 10 s and pool the sample with the first collection (150 μL in total).

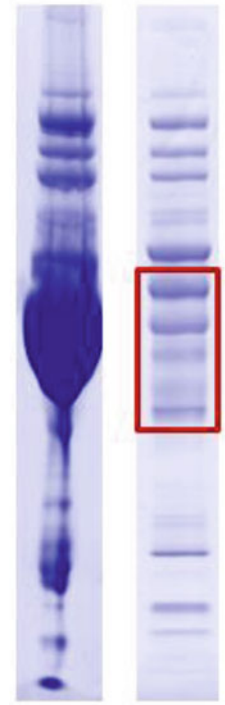

Fig. 1 Non-depleted (left) versus Serum/IgG depleted sample (right) from a serum sample separated on a Coomassie stained SDS-PAGE gel. The region highlighted in red shows proteins no longer masked by very high levels of albumin and IgG

6. Determine the protein concentration prior to separation by SDS-PAGE. Pipette 5 μL of standard and unknown samples into individual wells of a microplate in triplicate. Add 250 μL of Bradford dye reagent to each sample and mix. Incubate at room temperature for a minimum of 5 min and a maximum of 1 h. Set spectrophotometer to 595 nm and zero against the blank samples (*see* **Note 6**).
7. Run samples on a standard 1D SDS-PAGE gel and stain with Coomassie blue, for example, Brilliant Blue G–Colloidal.

Figure 1 shows a Coomassie blue stained SDS-PAGE gel comparing a 10 μg serum sample before and after albumin and IgG depletion and shows the reduction in the amounts of highly abundant IgG and albumin.

3.2 Protein In-Gel Digestion Protocol

1. Excise the protein bands of interest from the stained gel (*see* **Note 7**) using a sterile polypropylene Pasteur pipette and transfer the cored gel spots to a 1.7 mL microcentrifuge tube (*see* **Note 8**).

2. Wash each gel plug with 200 μL of LC-MS grade water.
3. Destain the gel plug with 100 μL of methanol:50 mM ammonium bicarbonate (1:1 vol:vol) to remove the stain.
4. Dehydrate the gel plug with 100 μL acetonitrile. Once the gel slice has dehydrated, remove the supernatant and dry in a vacuum centrifuge (e.g., SpeedVac™) until the sample is dry.
5. Reduce the sample with 100 μL of 25 mM DTT in 50 mM ammonium bicarbonate for 20 min at 56 °C.
6. Alkylate the sample with 100 μL of 55 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature in the dark for 20 min.
7. Dehydrate and dry the sample again (*see* Subheading 3.2, **step 4**).
8. Digest the sample by rehydrating with 20 μL of a 12.5 ng/μL trypsin (*see* **Note 9**) containing 0.01% ProteaseMAX for 10 min. Overlay the digesting sample with 30 μL of 50 mM ammonium bicarbonate and gently mix.
9. Incubate at 50 °C for 1 h or 37 °C for 2–4 h.
10. Centrifuge at 12,000 × *g* for 10 s to collect any condensate.
11. Transfer the digested peptides to a fresh tube and acidify the solution with the addition of TFA Trypsin stopping solution to a final concentration of 0.5%. If the amount of digested protein is sufficiently high, the digest can be analyzed directly by LC-MS, or it can be stored at −20 °C (if it is not going to be analyzed straight away).
12. However, if the peptide abundance is low, the peptide sample can be concentrated and purified with 10 μL C18 Millipore ZipTips®. Wet the C18 ZipTip by aspirating and dispensing 15 μL of wetting solution three times through the ZipTip and discarding the solution each time (*see* **Note 10**).
13. Wet the ZipTip again by aspirating and dispensing 15 μL of elution buffer three times discarding the solution each time. Equilibrate the ZipTip by aspirating and dispensing 15 μL of equilibration buffer three times discarding the solution each time.
14. Bind the low abundant peptide sample to the Zip Tip by aspirating and dispensing the sample through the ZipTip without discarding the sample.
15. After binding is completed, wash the sample on the Zip Tip by aspirating and dispensing 15 μL of equilibration buffer solution three times.

16. Elute the sample with 10 µL of elution buffer into a clean microcentrifuge tube and repeat this step. As the elution buffer contains acetonitrile, the sample must be evaporated by vacuum centrifugation (e.g., Speed Vac™) prior to LC-MS analysis. If the digest is not going to be analyzed straight away, freeze and store at –20 °C. If going straight to LC-MS analysis, rehydrate the sample in 20 µL of 2% acetonitrile containing 1% formic acid.

3.3 One-Dimensional (1D) Reverse-Phase Chromatography Using a 60 min Separation Time

1. Perform nano-flow chromatography with a suitable LC such as the Ultimate® 3000 RSLCnanoLC system, which is used here. The LC is configured, as shown in Fig. 2, using an Acclaim®PepMap100 75 µm × 50 cm column and a nanoViper C18, 3 µm, 100 Å and PepMap C18 trap cartridge (300 µm × 5 mm).
2. Prior to analysis, equilibrate the columns in Solvent A for 10 min and set the column temperatures to 45 °C using a column oven.
3. Using a 20 µL loop, pick up 1 µg injection of peptide sample at a flow rate of 25 µL/min using Sample loading buffer 1 and desalt for 3 min through the PepMap C18 trap cartridge.

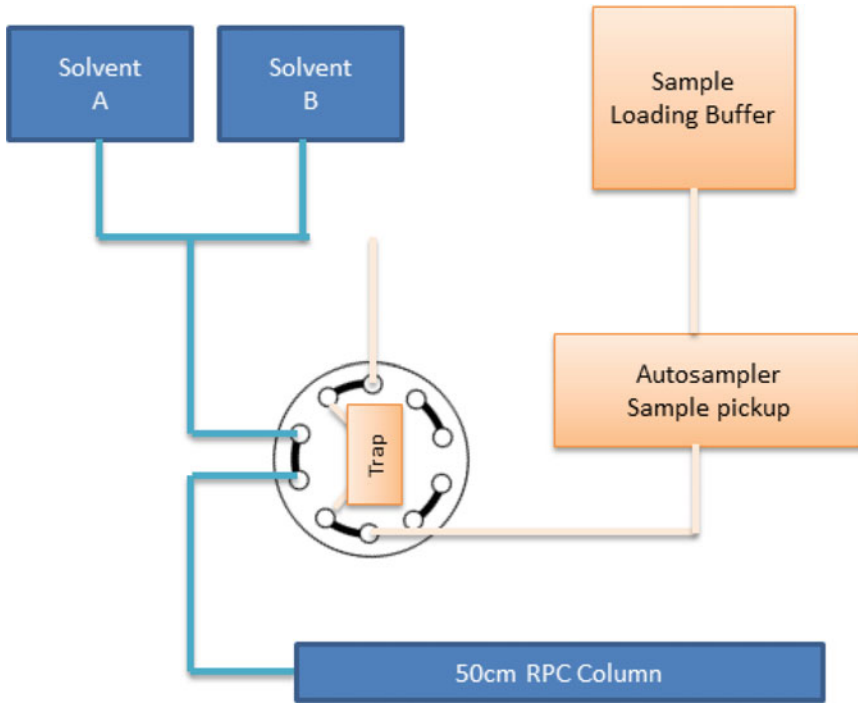

Fig. 2 Schematic using a ten port valve for the fluidic connections for one-dimensional (1D) reverse-phase chromatography for simple protein mixtures

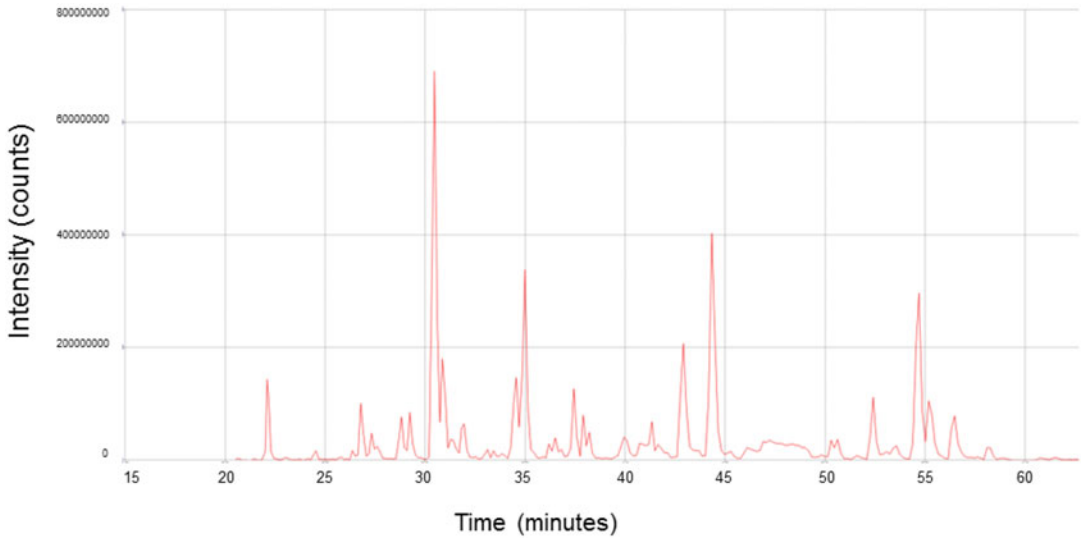

Fig. 3 Mass spectrometry trace showing the total ion intensity from all the mass spectra recorded during a 1D reverse-phase LC-MS using a 60 min reverse-phase separation, shown as a function of elution time. The sample shown is a serum protein digest from a 1D gel band

4. Separate the peptides using a 60 min linear gradient elution using Solvent A and Solvent B starting with 2% Solvent B to 90% B over 60 min at a flow rate of 350 nL/min. *See* **Note 11** for details of longer separation times used for complex peptide separations. Figure 3 shows the peptides obtained from a typical 1D LC-MS experimental run from a 1D PAGE gel band over a 60-min gradient from a patient serum sample after immunodepletion.
5. Use a stainless steel emitter (*see* **Note 12**) for nano-electrospraying the samples into the mass spectrometer with the ion spray voltage set at 1.8 kV.
6. Use a tandem mass spectrometer (MS/MS) to analyze the peptides eluting from the nano-flow chromatography system. We use an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific). The instrument is set up for data-dependent acquisition with a scan range of 380–1500 m/z using the Orbitrap mass analyzer with a resolution of 120,000 (at m/z 200) with a maximum injection time of 50 ms and an automatic gain control (AGC) value of 4.0×10^5. The top-speed acquisition algorithm is used to determine the number of selected precursor ions for fragmentation. An isolation width of 1.6 m/z is used to isolate selected precursor ions in the quadrupole. A dynamic exclusion is applied to the analyzed peptides after 60 s, and only peptides with a charge state between 2+ and 7+ are analyzed. Higher energy collision-induced dissociation (HCD) with a normalized collision energy of 28% is used for fragmenting precursor

ions, and the resulting MS/MS ions are also measured in the Orbitrap at 30,000 resolution. MS/MS scan conditions are typically the following: a targeted AGC value of 5.0×10^4 and a maximum fill time of 300 ms.

7. Use computer software search engine(s) to analyze data from tandem mass-spectrometry samples to match mass spectra with peptide sequences. The search engine will match the tandem mass spectra with peptide sequences from a protein sequence database and use the identified peptides to infer the protein content of the sample. Through Proteome Discoverer 2.2.0 (Thermo Fisher Scientific) using the search engine SEQUEST HT (Thermo Fisher Scientific) against the UniProt/SwissProt fasta sequence database using the *Homo sapiens* (human) taxonomy when searching the data from patient samples. Set cysteine carbamylation as a fixed modification and set oxidation of methionine as a variable modification. For database analysis, set a precursor mass tolerance of 10 ppm and an MS/MS tolerance at 0.02 Da using full trypsin specificity and allow for two missed cleavages.
8. We use strict scoring criteria for our protein identification search engine results (*see* **Note 13**).

3.4 Protein In-Solution Digestion Using PreOmics iST Proteomic Sample Preparation Kit

1. PreOmics iST kit recommends starting amounts of material of approximately 6×10^5 cells for mammalian cell lines (protein amount 100 μg), 2 μL (protein amount 100 μg) of blood, serum or plasma or 1×10^9 μm^3 (protein amount 100 μg) of dissected tissue.
2. Add 50 μL of Lyse buffer to each sample and heat at 95 °C for 10 min. Protein samples will be denatured, reduced, and alkylated during this step.
3. Transfer each sample to an individual labeled iST cartridge.
4. Add 50 μL of the freshly prepared digest buffer to each sample. Protein samples are digested for 2 h at 37 °C.
5. The enzymatic digestion is stopped by the addition of 100 μL of stop solution.
6. Peptide samples are washed twice with 200 μL of wash solution.
7. Peptides are eluted with two elution washes of 100 μL of elute solution.
8. Eluted peptides are evaporated using a SpeedVac.
9. Dried peptides are either resuspended in 50 μL of LC-Load solution for direct LC-MS analysis or remain dried for high pH reversed-phase peptide fractionation.
10. If direct LC-MS analysis is being carried out, determine the peptide concentration using a NanoDrop One (Thermo Fisher Scientific).

3.5 High pH Reversed-Phase Peptide Fractionation

1. Dried peptides with a concentration between 10 and 100 μg are dissolved in 300 μL of 0.1% TFA solution.
2. Place a spin column into a 2.0 mL sample tube and add 300 μL of the sample solution onto the column, replace the top cap, and centrifuge at 3000 × g for 2 min.
   Retain eluate as the "unbound flow-through" fraction.
3. Place the column into a new 2.0 mL sample tube and wash with 300 μL of LC-MS water.
   Retain eluate as "wash" fraction.
4. Place the column into a new 2.0 mL sample tube and add 300 μL of the first elution solution (fraction 1) and centrifuge at 3000 × g for 2 min. Collect the fraction and label as fraction 1.
5. Repeat **step 4** for the remaining step gradient fractions using the appropriate elution in a new 2.0 mL sample tubes and label accordingly.
6. Peptide fractions are evaporated to dryness using a SpeedVac.
7. Dried peptide can either be stored at −20 °C or resuspended in 50 μL of LC-Load solution from the PreOmics iST kit.
8. Determine the peptide concentration using a Nanodrop One.

See Fig. 4 for the diagrammatic procedure for high pH reversed-phase fractionation method to collect 8 fractions. *See* Fig. 5 for high pH reversed-phase fractionation mass spectrum base peak chromatograms overlapping eight fractions from 100 μg depleted protein sample.

4 Notes

1. There are many commercially available immunodepletion kits and columns that can remove high abundant proteins in serum such as IgG and serum albumin. Examples include Proteo-Prep® (Merck), ProteoSpin™ (NORGEN BioTek Corp), Proteome Purify (R&D Systems), High Select™ Top14 Abundant Protein Depletion Mini Spin Columns (Thermo Fisher Scientific), Multiple Affinity Removal (MARS) range (Agilent). These kits and columns are not just confined to use on serum but can be used for immunodepletion of other biological fluids (e.g., plasma, vitreous fluid, and wound exudate).
2. Proteins separated by either 1D or 2D PAGE for protein identification are normally visualized by staining with dyes such as Coomassie Brilliant Blue (CBB) R250 and silver stain (gluteraldehyde-free), or fluorescence-based methods such as Sypro Ruby and Deep Purple. For mass spectrometry analysis, the silver stain method should not use glutaraldehyde.

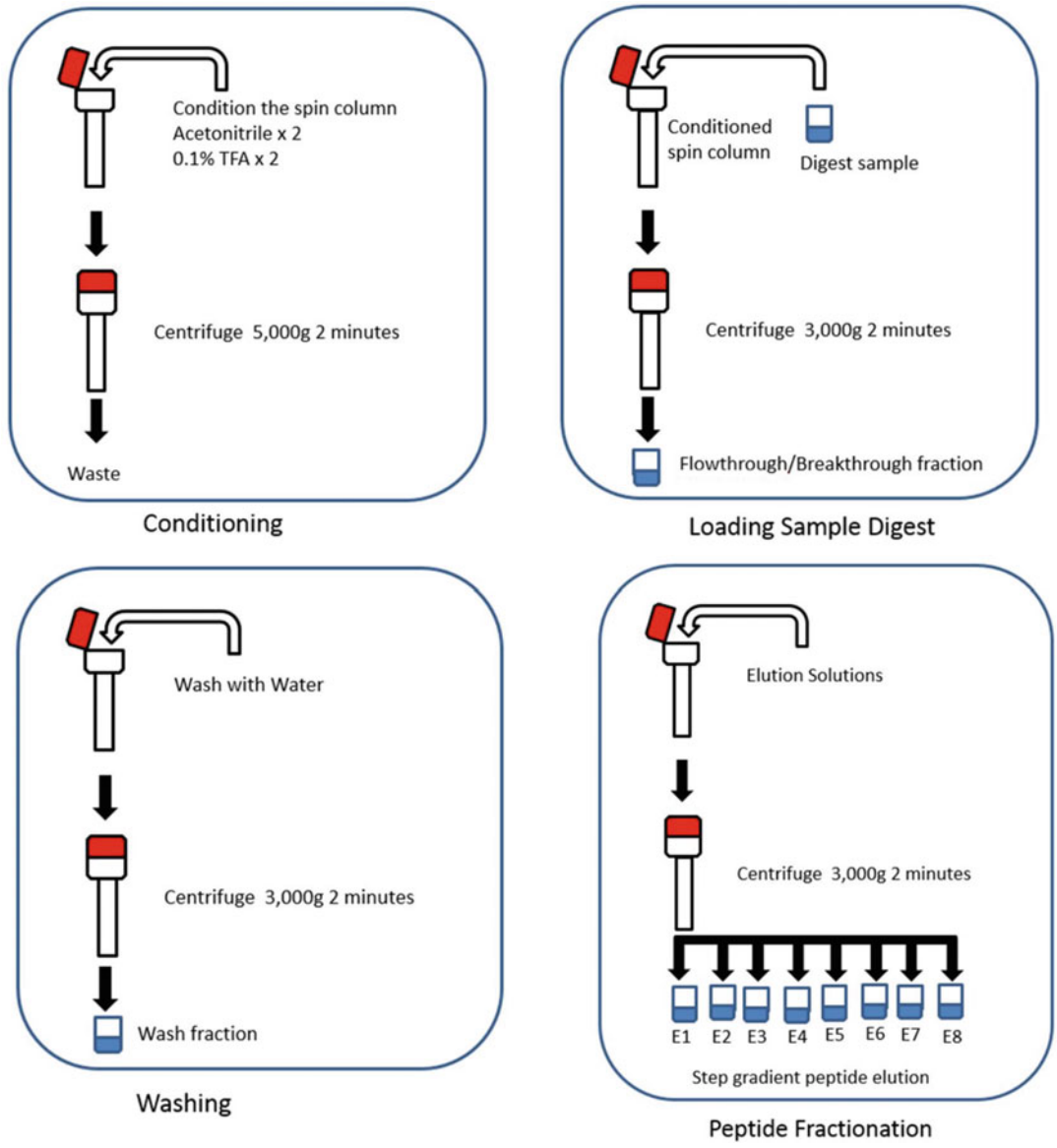

Fig. 4 Procedure summary for high pH reverse-phase peptide fractionation

3. All solvents and water used must be LC-MS grade. All chemicals must be of the highest purity.
4. The most common enzyme used for peptide generation for LC-MS analysis is trypsin, which has been modified by reductive alkylation to suppress trypsin autolysis. Trypsin is a robust enzyme, and digestions can be performed under denaturing conditions. We use modified porcine trypsin (Promega) and also the digest buffer from PreOmics (Trypsin/LysC mix).

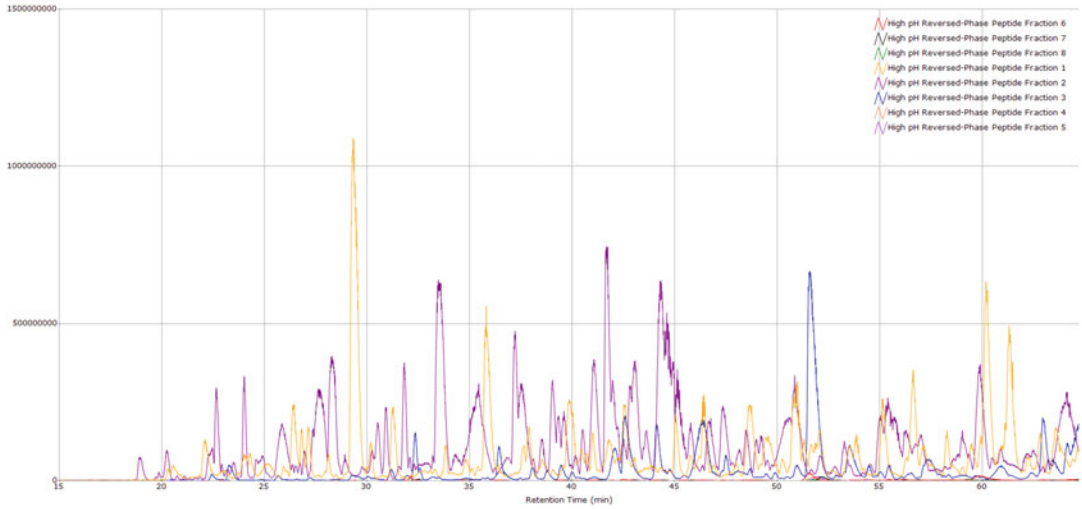

Fig. 5 Mass spectrometry traces overlaid from eight peptide fractions generated from for high pH reverse-phase peptide fractionation. Each fraction is designated by a different color chromatogram

5. We use ProteaseMax™ Surfactant Trypsin Enhancer as it removes the need to extract peptides following In-gel digestion. We also find there is improved peptide recovery from gels compared to when not using it. When we carry out protein In-solution digestions, we find protein solubilization is improved. ProteaseMax™ Surfactant Trypsin Enhancer degrades over the course of the digestion so samples are ready for LC-MS without the need to remove the detergent.
6. To ensure precision in determining protein concentrations when using a Bradford protein assay, the relative standard deviation (RSD) for each triplicate sample UV value must be very low <1%.
7. When working with SDS-PAGE gels, gloves must be worn at all times to minimize keratin and dust contamination which can affect your LC-MS results. When excising protein from gels, this work should be done in a laminar flow cabinet to minimize the possibility of any hair, dust, or skin flakes contaminating the sample with unwanted keratins.
8. The use of low protein-binding microcentrifuge tubes is recommended to minimize protein loss by adsorption.
9. There are enzymes other than trypsin that can be used for protein digestion, which have different cleavage specificities, such as Asp-N and Glu-C.
10. Avoid introducing any air bubbles into the ZipTip at any stage of the process.
11. Complex peptide mixtures can require long separation times. We use 60 or 120 min separation times depending on the complexity of the sample.

12. We use stainless steel emitters to maintain a stable spray into our mass spectrometer; however, stainless steel emitters are not suitable when looking at phosphorylated peptides, so fused silica emitters are recommended instead.
13. We use SEQUEST HT and Percolator and set a false discovery rate (FDR) cutoff of 1% at the protein level.

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Chapter 15

Immunoprecipitation: Variations, Considerations, and Applications

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Abstract

Immunoprecipitation (IP) refers to methods of affinity chromatography that enrich and/or purify a specific protein from a complex mixture using a specific antibody immobilized on a solid support. Several operations and processes that are dependent on the isolation, concentration, and modification of proteins have seen improvement in their selectivity and separation based on the integration of IP-specific reactions into their workflows. This relatively simple principle has contributed significantly to our understanding of proteins and their behaviors and has become increasingly fundamental to most protein characterization studies today. In this chapter, we review the basic principles of IP and the several factors that influence each stage, and subsequently the success, of an IP experiment. Moreover, variations in application of the IP principle are discussed, and the adaptability of the techniques based on such is highlighted in the provision of two IP workflows to purify a particular protein from an entire cellular proteosome. These workflows cover the preparation and fractionation of crude cellular lysate into individual subcellular fractions, through to both "batch" and "column"-based extractions of the target protein of interest. Protocols for determining the validity of the workflows, and the presence/abundance of the protein of interest, are also briefly described.

Key words Immunoprecipitation, Fractionation, Centrifugation, Antibody, Immunocomplex, Affinity chromatography, Batch immunoprecipitation, Column immunoprecipitation

1 Introduction

Over time, there has been an increasing correlation in discoveries in proteomics with advancements in the separation sciences. Initially, breakthroughs were limited to the profiling and utilization of small, extracellular proteins that were stable, plentiful, and easily isolated without the need to disrupt the host system. As such, early methods and discoveries predominantly revolved around the determination of the presence of a target within a sample matrix. Coupled with the complexity of total proteosomes, a bottleneck emerged in that advancements beyond these initial discoveries were limited by an inability to isolate low-abundance proteins effectively and

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efficiently from a complex mixture toward quantitative and qualitative analyses. In time however, methodologies for preparing partial proteosomes based on the divergence of protein properties were established and have evolved rapidly over the last number of decades to allow for not only the identification but also subsequent isolation, concentration, and modification of protein targets of interest, toward downstream processes and applications [1]. In this way, there now exist several methodologies for the recovery of a diverse range of target biomolecules such as recombinant proteins, antibodies, and genetic material [2]. These advancements have enabled the profiling of the in vivo nature and behavior of biomolecules of interest in an in vitro environment, and have accelerated the discovery of numerous important scientific breakthroughs [3]. These methods have contributed significantly to our ability to characterize biomolecules in different expression systems: their relative abundance, size, regulation dynamics, stability, post-translational modifications [4], and interactions with other biomolecules [5], revolutionizing how we understand biomolecule behavior today.

More than ever, said methodologies are becoming increasingly crucial and indeed fundamental steps in many proteomic workflows in both research and industrial settings. As such, existing methodologies are continuously evolving to meet these increasing demands: fostering the development, optimization and refinement of reagents, and instrumentation pertinent to such at both the micro- and macroscale [6]. Over time, these improvements include, but are not limited to, the preservation of the physiological nature of the target protein ex vivo, their performance at an economically sound costing, and the ability to have a high-resolution, high-throughput capacity at larger scales. Despite these advancements, the fundamentals have stayed the same in that the optimal approach, which allows for direct, selective enrichment of a target protein, while also achieving the aforementioned standards, are those which exploit bio-specific interactions [7].

Several well-known operations and processes have seen improvement over time in terms of selectivity and separation with an increased understanding and incorporation of "affinity motifs" [8, 9]. An "affinity motif" allows for a specific interaction between two components enabling the purification of one of the components. Immunoaffinity precipitation, or "immunoprecipitation" (IP), is one such process, which is based on this principle and will be the key focus of this chapter.

1.1 General Description of Immunoprecipitation

IP generally refers to a method of affinity chromatography used to enrich and/or purify a specific protein from a complex mixture in its active form using a specific antibody or antibody fragment immobilized on a solid support. The highly specific interaction of an antibody with a specific target protein to which it has a natural affinity forms what is essentially an "immunocomplex" - an

assembly central to all forms and iterations of IP, which are employed today [10]. Assembly of the immunocomplex can be accomplished sequentially or in a single step. A common sequential method is to initially incubate the antibody with the sample of interest, before introducing the support material, which captures any resultant immunocomplexes that have assembled. Alternatively, the initial reaction may be between the antibody and the support material to first immobilize it, before the sample of interest is then is added. Irrespective of the order of addition, after the assembly of any immunocomplexes between antibody and proteins has occurred, the remaining unbound contaminating proteins that formed part of the original complex crude mixture are then separated and removed from the system using a series of pre-optimized wash/purification steps [11]. The remaining immunocomplexes of the system may then be dissociated using an elution buffer, which weakens the antibody-protein interaction, and the purified target protein/s can be recovered for subsequent downstream processing and/or analysis [12].

The incorporation of the antibody molecule imbues methods of IP with an extremely high degree of selectivity and separation, with most forms offering a limit of detection in the range of 100 picograms for a tagged or labeled protein (condition-dependent). The simplicity of the principle has seen the method employed extensively in both industry and research settings in the successful targeting and purification of a range of biomolecules; substrates, coenzymes, hormones, antibodies, and even nucleic acids [3]. Moreover, the formation of the immunocomplex has been exploited in its functional capacity beyond its initial applications for the detection, quantitation, and isolation of target proteins from complex mixtures in the context of IP. Implementation of this principle in other ways has led to the development of variations of the classic IP method, broadening its applications and in turn making it the incredibly versatile method which it is recognized as today. For example, improvements to the purification processes inherent to IP have allowed for the concentration of target proteins from a dilute solution to a more concentrated extract with a high degree of purity—typical IP setups have demonstrated 10^3- to 10^4-fold purification factors for a protein in a single chromatographic step. Conversely, alternative adaptations to the purification steps have led to the process of co-immunoprecipitation, whereby the target protein of interest isolated along with any proteins or ligands that have interacted and are subsequently bound to it. The number and complexity of these binding partners can be varied, with signaling molecules, structural proteins, and/or cofactors; the level of interaction range from highly transient to very stable, allowing for even more in-depth analyses of target protein behavior [5]. Moreover, while

the technique typically involves the immobilization of the antibody to the solid support to target proteins of interest, the inverse (immobilizing a protein of interest) has been used for the identification, quantification, and purification of target antibodies [13].

1.2 Methodological Considerations

While the principle of IP and the core steps inherent in the process are logically and procedurally simple, there are currently a multitude of approaches incorporating this principle as part of their purification processes and workflows today. The sheer variety and diversity in protocols and procedures for carrying out IP means there are distinct options available, which are entirely dependent on what the intended use for isolated target protein of interest is. For example, what is the isolated protein to be used for? What quantity is needed for the intended downstream analyses/application? What should the state of purity be? Must it be folded and/or be associated with other peptides or cofactors? This point is of particular importance as many IP approaches will, for instance, deliver a high yield of target protein but may perhaps alter protein's activity and/or structure. As most antibodies designed for IP purposes target epitopes on the surface of native proteins, a loss of shape inherently disrupts formation of the immunocomplex. The answers to these questions can inform one in the selection of a means to immunoprecipitate a target protein.

As a consequence, empirical testing is nearly always needed before the selected means of IP can successfully isolate adequate amounts of pure target proteins. Conscious consideration of the variables inherent to the many steps of an IP process can help identify those that are most likely to affect a particular experiment and expedite the rate of success, while avoiding the tedious process of having to constantly redesign the process. In that way, while it is paramount to find the optimal experimental conditions relative to the protein of interest (i.e., stability and activity), one is often limited by the ability of the antibody that is employed to form that critical immunocomplex. As such, one should anticipate variability in methods employed for each individual protein of interest and remain flexible in adopting changes to procedures in a protein-specific manner as the need arises [14–16]. Taken together, there are numerous factors that ultimately influence the success of any IP experiment, and these variables are as numerous and diverse as the specific differences between individual proteins and primary antibodies. Appropriate planning in selecting the assay, with all considerations taken into account, is therefore imperative to the success of an IP [14–16].

In the following subheadings (1.2.1, 1.2.2, 1.2.3, 1.2.4, 1.3, 1.3.1, 1.3.2, 1.3.3, 1.3.4, and 1.4), the most pertinent considerations one should take for selecting an IP method are discussed in order to allow one to better comprehend and select a suitable method. Thereafter, the ensuing materials and methods subheadings (2 and 3) firstly outline a protocol for the fractionation and

isolation of cell organelles from a mammalian cell culture, for reducing the presence of contaminant proteins in a crude sample for IP. Thereafter, protocols for both batch (subheading 3.2) and spin-column (subheading 3.3) methods of IP are described for the isolation of COX IV, a 17 kDa enzyme responsible for regulating the proton electrochemical gradient across the inner mitochondrial membrane [17], with a brief discussion on validating the resultant fractions/isolated protein of interest using Western blot to close.

1.2.1 Sample Preparation

The initial step in the purification of a protein, or a protein-complex, is the preparation of a suitable extract, which contains the target protein in a soluble form. This step should be highly reproducible and be capable of disrupting the crude sample to the highest degree (>90%), while minimizing the loss of/alterations to the target protein/s. Thus, in selecting an appropriate method, several considerations should be taken, for example, assessing whether the protein of interest is in fact soluble and, if not, what approach is most suited to achieving this goal. In that way, the influence of the selected buffer system on the protein of interest is often critical in the success of the intended IP experiment, namely, salt concentration, temperature, pH, and the presence of oxidants should be evaluated when choosing the method of preparing an extract. Thus, each protein in the proteosome of interest should be considered as individual and ideally would command its own extraction method tailored toward its own biochemical requirements and intended application post-IP [18].

This approach can be tedious and time-consuming, and compromises are often made in selecting a method of extraction in the full knowledge that losses (i.e., abundance and activity) will be incurred. Consideration of at what point such losses directly stemming from the selected environmental influences becomes unacceptable, and implementation of measures to reduce and/or eliminate such losses is essential in selecting an extraction method upstream of an IP workflow [19].

A confounding aspect of this process however, aside from the sheer uniqueness of proteins themselves, is the divergence in the nature and structure of the host cells in which they reside. The extraction of proteins from most mammalian cells is straightforward as animal cells are enclosed by only the surface plasma membrane, which is relatively fragile and easily disrupted by liquid shear forces for homogenization. In contrast, cells isolated from hard muscle tissue (e.g., cardiac or skeletal) require strong mechanical forces and require the use of instruments with mincing and blending capabilities. Sonication, chemical reagents, detergents, enzymatic treatments, and freeze-thaw step, whether employed individually or in combination, are among the available options for preparing a sample. The procedures and methods that utilize

such steps are constantly evolving in an effort to meet the demands of modern proteomics [19, 20]. The selection and optimization of cell disruption and protein extract preparations can thus be quite an empirical process.

Considerations for cell disruption methods, reagents, and instrumentation (subheading 2.1, **items 1–3**) serve as starting points to produce quality cellular extracts suitable for most downstream IP and analysis processes. While these subheadings focus on the disruption of mammalian cells at microscale, many of the points and techniques discussed here are pertinent and applicable to the disruption and isolation of intracellular proteins from other sources and at different scales.

1.2.2 Subcellular Fractionation

Subcellular fractionation is a useful process that can be used upstream of IP aimed at reducing sample complexity. If the protein of interest is in a specific organelle or cellular compartment (i.e., the nucleus, mitochondria, and cytosol), subcellular fractionation approaches can isolate the specific organelle of interest to take forward for IP [21]. Ultimately, it is a flexible and adjustable process that acts as a partial purification, reducing the presence of nonspecific proteins in a sample and thus significantly improving the chance of success of an IP experiment [22].

Subcellular fractionation involves three successive steps: (1) convert the tissue or cell suspension into a homogenate; (2) group components of the homogenate together into separate, individual fractions by exploiting similarities in a particular physical or biochemical property (i.e., density and sedimentation); and (3) analyze/process the resultant isolated fractions [23, 24].

Disruption of tissues may require instruments such as a Potter-Elveljem homogenizer, or a Chaikoff Press, to initially reduce the material into smaller, multicellular fragments. Homogenization of cells can subsequently be achieved using chemical and/or enzymatic buffers (subheading 1.2.3) and/or further mechanical disruption (i.e., sonicator). Ultimately, selection of the homogenization process should be chosen such as to sufficiently force the opening of the plasma membrane and release of the cytosol, but not exert a force capable of causing damage to the subcellular organelles, structures, and membrane vesicles, that have been released as a result.

There are several methods for subsequently separating the resultant homogenate that comprises whole cells [25, 26], partially broken cells, and subcellular components, into the individual, uniform fractions. Separation is achieved by utilizing methods that exploit differences in the physicochemical properties of the constituents of the homogenate, with centrifugation being the most common sample preparation technique. Alternatives such as commercial kits can be prohibitively expensive, and limiting in the volumes of kit components should further optimization be

required. Employing electrophoretic and countercurrent approaches can thus be impractical if a large number of samples are involved. In contrast, using differential centrifugation to separate subcellular components relative to their density and size differences offers a number of distinct advantages. The equipment required to carry out centrifugation is widely available in laboratories and presents the opportunity to process large volumes of material, and subsequent recovery of the fraction/s of interest is relatively straightforward [23] (subheading 3.1).

1.2.3 Lysis Buffer

The aim of purposely releasing the protein constituents in the homogenate is to solubilize the target protein, to produce an undegraded, biologically active protein that will ultimately be immunoreactive in the IP process [27]. Mechanical and physical means of disrupting tissues, from the Potter-Elveljem homogenizer to more industrial blenders and mincers, can effectively lyse cells and expose their contents for interaction by processes such as IP. However, these mechanical methods are not without their drawbacks, and while capable of lysing cells, they can generate very high temperatures and forces, which can impact negatively upon each protein that is subsequently released [28]. Therefore, the selection of an effective means of lysing cells and their respective constituents, with the minimum amount of impact on the native state of the proteins, is thus considered one of the most crucial parts of an IP, and several buffer-based approaches are available, which address this aspect.

Depending on the sample and the downstream application (i.e., IP), lysis buffers will vary in nature: from ionic strength, salt concentration, pH, and the concentration and type of stabilizing ligand detergents used [29]. The ability to control the respective concentrations of each in a formulation allows one to tailor a lysis buffer toward the extraction of a particular target protein—albeit optimization of buffer conditions can prove tedious. Fortunately, the development of generic lysis buffers containing bioactive compounds such as Triton-X-100, NP-40, and sodium dodecyl sulfate (SDS) has enabled rapid, efficient means of disrupting the cell membrane/wall of most cell types, weakening them for lysis by osmotic shock, freeze-thaw, or enzymatic attack, the result of which is sample protein solubilization. The emergence of these lysis buffers has significantly simplified the extraction and enrichment of proteins from a range of host species including bacteria, yeast, plants, and insect cells, in addition to those derived from mammalian sources. A distinct advantage of these formulations is that in addition to releasing and solubilizing the proteins from the homogenate sample, they also preserve the proteins in their native conformation. This minimizes denaturation of the antibody-binding sites and concomitantly increases the chance of success for IP. Another advantage is that most lysis buffers do not require expensive

equipment to process a sample (though some cell types may require sonication) and are typically quite fast in action and easy to use. As such, lysis buffers are the most practical and effective means of high-throughput protein extraction, though care should be taken in the selection of the buffer to be used on a sample upstream of an IP. Common compounds that act as reducing agents, protease inhibitors, and detergents have been shown to interfere with certain IP methods and increase the risk of IP failure [30, 31].

It should be noted that every lysed protein sample will contain varying levels of contaminating proteolytic enzymes. Every cell type has its own complement of proteolytic enzymes, and under normal circumstances, the respective activities of each enzyme are regulated. In a disrupted cell, however, the processes regulating the activities are lost, partially explaining the observable loss of material/biological activity that is apparent in all lysis-based approaches. This can lead to undesirable changes in protein post-translational modifications such as methylation, ubiquitination, or phosphorylation, characteristics that may be responsible for pertinent protein-protein interactions specific to the target protein of interest, which are subsequently undetected in downstream analyses [32]. Supplementation of lysis buffers with inhibitors that maintain the proteins of a homogenate in their native states is subsequently commonplace. Table 1 highlights some of the most commonly used inhibitors added to lysis buffers for maintaining in vivo interactions in vitro. These inhibitors are critical for maintaining the stability of sample constituents during storage as even in the presence of some of the aforementioned inhibitors, proteolytic degradation can occur in frozen samples—albeit slowly [33].

1.2.4 Lysate Pre-clearing

Pre-clearing a sample to remove proteins and ligands that nonspecifically bind to components of the IP system is an optional step routinely incorporated to remove the background presence of contaminating proteins. Pre-clearing typically involves incubating the sample with the base support material that is to be used in the IP protocol (i.e., agarose-derived resin materials) before it is separated from the mixture (typically using centrifugal force). Off-target, contaminant proteins, or other components of the sample that have an affinity for the material in question can thus be removed from the sample, ensuring they will not be present and potentially precipitated with the target protein of interest in the actual IP experiment.

Pre-clearing approaches can also include a combination of the support material with binding proteins (e.g., protein A/G) and even the support material with the binding protein and a nonspecific immunoglobulin antibody to account for nonspecific interactions with immunoglobulin molecules in general [34]. These setups are becoming increasingly important in use and often act as negative controls for IP-based experiments when interpreting results.

Table 1
Comparison of routinely employed compounds of cell lysis buffers and their inhibitory function that can be used individually, or in combination, for preserving particular characteristics of protein native state

Type	Inhibitor	Working concentration	Solvent	Inhibition of
Phosphatase	Sodium orthovanadate	1–100 mM	Water	Tyrosine phosphatases
	B-Glycerophosphatase	1–100 mM	Water	Serine and threonine phosphatases
	Okadaic acid	10–1000 nM	DMSO	Protein phosphatase 1 and 2A
	Sodium fluoride	1–20 mM	Water	Serine and threonine phsophatases
Protease	Aprotinin	1–2 μg/mL	Water	Pancreatic trypsin
	Benzamidine	15 μg/mL	Water	Serine proteases
	EDTA/EGTA	1–10 mM	Water	Metallo-proteases
	Leupeptin	1–2 μg/mL	Water	Serine and cysteine proteases
	PMSF	0.1–1 mM	Isopropanol	Serine and cysteine proteases
	Pepstatin A	1 μg/mL	Methanol	Acid proteases
Deubiquitination	TAME	1–200 μM	DMSO	Ubiquitin ligase
	IU1	1–30 mM	DMSO	USP14 deubiquitinase
	b-AP15	0.1–1 μM	DMSO	19S deubiquitinase
	P22077	5–50 mM	DMSO	USP7 and USP47 deubiquitinase
Methylation	RG108	0.1–1 μM	DMSO	DNA methyltransferase
	5-Azacytidine	0.1–50 μM	Water	DNA methyltransferase

1.3 Method Format

There are several formats of IP, which allow for the assembly of the immunocomplex central to the IP method. These methods broadly differ in the order in which the components responsible for the immunocomplex formation, isolation, and extraction are combined. For example, the most straightforward approach is to combine the antibody, the protein sample, and the support material together in a single reaction. While this approach is without doubt the most direct and is significantly quicker than some alternative approaches to be discussed, the initial complexity and competition between components of the mixtures generally produces an isolation with low purity and yield of target protein. To address this, an initial reaction between the antibody and protein sample can improve the amount of immunocomplex formed as the omission of the support material increases antibody-protein interaction. A second reaction then introduces the support material to then precipitate whatever amount of immunocomplex has formed. While this approach significantly increases the yield of target protein in the

final precipitated sample in comparison to the first approach, it also presents its own respective drawback. This second reaction is known to result in contamination of the eluted sample, typically with the capture antibody, which forms part of the immunocomplex. This aspect can be addressed by employing a third and perhaps most adopted approach, which involves an initial reaction where the capture antibody is immobilized to the support material. The protein sample is then added as part of the second reaction. This approach gives comparable, if not slightly reduced yields of target protein to the second approach, but it significantly decreases the presence of contaminants/increases the purity of the final eluted samples.

Irrespective of the order of addition of the IP reaction components, the mode by which the reaction is carried out, and thus processed, broadens the choice of method further (*see* Fig. 1). For example, the most straightforward method is the "batch" method, which simply involves mixing the components of the overall IP process, either together or sequentially, for predetermined periods of time, in a single reaction vessel. Reactants and nonspecific, contaminating biomolecules are separated typically using centrifugation-based methods. In comparison, "column"-based methods revolve around the interaction of IP components with a stationary packing material (i.e., resin beads) as they transition through the column. Often, upon the addition of a sample, a column may be "capped" to reduce passage of the material through the column and increase the time for potential "binding" toward formation of the immunocomplexes. Irrespective of the approach employed, both the "batch" and "column" methods of IP involve separation of the sample performed by either gravity, or another force (i.e., centrifugation using spin columns).

Choosing whether "gravity" or "centrifugal" force is most appropriate for a particular experimental setup is typically determined by the initial starting volume of material. Large volume samples are generally restricted to gravity-based approaches owing to the impracticality of having to centrifuge large-scale spin columns. Conversely, in situations where the starting volume of material is quite small (i.e., microliter range), spin columns represent the only choice as such small volumes will not filter through a column and filter setup by gravity alone. Overall, the spin-column approach has emerged as the more popular mode of IP owing to several distinct advantages it has over "batch" and "gravity"-based methods. The use of centrifugal force greatly reduces the time taken to process fractions, facilitating the complete scrutiny of the sample by ensuring all the sample passes through the column material. This results in a cleaner separation of the column components and the sample of interest, increasing target yields and purity. In contrast, while effective, "gravity"-based approaches take significantly longer to fully resolve a sample and require constant monitoring to reduce

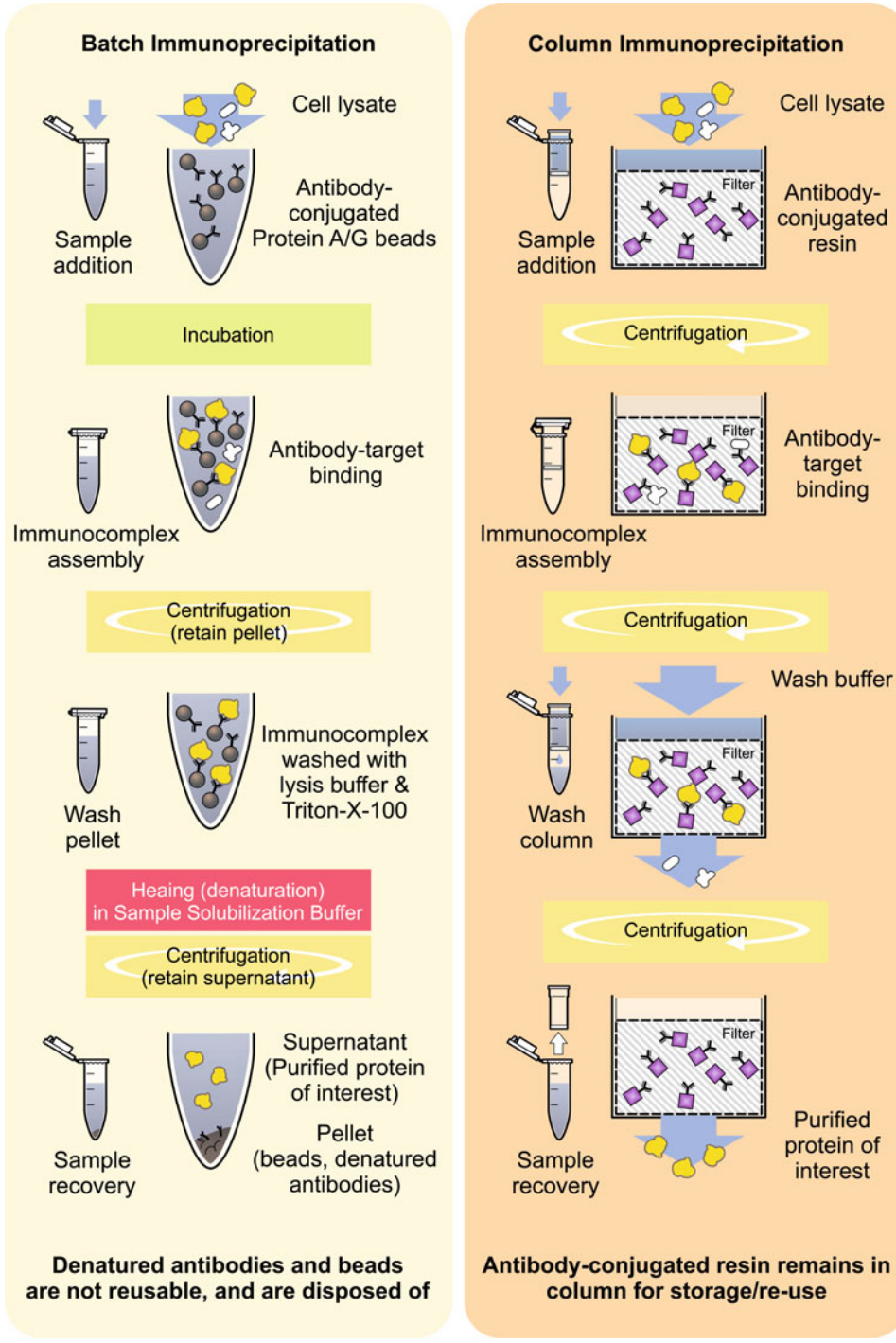

Fig. 1 Variations in immunoprecipitation. Immunoprecipitation refers to approaches that are used to enrich and purify a specific protein from a complex mixture using a specific antibody toward that protein of interest. "Batch" approaches (left) see the mixing of the antibody, sample containing protein of interest, and the binding material together, or sequentially, in a single reaction vessel before the components are separated using centrifugation-based methods. "Column" approaches (right) in contrast see the interaction of the same components (antibody, sample containing protein of interest, and binding material) with one another, though

the risk of error (i.e., ensuring the resin never dries out and air bubbles do not form). "Batch" methods in comparison command less supervision, though they often incur the greatest loss of sample by way of the resin retaining a percentage of the sample in forming the resin pellets that are generated from the separation process. Additional wash steps involving resuspension of the pellet, coupled with subsequent separations and elutions, can improve purity and yield of the target protein, but not to levels comparable to "spin-column"-based approaches.

1.3.1 Type of Support Material

Today, the most prevalent support material used in research scale IP experiments is beaded agarose. Agarose and cross-linked beaded derivatives of such (i.e., Sepharose) have a very well understood chemistry. This makes them relatively easy to implement in separation systems, while also being quite adaptable given their versatility in coupling to a ligand of interest (i.e., immunoglobulins). Moreover, agarose has been demonstrated to exhibit low nonspecific binding in complex protein samples and can tolerate the moderate levels of detergents, salts, solvents, and pH that may form part of the buffer systems used to reduce nonspecific binding in an IP system [35]. However, there are a variety of alternative support materials that are used in IP scale affinity purification today, including cellulose resins, polystyrene particles, porous glass, microplate wells, and ferrous magnetic beads. The latter has become particularly popular in recent years, as protocols utilizing magnetic beads have created an effective means of separation that does not require a centrifuge. In utilizing magnetic forces to separate a complex mixture, high-throughput automated workflows have been developed, which are almost equal in efficacy, while also seeing the process time significantly reduced [36, 37]. While increasing in prominence, the cost of the materials needed for this approach has limited its growth, further highlighting the mainstream implementation of traditional materials like agarose.

Irrespective of the support material chosen for the basis of the IP experiment, it is important to highlight that depending on the method employed, the support material may influence the formation of immunocomplexes and/or demonstrate an affinity for nonspecific proteins, which make up the sample of interest. It is thus incredibly important to incorporate appropriate controls into an experimental protocol to ensure the protein of interest is isolated from being part of the immunocomplex, or else through a nonspecific interaction with the support material.

Fig. 1 (continued) in this setup, one or more components are stationary (typically the binding antibody-protein complex), with the remaining (sample containing protein of interest) interacting with such as it works its way through the column. With either format, sample dissociation from the immunocomplex can be achieved by disrupting the bonds between antibody and bound proteins by physical and/or chemical means

1.3.2 Choice of Antibody

A significant level of control can be exerted over the processing, and resultant nature, of the sample of interest. In contrast, there is significantly less control in the selection and performance of the antibody, which forms the appropriate immunocomplex, that is, is it of sufficient capability to capture the protein of interest (and in the case of co-IP, all possible binding partners), while displaying no interactions or affinity toward nonspecific molecules that form the remainder of the original sample.

While a percentage of laboratories can produce their own immunoglobulin molecules toward targets of interest for IP application [38], most investigators will be dependent on commercially available antibodies. As such, selection and subsequent incorporation of an antibody into an IP workflow can command its own degree of empiricism, and often, one is dependent on the information pertaining to the antibody of interest provided by the respective vendor.

Measures can be taken however to reduce the risk associated with selection of an antibody for IP purposes and subsequently increase the chance of your selected antibody forming an effective immunocomplex with the target of interest as part of this process. An antibody intended for use in IP processes needs to be available in sufficient quantity and be of high purity. Compromising on either of these aspects immediately reduces the performance and efficacy of the intended IP reaction and poses particular risks to the success of the experiment—predominantly in situations where the sample to be processed is dilute in nature and/or the target protein of interest is in low concentration. Care should be taken to select an antibody that targets an epitope exposed on the surface of the native form of the protein of interest. In this way, if appropriate care is taken in preparing the sample (subheadings 1.2.1, 1.2.2, 1.2.3, 1.2.4, 1.3, 1.3.1, 1.3.2, 1.3.3, 1.3.4, and 1.4), formation of the immunocomplex between the selected antibody and the target of interest should be relatively straightforward (provided other environmental factors are conducive) [39]. Finally, the choice of antibody type can present its own unique opportunities and challenges. Polyclonal antibodies consist of a pool of antibodies targeting different epitopes on the same target protein. Each antibody in this pool exhibits its own distinct affinity to its respective epitope, and as such, each antibody is individually influenced by changes in the workflow. The recovery of a target protein from a polyclonal population subsequently requires very harsh, denaturing conditions to ensure the "complete" disruption of each individual immunocomplex stemming from each individual antibody clone, an aspect that can impact on the profile of the proteins found in the final eluted sample. The nature of polyclonal antibodies therefore infers that the efficacy of an IP employing such is the culmination of the summative performances of the entire antibody population.

This can be advantageous in that the multi-targeting aspect of the population increases the likelihood of capturing the target protein of interest; however, the respective nonspecific binding of each antibody in the polyclonal population can confound the purity of the final sample and subsequent analyses of such. Moreover, as supplies of polyclonal antibodies can be "finite," there can be considerable variation between batches or preparations, meaning conditions may need to be optimized on a continuous basis. Monoclonal antibodies, in contrast, are specific to a single target epitope: each clone exhibiting equal affinity, any change to the workflow will ultimately influence the performance of the antibody population in its entirety. As such, monoclonal antibodies themselves may command the use of harsh buffer conditions in recovering the target protein of interest, and the uniformity of the antibody population significantly reduces the complexity of this process [40]. In turn, owing to the "infinite" source of such, once optimized, there is no significant difference encountered between respective batches or preparations. In that way, the emergence of monoclonal antibodies and their continuous supply of highly uniform antibodies has revolutionized the field of IP [41].

The selected antibody will still require optimization, for example, formation of the immunocomplex will depend on the concentration of the antibody employed with respect to the abundance of the target protein in the sample being processed. In many cases, the abundance of the target protein will be unknown; therefore, optimization of not only the concentration of capture antibody will be needed (dependent on the affinity properties of the antibody), but so too the allotted incubation time between the capture antibody and sample being processed. Generally, a starting concentration of 1–10 μg of antibody for every 500 μg of sample extract can inform you of the potential of an antibody for one's IP purposes. Thereafter, incubation time (1–12 h) can be trialed, bearing in mind longer incubation times may encourage nonspecific interactions. Moreover, additional considerations such as the ability to immobilize the antibody (subheading 1.3.3) and the influence of the buffer system (subheading 1.3.4) need to be taken into account in achieving the optimal performance of the antibody.

As with every other aspect of IP, control measures should be employed when trialing and optimizing an antibody for use in IP. For example, an isotype control, in an IP reaction that mirrors the intended experimental IP system but employs an antibody, which matches the class of capture antibody while lacking specificity to the target of interest, should be run in parallel. The results of such determine if the purification of the target protein of interest is due to the capture antibody specifically, or the mere presence of an immunoglobulin molecule.

1.3.3 Immobilization of Antibody

As detailed previously, isolating the immunocomplex formed between the capture antibody and the protein/s of interest involves the separation and extraction of such via an additional interaction between the antibody and the solid support material. As such, the support material must possess the ability to interact, bind, and immobilize the immunocomplex, and this is a process typically achieved via the direct or indirect application of a binding protein, which facilitates the interaction between the respective components [42].

Protein A and Protein G are both surface proteins isolated from bacteria (*Staphylococcus aureus* and *Streptococcal* bacteria, respectively) that are routinely utilized in IP workflows owing to their strong affinity toward antibody molecules. Protein A exhibits two, high-affinity binding sites, which exclusively bind to the constant region of immunoglobulin G [43]. Protein G in comparison preferentially binds to the same region (but has also been shown to exhibit multiple secondary binding sites for the antigen-binding fragment) [44]. The binding of Protein A and G to residues located in the constant region of an antibody effectively orientates the antibody with the antigen-binding sites freely accessible, ideal for promoting the assembly of the immunocomplex central to IP. Both proteins bind their own respective libraries of antibodies and their subclasses, across a host of different species, including but not limited to human, rabbit, and mouse [43, 44], while the recombinant Protein A/G fusion molecule exhibits the combined capabilities of each and is thus the most often binding protein implemented in IP workflows.

Irrespective of the binding protein used, considerations must be made for how the IP workflow will impact the interaction between the binding protein and the support material/antibody. For example, the performance of the capture antibody can be influenced by the "binding environment," such as the chemical composition of the binding, wash, elution buffers (subheading 1.3.4), the pH, temperature, and the presence of reducing agents. However, the binding of antibody-protein complexes themselves may subsequently dissociate in those conditions, which favor immunocomplex formation. In these instances, the support material may lose a percentage of the capture antibody, reducing the efficacy of the system, increasing the likelihood of antibody contamination of any isolated fractions. Fortunately, it is possible to covalently cross-link an antibody to the support material, increasing its stability further. Cross-linkers such as disuccinimidyl suberate (DSS) and bis(sulfosuccinimidyl)suberate (BS3) can covalently link adjacent amines located on the intended capture antibody and Protein A, G, or A/G, for example. This increases the stability of the capture system, reducing the presence of antibody/fragments that may elute in response to buffers and reagents that come into contact with it and preserves its activity allowing for repeated use.

This practice does require optimization as an insufficient cross-linker may result in only a fraction of the antibody being linked to the binding protein, while excessive amounts may irreversibly modify amine groups throughout the antibody, rendering it unable to bind the protein of interest [45].

Of note, while Protein A, G, and A/G are often central to a number of routine IP workflows in use today, these binding proteins are not always compatible in an IP workflow. For example, samples rich in immunoglobulins (i.e., serum) can create competition between the intended capture antibody and those antibodies present in the serum for binding to Protein A, G, and A/G [46], reducing the formation of immunocomplexes, and in turn, the resultant target protein/s yield. Similarly, care should be taken when the experiment involves a species or subclass of antibody, which does not have a natural affinity for Protein A, G, or A/G—in this way no immobilization can occur, and the system will effectively be devoid of any capture antibody. Fortunately, there are approaches for overcoming these obstacles. Utilizing an anti-immunoglobulin antibody bound to Protein A, G, or A/G allows one to indirectly bind the antibody of interest to the support material. Similarly, there are protocols for chemically conjugating an antibody directly to the support material, eliminating the need for Protein A/G in the system. These methods immobilize the capture antibody population in random orientations based on interactions between chemical groups on the immunoglobulin molecule and reactive groups on the support material. These measures present a slight decrease in immunocomplex formation and in turn protein yield, but conversely, by eliminating the presence of Protein A/G in the system, there is a reduced risk of antibody contamination in the targeted fraction of interest.

1.3.4 Choice of Buffers

While much attention has been directed toward the components of the immunocomplex, the constituents and volume of buffers used throughout the IP workflow play a critical role in not only the efficient formation of the immunocomplex but also in the subsequent purification and preservation of the target protein stability and recovery. A brief discussion of buffers used to bind, wash, and elute proteins of interest in an IP system provides insight into the starting point for each, with information on additives, which may be employed to improve each buffer's respective process.

The assembly of the immunocomplex is not only dependent on the respective biochemically driven affinity interactions between the capture antibody with the target protein of interest and binding protein, but also the "binding" environment within which these interactions occur. From a biochemistry perspective, the reactions that lead to the formation of an immunocomplex between most antibodies and their target protein are incredibly robust in nature

and will occur at near neutral pH conditions. As such, phosphate-buffered saline (PBS) or tris-buffered saline (TBS), both of which have physiological levels of salt and pH, should exert negligible changes or interference in the formation of the immunocomplex [29]. As will be discussed, the subsequent implementation of buffers of an alkaline or acidic nature (i.e., wash buffer and elution buffer (subheading 1.3.4) can then be used to scrutinize these interactions that occurred in the binding phase and ensure a level of stringency in the IP process.

However, some approaches to IP command specific conditions of the binding buffer to facilitate particular reactions inherent in that setup. Specific interactions between particular antibodies, sample types, and indeed binding proteins (where necessary) may require specific reaction conditions of varying strength and duration, and such components may need to be added to the PBS/TBS base formulation to accomplish this. A pertinent example of this revolves around the contrast in binding buffer conditions required when employing the binding proteins Protein A and G in an IP workflow. Both proteins, as was discussed (subheading 1.3.3), when incorporated into an IP workflow, can significantly increase the binding capacity and thus improve the performance of the IP reaction. By incorporating them, the optimal pH of the binding buffer for Protein A is pH 8.2, while for Protein G, it is pH 5.0. Subsequent adjustment of the binding buffer to accommodate a single component of the IP system may have consequences in negatively influencing the performance of another—alkaline/acidic environments can interfere with the formation of the immunocomplex [29]. As such, empirical testing is once again central to determining the optimal binding buffer for each IP workflow—of note, once the optimal binding buffer has been determined, the exact same formulation is often employed as the "Wash" buffer, or at minimum, is the basis of such.

There are very few IP protocols, or protein purification protocols in general, that ultimately produce a "pure" protein as a product of the process—instead, they reduce the levels of contaminant proteins to levels that are not detectable by routine methods and are thus of negligible influence. The choice of wash buffer plays a critical role in this respect, by creating conditions in which the stability of the immunocomplex is maintained but the binding of nonspecific proteins is prevented, allowing them to subsequently be removed from the system. As discussed already, many of the components of an IP reaction demonstrate affinities of varying degree to biomolecules, which may be present in a sample. The interaction, and subsequent retention of these nonspecific proteins can create a "background"; the extent of which is dependent not only on the materials in question but also on the introduction and efficacy of measures employed to reduce such.

As with many steps of an IP workflow, empirical testing is necessary in finding a wash buffer and the conditions that are effective for a particular setup. The starting point in developing and optimizing a wash buffer for IP is either PBS or TBS. If the IP experiment demonstrates the successful formation of the immunocomplex (as indicated in the analyses of final eluted sample) while using these as the wash buffer, the formulation can then be further optimized to improve the purity of the target protein depending on the complexity/purity of the eluted sample. Low levels of mild detergents (i.e., 0.5–1.0% of NP-40, Triton-X-100, or 3-cholamidopropyl dimethylammonio 1-propanesulfonate (CHAPS)) can be added to increase the stringency of the separation process and further reduce potential background [47]. If the degree of background contaminants remains high, agents, which target ionic and electrostatic interactions (increasing sodium chloride levels up to 1 M) and disulfide and nucleophilic interactions (addition of 2 mM dithiothreitol (DTT) or β-mercaptoethanol), may be considered.

Elution buffers promote the disassembly of the immunocomplex, allowing the purification of the target protein. Examples of those buffers typically used in IP workflows include 0.1 M citric acid and 0.1 M glycine: pH 2.5–3. Both of these buffers act by adjusting the pH of the IP environment until the capture antibody releases the bound protein of interest. The appropriate buffer conditions for elution are however as varied as the types of proteins concerned and their specific chemical binding properties. In that way, low-pH buffers, while mild in action, may not be effective against immunocomplex formations of greater binding potential and will in those instances result in a low IP yield. In these instances, a high-pH buffer may be more effective, though this may subsequently cause irreversible functional damage to components of the IP workflow.

As such, the ideal elution buffer is one that facilitates the dissociation of the immunocomplex without irreversibly denaturing or inactivating the components of such. Considerations should therefore be given as to whether the mode of action of an elution buffer may negatively impact on any of the components responsible for the formation and maintenance of the immunocomplex. Elution buffers of varying pH, ionic strength, and ability to denature proteins are readily available for manipulating the bonds between antibody and the target protein of interest to aid in the latter's recovery [48]—though as already introduced, the impact of these buffers on protein structure in general can influence the IP workflow (i.e., capture antibody, binding protein, and sample of interest). Ultimately, this can result in changes in the structure, activity, and nature of biomolecules, which the elution buffer comes into

contact with—some of which can persist after the elution step has been completed and may be irreversible if not suitably addressed. As such, many IP workflows incorporate the addition of a "neutralizing" buffer to resultant eluted fractions to nullify any potential impact of the elution buffer in question and preserve protein structure and function inside and outside of the system.

In that way, irrespective of the choice of elution buffer, one can expect a certain degree of loss of antibody function, in addition to a loss of activity in the target protein of interest, given the action/s of elution buffers on protein biochemistry [49]. Sample purity can also be impacted upon—for example, the nature of most elution buffers can lead to dissociation of the antibody/binding protein complex (assuming measures have not been taken to stabilize it beyond their natural interaction) meaning binding proteins may have a presence in the resultant eluted fraction. Given the influence elution buffers can have at the closing stages of an IP, testing a variety of elution buffers to find the one that is most gentle, yet effective in its action, is strongly worth considering.

1.4 Downstream Processing

The methodology of IP is diverse, with many techniques and approaches adaptable to almost all proteins of interest. Implementation of any one of the diverse methods toward a protein of interest can subsequently generate samples varying in yields, purities, stabilities, and nature. Interpretation of the results obtained from IP protocols can be incredibly complex, and as such, most IP workflows incorporate methods of analysis to subsequently profile the eluted sample/s and perform characterizations for quality control and assurance purposes [50, 51]. For example, advancements within in silico -based approaches and modeling have supported many of these existing workflows with computational-based predicted protein-protein interactions. This division of research has proven incredibly useful in distinguishing between a legitimate biomolecular interaction and those nonspecific in nature [52]. Nevertheless, this means of analysis is not accessible or readily available to everyone who has need of IP in the experimental workflow, and in that way, there is still a reliance on the traditional methods of biomolecular analysis, with more advanced techniques supplementing such where needed/affordable.

For example, any laboratory interested in utilizing IP must have a means of determining the presence of the target protein (typically the first analysis conducted after the experiment has been performed). Thereafter, a means of determining the efficacy of the isolation technique is required. There are a multitude of distinct techniques for carrying this out [53]: x-ray crystallography, yeast two-hybrid system, enzyme-linked immunosorbent assay (ELISA),

mass spectrometry [54], and the most routine and accessible to most labs, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) coupled with Western blotting [55, 56]. Many experimental IP workflows utilize the elution of the captured protein/s directly into SDS-PAGE sample buffer for subsequent downstream analysis, though it should be noted that many other applications (i.e., mass spectrometry) are not compatible with this agent, and alternative buffers should be sought and application-dependent.

SDS is a strong anionic detergent, which upon binding to eluted proteins denatures them and infers on them a negative charge, the amount of which is relative to the size of the respective protein [57]. SDS-treated samples can then be electrophoretically separated, based on size, using polyacrylamide gels of a predetermined porosity in a discontinuous buffer setup. Molecular weight markers, coupled with staining of the resultant gel (with Coomassie Brilliant Blue or silver nitrate solution), then allow one to calculate the estimated molecular weights of each polypeptide, which is present in the respective sample. Addition of Western blotting to the workflow allows for further scrutiny, as it allows for the presence of the protein of interest to be determined among the complexity of polypeptides in the resolved biological sample. In this way, Western blotting enables most laboratories to detect not only their protein of interest in an isolated fraction, or its respective level, but also other parameters such as binding/affinity to other biomolecules and post-translational modifications. Thus, Western blotting is a versatile means of analyzing IP samples in allowing for comparisons of such between individual fractions and control samples (for more on Western blotting, *see* Chapter 16).

2 Materials

Unless otherwise indicated, all chemicals can be purchased from Merck Sigma-Aldrich.

2.1 Isolation of Cell Organelles

1. Lysis buffer; 0.025 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, 1% v/v NP-40, 5% glycerol, and 4% v/v cOmplete™, EDTA-free protease inhibitor cocktail, pH 7.4 protease inhibitor cocktail (*see* **Note 1**).
2. TBS (Tris-buffered saline); 50 mM Tris, 150 mM NaCl, pH 7.5.
3. Container with crushed ice.
4. Refrigerated centrifuge and rotor (e.g., Eppendorf 5430R/Rotor FA-48-45-11; 12,000 × *g*).
5. Refrigerated ultracentrifuge (e.g., Beckman L8-M/70Ti rotor; 100,000 × *g*).

2.2 Batch Immunoprecipitation

1. Lysis buffer; 0.025 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, 1% v/v NP-40, 5% glycerol, and 4% v/v cOmplete™, EDTA-free protease inhibitor cocktail, pH 7.4 (*see* **Note 1**).
2. Protein A/G Sepharose® Beads.
3. BSA (bovine serum albumin).
4. 2× SSB (sample solubilization buffer) (*see* **Note 2**).
5. 1% Triton X-100.
6. Refrigerated centrifuge and rotor (e.g., Eppendorf 5430R/Rotor FA-48-45-11; 12,000 × *g*).
7. Stuart™ Rotator SB3.
8. Stuart™ Block Heater.

2.3 Spin-Column Immunoprecipitation

1. AminoLink™ Plus Coupling Resin.
2. Control agarose resin slurry.
3. Coupling buffer; 0.01 M sodium phosphate, 0.15 M NaCl pH 7.2.
4. 5 M Sodium cyanoborohydride.
5. Quenching buffer; 1 M HCl (hydrochloric acid).
6. Wash solution; 1 M NaCl.
7. Wash buffer; 0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% v/v glycerol.
8. TBS (Tris-buffered saline); 50 mM Tris- 150 mM NaCl, pH 7.5.
9. Conditioning buffer; 50 mM Tris–HCl, 50 mM NaCl, pH 8.0.
10. Elution buffer; 0.1 M glycine pH 2.5.
11. Sodium azide.
12. Spin columns—screw cap (Pierce™, Cat. No. 69705).
13. Refrigerated centrifuge and rotor (e.g., Eppendorf 5430R/Rotor FA-48-45-11; 12,000 × *g*).
14. COX IV mouse mAb (Cell Signaling Technology®, #11967).
15. Stuart™ Rotator SB3.

2.4 Assessment of Post-precipitated Fractions

1. Lamin A mouse monoclonal antibody (Cell Signaling Technology®, #86846).
2. AIF rabbit polyclonal antibody (Cell Signaling Technology®, #4642).
3. Calnexin rabbit polyclonal antibody (Cell Signaling Technology®, #2433).
4. MEK1/2 rabbit polyclonal antibody (Cell Signaling Technology®, #9122).
5. COX IV rabbit polyclonal antibody (Cell Signaling Technology®, #4844).

3 Methods

All steps are to be performed at 4 °C unless otherwise stated. Latex gloves should also be worn.

3.1 Isolation of Cell Organelles

1. The following protocol will focus on the isolation and fractionation of cell organelles from adherent cells grown on a 100 mm diameter dish. For information on adapting this protocol for suspension cell, *see* **Note 3**. For information on adapting this protocol for tissue, *see* **Note 4**.
2. Place the culture vessel on crushed ice and aspirate the growth medium.
3. Wash the vessel with TBS and aspirate. Repeat this step two more times.
4. Add 2 μL of TBS per cm^2 of the culture dish.
5. Harvest the cells using a sterile cell scraper and transfer the liquid volume to a sterile microcentrifuge tube.
6. Centrifuge the mixture at 500 × *g* for 5 min. Resuspend the pellet in 500 μL of ice-cold lysis buffer in a fresh microcentrifuge tube.
7. Incubate the mixture for 1 h on a rotator at 4 °C with continuous tube inversion.
8. Incubate the tube containing the lysate on crushed ice for 1 h. Take a 100 μL sample for a control (whole cell lysate).
9. Centrifuge the mixture at 100 × *g* for 2 min to pellet hard-to-break cells/debris. Place the supernatant in a fresh 15 mL centrifuge tube.
10. Centrifuge the supernatant at 600 × *g* for 10 min to pellet cell nuclei. Place the supernatant in a fresh 15 mL centrifuge tube and resuspend the pelleted nuclei fraction (N) in 200 μL of TBS.
11. Centrifuge the supernatant at 12,000 × *g* for 5 min to pellet cell mitochondria, lysosomes, and peroxisomes. Place the supernatant in a fresh 15 mL centrifuge tube and resuspend the pelleted organelles (O) fraction in 200 μL of TBS.
12. Centrifuge the supernatant at 100,000 × *g* for 1 h to pellet the cell membrane. Place the supernatant (the cytosol; (C)) in a fresh 15 mL centrifuge tube and resuspend the pelleted cell membrane (M) fraction in 200 μL of TBS.
13. Store the samples at −20 °C until further analysis/processing can be conducted (*see* **Note 5**). The focus of the remainder of this chapter will be the "organelle" fraction (O) for the isolation of the COX IV protein. For "batch" method of IP, proceed to subheading 3.2. For "spin-column" method of IP, proceed to subheading 3.3.

3.2 Batch Immunoprecipitation

All steps are to be conducted at 4 °C unless stated otherwise. Latex gloves and appropriate PPE should be worn throughout in the interest of keeping the sample contaminant-free:

1. Add 200 μg of protein lysate to 20 μL of Protein A/G Sepharose® beads in a microcentrifuge tube before adding 0.2% (w/v) BSA (*see* **Note 6**). Allow the mixture to stand for 1 h (*see* **Note 7**).
2. Centrifuge the mixture at 12,000 × for 30 s to pellet the Protein A/G Sepharose® beads.
3. Transfer the supernatant (pre-cleared lysate) to a fresh microcentrifuge tube, and add (insert antibody here), 10 μL of 10% w/v BSA and 20 μL of fresh Protein A/G Sepharose® beads (*see* **Note 8**).
4. Incubate the mixture overnight on a rotator at 4 °C with continuous tube inversion.
5. Centrifuge the mixture at 12,000 × *g* for 5 min to pellet the Protein A/G Sepharose® beads.
6. Remove the supernatant and resuspend the pelleted beads in 500 μL of lysis buffer supplemented with 1% Triton X-100.
7. Repeat **steps 5** and **6**.
8. Centrifuge the mixture at 12,000 × *g* for 5 min to pellet the Protein A/G Sepharose® beads.
9. Remove the supernatant and resuspend the pelleted beads in 500 μL of lysis buffer.
10. Centrifuge the mixture at 12,000 × *g* for 5 min to pellet the Protein A/G Sepharose® beads once more and resuspend the pelleted beads in 2× SSB buffer (*see* **Note 2**).
11. Heat the mixture for 5 min at 95 °C.
12. Centrifuge the mixture at 12,000 × *g* for 5 min to pellet the beads and transfer the supernatant (containing solubilized proteins) to a fresh microcentrifuge tube (*see* **Note 9**).
13. Analyze the sample using methods outlined in subheading 3.4 (*see* **Note 10**).

3.3 Spin-Column Immunoprecipitation

Equilibrate all reagents to room temperature prior to use. *All* steps are to be conducted at 4 °C unless stated otherwise. Latex gloves and appropriate PPE should be worn throughout in the interest of keeping the sample contaminant-free.

3.3.1 Antibody Immobilization

1. Add 50 μL of AminoLink™ Plus Coupling Resin to a spin column housed in a microcentrifuge tube.
2. Centrifuge the tube at 1000 × *g* for 1 min and discard the flow-through.

3. Wash the spin column by adding 200 µL of coupling buffer and centrifuge the tube at 1000 × *g* for 1 min. Discard the flow-through.
4. Repeat **step 3** twice.
5. Remove the spin column from the microcentrifuge tube and gently remove excess flow-through by tapping the spin column against paper towel. Insert the column plug before proceeding.
6. Dilute 10 µg of COX IV mouse monoclonal antibody to 200 µL using coupling buffer and add to the spin column housed in a microcentrifuge tube (*see* **Note 8**).
7. In a fume hood, add 3 µL of sodium cyanoborohydride to the mixture contained in the spin column (*see* **Note 11**).
8. Attach the screw cap to the spin column and incubate the tube on a rotator with gentle inversion at room temperature for 2 h.
9. Remove the column plug from the spin column and loosen the screw cap. Place the spin column into a fresh microcentrifuge tube and centrifuge at 1000 × *g* for 1 min. Save the flow-through (label and store at –20 °C) and add the spin column a fresh microcentrifuge tube (*see* **Note 12**).
10. Add 200 µL of coupling buffer to the spin column. Centrifuge at 1000 × *g* for 1 min and discard the flow-through.
11. Repeat **step 10** two times.
12. Add 200 µL of quenching buffer to the spin column, centrifuge at 1000 × *g* for 1 min, and discard the flow-through.
13. Remove the spin column from the microcentrifuge tube and gently remove excess flow-through by tapping the spin column against paper towel. Insert the column plug before proceeding.
14. Add 200 µL of quenching buffer to the spin column.
15. In a fume hood, add 3 µL of sodium cyanoborohydride to the mixture contained in the spin column.
16. Attach the screw cap to the spin column and incubate the tube on a rotator with gentle inversion at room temperature for 15 min.
17. Remove the column plug from the spin column and loosen the screw cap. Place the spin column into a fresh microcentrifuge tube and centrifuge at 1000 × *g* for 1 min. Discard the flow-through.
18. Remove the screw cap and add 200 µL of coupling buffer to the spin column. Centrifuge at 1000 × *g* for 1 min and discard the flow-through.
19. Repeat **step 18** two times.

20. Add 150 µL of Wash Solution to the spin column and centrifuge at 1000 × *g* for 1 min. Discard the flow-through.
21. Repeat **step 20** six times.
22. Proceed to subheading 3.3.2 or subheading 3.3.3 or prepare the column for long-term storage (*see* **Note 13**).

3.3.2 Protein Sample Pre-clearance

1. Add 80 µL of Control Agarose Resin slurry for every 1 mg of protein lysate to be co-immunoprecipitated into a new spin column.
2. Centrifuge the spin column at 1000 × *g* for 1 min and discard the flow-through.
3. Add 100 µL of coupling buffer to the spin column, centrifuge at 1000 × *g* for 1 min, and discard the flow-through.
4. Add 1 mg of protein lysate to the spin column and incubate on a rotator with gentle inversion at room temperature for 1 h.
5. Centrifuge the spin column at 1000 × g for 1 min. Save the flow-through (pre-cleared lysate) and discard the spin column.
6. Proceed to subheading 3.3.3.

3.3.3 Co-immunoprecipitation

1. Pre-dilute the pre-cleared lysate in wash buffer to 500 µL.
2. Place the spin column prepared in subheading 3.3.1 in a fresh microcentrifuge tube and add 200 µL of wash buffer. Centrifuge at 1000 × *g* for 1 min and discard the flow-through.
3. Repeat **step 2** two more times.
4. Remove the spin column from the microcentrifuge tube and gently remove excess flow-through by tapping the spin column against paper towel. Insert the column plug before proceeding.
5. Add the diluted pre-cleared lysate from **step 1** to the spin column. Attach the screw cap to the spin column and incubate the tube on a rotator with gentle inversion at room temperature overnight.
6. Remove the column plug from the spin column and loosen the screw cap. Place the spin column into a fresh microcentrifuge tube and centrifuge at 1000 × *g* for 1 min. Save the flow-through (*see* **Note 14**).
7. Remove the screw cap and place the spin column in a fresh collection tube. Add 200 µL of wash buffer and centrifuge at 1000 × *g* for 1 min.
8. Repeat **step 7** two more times (*see* **Note 15**).
9. Proceed to subheading 3.3.4.

3.3.4 Elution of Target Protein/s

1. Place the spin column into a new microcentrifuge collection tube and add 10 µL of elution buffer.
2. Centrifuge the tube at 1000 × *g* for 1 min.
3. Add an additional 50 µL of elution buffer to the spin column and incubate for 5 min at room temperature (*see* **Note 16**).
4. Centrifuge the tube at 1000 × *g* for 1 min and collect the flow-through (*see* **Note 17**). Store the eluate samples at –20 °C until analysis can be conducted (subheading 3.4) (*see* **Note 18**).
5. To preserve the column for subsequent co-immunoprecipitations of target protein, add 100 µL of coupling buffer to the spin column and centrifuge the tube at 1000 × *g* for 1 min.
6. Discard the flow-through and repeat **step 5** once more.
7. Discard the flow-through and seal the bottom of the spin column with the corresponding column plug. Add 200 µL of coupling buffer to the tube supplemented with 0.02% sodium azide. Add the screw cap and store at 4 °C.

3.4 Assay of Protein Fractions

Determination of the total protein in pre- and post-fractionated samples can be done in a number of ways:

- Monitoring fraction absorbance at 280 nm (e.g., Tecan Infinite® M200 Pro, NanoDrop® One, WPA Biowave II)
- Colorimetric or fluorescent-based assays (e.g., Bradford assay, silver staining).
- Chromogenic-based assay (e.g., Bicinchoninic assay (BCA))
- Immunoassays (e.g., indirect ELISA)
- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with Coomassie brilliant blue staining and/or Western blot

Validation of the isolation of targeted protein fractions from the methods outlined in subheadings 3.1, 3.2, and 3.3 can utilize any one or combination of these approaches (for more information on protein quantitation methods, *see* Chapter 16). The utilization of SDS-PAGE analysis and Western blotting to validate fractionation methodologies described in this chapter are briefly described below:

1. If not already added, add 2× SSB to the protein sample of interest to make a 1× final solution.
2. Heat the sample to 95 °C for 5 min. Allow the sample to cool to room temperature.
3. Prepare and carry out SDS-PAGE of your samples as described (Chapter 16) (*see* **Note 19**).

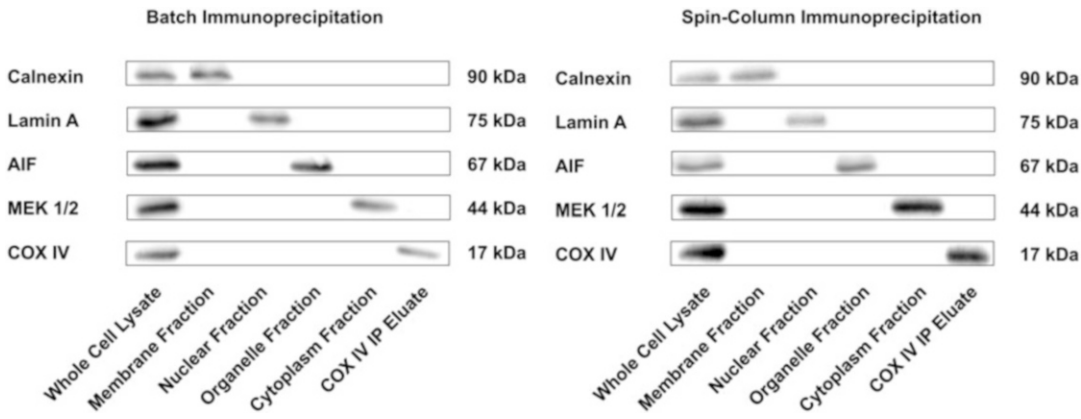

Fig. 2 Validation of the isolation of nuclear, membrane, organelle, and cytoplasm fractions, and purification of target protein of interest; COX IV, by subcellular fractionation and immunoprecipitation, respectively. Human brain microvascular endothelial cells were lysed, and fractionated using differential centrifugation, before the purified "organelle" fraction was subjected to independent IP workflows of both "batch" (left) and "spin column" (right) utilizing the COX IV antibody. Downstream Western blot analysis highlights the effective isolation of each subcellular fraction courtesy of the absence/presence of fraction-specific biomarkers. The successful isolation of COX IV using both variations of IP can also be observed, with the absence of mitochondrial protein AIF absent in the same sample corroborating such. All data courtesy of Dr. Keith Rochfort (unpublished observations)

Table 2
Western blot antibody concentrations for the validation of the isolation of individual cell fractions, and target protein of interest; COX IV

Cell fraction	Primary antibody	Working concentration	Secondary antibody	Working concentration
Nucleus	Lamin A mouse mAb, #86846	1 μg/mL	Anti-mouse IgG, HRP-Linked Antibody, #7076	1 μg/mL
Organelles	AIF rabbit pAb, #4642	100 ng/mL	Anti-rabbit IgG, HRP-Linked Antibody, #7074	1 μg/mL
Membrane	Calnexin rabbit pAb, #2433	100 ng/mL	Anti-rabbit IgG, HRP-Linked Antibody, #7074	1 μg/mL
Cytosol	MEK1/2 rabbit pAb, 9122	100 ng/mL	Anti-rabbit IgG, HRP-Linked Antibody, #7074	1 μg/mL
Mitochondria	COX IV rabbit pAb, #4844	100 ng/mL	Anti-rabbit IgG, HRP-Linked Antibody, #7074	1 μg/mL

4. Carry out Western blot analysis of your samples (*see* Chapter 16), *see* Fig. 2 for representative Western blots utilizing these antibodies on samples prepared using the protocols outlined in this chapter (*see* **Notes 10** and **20**). Information on the antibodies used to validate subcellular fractionation of the cell lysate, and successful IP of COX IV, are included in Table 2.

4 Notes

1. The individual components of lysis buffer can be prepared and stored at 4 °C; however, for optimal activity the cOmplete™, EDTA-free protease inhibitor cocktail should be prepared fresh on the day of the experiment. The 25× stock solution (1 tablet in 2 mL of distilled water) can remain stable for 1–2 weeks at 2–8 °C, or at least 12 weeks at –20 °C if made in advance.
2. SSB buffer is made to a 2× concentration: 120 mM Tris–HCl pH 6.8, 4% SDS (sodium dodecyl sulfate) (w/v), 20% glycerol (v/v), 0.02% bromophenol blue (w/v), and 4% β-mercaptoethanol (v/v). With the addition of β-mercaptoethanol, the buffer must be used immediately, or else aliquoted and stored at –20 °C until required. If the β-mercaptoethanol is omitted the buffer can be stored at room temperature/4 °C until required, at which point the relevant concentration of β-mercaptoethanol (4% v/v) must be added.
3. Harvest the cells from the culture vessel and place in a centrifuge tube. Centrifuge the suspension at 500 × *g* for 5 min. Resuspend the pellet in 500 μL of Dulbecco's PBS and centrifuge the suspension using the same settings. Repeat this "wash" step two more times. Resuspend the pellet in 500 μL of lysis buffer. Proceed to **step 6** of subheading 3.1.
4. Cut fresh, unfrozen tissue into 2–4 mm pieces and wash briefly with 1 mL of ice-cold TBS solution. Weigh 40–60 mg of tissue into a microcentrifuge tube and add 500 μL of ice-cold lysis buffer. Disrupt the tissue using a tissue homogenizer and transfer to a QIAshredder column (QIAGEN). Centrifuge at 500 × *g* for 10 min to filter the homogenate. Discard the QIAshredder column and resuspend the resultant pellet in the microcentrifuge with the filtrate by gently pipetting up and down, and transfer to 500 μL of ice-cold lysis buffer in a fresh microcentrifuge tube. Continue from **step 7** in subheading 3.1.
5. Depending on the intended use/analyses of the resultant eluates, addition of inhibitors may be necessary to preserve the native state and activity levels of the proteins held in that fraction. A list of traditionally used inhibitors is included in Table 1 for reference.
6. The addition of BSA is an optional step that aims to reduce the presence of nonspecific proteins, which can contaminate the eluate. BSA "blocks" the binding sites on the support material creating a competitive environment for subsequent binding to the materials. Inclusion of BSA in the reaction increases the risk

of it co-eluting with the target protein of interest—though this can be easily identified and accounted for in downstream analyses.

7. The length of incubation time needed to pre-clear the lysate of nonspecific interactions may need to be optimized. Increasing the time may improve the removal of background, nonspecific binding, but excessive incubation may promote additional, nonspecific interactions between the proteins of interest and the support material.
8. Inclusion of a control reaction which uses an unrelated antibody in place of the target protein of interest is useful in determining, and controlling for, the nonspecific interactions that may be occurring in the IP reaction. If proteins are detected in this control, pre-clear the lysate before starting the IP workflow, and/or optimize the wash steps further to decrease the presence of nonspecific binding. Ensure that the concentration of unrelated antibody matches that of the antibody raised against the target.
9. An increase in the strength/harshness of the elution buffer increases the risk of nonspecific proteins co-eluting with the target protein of interest. With SDS-PAGE sample buffer, fragments of the binding protein, resin, immunoglobulin, etc., may be released and appear as background in the final eluate subjected to downstream analyses.
10. If the target protein/s of interest is not present on the gel/-Western blot: (i) increase the amount of antibody coupled to the resin, (ii) increase the incubation time between the sample and the antibody-coupled resin, (iii) consider a more sensitive method of detecting the protein/s of interest, and (iv) consider a different capture antibody. If the antibody binds weakly to the target protein of interest, it may be very difficult to find conditions that are mild enough to establish and maintain the immunocomplex formed by it for the duration of the IP workflow.
11. The volume of sodium cyanoborohydride correlates with the reaction volume at this step. If the process requires greater volumes of antibody, the reaction volume of coupling buffer will correlatively increase. For every 200 μL of reaction volume at this step, add 3 μL of sodium cyanoborohydride.
12. Analyses of the flow-through for the presence of immunoglobulins can be useful for determining the success of the immobilization process. The choice of antibody, binding protein, and length of incubation time should be optimized such that there are negligible immunoglobulins detectable in the flow-through. Following immobilization, wash the antibody-coupled resin with elution buffer until no additional antibody

elutes from the column. This will reduce the presence of the antibody in the eluates resulting from an IP reaction but may reduce the presence of antibody bound to the resin and, in turn, the yield of target protein captured by such.

13. The components of the spin column (i.e., the antibody) can degrade over time unless properly stored, reducing the performance of such with subsequent uses. Following use, tap the column against a paper towel to remove as much excess liquid from the column as possible. Place the plug in the bottom of the column and add 200 μL of coupling buffer to keep the resin hydrated. Tighten the screw-cap to seal the system and store the column at 4 °C. For long-term storage, to prevent microbial contamination, add sodium azide (0.02% w/v) to the coupling buffer contained in the spin column before storing at 4 °C.
14. Analysis of this sample provides not only a profile of the nonspecific proteins in the original sample but also allows for an assessment of the column itself. Analyses for the presence of protein/s of interest, binding proteins, and/or immunoglobulins in this sample can be indicative of the column's quality, which in turn corresponds to its ability to subsequently perform an efficient IP extraction. This can be useful data when interpreting the resultant eluates of the protocol.
15. Analysis of the washes for the presence of proteins can be useful for determining the optimal washing protocol for a specific IP reaction. The formulation, volume, and resultant number of washes should be optimized such that there is no protein detectable in the final wash fraction (i.e., all nonspecific proteins are removed) before addition of the elution buffer takes place.
16. For a more concentrated protein sample, a smaller volume of elution buffer can be used—in reducing the volume, however, the risk of a reduced yield increases. This should be optimized relative to the components of the system being employed.
17. Additional elutions can be performed on the column. **Steps 3** and **4** of subheading 3.3.4 can be repeated, and the eluates collected, to ensure complete dissociation of any immunocomplexes within the column, and removal of all potential protein contaminants, should the column be used again.
18. In certain instances, the target protein of interest may bind to the antibody/resin to such a strong degree that conventional elution buffers may not be effective in breaking the interaction. If a low protein yield is obtained, it is possible the protein is still bound to the resin. To investigate this, a harsh buffer such as SDS-PAGE sample buffer can be used to cleanse the column by centrifugation. The resultant eluate can then be analyzed by

Western blot to confirm the presence or absence of the protein of interest. If the protein's presence is confirmed, optimization of elution conditions will be required. If the protein's absence is confirmed, the protein was never captured, and the problem lies in another aspect of the workflow.

19. Separation of the proteins contained within samples that are analyzed by SDS-PAGE is dependent on the relative size of the pores formed within the gel—as the total amount of acrylamide increases in the gel, the pore size decreases, respectively. It is important therefore that the percentage of acrylamide in the gel allows for not only the effective resolution of the target of interest but also any other nonspecific proteins that may be present in the sample being analyzed. This allows for a complete profiling of the sample of interest, and a thorough interpretation of results obtained.
20. To improve the results obtained from Western blotting, use an antibody specific for the target of interest that has been raised in a species different to that of the one used to isolate the protein of interest. As fragments of the capture antibody can co-elute with the target protein of interest, using an antibody from a different species can reduce the interaction between both when performing the Western blot of the sample.

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Chapter 16

Protein Quantitation and Analysis of Purity

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Abstract

The accurate quantitation of proteins and an analysis of their purity is essential in numerous areas of scientific research and is a critical factor in many clinical applications. The large number and variety of techniques employed for this purpose is therefore not surprising. The selection of a suitable assay is dependent on such factors as the level of sensitivity required, the presence of interfering agents, and the composition of the protein itself. In this chapter, protocols for the most commonly used protein determination methodologies are outlined, including an overview of the highly sensitive real-time quantitative immuno-polymerase chain reaction assay. In addition, an approach to validate the UV protein absorption assay is outlined, which can be applied to any procedure for method validation.

Key words Protein, Lowry, Bradford, BCA, ELISA, Quantitative immuno-PCR (qIPCR)

1 Introduction

The ability to easily and accurately quantitate total protein content in a given sample is a fundamental requirement of many biological studies. Indeed, the routine measurement of total protein content is a well-established essential step in many areas of basic biochemical research and routine clinical practice [1, 2].

Numerous and varied methods to assay total protein content have been described in the literature. The most commonly utilized methods rely on (i) the intrinsic ability of protein molecules to absorb ultraviolet (UV) light (UV absorption) [3], (ii) the use of protein-binding dyes exploiting either colorimetric or fluorescent-based detection (Bradford Assay, Silver staining, NanoOrange™) [4–7], and (iii) the reduction of copper in the presence of a chromogenic reagent [Lowry and bicinchoninic acid (BCA) assays] [3, 8, 9]. Since each of these methods has its own strengths and weaknesses (*see* **Note 1**) [10], none of these assays should be employed without first considering its suitability for the application in question (*see* Table 1). Even fluorescent assays developed in

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Table 1
A comparison of UV absorption, Bradford, Lowry, BCA, and fluorescent protein assay methods

Method name	Advantages	Disadvantages
UV absorption	Simple, fast Inexpensive Sample is recoverable	Many buffer components absorb strongly in this region The presence of nucleic acid can greatly influence the absorption Least sensitive method
Bradford Assay	Simple, fast Inexpensive Very sensitive Compatible with a wide range of buffers	Nonlinear standard curve over wide ranges Response to different proteins can vary widely: choice of standard is very important
Lowry Assay	Sensitive Commonly referenced procedure Easily adapted to microplate format	Time-consuming Susceptible to many interfering compounds Variation in the content of tyrosine and tryptophan residues will influence the assay
BCA Assay	Very sensitive Rapid Compatible with a wide range of buffers Little variation in response between different proteins	The reaction does not go to completion when performed at room temperature or 37 °C (difficult when prepping large numbers of samples)
NanoOrange™	Highly sensitive Little variation in response between different proteins Simple, fast	Compatible with reducing agents not detergents
Fluorescamine	Simple, fast Very sensitive	Sensitivity depends on number of amines present in sample Poor water solubility Not compatible with amine-containing buffers
OPA	Very sensitive Simple, fast Water soluble Inexpensive	Sensitivity depends on number of amines present in sample Not compatible with amine-containing buffers
CBQCA	Highly sensitive Linear over an extended range of protein concentration Compatible with detergents and lipophilic proteins	Sensitivity depends on number of amines present in sample Not compatible with buffers containing amines or thiols

recent years to alleviate difficulties experienced with absorbance-based assays (e.g., OPA (o-phthaldialdehyde), fluorescamine, and NanoOrange™) are not without their weaknesses (*see* Table 1) and the UV, Bradford and Coomassie, assays remain the most widely referenced in the literature [3, 11]. There is, in fact, no absolute method that produces accurate results in every instance, and often, it is necessary to employ more than one type of protein assay [12, 13]. In addition to total protein concentration, the specific activity of a particular target protein in a sample is of significance when proteins are being purified or when different protein samples are being compared [6]. In broad terms, measurement of the level of a specific protein of interest may be undertaken by one of two methods, a specific biological assay (or bioassay) or an immunoassay. The specificity of an immunoassay relies on the interaction between the protein and an antibody, and determining the quantity of bound antibody in an immunoassay is routinely achieved by virtue of either using a labeled primary antibody, or detecting the latter using a secondary antibody that is itself labeled [14]. There are many immunoassay formats possible, and labels that are used include enzymes (e.g., enzyme linked-immunosorbent assay (ELISA)), radioactive labels (radioimmunoassay (RIA)), magnetic labels (e.g., magnetic immunoassay (MIA)), fluorescent tags (as used in flow cytometry/fluorescence activated cell sorting (FACS) analysis and fluorescence microscopy), or a piece of DNA (as in real-time quantitative immuno-polymerase chain reaction (qIPCR)). More recently, protein mass spectrometry has become an emerging method for protein quantitation and is discussed elsewhere in this volume.

A pure protein is one that is free from quantifiable levels of impurities [15]. Any purity determination is only as reliable as the analytical methods used, and factors such as the structural properties of the protein itself, the amount of protein available, the nature of potential contaminants in the sample, the capabilities of the particular assay being considered, and the accuracy of the estimate required should always be assessed when selecting the method of analysis [3, 16]. In reality, it may only be necessary to ensure the sample is free of contaminating products that may affect the application in question, and thus, aspects of the process such as the intended use (e.g., bulk enzyme preparations, protein crystallography, primary sequence analysis, or therapeutic applications), the source of the protein (animal tissue, human serum, recombinant-microorganisms or hybridomas), and the purification processes employed should all accordingly dictate the extent of analysis required [6]. Here, general protocols for the following most commonly employed methods of protein estimation and purity are described: Ultraviolet (UV) Protein Absorption Assays, the Bradford and Lowry assays, Macro- and Micro-Bicinchoninic acid (BCA) assays, ELISA, sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and associated staining methods, Western Immunoblotting, and several formats for qIPCR. In addition to the commonly used methods outlined in this chapter, an approach to validate the UV Protein Absorption Assay procedure is outlined. This approach can be applied to any procedure for method validation to confirm that the assay employed is suitable for its intended use. Results from method validation can be used to judge the quality, reliability, and consistency of results.

2 Materials

2.1 Protein Determination by Ultraviolet (UV) Light Absorption

1. Quartz Cuvettes (1 mL volume). Alternatively, plastic disposable UV-Cuvettes for the UV/VIS range are now available.
2. Buffer solution in which the protein sample is dissolved (for blanking, *see* **Note 2**).
3. UV spectrophotometer.

2.2 Preparation of a Standard Curve

1. Stock solution: prepare a stock solution of a standard protein, for example, bovine serum albumin (BSA), lysozyme, albumin, immunoglobulin G (IgG), or γ-globulin (*see* **Note 3**) at a suitable concentration (1–5 mg/mL *see* **Note 4**) and in the same buffer as the protein of unknown concentration.
2. Buffer (for blanking) (*see* **Note 5**).
3. Quartz Cuvettes (1 mL volume). Alternatively, plastic disposable UV-Cuvettes for the UV/VIS range are now available (*see* **Note 6**).
4. UV spectrophotometer.

2.3 UV Protein Absorption Assay Using Microvolume Spectroscopy

1. Distilled H_2O (dH_2O).
2. Lab-wipes (Thermo Fisher Scientific).
3. Buffer for blanking (*see* **Note 5**).
4. Nanodrop® One Spectrophotometer or other microvolume spectrophotometer (Thermo Fisher Scientific).

2.4 The Bradford Protein Assay

1. Protein standard solutions (e.g., 1 mg/mL IgG) (*see* **Note 7**): dilute in the range 20–100 μg/mL in a total volume of 100 μL. Aliquot and store at −20 °C.
2. Buffer (for blanking, *see* **Note 8**).
3. 1 M sodium hydroxide (NaOH) (*see* **Note 9**).
4. Bradford reagent available from Merck, or prepare by adding 100 mg Coomassie brilliant blue G-250 to 50 mL 95% (v/v) ethanol. When Coomassie brilliant blue G-250 has dissolved, add 100 mL 85% (w/v) phosphoric acid and stir overnight. Dilute to 1 L with dH_2O. Store for up to 3 months at 4 °C in a brown glass bottle (*see* **Notes 10** and **11**).

5. Disposable cuvettes (*see* **Note 6**) (a microtiter plate should be used if adopting a microassay format (*see* **Note 12**)
6. UV spectrophotometer (or microplate reader).

2.5 The Lowry Protein Assay

1. Lowry solution A: 2% (w/v) sodium carbonate (Na_2CO_3) in 0.1 M sodium hydroxide (NaOH). Store for up to 3 months at room temperature.
2. Lowry solution B: 1% (w/v) copper sulfate pentahydrate ($CuSO_4.5H_2O$, Merck) in dH_2O. Store for up to 1 year at room temperature.
3. Lowry solution C: 2% (w/v) sodium potassium tartrate ($NaKC_4H_4O_6.\ 4H_2O$) in dH_2O. Store for up to 3 months at room temperature.
4. Lowry working solution: Prepare immediately before use; Lowry Solution A: Lowry solution B: Lowry Solution C in the ratio 100:1:1 (v:v:v), respectively.
5. Folin-Ciocalteu Reagent: Available as 2 N reagent (Merck). Dilute 1:1 in dH_2O. This solution is light sensitive and should be prepared just prior to use and kept in a light-protected container.
6. Protein standards: Prepare a dilution series of standard protein, for example, albumin in the range 0–100 μg/mL in a total volume of 1 mL.
7. Buffer for blanking (*see* **Note 13**).
8. Disposable cuvettes.
9. UV spectrophotometer.

2.6 The Bicinchoninic Acid (BCA) Assay

2.6.1 Macro-BCA Assay

1. Macro-BCA Reagent A: Dissolve 1 g sodium bicinchoninate (BCA, Pierce), 2 g sodium carbonate, 0.16 g sodium tartrate, 0.4 g NaOH, and 0.95 g sodium bicarbonate in dH_2O. Adjust the pH to 11.25 with 10 M NaOH and bring to 100 mL with dH_2O. Stable for 1 year at room temperature.
2. Macro-BCA Reagent B: Dissolve 0.4 g $CuSO_4.5H_2O$ in 10 mL distilled water. Stable for 1 year at room temperature.
3. Macro-BCA Working solution: Mix 50 volumes of Macro-BCA reagent A with 1 volume of Macro-BCA reagent B (prepare fresh before use). The working solution should be green in color. The solution is stable for a week at room temperature.
4. Glass or disposable polystyrene cuvettes.
5. Buffer for blanking (*see* **Note 14**).
6. UV spectrophotometer.

2.6.2 Micro-BCA Assay

The Micro-BCA assay uses three reagents whose concentrations are significantly higher than the two reagents used in the Macro-BCA format. Here, BCA is prepared as a separate reagent in order to avoid its precipitation.

1. Micro-BCA assay reagent A: Dissolve 8 g sodium carbonate monohydrate, 1.6 g sodium tartrate in dH_2O, adjust the pH to 11.25 with 10 M NaOH, and bring to 100 mL with dH_2O. Stable for 1 year at room temperature.
2. Micro-BCA assay reagent B: Dissolve 4 g BCA in 100 mL dH_2O. Stable for 1 year at room temperature.
3. Micro-BCA assay reagent C: Dissolve 0.4 g $CuSO_4.5\ H_2O$ in 10 mL dH_2O. Stable for 1 year at room temperature.
4. Micro-BCA assay solution (prepare fresh): 25:25:1 (v/v/v) Micro-BCA assay reagent A/Micro-BCA assay reagent B/ Micro-BCA assay reagent C.
5. Glass or disposable polystyrene cuvettes.
6. Buffer for blanking (*see* **Note 14**).
7. UV spectrophotometer.

2.7 Immunoassay: Indirect ELISA

1. Microtiter plates.
2. Antigen-coating buffer: 0.1 M $Na_2CO_3/NaHCO_3$ pH 9.6 (adjust the pH if necessary, using HCl).
3. Phosphate buffered saline (PBS).
4. Assay buffer: 20 mM PBS pH 7.4 (adjust pH to 7.4 by adding dilute HCl or NaOH if necessary), 0.05% (v/v) Tween 20.
5. Blocking buffer: 20 mM PBS, 5% (w/v) nonfat dried milk (or other suitable blocking agent *see* **Note 15**).
6. Primary antibody (diluted to the optimal concentration in blocking buffer immediately before use. This concentration should be experimentally determined, for most applications dilution in the range 1:200 to 1:1000 is sufficient, consult manufacturer's guidelines).
7. Conjugated secondary antibodies directed toward the primary antibody (diluted to the optimal concentration in blocking buffer immediately before use).
8. Substrate, for example, for peroxidase system: add 100 μL of 0.8 mg/mL *o*-phenylenediamine dihydrochloride (OPD) dissolved in 0.1 M phosphate-citrate buffer pH 5.0, containing 0.4 mg/mL urea hydrogen peroxide (UHP) and incubate at room temperature for 30 min.
9. Stop solution: alkaline phosphatase 3 M NaOH; peroxidase 3 M HCl or 3 M H_2SO_4.
10. Microplate reader.

2.8 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

1. Leupeptin: Dissolve 2 mg/mL leupeptin in dH_2O and store at −20 °C.
2. Aprotinin: Make 0.1 M stock solution of aprotinin in dH_2O and store at −20 °C.
3. Phenylmethanesulfonyl fluoride (PMSF): Make 100 mg/mL PMSF in isopropanol and store at −20 °C in the dark.
4. Suspension buffer: 0.1 M NaCl, 0.01 M Tris–HCl (pH 7.6), 0.001 M NaOH-EDTA (pH 8.0), 1 μg/mL leupeptin, 1 μg/mL aprotinin, 100 μg/mL PMSF. Store at 4 °C.
5. 2× SDS loading buffer (Sample Buffer): 100 mM Tris–HCl (pH 7.6), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue. Store at room temperature.
6. Acrylagel (National Diagnostics). Acrylagel is toxic and a known carcinogen. Consult the corresponding material safety data sheet (MSDS) before use.
7. Bis-Acrylagel (National Diagnostics). Bis-acrylagel is an irritant. Consult the corresponding MSDS before use.
8. 1.5 M Tris–HCl (pH 8.8).
9. 1 M Tris–HCl (pH 6.8).
10. dH_2O.
11. 10% (w/v) sodium dodecyl sulfate (SDS) in dH_2O.
12. 10% (w/v) ammonium persulfate (APS) dH_2O (prepare freshly). APS is a strong and harmful oxidizing agent. Consult the corresponding MSDS before use.
13. TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine).
14. 5× Tris-glycine running buffer (Electrode Buffer): 15.1 g Tris base, 95.4 g glycine, 50 mL 10% (w/v) SDS. Make up to 1 L with dH_2O and store at room temperature.
15. 1× Tris-glycine running buffer: 200 mL 5× Tris-glycine running buffer, 800 mL dH_2O. Store at room temperature.
16. Molecular weight marker (prestained) (Cytiva).
17. ATTO protein gel electrophoresis system or other electrophoresis system.

2.9 Staining of SDS-PAGE Gels

2.9.1 Coomassie Brilliant Blue Staining of SDS-PAGE Gels

1. Coomassie brilliant blue G-250 solution (Merck).
2. Destain: 450 mL methanol, 450 mL dH_2O, 100 mL glacial acetic acid. Store at room temperature.

2.9.2 Silver Staining of SDS-PAGE Gels

1. Fixing Solution: methanol, acetic acid and formalin (40: 10:0.05 by volume).
2. Wash Solution: ethanol, acetic acid, and water (10:5:85, by volume).
3. Silver nitrate solution: or 0.2% (w/v) $AgNO_3$, 0.076% formalin (prepare fresh). Silver nitrate is harmful. Consult the corresponding Material Safety Data Sheet (MSDS) before use.
4. Oxidizing solution: 3.4 mM potassium dichromate and 3.2 mM nitric acid
5. Developing solution: 0.28 mM sodium carbonate and 1.9% (v/v) formaldehyde. Formaldehyde is toxic, consult the corresponding MSDS prior to use.
6. Stop solution: methanol, acetic acid (50:12).
7. 1% (v/v) acetic acid.

2.10 Western Blotting

1. Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell.
2. Transfer buffer: 750 mL dH_2O, 2.9 g glycine, 5.8 g Tris base, 3.7 mL 10% (w/v) SDS, 200 mL methanol. Adjust volume to 1 L with dH_2O and store at 4 °C (storing at this temperature is critical).
3. 1× Tris buffered saline (TBS): 6.1 g Tris base, 8.8 g NaCl, 800 mL dH_2O. Adjust the pH to 7.5 with HCl and adjust the volume to 1 L with dH_2O. Store at room temperature.
4. TBS-T: 1 L 1× TBS, 1 mL Tween 20 (Merck). Store at room temperature.
5. Blocking buffer: 5 g nonfat dry milk powder (or other appropriate blocking agent *see* **Note 15**), 100 mL TBS-T. Store at 4 °C.
6. Nitrocellulose blotting membrane.
7. 3MM filter paper (Whatman®).
8. Scalpel blade.
9. Ponceau S.
10. dH_2O.
11. Primary antibody (diluted to optimal working concentration in blocking buffer immediately before use. This concentration should be experimentally determined, for most applications dilution in the range 1:200 to 1:1000 is sufficient; consult manufacturers' guidelines).
12. Conjugated secondary antibody directed toward the primary antibody (diluted to an optimal working concentration in blocking buffer immediately before use).
13. Substrate (e.g., 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT, Merck) or 3,3′,5,5′--tetramethylbenzidine (TMB).

2.11 qIPCR

2.11.1 Preparation of Biotinylated DNA Label

1. Template DNA (e.g., linearized pUC19 plasmid DNA).
2. Template-specific oligonucleotide primers: 5′-biotinylated forward primer and unmodified reverse primer.
3. PCR purification kit, for example, QIAquick PCR purification kit (Qiagen).
4. Agarose powder.
5. 50× Tris-acetate/EDTA electrophoresis buffer (TAE): 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M NaOH-EDTA (pH 8.0). Adjust to 1 L with dH_2O and store at room temperature.
6. 1× TAE buffer: 20 mL 50× TAE, 980 mL dH_2O. Store at room temperature.
7. Loading dye: 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue. Store at room temperature.
8. SYBR Safe DNA gel stain (Invitrogen) (or other suitable DNA gel stain).
9. Horizontal agarose gel electrophoresis system (e.g., MSMINI-DUO horizontal gel electrophoresis system, Merck).
10. DNA size markers (e.g., 100 bp ladder from Invitrogen).
11. PCR reaction mixture: 5 μL 10× PCR buffer, 1 μL of each dNTP (200 μM), 1 μL of each primer (100 μmol/L), 1.25 U *Taq* polymerase, 50 ng template DNA, bring to 50 μL with molecular grade H_2O.
12. Agarose gel 1.5% (w/v): Dissolve the agarose in 1× TAE by boiling, with intermittent mixing until completely dissolved. Add SYBR Safe DNA gel stain according to the manufacturer's instructions. Cast the gel according to the instructions accompanying the apparatus being used.
13. G-50 Sephadex column (Roche).
14. Cuvettes.

2.11.2 Biotinylation of Antibody

1. Capture antibody in amine-free buffer, pH 7.2–8.0.
2. Biotinamido-caproate-*N*-hydroxysuccinimide ester (BNHS) 1 mg/mL: Dissolve 1 mg of BNHS in 1 mL dimethyl sulfoxide (DMSO) immediately before use.
3. 1 M $NaHCO_3$ pH 8.5 (adjust the pH if necessary, using NaOH or H_2SO_4).
4. PD-10 gel filtration column (Sephadex G-25M, Cytiva Healthcare) *or* dialysis tubing (Merck).
5. PBS pH 7.4, adjust pH to 7.4 by adding dilute HCl or NaOH if necessary.

2.11.3 Preparation of DNA-Antibody Conjugate

1. Detection antibody in PBS, 1 mM EDTA, pH 8.0, adjust the pH of buffer by adding dilute HCl/ NaOH if necessary.
2. 2-iminothiolane (Traut's reagent).
3. 1 M glycine-NaOH pH 7.3.
4. 5′ amino-modified DNA in PBS, 1 mM EDTA, pH 7.2, adjust the pH of the buffer by adding dilute HCl or NaOH if necessary.
5. Sulfo-succinimidyl4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC).
6. Zeba™ 2 mL desalt spin column (Thermo Fisher Scientific).
7. Ion exchange column (1 mL, Biosciences).
8. 10 mM Tris–HCl pH 8.0.
9. 1.5 M NaCl.
10. Centricon® YM-100 (Millipore).

2.11.4 qIPCR Assay (Common Components)

1. Wash buffer: 0.154 M NaCl, 5 mM Tris–HCl pH 7.75, 0.02% (w/v) sodium azide (NaN_3) (*see* **Note 16**).
2. Blocking buffer: PBS, 0.1–1% (w/v) BSA (or other suitable blocking agent, *see* **Note 17**) and 0.05% (v/v) Tween 20.
3. Antigen/ unknown samples: dilute the antigen standard, preparing a suitable concentration range (*see* **Note 18**).
4. Ultrapure water (*see* **Note 19**).

2.11.5 Assay Format I (Additional Components)

1. Capture antibody (diluted to optimal working concentration in 0.2 M NaH_2PO_4; *see* **Note 20**).
2. Polypropylene PCR plate (*see* **Note 21**).
3. Biotinylated detection antibody (diluted to optimal working concentration in blocking buffer) (*see* Subheading 3.11.2).
4. Streptavidin: 5 nM in blocking buffer.
5. Biotinylated DNA label: 0.7 pM in blocking buffer (*see* Subheading 3.11.1).

2.11.6 Assay Format II (Additional Components)

1. Capture antibody: diluted at optimal concentration in 0.2 M NaH_2PO_4.
2. Polypropylene PCR plate (*see* **Note 21**).
3. DNA-conjugated detection antibody (*see* Subheading 3.11.3) (diluted to optimal working concentration).

2.11.7 Assay Format III (Additional Components)

1. Streptavidin-coated microtiter plates (Roche).
2. DNA-conjugated detection antibody (*see* Subheading 3.11.3) (diluted to optimal working concentration *see* **Note 20**).
3. Biotinylated capture antibody (diluted to optimal working concentration) (*see* Subheading 3.11.2).

2.11.8 Assay Format IV (Additional Components)

1. Polypropylene PCR plate (*see* **Note 21**).
2. DNA-conjugated detection antibody (*see* Subheading 3.11.3) (diluted at optimal concentration).
3. Antigen-coating buffer: 0.1 M Na_2CO_3/$NaHCO_3$ pH 9.6 (adjust the pH if necessary using HCl).

2.11.9 Real-Time PCR (qPCR) Assay

Real-Time PCR mixture (assemble on ice):

1. 2 μL 10× PCR buffer (4 mM $MgCl_2$) (Promega).
2. 200 μM dNTPs (dUTP, dGTP, dCTP, dATP) (Invitrogen).
3. Hot start *Taq* polymerase (Promega).
4. 200–400 nM primers [specific for DNA label (*see* Subheading 3.11.1)] (Eurofins MWG operon).
5. 0.5 μL 50× SYBR green solution (vortex for 30 s before use) (Bioline).
6. Uracil-N-glycosylase (UNG) (Bioline) (*see* **Note 22**).
7. Bring to 20 μL with molecular grade H_2O.
8. Filter/barrier tips.

3 Methods

3.1 Protein Determination by UV Light Absorption

Simple and rapid estimations of protein concentration can be made by monitoring the absorbance of ultraviolet light. Absorbance of near-UV light at 280 nm depends largely on the presence of aromatic amino acids, in particular tryptophan and tyrosine, and to a much more minor extent on phenylalanine and disulfide bonds. Absorption is affected by pH and ionic strength. In addition, strong interference from nucleic acids is a particular problem at this wavelength. As a method for determining protein concentration, UV absorption gives no more than a quick and rough estimate unless the protein preparation is pure, and its molar extinction coefficient is known. The sample is recoverable however, and the user should proceed to one of the other assays below when a more accurate determination of protein concentration is required.

1. Switch on the UV spectrophotometer and set the wavelength to 280 nm. Leave the instrument to stabilize for 15–20 min.
2. Calibrate the instrument to zero absorbance using a water blank. Ensure to use suitable cuvettes (i.e., quartz or other cuvettes known to be transparent at the given wavelength). The cuvette should be filled with a sufficient volume to cover the aperture through which the light beam passes (do not allow any bubbles to inhibit the path of the light).
3. Measure the A_{280} of the buffer used to prepare the sample to correct for background absorbance.

Table 2
Preparation of protein standards for standard curve generation

Final concentration (e.g., BSA μg/mL)	Volume of stock μL (BSA 0.1 mg/mL)	Volume of buffer (μL)
0	0	100
10	10	90
20	20	80
30	30	70
40	40	60
50	50	50

4. Measure the absorbance of the protein sample by replacing the buffer blank with a cuvette containing the protein sample. This step can be repeated in order to obtain duplicate readings.
5. If the A_{280} exceeds 2, dilute the sample using buffer and read the absorbance again (*see* **Note 23**).

3.2 Preparation of a Standard Curve

1. Using the stock solution of a protein standard (e.g., BSA 0.1 mg/mL, Subheading 2.2), set up dilutions for the preparation of a standard curve (*see* Table 2). The concentration of protein in the samples must fall within the linear range of the standard curve.
2. Pipette duplicate aliquots of protein standards into microfuge tubes and dilute appropriately (*see* **Note 24**) with a suitable buffer, for example, 0.15 M NaCl.
3. Mix well by inversion or using a vortex.
4. Carry out the assay as per Subheading 3.1.
5. Prepare a standard curve by plotting absorbance versus protein concentration (*see* **Note 25**).
6. Use the equation of the line to calculate the protein concentrations of the unknown samples (*see* **Note 26**).

3.3 UV Protein Absorption Assay Using Microvolume Spectroscopy

Microvolume spectrophotometers measure protein sample volumes in the range of 0.5–2 μL. These instruments allow fast, precise quantification of protein, while also preserving precious sample. There are numerous microvolume spectrophotometers on the market—all with similar modus operandi. Generally, microvolume spectrophotometers employ fiber-optic technology and use the inherent surface tension of the liquids being analyzed to create a column between the ends of the optical fibers (the sensors). In this way, the measurement optical path is formed. Very small protein sample volumes can therefore be rapidly analyzed

spectrophotometrically without the need for cuvettes or capillaries. The procedure for use with the Nanodrop® One instrument is outlined below.

1. Raise the sampling arm and clean the upper and lower sensor surfaces with a lab-wipe. Pipette 3–5 μL of dH_2O water onto the lower pedestal. Lower the arm and wait for 2–3 min, before cleaning with a new lab-wipe (cleaning in this manner is sufficient to prevent any sample carryover) (*see* **Note 27**).
2. Open the NanoDrop® software program and select the "Proteins" tab and choose "Protein A280" (*see* **Note 28**). Select the appropriate sample type and baseline correction to correct for any offset.
3. Pipette 2 μL (*see* **Note 29**) of the buffer (for blanking) onto the sample surface. Lower the arm.
4. Follow the onscreen prompts to blank the instrument (*see* **Note 30**).
5. Clean the upper and lower pedestal surfaces and pipette 2 μL of the sample (*see* **Note 31**) onto the sensor and lower the arm.
6. Click "measure." Once complete the spectrum and reported values are displayed onscreen, record the concentration of the sample.
7. In order to analyze multiple samples, clean the sensor between measurements. (Recalibration or re-blanking is not necessary.)
8. Once finished, select "End Experiment," clean the sensors and switch off the instrument.

3.4 The Bradford Protein Assay

The Bradford Assay is based on the formation of a complex between the dye, Coomassie brilliant blue G-250, and the proteins in a solution. The absorption maximum of the dye shifts from 465 to 595 nm when it complexes with protein, and the amount of absorption observed is proportional to the quantity of protein present. This is a simple, rapid, inexpensive assay, and unlike other protein assay procedures such as Lowry and BCA, the Bradford assay is compatible with reducing agents that are often used for the purposes of stabilizing proteins in solution. The Bradford Assay is not suitable however, if even low concentrations of detergents are present in the sample, and in that case, the BCA protein determination procedure is recommended. Although several modified protocols exist [17, 18], the original method as described by Bradford is still the most widely used formulation [4]. The standard protocol can be performed in three different formats: a 5 mL and a 1 mL assay using cuvettes and a 250 μL microplate assay (*see* **Notes 12** and **32**). The 5 mL assay is laid out here. The linear concentration range of the Bradford assay when using BSA as standard is 0.1–1.5 mg/mL of protein.

1. Prepare the protein standards in the range 20–100 μg (*see* **Note 32**) diluted in the same buffer as the unknown (e.g., H_2O/0.15 M NaCl) in a final volume of 100 μL as per Subheading 3.2.
2. Prepare two blanks by pipetting 100 μL of buffer into two microfuge tubes.
3. Pipette 20–100 μg of the protein sample of unknown concentration into microfuge tubes in a total volume of 100 μL (*see* **Note 33**) (an equal volume of 1 M NaOH may be added to samples and standards to prevent precipitation upon the addition of Bradford reagent).
4. Add 5 mL Bradford reagent and mix well by inversion or using a vortex. Allow the samples to stand for 2–60 min at room temperature (avoid foaming as this will lead to poor reproducibility).
5. Meanwhile, switch on the UV spectrophotometer and set the wavelength at 595 nm. Leave the instrument to stabilize for 15–20 min.
6. Calibrate the instrument to zero absorbance using air as a blank.
7. Transfer samples and standards to cuvettes (*see* **Note 34**) and determine the absorption at 595 nm.
8. Make a standard curve by plotting absorbance at 595 nm versus protein concentration. Use the standard curve to determine the concentration of protein in the unknown sample (*see* **Notes 26** and **35**).

3.5 The Lowry Protein Assay

The Lowry assay involves two reactions, the first resulting in the formation of a copper ion complex with amide bonds, which forms reduced copper in alkaline solution. The second reaction is the reduction of the Folin-Ciocalteu reagent, mainly by the reduced copper-amide bond complex and also by tyrosine and tryptophan residues. The reduced reagent is blue and is thus detectable using a spectrophotometer, detectable in the region of 650–750 nm. The assay has a dynamic range of 1–100 μg of protein and exhibits less variability than the Bradford assay. However, a major limitation of the Lowry assay is the fact that it is sensitive to a considerable range of agents that are frequently found in many buffers commonly used during cell lysis. In addition, this assay is strongly biased to those proteins that are rich in tyrosine and tryptophan. There are several commercial suppliers of the modified Lowry assay (Roche, Pierce, Bio-Rad, Merck and Thermo Fisher Scientific), which may be employed here. However, different formulations often do not give equivalent results even when using the same standards, dilution buffers, and in the presence of the same interfering substances. For this reason, it is important to be consistent with the choice of commercial assay [7].

1. Set the spectrophotometer to 660 nm and leave to stabilize for 15–20 min (*see* **Note 36**).
2. Prepare the protein standards (*see* **Note 37**) in the range 0–100 μg (as per Subheading 3.2) in a total volume of 1 mL.
3. Pipette 200 μL of each standard and the samples of unknown protein concentration into microfuge tubes. Prepare two blank tubes using 200 μL water/buffer.
4. To 200 μL of sample, blank or standard, add 1 mL of freshly prepared Lowry working solution. Let the solution stand at room temperature for 10–30 min (*see* **Note 38**).
5. Add 100 μL of diluted Folin-Ciocalteu reagent, vortex immediately (essential for obtaining reproducible results), and stand for 30–60 min (do not exceed 60 min) at room temperature in the dark (complete mixing of the reagent must be accomplished quickly to avoid decomposition of the reagent before it reacts with protein) (*see* **Note 39**).
6. Transfer the samples to cuvettes.
7. Zero the spectrophotometer using the blank sample. Measure the absorbance of all the samples in turn.
8. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations (*see* **Notes 26** and **40**).

3.6 The Bicinchoninic Acid (BCA) Assay

The principle of the bicinchoninic acid (BCA) assay is similar to that of the Lowry procedure, in that both rely on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. Here, BCA reagent replaces the Folin-Ciocalteu reagent, and the amount of reduction is proportional to the protein present. BCA forms a blue-purple complex with Cu^{1+} in alkaline solutions, the appearance of which can be monitored by absorbance at 562 nm. Unlike the Lowry assay, BCA does not interact with detergents and is less susceptible to interference from other compounds that may be found in buffers used for cell lysis and protein preparation. Some reducing or chelating agents such as DTT and EGTA are best avoided however as they interfere by either reducing or sequestering Cu^{2+}.

3.6.1 Macro-BCA Assay

1. Set the spectrophotometer to read A_{562} and leave to stabilize for 15–20 min.
2. Prepare the protein standards in the range 0–100 μg diluted in dH_2O (as per Subheading 3.2) in a final volume of 100 μL. Prepare a blank tube using 100 μL water/buffer.
3. Pipette 100 μL of sample/blank/standard into test tubes, add 2 mL of the working solution and mix thoroughly.
4. Incubate for 15 min at 60 °C (*see* **Note 41**).

5. Cool the samples to room temperature (*see* **Note 42**) (the color is stable up to 1 h following incubation at 60 °C).
6. Mix the samples using a vortex and transfer to cuvettes.
7. Zero the spectrophotometer, blank and measure the absorbance of every sample in turn.
8. Plot a standard curve of absorbance as a function of protein concentration and use it to determine the protein concentrations in the samples (*see* **Note 26**).

3.6.2 Micro-BCA Assay

1. Set up microfuge tubes containing samples and known amounts of standard protein in the range of 0.5–20 μg with a final sample volume of 500 μL. Prepare two blank tubes using 500 μL water/buffer.
2. Add 500 μL of micro-BCA assay solution to each tube, vortex, and incubate the sample for 15 min at 60 °C (*see* **Notes 41** and **43**).
3. Cool samples to room temperature (*see* **Note 42**).
4. Vortex the samples and read the absorbance at 562 nm.
5. Plot a standard curve of absorbance as a function of protein concentration and use it to determine the protein concentrations in the samples (*see* **Note 26**).

3.7 Immunoassay: Indirect ELISA

ELISAs are common antibody-based tests that are used to detect antigens or other antibodies in a sample. It is imperative that a robust antibody directed against the protein of interest (i.e., the antigen) be available to the investigator for the purpose of target protein quantitation. There are many direct and indirect formats available, and the specific detection antibody can be covalently linked to a reporter enzyme or can alternatively be itself detected using an enzyme-labeled secondary antibody. Many ELISAs utilize chromogenic substrates, though newer assays employ fluorogenic markers enabling much higher sensitivities to be achieved. The amount of target protein in a sample is inferred from the level of activity/signal from the reporter. Traditional ELISA can be performed in four different formats: direct, indirect, sandwich, and competitive [19–22]. The detection limit of an ELISA can vary in a wide range from 0.01 pg/mL to 100 ng/mL [23], and thus, the ability of the assay to detect very low quantities of protein can be limited [24]. Many attempts have been made to develop methods to improve ELISA sensitivity, and these have met with some success. In particular, gold nanoparticle-enhanced ELISA was shown to increase the sensitivity of the traditional ELISA, shorten the reaction time, and in some instances also improve the limit of detection [25–27]. In addition, a combined sandwich ELISA and

enzyme cycling has also been described [24, 28] with a reported limit of detection of just 8.0×10^{-19} mol/assay. Thus, if ultrasensitive detection is a necessary assay requirement, then these additions to the basic ELISA format could be considered.

Here, the format typical of an indirect ELISA is outlined.

3.7.1 Antigen Coating to Microtiter Plates

1. Dilute the antigen standard and the unknown sample in the antigen-coating buffer to set up a concentration range of 0.1–10 μg/mL (*see* **Note 44**).
2. Add 50 μL of these to separate wells of the microtiter plate. Set up control wells containing no antigen and standard wells containing serially diluted antigen to provide data to construct a calibration curve. It is advisable to prepare triplicate wells in all cases.
3. Incubate the plates at room temperature for 5–6 h on a microtiter plate shaker, or alternatively overnight at 4 °C.

3.7.2 Blocking

1. Empty the wells and wash three times with assay buffer followed by three further washes with PBS (*see* **Note 45** for washing procedure). Ensure the wells are washed sufficiently by completely filling them with wash buffer. It is important not to let the wells dry out or enzyme activity will be lost.
2. Add 200 μL of blocking buffer and incubate overnight (or alternatively for 1–2 h at 37 °C).
3. Thoroughly wash the plate again three times with the assay buffer followed by three washes with PBS as before (*see* **Note 45**).

3.7.3 Primary Antibody Binding Step

1. Add 100 μL of primary antibody (at the experimentally determined optimal working concentration) to each well and incubate for 5–6 h at room temperature (*see* **Note 46**).
2. Wash the plates as before (*see* **Note 45**).

3.7.4 Secondary Antibody Binding Step

1. Add 100 μL of the diluted secondary antibody (at the experimentally determined optimal working concentration) to each well and incubate at room temperature for 1 h.
2. Thoroughly wash the plates with assay buffer, as before (*see* **Note 45**).

3.7.5 Detection Step

1. Detect bound antibody by the addition of a suitable substrate solution to the wells (*see* **Note 47**).
2. As soon as the color develops add stop solution.
3. Read the plates immediately at A_{450} nm on a microtiter plate reader (*see* **Note 48**).
4. Plot a standard curve of absorbance versus protein concentration and use the curve to estimate the protein concentration in the unknown sample (*see* **Notes 26** and **49**).

3.8 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins

SDS-PAGE is a widely used method for fractionating proteins based on their mobility in an electric field. Binding of the detergent SDS confers different proteins with similar charge per unit mass ratios, and separation by PAGE is therefore based on protein size. Many variations of this protocol have been described in the literature, which become useful when the limits of the classical protocols are reached [29] (*see* **Note 50**). Instructions for use with an ATTO protein gel electrophoresis system are laid out here and can easily be adapted to other equivalent systems. The method can be simplified greatly by using commercially available precast gels.

1. Wash glass plates with detergent, rinse first with tap water, then with dH_2O and finally wipe in one direction with tissue soaked in 100% ethanol.
2. Place the gasket around the ridged plate, assemble the plates, and secure with clamps.
3. Prepare a 10% resolving gel by mixing 3.3 mL acrylagel, 1.35 mL bis-acrylagel, 2.5 mL 1.5 M Tris–HCl (pH 8.8), 2.61 mL dH_2O, 0.10 mL 10% (w/v) SDS, 0.10 mL 10% (w/v) APS (freshly prepared), and 0.01 mL TEMED (*see* **Note 51**).
4. Pour the gel to within 2 cm of the top of the larger plate (allowing space for the stacking gel) and overlay with 100% ethanol immediately. The resolving gel should be fully polymerized within 30 min.
5. Prepare the 5% stacking gel by mixing 0.42 mL of acrylagel with 0.168 mL bis-Acrylagel, 0.312 mL 1 M Tris–HCl (pH 6.8), 1.55 mL dH_2O, 0.025 mL 10% (w/v) SDS, 0.025 mL 10% (w/v) APS, and 0.0025 mL TEMED (*see* **Note 52**).
6. Remove the ethanol, pour the stacking gel, and immediately insert a clean comb (wiped with 100% ethanol). Allow the gel to polymerize for at least 20 min.
7. Fill the electrophoresis tank with 1× Tris-glycine running buffer to a level of about 5 cm deep.
8. After polymerization, remove the clamps and gaskets and lower the pre-poured gels into the buffer (place the gels into the buffer at an angle to exclude air bubbles from the gel interface) notched plate facing inward.
9. Fix the gel plates firmly in place using the pressure plates. Completely fill the tank (including the chamber formed by the inner plates) with 1× running buffer and carefully remove the comb from the gel (*see* **Note 53**).
10. Load the sample into the wells. Include at least one well with a prestained molecular weight marker.

11. Complete the assembly of the gel unit and connect to a power supply.
12. Electrophorese at a constant current of 30 mA per gel until the blue dye front has reached the bottom of the gel.
13. When complete, remove the plates, separate and process the gel as required (*see* Subheadings 3.9/3.10).

3.9 Staining of SDS-PAGE Gels

While a wide range of commercially available preparations for colorimetric and fluorescent staining of SDS-PAGE gels are available [30], visible stains such as Coomassie Blue and Silver discussed here remain the most widely used for routine visualization [31, 32] since they do not require costly reagents or expensive equipment for detection and quantification. A crystal violet staining protocol has also recently been reported. This novel method does not require a destaining step and detects 2–16 ng of protein with visible protein bands after just 5 mins [31].

3.9.1 Coomassie Brilliant Blue Staining of SDS-PAGE Gels

Coomassie Brilliant Blue remains the most frequently used stain for total protein detection [30]. The dye Coomassie Brilliant Blue G-250 binds nonspecifically to virtually all proteins and is commonly used as a convenient stain for proteins following fractionation by PAGE. Gels are first soaked in a solution of the dye, and any dye that is not bound to protein diffuses out of the gel during the destain steps. This stain binds to proteins more or less stoichiometrically, and so, this method can be used when relative amounts of protein need to be established by densitometry.

1. After electrophoresis, disconnect the gel unit from the power supply and disassemble the apparatus. Carefully separate the plates and remove and discard the stacking gel. Cut one corner from the resolving gel to allow the orientation of the gel to be followed.
2. Immerse the gel in Coomassie blue solution for 30 min with constant gentle agitation.
3. After staining, immerse the gel in destain with constant agitation. Refresh the destain four or five times at 1 h intervals until all of the background staining has been removed from the gel (*see* **Note 54**).
4. An image of the gel in black and white can be captured using a UV trans-illuminator (switched to white light only and using a white tray), or alternatively the gel can be imaged or scanned onto a computer (*see* **Note 55**).

3.9.2 Silver Staining of SDS-PAGE Gels

Silver staining is a highly sensitive method for visualizing proteins following electrophoresis and continues to be a popular alternative colorimetric method. Detection of 0.3–10 ng of protein is possible, making it up to 100 times more sensitive than Coomassie Blue staining. Although sensitive, not all proteins stain equally using this

method, and the linear dynamic range is rather limited. Silver staining is therefore not favored when quantitative protein expression analysis is required. Commercially available silver staining kits that offer improved compatibility with subsequent mass spectrometric work include SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific), Pierce™ Silver Stain for Mass Spectrometry, ProteoSilver™ Plus Silver Stain Kit (Merck), and PlusOne™ Silver Staining Kit for protein (Cytiva).

1. Following electrophoresis (*see* **Note 56**), disconnect the gel unit from the power supply and disassemble the apparatus. Carefully separate the plates and remove and discard the stacking gel. Cut one corner from the resolving gel to allow the orientation of the gel to be followed.
2. Place the gel in a clean container in fixing solution for 1–2 h with gentle agitation at room temperature (for gels previously stained with Coomassie blue, wash overnight with dH_2O and start from point 3).
3. Discard the fixing solution and wash the gel three times for 20 min each in wash solution.
4. Incubate the gel in oxidizing solution for 10 min.
5. Remove the oxidizing solution and wash the gel for 10 min in copious amounts of water. Repeat the washing procedure until the pale-yellow color has completely faded.
6. Soak the gel in silver nitrate solution for 30 min to stain.
7. Following staining, wash the gel twice for 2 min each using plenty of water.
8. Place the gel in developing solution for 1 min. Replenish the developing solution and incubate for a further 5 min. Repeat this process until bands are stained satisfactorily.
9. Immerse the gel in stop solution for 5 min.
10. Store the gel at 4 °C in 1% (v/v) acetic acid. *See* **Note 55** for analysis.

3.10 Western Blotting

Western blotting (also known as immunoblotting) is a method used to detect specific proteins, for which an antibody is available, in a cell culture or tissue extract. Total cellular proteins are first fractionated by gel electrophoresis under denaturing or non-denaturing/native conditions. The proteins are then transferred to a nitrocellulose or nylon membrane onto which they are then immobilized (the blot). Primary antibodies, (either monoclonal or polyclonal) that are specific to the protein under investigation are then used to "probe" the blot. When bound to the blot, these antibodies are then in turn located using a secondary antibody that is labeled with a reporter enzyme such as a peroxidase (POD) or alkaline phosphatase. However, conjugated primary antibodies are also available,

negating the need for the secondary antibody incubation step. Reporter enzyme activity is then detected by incubation of the blot with an appropriate substrate, yielding a detectable product that maps to the location of the protein of interest. The use of fluorescently labeled secondary antibodies has extended the dynamic range of quantitation [33] and has become a popular alternative to reporter enzymes. Specific proteins can thus be detected, even when present at very low levels in cell extracts. The outcome of a Western blot experiment depends heavily on the quality of the primary antibody available for the protein under study, and there are now many companies that offer specific antibodies against a vast array of protein targets, enzyme-conjugated secondary antibodies, and detection kits. The user should adhere to any recommendations made by the manufacturers of the apparatus, antibodies, transfer membrane, and immunodetection kits being employed. One should also survey any published literature on the protein under study and take note of the reagents and immunoblotting conditions that are most frequently described. The directions given here are generic and assume the use of a Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell.

3.10.1 Electrophoretic Transfer of Proteins from PAGE Gels to Nitrocellulose Filters

1. Carry out SDS-PAGE as previously described (*see* Subheading 3.8). The use of commercially available prestained protein size markers is recommended.
2. Disconnect the gel unit from the power supply and disassemble the apparatus. Carefully separate the plates and remove and discard the stacking gel. Cut one corner from the resolving gel to allow the orientation of the gel to be followed.
3. Soak the gel in transfer buffer for 15 min to equilibrate the gel removing salts and detergents.
4. Cut the transfer membrane (e.g., nitrocellulose) to the same dimensions as the gel, along with six pieces of 3MM filter paper required to complete the gel/membrane sandwich.
5. Place a pre-soaked sheet of filter paper onto the platinum anode. Roll a pipette over the surface of the filter paper to exclude all air bubbles. Repeat this step with two more sheets of filter paper.
6. Place pre-wetted blotting membrane on top of the filter paper and exclude all air bubbles as before (not all types of membrane require pre-wetting).
7. Place the equilibrated gel carefully on top of the nitrocellulose membrane, align the gel on the center of the membrane, and ensure all the air bubbles removed.
8. Place another three sheets of pre-wetted filter paper on top of the gel, again remove air bubbles (*see* **Note 57**).

9. Put the cathode on top of the stack and place the safety cover on the transfer unit. Transfer the gel for 30 min at 17 V (transfer conditions should be experimentally determined for optimal results).
10. Once the transfer is complete, disassemble the apparatus. Leave the gel in place on top of the nitrocellulose membrane so that the membrane can be cut to the shape of the gel (including the cut corner for orientation) using a sharp blade. The gel and any excess membrane can then be discarded.
11. The colored molecular weight markers should be clearly visible on the membrane.

3.10.2 Staining of Proteins Immobilized on Nitrocellulose Filters

1. Following electrophoretic transfer, immerse the nitrocellulose membrane in 20 mL Ponceau S solution and stain for 5 min with constant agitation.
2. After proteins are visualized, wash the membrane in several changes of dH_2O until all the excess stain has been washed away. The loading of equal quantities of sample across all lanes—or otherwise as the case may be—will be apparent. The membrane can now be used for immunological probing.

3.10.3 Immunological Detection

1. Following Ponceau S staining, incubate the membrane in blocking buffer (*see* Subheading 2.10) for 3 h at room temperature with constant agitation.
2. Discard the blocking buffer and quickly rinse the membrane prior to the addition of the primary antibody (diluted appropriately in blocking buffer) at 4 °C overnight on a rocking platform.
3. Remove the primary antibody (*see* **Note 58**) and wash the membrane three times for 15 min each with 50 mL TBS-T [if using conjugated primary antibodies then proceed to develop the blot (i.e., proceed to **step 6**)].
4. Add the secondary antibody (diluted in blocking buffer) to the membrane and incubate for 90 min at room temperature with constant agitation.
5. Remove the secondary antibody and wash the membrane three times for 15 min each with 50 mL TBS-T.
6. During the final wash, bring 2 mL aliquots of the appropriate substrate (e.g., BCIP/NBT, or TMB) to room temperature.
7. Place the membrane in a clean container and cover with the substrate (if using a chemiluminescent or fluorescent assay then follow the manufacturer's instructions accordingly).

8. When sufficient chromogenic development has occurred (usually within 10 min, but in some cases, it may take several hours) revealing bands of satisfactory intensity, rinse the membrane in dH_2O to stop the reaction.
9. Photograph or scan the blot.

3.11 qIPCR Assay

Nucleic acid amplification methods can be used for signal generation in antibody-based immunoassays [34]. Immuno-PCR (IPCR) is based on the use of specific antibodies that have been conjugated to a nucleic acid molecule. This DNA fragment is then targeted for amplification by PCR. Quantitative IPCR (qIPCR) is an evolution of IPCR and specifically employs real-time PCR, the method of choice for quantitative determinations of low levels of DNA. The benefits that are brought to bear include dramatically improved limits of detection (by a factor of up to 10,000), the capacity to work with small sample volumes, and assay formats that are compatible with complex biological matrices [35]. It has been suggested that qIPCR has the potential to become the most sensitive, specific, and robust method for protein detection [36].

Various synthetic DNA sequences and the corresponding PCR primers may be used as a target region [37]. Several possible strategies are available for linking antibody recognition with nucleic acid amplification. These strategies have evolved to combine the advantages of several of the latest technologies with those of qIPCR. The options here are numerous and include phage display (where the phage particle supplies both the detection antibody and the DNA, known as PD-IPCR) [38, 39], the use of asymmetric PCR (for higher sensitivity of detection) [40], the incorporation of gold nanoparticles (to allow easier coupling between antibodies and DNA) [41], the addition of magnetic beads (allowing more thorough wash steps leading to less nonspecific binding) [42], the use of liposomes allowing encapsulation of the DNA reporters [43], using modified double-stranded DNA molecules (for increased assay sensitivity) [44], or the use of the Tus–Ter-lock system (which allows site-specific covalent attachment of DNA to proteins using a Tus–Ter protein-oligonucleotide complex) [45]. The pairing of a qIPCR assay with one of these technologies has resulted in an "ultrasensitive" and powerful technique with a detection limit—demonstrated in at least two separate studies—to be as low as 0.01 fg/mL [46, 47]. As is the case for ELISA, there are several formats used for qIPCR. Various assay formats demonstrate varying degrees of sensitivity and reproducibility [48], and so, the format chosen for assay design will depend on the specific requirements of the assay and should be given careful consideration. Here, we describe four basic formats for the assembly of a qIPCR detection system including the so-called "universal system of qIPCR," which employs streptavidin–biotin systems and has become one of the main formats in immuno-PCR [50]. The

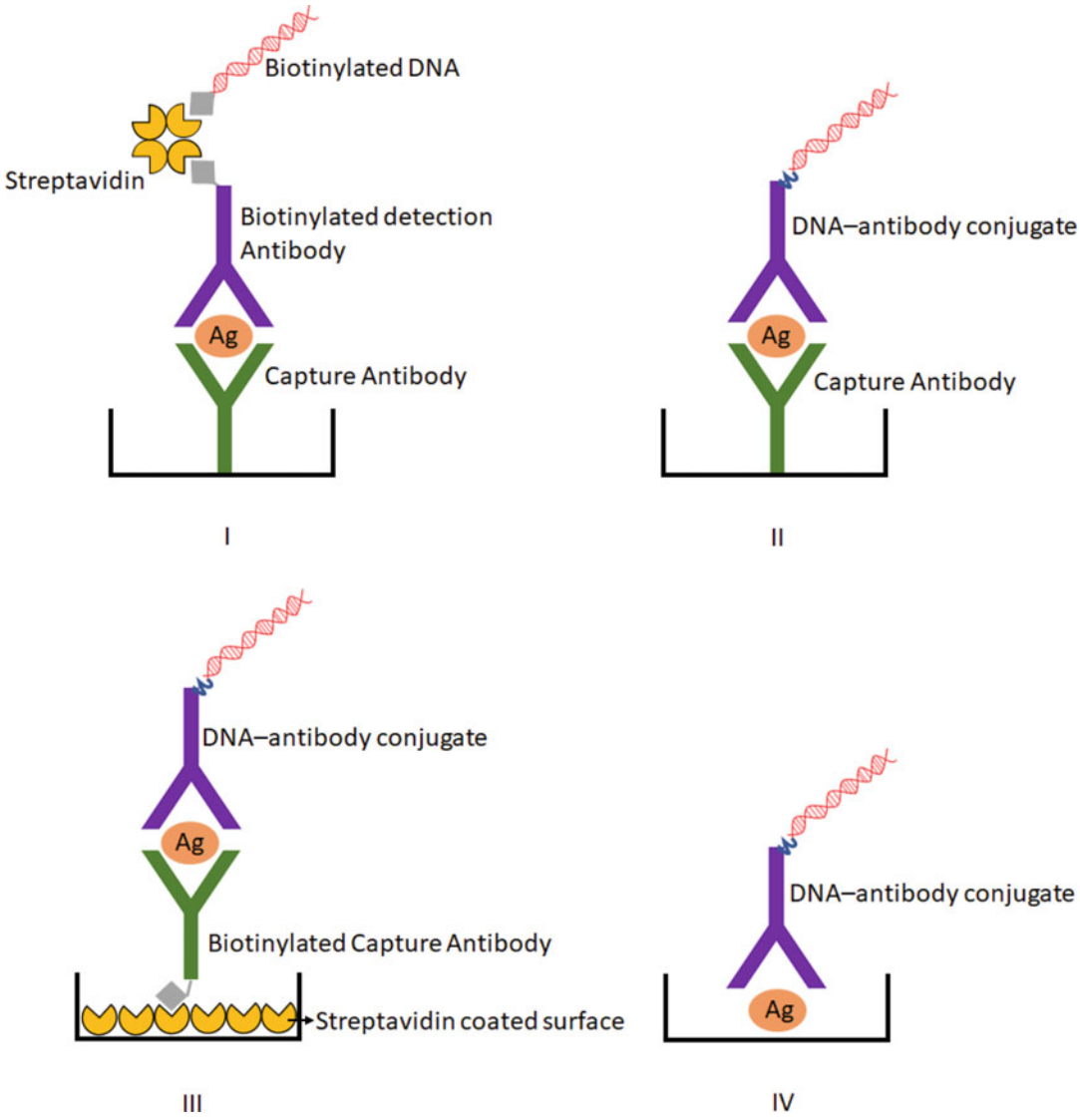

Fig. 1 Schematic diagram of the various Immuno-qPCR assay formats presented here. (I) The capture antibody is first adsorbed to the PCR tube surface, and streptavidin is used to couple biotinylated DNA to biotinylated detection antibody. (II) The capture antibody is first adsorbed to the PCR tube surface, and a DNA-antibody conjugate is mixed with the antigen or sample before addition to the well. (III) Streptavidin-coated microtiter plates are used, and biotinylated capture antibody, DNA-antibody conjugate, and antigen or sample are pre-mixed before addition to the well. (IV) Antigen and samples are adsorbed to the surface of the well prior to the addition of DNA-antibody conjugate

remaining protocols presented make use of a chemically conjugated antibody/DNA label complex (*see* Fig. 1). To further increase the sensitivity of the assay a combination of the qIPCR assay with the technologies mentioned above should be considered.

The assay formats presented here are as follows: *Assay Format (I)*: The capture antibody is first adsorbed to the PCR tube surface, and streptavidin is used to couple biotinylated DNA to biotinylated detection antibody. *Assay Format (II)*: The capture antibody is first adsorbed to the PCR tube surface, and a DNA-antibody conjugate is mixed with the antigen or sample before addition to the well. *Assay Format (III)*: Streptavidin-coated microtiter plates are used, and biotinylated capture antibody, DNA-antibody conjugate, and antigen or sample are pre-mixed before addition to the well. *Assay Format (IV)*: Antigen and samples are adsorbed to the surface of the well prior to the addition of DNA-antibody conjugate.

All methods require some prior knowledge of real-time PCR and for the appropriate instrumentation and software to be present in the laboratory [The procedure described below employs the ABI 7500 and the corresponding 7500 software (Applied Biosystems)].

3.11.1 Preparation of Biotinylated DNA Label

1. Use a 5′-biotinylated forward primer and an unmodified reverse primer when using PCR to prepare biotinylated DNA.
2. Assemble the PCR component mixture in a total volume of 50 µL (*see* **Note 59**).
3. Perform PCR using the following temperature and time profile: hold at 95 °C for 5 min (1 cycle); denature at 95 °C for 30 s, anneal at X °C for 30 s (X is the annealing temperature specific to the primer set), extend at 72 °C for 40 s, (40 cycles); then perform a final extension step at 72 °C for 5 min.
4. Analyze the PCR product obtained by standard agarose gel electrophoresis in conjunction with appropriate DNA size markers.
5. Locate, excise, and purify the PCR product using a PCR product Clean-Up kit according to the manufacturer's instructions.
6. Concentrate the purified biotinylated DNA label using a G-50 Sephadex Column according to the manufacturer's instructions.
7. Determine the concentration of the DNA label using UV-visible spectrophotometry.
8. Aliquot appropriately and store at −20 °C. Biotinylated DNA labels are stable at −20 °C for up to 1 year.

3.11.2 Biotinylation of Antibody

1. Pipette 2.5 mg/mL of antibody into a microfuge tube.
2. Add BNHS (1 mg/mL in DMSO) in five to ten times molar excess and mix gently but thoroughly.
3. Add one tenth volume of 1 M $NaHCO_3$, pH 8.5 to the antibody solution.
4. Incubate at room temperature for 1–4 h.

5. Purify the biotinylated antibody either by dialysis (for 24 h with three changes of PBS pH 7.4) or using a PD-10 gel filtration column according to the manufacturer's instructions.
6. Store aliquots of biotinylated antibody at −20 °C (or under conditions consistent with the stability properties of the antibody).

3.11.3 Preparation of DNA-Antibody Conjugate

1. Activate the antibody with thiol groups by mixing ~5 mg of antibody (in PBS, 1 mM EDTA, pH 8.0) with 2-iminothiolane Traut's reagent in tenfold molar excess in a total volume of 1 mL.
2. Incubate the reaction for 1 h at room temperature.
3. Terminate the reaction with 30 μL of 1 M glycine-NaOH pH 7.3.
4. Meanwhile, mix 40–50 nanomoles of amino-modified DNA (in PBS, 1 mM EDTA, pH 7.2) with 2 mM of sulfo-SMCC in a total volume of 500 μL.
5. Incubate for 30 min at room temperature.
6. Terminate the reaction with 13 μL of 1 M glycine-NaOH pH 7.3.
7. Purify the activated antibody and the activated DNA label using a 2 mL Zeba desalt spin column according to the manufacturer's instructions.
8. Mix together and incubate on a shaker for 3 h at room temperature.
9. Incubate overnight at 4 °C on a shaker.
10. Purify the conjugate using an ion exchange column (Resource Q 1 mL), according to the manufacturer's instructions, using 10 mM Tris–HCl pH 8.0 and elute with a 0–1.5 M NaCl gradient.
11. Pool fractions that contain the antibody/DNA conjugate and concentrate to 0.3 mL using a Centricon YM-100.
12. Test the concentrated fraction by ELISA and qPCR (*see* Subheading 3.7 and 3.11.9).

3.11.4 qIPCR Assay Setup

General comments: (i) In all assay formats, set up a no-antigen control (to control for unspecific binding of antibody and DNA to the surface), a negative sample control (to determine that the sample matrix does not give rise to a signal) and a no antibody control (to check for any unspecific binding between antibodies). (ii) Prepare a standard curve by making a dilution series of antigen (0.1–10 μg/mL) including 6–7 concentrations. Vortex and spin between every dilution. (iii) Set up two or three replicates of all standards and unknowns that are to be assayed.

3.11.5 Assay Format I

1. Add 25 μL of diluted capture antibody to a microtiter plate and incubate overnight on microtiter plate shaker at 4 °C.
2. Wash the wells three times with wash buffer (*see* **Note 45**). Ensure the wells are washed sufficiently by filling the wells with wash buffer.
3. Add 200 μL of blocking buffer and incubate for 1 h at 37 °C.
4. Wash the wells three times with wash buffer (*see* **Note 45**).
5. Set up dilutions of the antigen standard and the unknown sample across a concentration range of 0.1–10 μg/mL in the antigen-coating buffer.
6. Add 25 μL of antigen or unknown sample to each well as appropriate and incubate at room temperature for 1 h.
7. Wash the wells three times with wash buffer (*see* **Note 45**).
8. Add 25 μL diluted biotinylated detection antibody and incubate for 1 h at room temperature.
9. Wash the wells six times with wash buffer.
10. Incubate with 25 μL streptavidin (5 nM) for 30 min at room temperature.
11. Add 25 μL of the diluted biotinylated DNA label and incubate at room temperature for 1 h.
12. Wash the wells six times with wash buffer (*see* **Note 45**).
13. Wash six times with ultrapure water.
14. Quantify using qPCR (*see* Subheading 3.11.9).

3.11.6 Assay Format II

1. Add 25 μL of the diluted capture antibody to a microtiter plate and incubate overnight on a microtiter plate shaker at 4 °C.
2. Wash the wells three times with wash buffer (*see* **Note 45**). Ensure the wells are washed sufficiently by filling the wells with wash buffer.
3. Add 200 μL of blocking buffer and incubate for 1 h at 37 °C.
4. Wash the wells three times with wash buffer (*see* **Note 45**).
5. At the same time, dilute the antigen standard and the unknown sample in a concentration range of 0.1–10 μg/mL.
6. Mix the antigen or sample together with the diluted DNA-antibody conjugate. Incubate for 1 h at 37 °C.
7. Add 25 μL of this mix to the wells and incubate for 30 min at room temperature.
8. Wash the wells six times with wash buffer (*see* **Note 45**).
9. Wash six times with ultrapure water.
10. Quantify using qPCR (*see* Subheading 3.11.9).

3.11.7 Assay Format III

1. Prepare dilutions of the antigen standard and the unknown sample in the antigen-coating buffer across a concentration range of 0.1–10 μg/mL.
2. Block the streptavidin-coated microtiter plates with 200 μL blocking buffer for 1 h at 37 °C.
3. At the same time, mix 5.5 μL antigen or sample with 11 μL of diluted DNA-antibody conjugate, and 11 μL of the diluted biotinylated capture antibody (per well). Incubate this mix at room temperature for 1 h.
4. Wash the wells three times with wash buffer (*see* **Note 45**).
5. Add 25 μL of the mix to the wells and incubate for 30 min at room temperature.
6. Wash the wells six times with wash buffer (*see* **Note 45**).
7. Wash six times with ultrapure water.
8. Quantify using qPCR (*see* Subheading 3.11.9).

3.11.8 Assay Format IV

1. Dilute the antigen standard and the unknown sample in a concentration range of 0.1–10 μg/mL in antigen-coating buffer.
2. Add 50 μL to each well.
3. Incubate the plates on microtiter plate shaker, overnight at 4 °C.
4. Wash the wells three times with wash buffer (*see* **Note 45**).
5. Add 200 μL of blocking buffer and incubate for 1 h at 37 °C.
6. Wash the wells three times with wash buffer.
7. Add 25 μl of the diluted DNA-antibody conjugate to the wells. Incubate for 1 h at 37 °C.
8. Wash the wells six times with wash buffer.
9. Wash six times with ultrapure water.
10. Quantify using qPCR (*see* Subheading 3.11.9).

3.11.9 Real-Time PCR (qPCR) Assay

1. Prepare the reagents for the PCR reaction as a master-mix (make up 10% more than the volume required so as to account for potential pipetting error).
2. Include three "no template controls" (NTC, a minus sample control) in each reaction plate. Set up a positive control sample using DNA/conjugate as template.
3. Pipette each master-mix up and down to mix and aliquot 20 μL of PCR master-mix per reaction into the individual wells of the PCR plate containing the immobilized DNA.

4. Immediately seal the plate with an optical adhesive cover using a plate sealer (*see* **Note 60**) and protect from light. Centrifuge at 500 × *g* for 2 min to eliminate air bubbles. It is important to check to ensure that no bubbles remain in any well (*see* **Note 61**).
5. Place the plate in the qPCR instrument and perform amplification and detection under the following cycling conditions: hold at 50° C for 2 min (1 cycle); hold at 95 °C for 5 min (1 cycle); denature at 95 °C for 20 s, anneal at X°C for 20 s (X is the annealing temperature specific to the primer set), extend at 72 °C for 25 s (45 cycles).
6. Record fluorescence data during the 72 °C step.
7. Include a dissociation stage to analyze PCR product melting temperature.
8. Analyze the qPCR data obtained with the appropriate software. Check for bimodal dissociation curves or an abnormal amplification plot (*see* **Note 62**) and for amplification in the NTC wells (*see* **Note 63**).

3.12 Method Validation

As can be seen above, there exists a good deal of choice when it comes to selecting an assay for quantitation of a given protein. The decision is usually based on the compatibility of the particular assay with the nature of the protein sample. When assessing compatibility, there are several important considerations: potential interfering substances that may be present in the samples that could affect certain assays, the repeatability and reproducibility of results, the linearity of response, the accuracy of the assay, and any limitations of the assay.

In this section, procedures for validation of the UV Protein Absorption assay are set out, but these can be adapted for the validation of other procedures/assays. The validation parameters used here to assess assay suitability are specificity, precision (repeatability and reproducibility), linearity, accuracy, and quantitation limit (*see* Table 3 for definitions, equations, and acceptance criteria). The main objective of the method validation procedures set out here is to demonstrate the reliability of a particular method for the determination of protein concentration.

3.12.1 Specificity Testing (Absence of Interference)

A valid protein assay is one that will measure only the protein content of the sample and avoid the influence of buffer components on the quantification results. Proof of specificity can be achieved by comparing the results obtained from a placebo (dilution buffer) and a sample solution.

1. Switch on the UV spectrophotometer, select the "Spectrum" mode and select to scan the wavelength range from 200–400 nm (if scanning is not possible on a given instrument, this procedure can be adapted to examine response at 280 nm

Table 3
Validation parameters that can be used to demonstrate the reliability of a particular method for quantitation (based on the guidelines of International Conference on Harmonization (ICH) [49]

Parameter	Definition	Equation	Acceptance criteria
Specificity	Specificity is the ability to assess unequivocally the analyte of interest in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, and matrix		For a method to be specific there should be no significant interference at the wavelength of interest from diluents, impurities, degradants, etc. Allowable interference is application dependent and could be in the region of 0.5%
Repeatability	Precision under the *same conditions* over a *short interval* of time Repeat tests with the same operator, same instrument, same batches of materials, same day, etc. Standard deviation (s) and relative standard deviation (RSD) calculations on these absorbance results will indicate the repeatability	$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$ $\text{RSD} = \frac{s}{\bar{x}} \times 100$	% RSD for method precision ($n = 6$ samples) ≤3.0% (*see* **Note 64**) Intra-assay RSD values should ideally be lower than 10%
Intermediate precision	Precision under the *different conditions* within the *same lab.* Intermediate precision expresses "within laboratories variations": different days, different analysts, different equipment, etc. Repeat tests with different operators, or batches of materials, or on different days. Standard deviation and RSD calculations on these absorbance results will indicate the intermediate precision	$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$ $\text{RSD} = \frac{s}{\bar{x}} \times 100$	% RSD (Analyst 1 and Analyst 2 ($n = 12$)) ≤3.0% (*see* **Note 64**) Inter-assay RSD is the variation of the sample measurement on different runs. Inter-assay CV values should ideally be less than 15%
Reproducibility	Precision under *different conditions not within the same lab.* Repeat tests in different	$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}}$ $\text{RSD} = \frac{s}{\bar{x}} \times 100$	

(continued)

Table 3 (continued)

Parameter	Definition	Equation	Acceptance criteria
	labs, in different countries with different equipment. Standard deviation and RSD calculations on these absorbance results will indicate the intermediate precision		
Linearity	The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample	Plot the peak response versus nominal concentration for the standard to obtain a correlation coefficient (R^2, the lowest possible value of R^2 is 0 and the highest possible value is 1), slope, and the y-intercept	Coefficient of Determination (R^2) / Regression coefficient should be ≥0.999 The y-intercept must ≤2% of the target concentration response
Accuracy	The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found	Accuracy can be measured as the ratio of the mean ($\overline{x}$) of n parameters to the true mean μ (population mean/ accepted value) Accuracy $= \frac{\overline{x}}{\mu} \times 100$ Recovery $= \frac{\text{Analytical result}}{\text{True value}} \times 100$	Accuracy for each concentration level 95–105% % RSD for each concentration level ≤3% Overall accuracy 95–105% Overall RSD ≤3% (*see* **Note 65**)
Limit of Quantitation (LOQ)	The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy	LOQ $= \frac{10 \times s}{\text{Slope}}$	The % RSD of the LOQ should be ≤15% between 6 replicates

(select "Photometric" instead of "Spectrum") for the presence of interference). Leave the instrument to stabilize for 15–20 min.

2. Calibrate the baseline absorbance using a water blank. Ensure to use suitable cuvettes (i.e., quartz or other cuvettes known to be transparent at the given wavelength). The cuvette should be filled with a sufficient volume to cover the aperture through which the light beam passes (do not allow any bubbles to inhibit the path of the light).
3. Conduct a scan on the buffer used to prepare the sample and record the spectrum.

4. Conduct a scan on the protein sample and record the spectrum.
5. Compare the spectra from **Step 3** and **Step 4** to identify if any absorbance occurs in the buffer solution, particularly in the region of 280 nm (interference).

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions and is usually expressed as the variance, standard deviation, or coefficient of determination of a series of measurements. Precision may be considered at three levels: repeatability (*see* Table 3 for definition and Subheading 3.12.2 for a procedure), intermediate precision (*see* Table 3 for definition and Subheading 3.12.3 for a procedure), and reproducibility (*see* Table 3 for definition). The coefficient of variation (CV) or the relative standard deviation (RSD) is a measure of precision from repeated measures and is expressed as a percentage (*see* Table 3 for equation). It can be used to determine how reliable assays are by determining the ratio of the standard deviation to the mean.

3.12.2 Repeatability

1. Switch on the UV spectrophotometer and set the wavelength to 280 nm. Leave the instrument to stabilize for 15–20 min.
2. Calibrate the instrument to zero absorbance using a water blank. Ensure to use suitable cuvettes (i.e., quartz or other cuvettes known to be transparent at the given wavelength). The cuvette should be filled with a sufficient volume to cover the aperture through which the light beam passes (do not allow any bubbles to inhibit the path of the light).
3. Measure the A_{280} of the buffer used to prepare the sample to correct for background absorbance.
4. Measure the absorbance of the protein sample by replacing the buffer blank with a cuvette containing the protein sample. If the A280 exceeds 2, dilute the sample using buffer and read the absorbance again (*see* **Note 23**).
5. Repeat **step 4** five times for a total of 6 replicate readings ($n = 6$).
6. Calculate the mean, standard deviation, and the relative standard deviation using the appropriate equations (*see* Table 3) and check findings against the acceptance criteria in Table 3.

3.12.3 Intermediate Precision

Intermediate precision is a measure of precision under the different conditions within the same lab and expresses "within laboratories variations": different days, different analysts, different equipment, etc. For example, the procedure below can be performed with different operators, or batches of materials, or on different days from the replicate tests performed for repeatability in Subheading 3.12.2.

1. Prepare and assay six sample solutions as in Subheading 3.12.2.
2. Repeat **steps 1–5** of Subheading 3.12.2 but with a different operator, or batch of materials, or on a different day. For intermediate precision analysis, it is recommended that the second analyst should vary some (or possibly all) of the conditions.
3. Using the 12 replicate results, calculate the standard deviation, and the relative standard deviation using the appropriate equations (*see* Table 3) and check findings against the acceptance criteria in Table 3. Results from intermediate precision should meet precision acceptance criteria that analyst 1 results are subject to (*see* Table 3).

3.12.4 Linearity

Linearity is assessed using the calibration curves across protein concentrations. Generally, a minimum of five linearity solutions must be prepared over the concentration range. The signal output (absorbance) is plotted against known protein concentration to determine the equation of the straight line and regression coefficient (R^2).

1. Prepare a stock solution of a protein standard (e.g., BSA 0.1 mg/mL, Subheading 2.2), and set up dilutions for the preparation of a standard curve (*see* Table 2).
2. Pipette duplicate aliquots of protein standards into microfuge tubes and dilute appropriately (*see* **Note 24**) with a suitable buffer.
3. Mix well by inversion or using a vortex.
4. Carry out the assay as per **steps 1–4** in Subheading 3.12.2.
5. Prepare a standard curve by plotting absorbance versus protein concentration (*see* **Note 26**). Check the findings against the acceptance criteria in Table 3

The limit of quantitation (LOQ) can also be determined using the equation outlined in Table 3 by inputting the standard deviation and the slope values for the method being validated.

3.12.5 Accuracy (Reference Material Comparison)

Accuracy can be measured by adding to the sample matrix, a known concentration of analyte (in this case, protein) standard and analyzing the sample using the method being validated.

1. Using a well-characterized protein sample or standard protein (*see* **Note 66**), prepare a sample covering three concentration ranges (e.g., 70%, 100%, and 130%, i.e., a minimum of nine determinations over a minimum of the three concentration levels) relative to the range of the standard curve. Assay the samples in triplicate as per **steps 1–4** in Subheading 3.12.2 (or as per the method being validated).
2. Calculate the Recovery using the equation in Table 3 and check findings against the acceptance criteria in Table 3.

4 Notes

1. When selecting the method to be used for total protein determination, care should be taken to consider sample and buffer properties and the objective of the analysis being undertaken. The advantages and limitations of the most commonly employed methods are compared in Table 1, as an aid to the user in the selection of the most suitable assay for the sample in question.
2. All solutions should be stored at room temperature prior to the assay (cold solutions can cause condensation on the surface of the cuvette, whereas warm solutions often lead to bubbles causing inaccuracy in the readings). Use particle-free solutions, for best results, filter buffers/solutions (e.g., pore size 5 μM) to remove particulates.
3. The selection of a protein standard is an important step in every protein assay as the standard and sample should be of a similar molecular weight and should respond in the same way to the assay in order to minimize errors in estimations.
4. Protein concentrations are measured in milligrams per milliliter (mg/mL) and micrograms/microliter (μg/μL). Concentrations can also be recorded in micrograms/ milliliter (μg/mL) for very small concentrations.
5. Common buffer components such as acetate, Brij™ 35 detergent, deoxycholate, SDS, Triton X-100, Tween 20, and urea are known to absorb strongly at 280 nm and should be avoided. Nucleic acid contamination of samples can also greatly influence the absorption.
6. It is advisable to use a double beam spectrophotometer and matched cuvettes for samples and controls wherever possible. Matched cuvettes have identical optical properties, meaning that one can be filled with the blank and left in the reference beam while all other samples are measured against it, saving time and improving accuracy.
7. An important consideration when employing the Bradford assay is the variation in response that is seen between proteins with dissimilar compositions. This anomaly is explained by the favored interaction of the Coomassie brilliant blue G 250 dye to specific amino acid residues (e.g., arginine and lysine) in a protein molecule. A suitable protein standard will be one that is likely to give absorbance values close to those for the protein samples of interest (e.g., if you wish to determine the concentration of an immunoglobulin, IgG can be used as a protein standard) [5].

8. The buffer composition should match that of the protein storage buffer. Common buffer components such as acetic acid, ammonium sulfate, 2-glycerol, mercaptoethanol, Tris, and SDS are known not to be compatible with the Bradford Assay.
9. NaOH is added to ensure that the sample does not precipitate upon addition of Bradford reagent.
10. During storage, dye may precipitate from solution. If this should happen filter the reagent using a general-purpose filter paper (e.g., Whatman No. 1) before use. Bradford stock solution is stable for several weeks when stored in a brown glass bottle at room temperature
11. Reagent made in-house generally works well but is usually not as sensitive as the commercial product.
12. Scale down the volumes of the reagents for the microassay procedure. A commercial source of Bradford reagent is recommended for use with the microassay procedure since the sensitivity of the assay depends on the dye quality.
13. Many substances are known to interfere with the Lowry protein assay including CAPS, barbital, cesium chloride, EDTA, citrate, cysteine, diethanolamine, dithiothreitol, EGTA, HEPES, mercaptoethanol, nonidet P-40, phenol, sodium deoxycholate, polyvinyl pyrrolidone, sodium salicylate, thimerosol, tricine, Tris, and Triton X-100.
14. Substances known to interfere with the BCA assay include Tris, ammonium sulfate, EDTA, DTT, EGTA, and SDS.
15. Commonly used blocking agents include BSA, serum, nonfat dry milk, casein, and gelatin. The selection of blocking agent is dependent on the antibody being used (see the manufacturer's guidelines).
16. Allow wash buffer to equilibrate to room temperature before use.
17. Nonfat dried milk is not compatible for use with streptavidin/biotin.
18. For accuracy, prepare standards that span the full range of the assay.
19. Use ultrapure water for preparation of all buffers.
20. Optimal concentrations are determined experimentally or following manufacturer's guidelines.
21. A plate material that can bind biological materials should be used. For samples containing low concentrations of target antigens, the adsorption to reaction vessels can be minimized using silanized cups. PCR plates can also be immersed in 0.8% glutaraldehyde solution at 37 °C for more than 6 h in order to improve absorbability.

22. UNG is included in the PCR to prevent the reamplification of carryover PCR product. Using dUTP rather than dTTP, the uracil incorporated into amplicons during PCR can be destroyed by UNG, thus destroying any contaminating PCR product present at the outset. The UNG incubation step involves incubating reactions at 50 °C for 2 min and precedes the HotStart *Taq* DNA polymerase activation step (95 °C for 5 min). UNG is then thermally inactivated during the first denaturation step of the PCR.
23. An initial 1 in 10 dilution is suggested. The dilution and readings of samples should be performed in duplicate.
24. Concentrated protein solutions should be diluted so they are within the linear range of the instrument being used. If the approximate sample concentration is unknown, a range of dilutions (1, 1:10, 1:100, 1:1000) should be assayed. When working with dilute samples, the addition of a nonionic detergent to the buffer may help to prevent loss of protein through adsorption of protein on the cuvette.
25. Protein concentration can be determined by comparing the absorbance value of the unknown samples to a standard curve. However, if a clear linear relationship is observed, a standard curve is not necessary. Amounts can be determined using interpolation. A standard curve should be prepared to check for accuracy and linearity the first time that an assay is performed.
26. The scatter plot chart feature in Microsoft Excel can be used to prepare a standard curve by plotting absorbance (y-axis) versus protein concentrations (x-axis). In order to generate the equation of the line right click on the graph and select "Add Trendline" from the menu. Choose an appropriate trendline from the options displayed, (e.g., linear, or polynomial (order 2, 3, or 4)) until the best-fit for the data appears. Check the box to display the equation on the chart and check the box to display the regression (R^2) of the line. Use the resulting equation to determine protein concentration (x) of an unknown sample by inserting the sample absorbance value (y). The plot of the data should be inspected for possible outliers and points of influence. Many modern spectrophotometers (and microplate readers) will automatically plot a regression line using the standard samples and will interpolate the unknown samples, reporting the calculated protein concentrations.
27. From time to time more thorough cleaning of the pedestals may be required (e.g., to remove dried sample on the pedestals), substitute 0.5 M HCl for the dH_2O and follow with 3–5 μL dH_2O.

28. Choose the appropriate protein reference (e.g., BSA, IgG, and lysozyme) for calculations of protein concentration in order to obtain the desired molar extinction coefficient.
29. It is recommended to use a precision pipette (0–2 μL) with precision tips to ensure that sufficient sample is delivered to the measurement pedestal.
30. It is recommended that an aliquot of the blanking buffer be measured as a sample to confirm that the instrument is working well and that dried-down sample on the pedestals is not a concern.
31. For Bradford, Lowry, or BCA assays or when working with purified protein, a 2 μL sample size will ensure proper column formation.
32. A 1 mL microassay format can be used for protein concentrations between 1 and 20 μg/mL. The protocol followed is the same as that of the basic protocol except with a reduced total volume of 1 mL. Similarly, a 250 μL microplate assay can also be performed with a reduced total volume of 250 μL (linear range: 1.25–10 μg/mL). The extent of color yielded in this assay is dependent on the amino acid composition of the sample. Thus, two different proteins at the same concentration can give significantly different color yields. Hence, the choice of a standard similar in composition to that of the sample is an important consideration in the design of this assay. The assay responds primarily to arginine residues, and an arginine-rich standard may be preferable if the sample is rich in that amino acid. If this is not possible for any given assay, a normalized protein quantitation model based on the target protein sequence has also been suggested [51].
33. The unknown sample can be diluted in the case of a high protein concentration.
34. Quartz cuvettes cannot be used as the Coomassie brilliant blue G-250 dye binds to this material.
35. The standard curve is not linear, and the precise absorbance varies depending on the age of the assay reagent. As a result, it is important to construct a calibration curve for each assay performed.
36. If the A_{660} values are low, then re-read the samples at A_{750}, which may increase the sensitivity of the assay, or at A_{550} if the sample concentration is between 100 and 2000 μg/mL.
37. There is a variation in response from proteins with differing amino acid compositions with this assay. Standards should always be considered based on the amino acid composition of the protein of interest.

38. This incubation period is not a critical parameter and can vary from 10 min to several hours with no effect on final absorbance values.
39. A modification of the Lowry assay, using a microwave oven, has been described that allows protein determination with much reduced incubation times of just seconds [52].
40. This assay is not linear at concentrations >0.10 mg/mL, and linearity should be monitored using a standard curve.
41. The assay can be run at room temperature or sensitivity can be increased at 60 °C [2]. A modification of the BCA assay, utilizing a microwave oven, has been described that allows protein determination with much reduced incubation times of just seconds [52].
42. This can easily be achieved by placing the samples in a water bath at room temperature.
43. Increase the incubation time in order to improve the sensitivity of this assay. The incubation temperature should be lowered to ambient room temperature to obtain a decrease in sensitivity.
44. All reagents should be brought to room temperature by allowing to sit for 15–20 min on the bench before starting the assay.
45. Washing procedure:

    Wash step: use a squirt bottle/multi-channel pipette to fill the wells with wash solution. Do not touch the inside surface of the wells with the pipette tips/or bottle nozzle. *Expulsion of wash solution:* holding the microtiter plate firmly (from the underneath) over a sink, turn the plate rapidly, and directly upside down causing the liquid to be forced out of the wells and into the sink. Repeat this "dumping motion" a second time. *Blotting and banging the plate*: immediately blot the upside-down plate (wells face down) on blotting/tissue paper. Move the plate to an unused section of the blotting paper and allow to drain for 5–10 s. Repeatedly "bang" the plate very hard four to five times over unused areas of the paper. *Wash step*: Use a squirt bottle/multi-channel pipette to refill the wells with wash solution as before and immediately dump and bang the plate again as described above. Following the last wash, leave the plate upside down for 30 s to drain. Bang again for three to four times, rotating the plate between each bang.
46. This incubation time can be optimized. Excessive incubation time can cause issues with excessively high signal in ELISA; be sure to follow incubation times as recommended by the antibody manufacturer.
47. Do not add substrate near the sink as the washing procedure can create aerosols which could re-contaminate the wells or substrate.

48. *Troubleshooting considerations with ELISA readings:*

    Weak or absent signal: may indicate the incorrect storage of components or that the assay steps/components may require further optimization. The antibodies used in the assay may lack affinity for each other or for the target or the reagents may have been prepared or added incorrectly.

    High background signal: may be due to insufficient washing/blocking or further optimization of assay steps/components may be required.

    High sample signal: may indicated insufficient washing, excessive incubation times, or contamination of the assay.

49. Variation between samples: If the standard curve is a good fit for the data but there is a high standard deviation, there might be a technical problem such as pipetting error. Including samples and standards in triplicate should help to mitigate this issue.

50. Chemical variations possible with this protocol include changes to the recipes for gel structure, buffers, and detergents.

51. The appropriate polyacrylamide percentage will depend on the percentage of gel required to best resolve your protein of interest, based on molecular weight. A 10% gel is generally recommended for a protein size of 15–100 kDa. 8, 10, 12.5, and 15% gels are commonly employed for protein resolution. The smaller the size of the protein of interest the higher the percentage polyacrylamide required. A gradient gel can also be used for the analysis of proteins of varying sizes.

52. It is possible to include a small amount of bromophenol blue in the stacking gel mix to give the gel a pale blue color. This allows for easier visualization and loading of the wells [53].

53. Use a 3 mL syringe fitted with a 22-gauge needle to wash the wells with running buffer and remove any un-polymerized gel. Straighten wells using a loading tip.

54. Extensive destaining can lead to the loss of some low molecular weight bands and the fading of other bands. Destaining can be accelerated by addition of absorbent paper at the edge of the destaining container. Note: when staining IEF gels with Coomassie Blue, the gel must first be fixed in a solution of trichloroacetic acid. This leaches out the carrier ampholytes, which would otherwise produce background staining. When staining small peptides (less than 10 kDa), the gel is first fixed in a solution containing glutaraldehyde to cross-link the peptides and prevent them from diffusing out of the gel during subsequent staining steps.

55. *Analysis of SDS-PAGE Gels*: Determine the correct orientation of the gel, using the cut corner to identify the top/bottom and left/right ends. Locate the lanes corresponding to each sample loaded on the gel. Identify the polypeptide bands of interest. Estimate the approximate molecular mass or relative molecular mass for each band of interest using the molecular weight markers as a guide. Note differences in the intensity of band staining. This may be indicative of disparity in abundance between individual polypeptides.

    Identify unusual patterns that might reflect incomplete denaturation or degradation of the proteins being analyzed (overloading or underloading, distortion of lanes, smears, and streaks can all limit the interpretation of results).

56. Maintain a high level of cleanliness during electrophoresis and silver staining to minimize spurious artifacts. Wear gloves to prevent staining of the gel due to finger marks.

57. Ensure that the nitrocellulose membrane is between the gel and the anode or the proteins will be lost from the gel into the buffer rather than transferred to the nitrocellulose.

58. Diluted antibody can usually be retained and stored at −20 °C for a further four to five uses.

59. Use filter/barrier tips to avoid cross-contamination during pipetting. Do not touch the microplate wells with the tips during pipetting.

60. The edge of a lid from a pipette tip box may be used to seal the plate if a plate sealer is not available. Ill-sealed qPCR plates leads to the evaporation of the sample from the wells of the plate during the reaction.

61. If no suitable centrifuge is available air bubbles can also be removed by gently tapping the plate.

62. Dissociation/melting curve analysis is used to monitor for the presence of nonspecific product during a SYBR green qPCR assay. During this stage of the reaction, the temperature is gradually increased, causing denaturation of the dsDNA. Since SYBR green binds to double-stranded DNA, this separation of the strands results in dissociation of the SYBR green and a sudden decrease in fluorescence. Melting temperatures (T_m) are specific to each individual amplicon (dependent on the base composition and size of the amplicon), and so the T_m of each individual PCR product can be detected through fluorescence intensity monitoring. A single, discrete peak suggests that only one PCR product is being amplified and detected. The presence of multiple peaks may indicate the presence of nonspecific products and/or the formation of primer dimers. A melt point with a lower T_m than predicted for the desired amplicon may suggest the formation of primer dimers during the reaction, while a higher T_m can be indicative of genomic DNA

contamination. However, multiple peaks may also sometimes appear if distinct regions of the product melt at different temperatures, and this possibility can be investigated using agarose gel electrophoresis.

63. Amplification in the NTC wells can indicate potential contamination that may have occurred during the reaction set up (and so the results must be discarded) or that the assay itself is nonspecific (the assay may need to be optimized).
64. Specifications are dependent on the analytical technique employed and the analyte to be quantitated, for example, a higher RSD specification may be justified for some assays.
65. Accuracy can be inferred if precision, linearity, and specificity have been established.
66. A well-characterized protein sample is one where the levels of the protein of interest have been determined with an alternative validated method.

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Chapter 17

Protein Extraction and Purification by Differential Solubilization

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Abstract

The preparation of purified soluble proteins for biochemical studies is essential and the solubility of a protein of interest in various media is central to this process. Selectively altering the solubility of a protein is a rapid and economical step in protein purification and is based on exploiting the inherent physicochemical properties of a polypeptide. Precipitation of proteins, released from cells upon lysis, is often used to concentrate a protein of interest before further purification steps (e.g., ion exchange chromatography, size exclusion chromatography etc).

Recombinant proteins may be expressed in host cells as insoluble inclusion bodies due to various influences during overexpression. Such inclusion bodies can often be solubilized to be reconstituted as functional, correctly folded proteins.

In this chapter, we examine strategies for extraction/precipitation/solubilization of proteins for protein purification. We also present bioinformatic tools to aid in understanding a protein's propensity to aggregate/solubilize that will be a useful starting point for the development of protein extraction, precipitation, and selective re-solubilization procedures.

Key words Protein purification, Protein extraction, Ammonium sulfate precipitation, Bioinformatics, Inclusion body solubilization, Protein refolding, Three-phase partitioning

1 Introduction

Proteins are polymers of amino acids bearing side chains of varying polarity and charge (Fig. 1). In general, proteins are folded such that the charged amino acid side chains are oriented toward the outside of the protein while hydrophobic amino acids are buried within the protein (with the exception of membrane proteins where hydrophobic residues may be on the surface to allow interaction with lipid components). Denaturation is a process that involves unfolding of the tertiary structure of a protein. The unfolding will expose the hydrophobic core of protein molecules causing them to aggregate and precipitate from solution. Denaturation may occur due to exposure to extremes of heat and pH or as a result of shear

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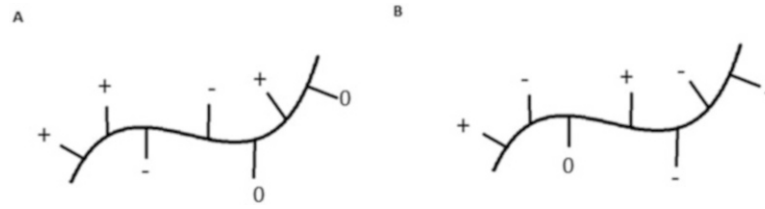

Fig. 1 A section of a protein with positively and negatively charged amino acid side chains. (**a**) Protein with more positive than negative charges which will make the protein positively charged overall (note the side chain with 0 charge). (**b**) The positive and negative charges equally balanced (isoelectric point)

stress [1, 2]. Exploiting differences in protein solubility can be a useful method to purify a selected protein from a crude cell extract.

Some aspects to consider when embarking on the purification of a protein of interest are in silico analysis, protein extraction and solubility, protein precipitation/salting out, inclusion body solubilization, and protein stabilization:

In silico analysis Protein modeling and in silico analysis can help in the experimental design process. When the DNA sequence for a protein is known, bioinformatic analyses can be used at the outset of a purification process to gain an understanding of the physicochemical properties of the target protein. Such tools can establish amino acid composition, provide secondary structure prediction, and identify the protein's isoelectric point. These bioinformatic tools are freely available online, for example at the Swiss Institute of Bioinformatics resource portal, Expasy (*see* Subheading 3.1).

The solubility of a protein upon recombinant expression depends on its primary amino acid sequence. Early solubility prediction methods were based on the content of charged and turn-forming residues. In recent years, however, more advanced prediction software has been developed utilizing training sets of soluble and insoluble proteins. These tools (*see* Subheading 3.2) are used to predict solubility before performing wet lab experiments, thus saving time, effort, and cost [3, 4].

Protein extraction and solubility The interactions between solvents and proteins determine the solubility of any given protein. Interactions can be classified as either attractive or repulsive. A protein will be soluble in a particular solvent if the net free energy of the protein's interactions is negative (i.e., attractive). Additionally, protein solubility is improved if protein-protein interactions have sufficiently positive net free energy (i.e. repulsive), although it should be noted that a protein-protein interaction is modulated by the chemical nature of the solvent of choice. Insolubility typically

results from attractive forces between proteins and repulsive forces between the solvent of choice and the protein.

When extracting a protein from lysed cells, the objective is to solubilize the protein and minimize contamination with unwanted proteins. In the case of purification from tissues, the extraction buffer should also aim to minimize disruption of subcellular compartments such as lysosomes and mitochondria. Some important considerations for protein extraction are discussed by Peach et al. [5]. In addition, Cordwell (2008) has described a process of progressive protein extraction from *E. coli* cells using buffers with increasing levels of solubilizing chaotropes and detergents [6]. In the special case of extraction of a membrane protein, a detergent such as Triton X-100 is often added to the extraction buffer. More recently, the use of detergent-free protocols for membrane protein extraction has emerged [7, 8].

Protein precipitation/salting out Soluble proteins can be precipitated by interaction with a precipitant that decreases the protein's attraction to the solvent and increases the protein's attraction to other protein molecules: this results in protein aggregation and eventually precipitation. One of the simplest ways to do this is by adjusting the pH of a cell extract to the isoelectric point of the protein of interest. At this pH, the protein is at its least soluble and will often precipitate from solution. It can subsequently be solubilized in a suitable buffer at a different pH.

Certain salts such as ammonium sulfate can influence solubility by "salting out" hydrophobic residues of a protein. The presence of high salt causes the protein molecule to adopt a more compact structure exposing hydrophobic regions thereby resulting in precipitation. The appropriate use of such protein-precipitating salts must be determined experimentally (*see* Subheadings 2.2 and 3.4). Protein precipitation can be used as a convenient method to concentrate proteins from cell lysates [9].

Inclusion body solubilization Differential solubilization of proteins is often employed for proteomic analyses [10–12], but it can also offer an alternative purification technique for non-soluble recombinant proteins expressed in heterologous hosts. Thus, recombinant proteins expressed as inclusion bodies (IB) can be readily separated from the host cell protein matrix by centrifugation; however, in many cases, careful solubilization and refolding are important for obtaining suitable recombinant protein for further downstream processes (*see* Subheadings 3.6 and 3.7) [13].

Protein stabilization Polyols, such as glycerol, polyethylene glycol, and sucrose, as well as high molecular weight substances such as serum albumin can have significant effects on protein structure and stability. Preferential hydration of a protein molecule caused by the presence of these additives can increase the protein's stability

and solubility. However, this stabilization may not apply to all proteins and must be judged on a case-by-case basis (*see* [14]).

Protein extraction and solubilization procedures, preceded by bioinformatic analyses, are considered herein (see below). Most of the methods described refer to recombinant proteins as expressed in *E. coli* with reference to other organisms where appropriate.

The control of solubility for protein purification is a rapidly growing field and new solvents and procedures are emerging constantly. The potential to realize simple, one-step, processes in place of traditional chromatography will revolutionize industrial protein production.

2 Materials

In the case of bioinformatic analyses, a computer and Internet access are all that is required.

2.1 Protein Extraction

1. pH meter.
2. Extraction buffer: 50 mM Na_2HPO_4-NaH_2PO_4, pH 5.8–8.0, and 0.3 M NaCl (*see* **Note 1**).
3. Homogenizer (e.g., PRO200 Homogenizer, ProScientific).
4. Sonicator (e.g., *Vibra-Cell*™, Sonics Scientific).
5. Centrifuge (Bucket Type, e.g., *J2–21*, Beckman) and Microfuge (e.g., *5415D* Eppendorf).

2.2 Protein Precipitation Using Ammonium Sulfate

1. Saturated ammonium sulfate solution: Add 750 g of $(NH_4)_2SO_4$ to 1 L of double-distilled water in a beaker. Stir the solution at room temperature with a magnetic stirrer for 15 min or until saturation. Allow any undissolved solids to settle on the bottom of the flask and gently decant the clear supernatant solution.
2. Graduated pipette (10 mL) for gradual addition to cell extract.

2.3 Protein Purification Using Three-Phase Partitioning

1. Saturated ammonium sulfate solution (*see* Subheading 2.2).
2. 1.6 M ammonium sulfate solution buffered at pH 8.0 with 50 mM Tris–HCl, pH 8.0.
3. *t*-Butanol (HPLC grade).
4. 50 mM phosphate buffer pH 7.5.
5. Centrifuge (Bucket Type, e.g., Beckman J2–21).

2.4 Inclusion Body Solubilization

1. DN*ase* I (100 U/mL).
2. Resuspension buffer: 50 mM Na_2HPO_4-NaH_2PO_4, pH 8.0, 0.3 M NaCl, 5 mM DTT, 0.35 mg/mL lysozyme, and proteinase inhibitor cocktail (*see also* Chapter 6). Make up immediately prior to use (*see* **Note 1**).

3. Triton X-100.
4. Solubilization buffer: 50 mM Na_2HPO_4-NaH_2PO_4, pH 8.0, 0.3 M NaCl, 25 mM DTT, and 6 M Guanidine HCl.
5. PBS-T: Phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100.

2.5 Protein Refolding

1. Refolding buffer: 50 mM Na_2HPO_4--NaH_2PO_4, pH 8.0, 0.3 M NaCl, 2.5 mM reduced glutathione, 0.25 mM oxidized glutathione, and 0.2 M arginine.
2. Dialysis buffer: 50 mM Na_2HPO_4--NaH_2PO_4, pH 8.0, and 0.3 M NaCl.
3. Guanidine hydrochloride: 6 M stock, made in double-distilled H_2O.
4. Amicon® protein concentration device (e.g., *Ultra-15 Centrifugal Filter Units*, Amicon®).
5. Gradient maker apparatus (*see* Fig. 2).

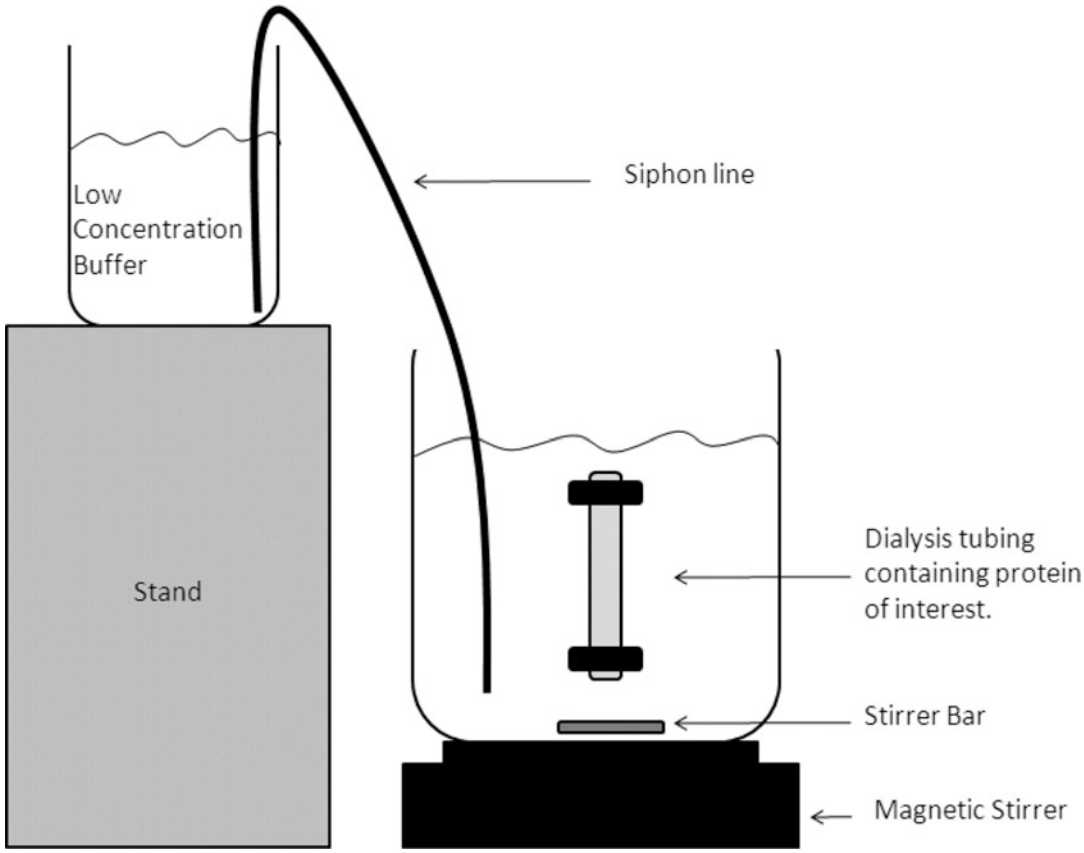

Fig. 2 Schematic diagram of a simple gradient maker

3 Methods

3.1 Computational Prediction of Protein Solubility

Substantial information on a protein's behavior in solution can be predicted by examining its DNA sequence in silico. For a given protein, this may be approached as follows:

1. Obtain the protein's accession number (a unique identifier) and its amino acid sequence (FASTA format) using the UniProt database (https://www.uniprot.org/).
2. Use TMHMM 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) or another membrane protein webserver to predict the protein's secondary structure and to deduce whether it is expected to be membrane bound.
3. If the protein of interest is not a membrane protein, utilize Protein-Sol (https://protein-sol.manchester.ac.uk/) to classify the protein as soluble or insoluble (*see* **Notes 2** and **3**).

3.2 Optimization of Recombinant Protein Solubility

After bioinformatic analysis, if the protein is predicted to be insoluble, there may be a risk of inclusion body formation. An approach toward preventing this is through random or rational mutagenesis of specific amino acids to enhance protein solubility (*see* **Note 4**). The success of rational molecular engineering depends on the availability of a suitably high-resolution three-dimensional structure for the candidate protein to identify the surface residues to mutate. One possible approach is described here:

1. Carry out a search of the Protein Databank (PDB, https://www.rcsb.org/) for a structure (crystal/nuclear magnetic resonance, etc.) of the protein of interest, and download the relevant PDB file. If a template structure is unavailable, a comparative or predicted model may be available (e.g., Alphafold, https://alphafold.ebi.ac.uk/, or ModBase, https://modbase.compbio.ucsf.edu/) or modeled.
2. Visualize the protein structure using suitable software, for example, Pymol software (www.pymol.org) or DeepView (http://spdbv.vital-it.ch/).
3. Analyze solvent accessible residues (e.g., PISA, https://www.ebi.ac.uk/msd-srv/prot_int/pistart.html), and determine possible hydrophobic surface amino acids for mutation (*see* **Note 5**).
4. Design a mutated gene for improved protein solubility (*see* **Note 6**). Translation software such as GeneDesigner is available to aid in this process [15].

3.3 Recombinant Cell Breakage

The source of the protein will determine the optimal technique to release the protein from the tissue or cells in which it is contained. The typical freeze-thaw cell lysis procedure (below) is sufficient to lyse most bacterial cell types although other options can be used (*see* **Notes 7–9**).

The correct choice of buffer for extraction of a protein of interest from tissues (e.g., liver, kidney) can be problematic [16]. The extraction buffer and materials used here are a useful starting point. The pH chosen will depend on the target protein but is usually between 7.0 and 7.5. The same buffer can also be used for extraction of recombinant proteins [5]. A typical freeze-thaw process is described here:

1. Collect the bacterial cells by transferring the bacterial culture to a prechilled sterile centrifuge tube, and centrifuge at low speed (5 min, 800 × *g*) in a cooled centrifuge (4 °C).
2. Carefully remove the culture media from the bacterial cell pellet, ensuring the pellet is not disturbed.
3. Resuspend the cell pellet in extraction buffer (Subheading 2.1), at 10% of the original culture volume. The pH may be varied to suit the protein under study (*see* **Note 10**).
4. Freeze the resuspended cells at −80 °C by placing the resuspension (still in the plastic centrifuge tube) into a pre-equilibrated −80 °C freezer (or liquid Nitrogen), and then warm the cells to 37 °C (using a pre-equilibrated water bath) for 10 min. Repeat this freeze-thaw process three times (*see* **Note 11**).

If the protein of interest is not released during the freeze-thaw steps, additional methods such as sonication or electroporation may be used [17, 18]. Of these methods, sonication is the most widely used. It is crucial to maintain the cell suspension on ice during the sonication process to avoid overheating (*see* **Notes 12–14**). A typical procedure is as follows:

5. Sonicate at 10 amplitude microns for 10–20 s.
6. Allow the cell suspension to cool on ice for 30 s.
7. Repeat **steps 5** and **6** three more times.
8. Check the recombinant protein induction/expression by loading and analyzing a representative sample (typically 50 μg protein) onto a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (*see* **Note 15**).

3.4 Protein Precipitation Using Ammonium Sulfate

In cases where a protein of interest does not have a "tag" such as a HIS-tag to aid purification, it is useful to concentrate the protein by precipitation. A common, inexpensive approach is to use a salt, which can cause the protein to precipitate out of solution [19, 20], so-called salting out. The addition of salt to a protein

solution removes water from hydrophobic patches on the protein's surface, resulting in aggregation which causes the protein to come out of solution. Several salts can be used for this process, NaCl, Na_2SO_4, KCl, $CaCl_2$, and $MgSO_4$; however $(NH_4)_2SO_4$ (ammonium sulfate) is by far the most widely used salt due to its high solubility in water (4 M at saturation) and its low density at saturation allowing precipitated proteins to be easily collected by centrifugation [9] (*see* **Note 16**):

1. Gently stir the protein mixture with the aid of a magnetic stirring bar at 4 °C. Add the saturated ammonium sulfate solution to the protein solution drop-wise until a precipitate starts to form (*see* **Notes 17** and **18**).
2. Once sufficient saturated salt solution has been added to cause precipitation of the protein of interest (indicated by collection of precipitate at the bottom of the container), centrifuge the mixture at 10,000 × *g* for 15 min in a precooled rotor. Collect the precipitate by carefully discarding as much supernatant as possible (*see* **Note 18**).
3. Resuspend the protein pellet at 4 °C in extraction buffer (Subheading 2.1) for further downstream processes (*see* **Note 1**).
4. Dialyze against an appropriate buffer to remove the excess salt if desired or the high salt soluble protein solution may be used in a subsequent hydrophobic interaction chromatography step (*see* **Note 19**).

3.5 Protein Purification by Three-Phase Partitioning (TPP)

Three-phase partitioning (TPP) is rapidly gaining ground as an effective alternative to column chromatography often achieving spectacular purification in a single step. It is cheaper and faster than chromatography [21–26] and does not require prior cell lysis in most cases. TPP is a batch method of protein separation that combines ammonium sulfate precipitation with solvent extraction. It utilizes saturated, buffered ammonium sulfate in combination with a water-miscible aliphatic alcohol, commonly *t*-butanol. The two solutions do not mix, and a heterogeneous, three-phase liquid is produced that can be used to partition proteins from whole cells without the need to lyse the cells (*see* **Note 20**). This process can be readily scaled up for larger-scale protein production. A typical procedure for recombinant proteins is described:

1. Whole cells in suspension are treated with aqueous 1.6 M ammonium sulfate solution buffered at pH 8.0 with 50 mM Tris–HCl, pH 8.0 (vary the volume as required), with shaking.
2. Add 1 volume of tertiary butanol (*t*-butanol). Shake vigorously for 1 min at room temperature (RT).
3. Centrifuge (10,000 × *g*) for 15 min at room temperature. Three phases separate during centrifugation.

4. Remove the upper (alcohol) layer by aspiration (*see* **Note 21**). Remove the lower precipitate layer, leaving an interfacial aqueous layer containing protein extract.
5. Add fresh *t*-butanol to the removed aqueous layer (with vigorous shaking once again).
6. Allow precipitation again and collect layers as above.
7. Add ammonium sulfate to the precipitate layer (*see* Subheading 3.4).
8. Resuspend the protein pellet at 4 °C in extraction buffer for further downstream processing.

3.6 Inclusion Body Solubilization

Recombinant protein expression in a heterologous host can frequently result in insoluble and inactive proteins (*see* **Note 22**) [27, 28]. These inclusion bodies (IB) are insoluble aggregates of misfolded protein. Although inclusion bodies can be purified, further characterization of this protein mass is often impossible without solubilization of the protein of interest and its refolding into an active form (*see* **Notes 23** and **24**). Formation of inclusion bodies is a persistent problem for recombinant protein expression in *E. coli* [29]. Resuspension, solubilization, and refolding are approaches to recovering proteins from inclusion. Buffers for IB suspension and solubilization are described here. Guanidine HCl is the agent that solubilizes the IBs and its slow removal by dialysis allows the protein to refold. If the protein contains cysteine residues, oxidation could lead to protein aggregation. A reducing agent, such as DTT (1.0 mM), is added to the buffer to prevent oxidation:

1. Carry out the cell lysis **steps 1**–**3** as outlined in Subheading 3.3; except resuspend the cell pellet in 10% of the original culture volume of resuspension buffer (*see* Subheading 2.4).
2. Slowly add Triton X-100 (to a final concentration of 1% v/v) (*see* **Note 25**) and mix gently.
3. Carry out the sonication procedure as outlined in Subheading 3.3, **steps 5**–7.
4. Incubate the cell debris with DN*ase* I (100 U/mL) for 1 h at 37 °C.
5. Collect the inclusion bodies by centrifugation at 30,000 × *g* for 30 min at 4 °C.
6. Wash the inclusion body pellet twice with PBS-T, followed by centrifugation at 30,000 × *g* for 30 min at 4 °C.
7. Solubilize the pelleted inclusion bodies in the solubilization buffer (50 mM Na_2HPO_4--NaH_2PO_4, pH 8.0, 0.3 M NaCl, 25 mM DTT, and 6 M guanidine HCl), and allow total solubilization to occur at 4 °C for 1 h, with occasional gentle mixing.
8. After 1 h solubilization, remove all remaining insoluble materials by centrifugation 30,000 × *g* for 10 min at 4 °C (*see* **Note 26**).

9. Determine the protein concentration and adjust to 1 mg/mL by dilution in solubilization buffer, and proceed directly to refolding at 4 °C (Subheading 3.7).

It is important to remove aggregates (**step 8**) that can act as nuclei to trigger aggregation during folding. The addition of a mild solubilizing agent (e.g., 1 M 3(1-pyridinio)-1-propane sulfonate) during the refolding steps limits reaggregation of refolding proteins. For further information, Tsumoto and co-workers [28] comprehensively detail the effect of small molecule additives on refolding and aggregation of proteins along with other considerations for refolding recombinant proteins.

3.6.1 Protein Solubilization Detergent Screen

Selecting an appropriate detergent can be crucial for effective purification of membrane proteins. A detergent screening methodology may be used:

1. Lyse cells as previously described (Subheading 3.3). A membrane preparation may be performed if required (*see* **Note 27**).
2. Small aliquots of lysed cells/cellular membranes are mixed with an equal volume of resuspension buffer two containing different detergents (e.g. DDM, LDAO, DM, FC-12, C12E9; *see* ref. 8). To avoid the production of foam, do not vortex the solubilization mixtures but mix by gently pipetting up and down.
3. Gently mix using an end-over-end rotator at 4 °C for 12–14 h (*see* **Note 28**).
4. Take a 10 μL sample for analysis by SDS-PAGE and store at 4 °C.
5. To pellet the un-solubilized material, centrifuge the remainder of the sample for 30 min at 100,000 × *g* and 4 °C.
6. Remove the supernatant from each tube taking care not to disturb the pellet and transfer it to another clean, chilled 1.5 mL tube.
7. Mix the contents of each tube by gentle pipetting up and down. Take a 10 μL sample for analysis by SDS-PAGE.
8. Examine samples by SDS-PAGE, and Western blot if appropriate, to identify the detergent and conditions that resulted in the best solubilization (*see* **Note 29**).
9. Incorporate these detergent and incubation parameters for the purification of the target protein.

3.7 Protein Refolding

Once inclusion bodies have been solubilized, the process of refolding the native protein can be addressed. In recent years, there have been many novel approaches developed to induce protein refolding. These include high hydrostatic pressure, dialysis against PE-PEG, solid-phase refolding on a cation-exchange resin with

a decreasing gradient of urea, and the use of micro-fluidic chips [30, 31]. However, one of the most cost-effective and easiest methods to execute is simple, low concentration, gradient dialysis, as detailed below:

1. Dilute the solubilized proteins as quickly as possible (to yield a final protein concentration of 0.1 mg/mL, *see* **Note 30**), into prechilled refolding buffer (*see* **Note 31**).
2. Dialyze the diluted solubilized protein overnight against a 200-fold volume of dialysis buffer with slowly decreasing concentrations of guanidine hydrochloride (GuHCl) (typically, it is appropriate to decrease GuHCl concentration stepwise as follows: 6 M, 4 M, 2 M, 1 M, 0.5 M, and then 0 M using a continual dialysis approach; *see* Fig. 2) (*see* **Notes 32** and **33**).
3. Centrifuge the dialysate at 4 °C for 30 min at 30,000 × *g*.
4. Carefully remove the liquid protein rich layer; concentrate (e.g., *Amicon*® filtration) and store at an appropriate temperature (*see* **Note 34**).

4 Notes

1. The exact pH chosen for extraction will depend on the protein but is usually between 7.0 and 7.5. The pH chosen should be one where the protein is stable. Various agents may be added to protect the extracted protein from proteolysis (protease inhibitors, *see* Chapter 6) or from aggregation (polyols, detergents, etc.). If the protein contains cysteine residues, oxidation could lead to protein aggregation or inactivation. A reducing agent, such as dithiothreitol (DTT) or mercaptoethanol (1–2 mM), may be added to the buffer to prevent oxidation. A detailed description of cell lysis and extraction is found in Subheading 3.3.
2. Protein-Sol (https://protein-sol.manchester.ac.uk/) is a machine learning model trained on an experimentally available dataset of proteins. The Protein-Sol software will take a single amino acid sequence and return the result of a set of solubility prediction calculations, compared to a solubility database.
3. If the protein is predicted to be soluble, proceed with recombinant expression and purification; if predicted as insoluble, mutagenesis (Subheading 3.2) is an option or the protein can be expressed as insoluble inclusion bodies and subsequently resolubilized (Subheading 3.3 onward).
4. In general, the amino acids on a protein's surface (those not affecting function) are primary mutagenesis targets to improve

solubility. The objective is to minimize the interactions that result in inclusion body formation. This is typically achieved by replacing hydrophobic surface amino acid residues with charged polar hydrophilic or less hydrophobic residues.

5. Computational prediction of a protein's aggregation propensity is based on its amino acid sequence. Available analysis tools include AGGRESCAN (http://bioinf.uab.es/aggrescan/) or AMYLPRED2 (http://biophysics.biol.uoa.gr/AMYLPRED2). Molecular engineering, by the addition of a fusion tag such as green fluorescent protein (GFP), can facilitate easier downstream analysis through its florescence detection.
6. An alternative approach is to determine the folding free energy estimations for any proposed mutated structures and focus on those site mutations likely to enhance solubility, for example, using PoPMuSiC software (http://dezyme.com/).
7. There are several methods to achieve cell lysis, including repeated freezing and thawing, sonication, homogenization at high pressures, enzymatic lysis, or permeabilization by organic solvents. The method of choice depends on how fragile the protein is and how robust the host cell is [32]. Common examples of cells with modified robustness include the use of easily lysed *E. coli* expression cell lines (e.g., pLYSs mutants or alternative mutants) or wall-less strains, L-form strains, lpp deletion strains, and the use of strains featuring the co-expression of lysis-promoting proteins [33, 34]. The use of molecular engineering to introduce a signal peptide (e.g., pelB from *Pectobacterium carotovorum*, formally known as *Erwinia carotovora*, or SP from *Bacillus*) may also offer a method to direct protein expression to a more easily extracted portion of the cell, the periplasmic space [35–37]. Methods of extraction from the various cellular, and extra cellular, compartments are detailed in **Notes 8** and **9**. Molecular methods can be further augmented by simple inclusion of specific additives and compounds to increase targeted protein accumulation and downstream solubility [38].
8. Molecular engineering to introduce a signal peptide such as *Pel*B or DsbA offers a method to direct protein expression to an easily extracted portion of the host cell, the periplasmic space [34–36]. Thus, proteins can be selectively released from the various compartments of a bacterial host, for example; proteins expressed in the periplasmic envelope can be selectively lysed as described by French and co-workers [37]. This process will yield an extract consisting of proteins from the periplasmic space of *E. coli:* to begin, pellet the bacterial cells to be disrupted by centrifugation (800 × *g* for 3 min). Resuspend the pellet in fractionation (F1) buffer at 20% of the original culture

volume. F1 buffer is made up (final concentrations) as follows: 500 μg/mL lysozyme, 20% w/v sucrose, 1 mM EDTA, 200 mM guanidine hydrochloride, and 200 mM Tris–HCl, pH 8.0. Incubate the resuspended cells at room temperature for 15 min, and then add an equal volume of ice-cold water. Stand at room temperature for 15 min. Remove cell debris by centrifugation at 10,400 × *g* for 10 min. Transfer the supernatant (containing the periplasmic fraction) to a clean container for further purification.

9. Proteins expressed and secreted extracellularly into the culture supernatant can also be conveniently concentrated for electrophoretic analysis by Pyrogallol precipitation. In brief, this method involves adding an equal volume of a PRMM solution (0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50.0 mM succinic acid, 20% v/v methanol in H_2O, adjusted to pH 2.0 with HCl) to a cleared (0.22 μm filtered) culture supernatant. Adjust the pH of the solution to 2.8 (±0.1), and allow the proteins to precipitate for 1–2 h at room temperature, followed by overnight incubation at 4 °C. Sediment the precipitate by centrifugation at 10,000 × *g* for 1 h, and carefully remove the supernatant. Repeatedly rinse the precipitate with 1 mL of acetone. Remove all traces of acetone by evaporation at room temperature. Solubilize the precipitate by adding an appropriate buffer or for electrophoresis add 100 μL of 2× SDS-PAGE sample buffer (25% glycerol, 8% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue, 100 mM Tris–HCl, pH 6.8). These samples can be applied directly to an SDS-PAGE gel, if this type of analysis is required.
10. A protein is least soluble when at its isoelectric point (pI). Hence, selection of an appropriate pH buffer range for protein extraction is important, for example, HEPES (pH 6.8–8.2), Tris–HCl (pH 7.5–9), and phosphate (pH 5.8–8) are commonly used. To determine a given protein's pI, its accession number (or amino acid sequence in FASTA format) should first be retrieved from the UniProt database (www.uniprot.org). Subsequently, a webserver such as the bioinformatics resource portal can be utilized to determine a theoretical pI (http://web.expasy.org/compute_pi/) and aid in appropriate buffer selection (e.g., choose a pH buffer one unit above or below the pI of the protein of interest).
11. If the expression host of choice is a yeast, one of the most widely used methods for the disruption is agitation with glass beads. Several cycles of agitation are interspersed with cooling cycles to avoid overheating.
12. The sonication process can generate large amounts of heat, which is why pulses are limited to ~20 s. In between pulses, cool the tube in ice or ice-water slurry for 30 s. If a large volume is required to be sonicated, split the cell suspension

into a number of smaller tubes, and alternate the sonication and cooling steps. Sonicate on ice where possible in order to maintain protein activity during sonication. *See* [17] for an in-depth review of sonication optimization during recombinant protein lysis.

13. The extraction process may also release proteases, which can digest proteins in the extract. If the protein of interest is sensitive to proteolysis, it is desirable to employ a protease inhibitor (*see* Chapter 6), to work quickly, to keep the extract cooled to minimize proteolysis, and to select an easily lysed cell line that is ideally protease-deficient (*see* **Note 7**).
14. Lysozyme (500 μg/mL, to assist cell wall degradation) and DN*ase* I (100 U/mL, to degrade genomic DNA) can be added to the lysis buffer.
15. An alternative approach to quantify protein induction and overexpression is to use a NanoDrop™ spectrophotometer (Thermo Scientific). Similar to SDS-PAGE gel analysis, it is best to compare an expressed sample lysate against a replicate of a non-induced sample as a control. If the protein of interest is catalytically active, a crude lysate test of functional activity or an appropriate bioassay may also give an indication of successful overexpression and cell lysis.
16. The first proteins to be purified during ammonium sulfate precipitation are the water-soluble proteins. The concentration of ammonium sulfate needed for precipitation varies from protein to protein and is dependent on salt/protein concentration, pH, temperature, and time. The required ammonium sulfate concentration should be determined empirically [9, 20]. In brief, this entails placing a volume of cell extract, for example, 10 mL, into each of five test tubes. Add, with gentle mixing, amounts of solid ammonium sulfate to yield 20%, 30%, 40%, 50%, and 60% w/v saturation, and allow to sit for 30 min to promote precipitation. The insoluble material is collected by centrifugation and the resulting pellets dissolved in an appropriate buffer and may be dialyzed if required and assayed for enzyme activity or analyzed by SDS-PAGE.

    An alternative method, using an empirically derived dual additive approach, has been used for selective protein precipitation based on the synergistic effect of electrostatic interactions combined with traditional salting-out effects (*see* [19]).
17. Final concentrations of ammonium sulfate must be calculated using standard nomograms or with online tools ("Ammonium Sulfate Calculator," available at www.encorbio.com/protocols/AM-SO4.htm). Adding increasing amounts of ammonium sulfate causes the different fractions of a protein mixture to precipitate at different rates. One advantage of this

method is that it can be performed inexpensively with very large volumes. Additionally, the high salt content of the precipitated protein permits its direct addition onto a hydrophobic interaction chromatography (HIC) purification column, thus speeding up the overall purification process.

18. Ammonium sulfate can be added either as a saturated solution or directly as salt crystals. It may be advantageous to add pre-ground ammonium sulfate directly into the protein mixture as a powdered solid during large-scale purification processes so that the effect of dilution by the salt solution is minimized. If a saturated salt solution is employed, the amount of ammonium sulfate solution added must be recorded accurately; often this is achieved by dispensing from a graduated pipette. It is critical to avoid nonuniformity in the salt concentration during the addition of the salt solution by thorough mixing. Localized concentration "hot spots" will prematurely initiate the precipitation of other proteins and inadvertently affect the precipitation process. Record the volume of the saturated ammonium sulfate solution required to precipitate the protein of interest. Also note that protein precipitation is not instantaneous; it may require more than 30 min to equilibrate.

    Other protein precipitation techniques: There are numerous options to effect other types of protein precipitation including (but not limited to) acetone precipitation (useful to simultaneously eliminate acetone-soluble components and increase protein concentration), ethanol precipitation (useful to simultaneously concentrate proteins and remove traces of GuHCl prior to SDS-PAGE analysis), acidified acetone/methanol (50/50 v/v; useful to simultaneously remove acetone and methanol soluble interferences such as the detergent SDS prior to analysis), and chloroform/methanol (50/50 v/v; useful to simultaneously remove salt and detergents) [19].

19. For many downstream processes to avoid protein degradation, it is important to desalt and to remove/inhibit proteases (*see* Chapter 6) either by dialysis or size exclusion chromatography (desalting column). Alternatively, this salt-rich preparation can be applied directly onto a hydrophobic interaction chromatography column, followed by a size exclusion column to effect purification and simultaneously remove precipitating salt. The high salt exposes *hydrophobic* regions and helps proteins to bind to hydrophobic resins (*see* **Note 17**).

20. Three-phase fractionation and derivatives such as ionic liquid-based three-phase partitioning are effective purification methods both singularly and as part of a combination approach [21–25]. Three-phase partitioning utilizes saturated, buffered ammonium sulfate in combination with an equal volume of

water-miscible aliphatic alcohol, commonly *t*-butanol. The two-phase liquid can be used to partition proteins from whole cells without the need for a separate cell lysis step. This process can be readily scaled up for larger-scale protein production and precipitation.

21. Although *t*-butanol is miscible with water, it is insoluble in aqueous solutions having high concentrations of salt, especially ammonium sulfate solutions. Three phases separate during centrifugation. The upper phase contains t-butanol, the lower layer is aqueous, and the protein precipitate settles out as a middle layer.

    A number of refinements of the basic method have been designed in recent years that incorporate sonication, ultrasound treatment, and aeration, for example, the liquid triphasic flotation system [23] and microwave-assisted multiphase partitioning [24]. A useful review of TPP and a range of protein precipitation techniques is provided by Goldring [25].

22. Expression of insoluble recombinant proteins has been noted as being greater than 30% of all recombinant proteins [26].

23. Protein solubility depends on interactions between protein molecules and between the protein and the solvent. Hence, additions or subtractions to a protein solution should be carefully assessed on a small scale [26].

24. The separation of one protein, or family of proteins, from other proteins by means of differential solubility with chemical reagents is based on the protein's differential affinity between a liquid phase and a solid phase. The optimization of this procedure is empirical, but Lindwall and colleagues [26] outline an optimization procedure based on a sparse matrix approach; solubilization buffers are composed based on "solubility space" which is related to accepted protein solubilization theories. This method assists in identifying suitable solubilization conditions for most overexpressed proteins.

25. Membrane proteins require detergents for solubilization during isolation to maintain solubility. Nonionic detergents such as Triton X-100 are less effective in solubilizing hydrophobic proteins [28]. An initial detergent screen may be required and can be performed to determine the optimum conditions for both solubilization efficiency and maintenance of protein function. During solubilization, a membrane protein is transferred from its natural environment to a buffered detergent solution.

26. It is important to remove aggregates (**step 8**) that can act as nuclei to trigger aggregation during folding. The addition of a mild solubilizing agent (e.g., 1 M 3(1-pyridinio)-1-propane

sulfonate) during the refolding steps limits reaggregation of proteins as they refold. For further information, *see* ref. [28] which examines the effect of additives on recombinant protein refolding.

27. For extraction of membrane proteins, it can be useful to isolate a membrane preparation. In brief, a typical membrane preparation protocol is as follows. Wash cells of interest three times with ice-cold PBS, and then pellet at 500 × *g* for 10 min at 4 °C. Discard the supernatant and resuspend the cells in 2 mL hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1× protease inhibitor cocktail containing 50 mM NaF, 2 mM Na_3VO_4, 1 mM $Na_4P_2O_7$). Sonicate cells on ice for 1 min. Spin the lysate at 3000 × *g* for 15 min at 4 °C, to remove cell debris. Transfer the supernatant to an Optiseal™ Polyallomer tube (Beckman 362185), and fill the tube with the lysate-rich hypotonic buffer (fill volume – 5 mL) and centrifuge at 100,000 × *g* for 1 h at 4 °C (in a NVT90 rotor using the Optima L-100 XP Ultracentrifuge, Beckman Coulter). After centrifugation, remove the supernatant and resuspend the membrane pellets in 50 μL MES buffer (25 mM MES, 150 mM M NaCl, 1% Triton X-100, pH 6.5). Finally, to aid complete solubilization, sonicate the sample on ice, using 10 s bursts.
28. The amount of time necessary for solubilization may be less than this and can be optimized to as little as 1 h once an appropriate detergent has been selected.
29. Detergent screening can be further facilitated if the recombinant protein of interest is tagged with Green Fluorescent Protein (GFP).
30. The final protein concentration should not exceed 0.05–0.1 mg/mL as dilute protein mixtures refold optimally at this concentration. A rapid and efficient mix is essential at this step. Literature descriptions of the fundamental refolding processes are available; *see* [28] and [30] for comprehensive and accessible reviews of refolding solubilized proteins. Cited protocols range from empirical to rationally designed [31]. Alternatively, an open source collection of protocols, *REFOLD*, is available at http://refold.med.monash.edu.au/. These are practitioner-derived standard protocols supplemented with hands-on practical considerations.
31. The addition of a mild solubilizing agent (e.g., 1 M 3-(1-pyridinio)-1-propane sulfonate) during the refolding steps limits reaggregation of refolding proteins. For further information on this, Tsumoto and co-workers [28] comprehensively detail the effect of small molecule additives on refolding along with other considerations for refolding recombinant proteins.

32. *Dialysis during protein refolding:* Continual dialysis can be set up by using a gradient maker (Fig. 2). In its simplest form, this consists of two containers connected by a siphon. One container contains the low concentration buffer, and the other contains high concentration buffer. The buffer is withdrawn from the low concentration container to the high concentration container. This will produce a linear gradient from high to low buffer concentrations. Once the "low concentration buffer" supply has been depleted, the dialysis tubing is removed from the larger vessel and placed in a similar, clean vessel containing fresh buffer at the same concentration as the original "low concentration buffer." The "low concentration buffer" vessel is replaced with a vessel containing buffer at the next lower concentration level, and the process is allowed to continue until the "low concentration buffer" supply is depleted again. This process is repeated until the vessel containing the dialysis tubing has reached the desired final concentration, typically 0 M guanidine HCl.
33. Recently, the awareness of inclusion bodies as a source of useful recombinant proteins has been growing, and technologies for separation and refolding have improved. Two informative and comprehensive reviews were recently published [31, 39].
34. *Storage of purified proteins:* Most proteins can be stored at 4 °C, without significant denaturation, for up to 24 h. For intermediate storage times (24 h to 1 week), the protein should be filter sterilized (through a 0.22 μm filter) and stored at 4 °C. For storage times greater than 1 week (up to several months), it is advisable to freeze the protein preparation. Rapid freezing helps reduce protein denaturation. It is useful to freeze the solution in small aliquots to avoid repeated freeze/thaw cycles which may reduce the biological activity of the protein. Stabilizing agents can also be added prior to freezing, such as glycerol (5–50% w/v), reducing agents (such as 1–2 mM DTT), and ligands/co-factors (depending on the nature of the target protein). Extended protein storage (several months to years) should be carried out at −80 °C or in liquid nitrogen. The addition of 50% (v/v) glycerol is recommended for storage at this temperature. Alternative strategies include storing the protein as an ammonium sulfate precipitate at 4 °C or at lower temperatures in a lyophilized form (*see* also Chapter 19 for protocols and discussion regarding the storage and lyophilization of proteins).

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Chapter 18

Protein Stability: Enhancement and Measurement

Ciarán Ó'Fágáin

Abstract

This chapter defines protein stability, emphasizes its importance, and surveys the field of protein stabilization, with summary reference to a selection of 2014–2021 publications. One can enhance stability, particularly by protein engineering strategies but also by chemical modification and by other means. General protocols are set out on how to measure a given protein's (i) kinetic thermal stability and (ii) oxidative stability and (iii) how to undertake chemical modification of a protein in solution.

Key words Protein stability, Thermal stability measurement, Oxidative stability measurement, Protein engineering, Directed evolution, Rational mutations, Chemical modification, PEGylation

1 The Importance of Protein Stability and Protein Stabilization

1.1 Protein Stability Is Vital

The usefulness of enzymes and proteins as analytical tools and as industrial catalysts is often limited by their requirements for "mild" reaction conditions. Deterioration of protein preparations over extended storage periods (i.e., a limiting "shelf life") is another serious drawback. Protein stability has great practical consequence throughout the biotechnology industry and is an important topic of interest for protein scientists. Even more importantly, however, biological fitness and evolution may depend on protein stability. In order to function, a protein must fold into a stable native structure and remain in that folded state under physiological conditions. Thus, a protein must attain a minimal stability threshold to function and evolve. Higher stability makes a protein more robust to the effects of mutations, since a given mutation is less likely to cause the protein to fall below the minimum stability threshold. Such a protein can tolerate a wider range of mutations (potentially beneficial but also perhaps destabilizing) while still maintaining its native structure. In this way, extra stability promotes evolvability [1].

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Other works have shown that decreased stability of any protein coded by an essential gene could lead to a lethal phenotype. Zeldovich et al. [2] demonstrate a relationship between mutation rate, genome size, and thermodynamic changes in proteins due to point mutations. Molecular evolution rates are subject to a universal "speed limit," above which populations become extinct due to lethal mutagenesis. For mesophiles, this critical rate is approximately six mutations per essential part of genome per replication; for thermophiles, it is one to two mutations per genome per replication. This relationship has implications for populations as well as for individuals: RNA viruses are close to the speed limit, while DNA viruses and many bacteria are well below it [2].

Kurahashi et al. [3] examined the protein activity/stability relationship in random mutant libraries of an esterase from the hyperthermophilic archaebacterium *Sulfolobus tokodaii*. Nearly 20% of the variants had higher activity than wild type (i.e., 20% evolvability). Evolvability depended on the stability of template proteins during these evolutionary processes. In addition, decreased activity could be recovered during evolution by maintaining the stability of variants. These findings indicate that evolutionary protein sequence space can expand during near-neutral evolution, where mutations slightly worsen activity but are seldom fatal for stability [3].

Rinaldi et al. [4] combined computational and experimental methods to investigate the structural-stability/functional-plasticity balance in homologous limonene-1,2-epoxide-hydrolases. They noticed the presence of a few highly conserved stabilization determinants that enabled structural stability linked to biological functionality, despite significant variations in sequence [4].

Two key factors that dictate the speed of changes in a protein sequence are the protein's folding stability and its tendency to aggregate. Considering the hydrophobic polar (HP) model together with Zwanzig-Szabo-Bagchi rate theory, Agozzino and Dill [5] propose the following:

(i) Selection pressure quickens adaptation.

(ii) Less stably folded proteins adapt the fastest, because they have the steepest fitness landscapes (cells should adapt more quickly under warmer rather than cooler conditions, because heat destabilizes folded proteins).

(iii) Evolution (the substitution rate in the sequence) slows as protein abundance increases: since a typical protein is not perfectly fit, a greater copy number of copies decreases the cell's fitness.

(iv) Chaperones can lessen this abundance effect and speed up evolution by enhancing protein stability ("evolutionary capacitance") [5].

A protein's evolutionary fitness will also depend on its energetic properties. Crippa et al. [6] studied the stabilizing interactions among 890 proteins, spanning 5 families, and how these had evolved over billions of years. A reconstruction algorithm enabled dating of the reconstructed proteins and calculation of the interaction network between their amino acids through a co-evolutionary approach. Special attention was paid to the networks of (i) most strongly attractive contacts and (ii) poorly optimized, frustrated contacts. Results suggested that the cluster of most attractive interactions extends its size along evolutionary time [6].

Newton et al. [7] have reviewed the use of big data and high-throughput methods to elucidate the evolutionary processes that resulted in the huge functional diversity of present-day enzymes. In addition to gene duplication and divergence, gene loss has been important in enzyme evolution. Despite the startling remark that "real-world enzymes are sloppy and mediocre," new and emerging knowledge of these "real-world" enzymes will inform protein engineering and synthetic biology into the future [7].

1.2 Definition and Measurement of Protein Stability

The term *stability* refers to a protein's resistance to adverse influences such as heat or denaturants, that is, to the persistence of its molecular integrity or biological function in the face of high temperatures or other deleterious influences. A folded, functional monomeric protein can lose its biological activity in vitro by *unfolding* of its tertiary structure to a disordered polypeptide, in which key residues are no longer aligned closely enough for continued participation in functional or structure-stabilizing interactions. Such unfolding is termed *denaturation*. It is usually cooperative and may be reversible if the denaturing influence is removed, since the polypeptide chain has not undergone any chemical changes [8].

In addition to unfolding, an isolated protein in an aqueous system can suffer various adverse reactions over time (due to physical, chemical, and biological factors) that lead to an irreversible loss of activity or *inactivation* [8]. Typical physical phenomena are aggregation [9, 10] and precipitation, while an individual chain "attempting" to refold may enter an incorrect, kinetically trapped conformation from which it cannot escape. There are many deleterious chemical reactions involving the side chains of amino acid residues, notably asparagine, aspartic acid, and cysteine/cystine [9–11] or the glycation of lysine residues with reducing sugars (the Maillard reaction) [12, 13]. Biological deterioration can result from loss of an essential cofactor or from the action of proteolytic enzymes, either endogenous or arising from microbial contamination. An unfolded, extended polypeptide will be much more prone to proteolysis than a tightly packed, globular protein [14].

These different molecular phenomena give rise to two distinct definitions of in vitro protein stability. These are *thermodynamic* (or *conformational*) stability and *long-term* (or *kinetic*) stability.

Table 1
Indices of protein stability

Parameter	Measures	Constraints	How estimated	References
T_{50}	Temperature of half-inactivation (over a fixed time)	None	$\text{Activity}_{\text{Elevated temperatures}}/\text{Activity}_{\text{"Normal" temperature}} \times 100\%$	–
% Activity	Residual activity at time t (at a fixed temperature)	None	$\text{Activity}_{\text{time } t}/\text{Activity}_{\text{time } 0} \times 100\%$	[15]
Half-life	Time to reach 50% activity	First-order decay	$0.693/k$ (k = first-order rate constant)	[15]
Accelerated degradation	Predicts lifetime at a given temperature	First-order decay at all temperatures used	Extrapolation of plot $\ln k$ v $1/T$ (Kelvin)	[17–19]
T_m	"Melting" temperature	None	Temperature where unfolding = 50%	[20]
$\Delta G_{(25\ ^\circ C)}$	Conformational stability	Two-state unfolding	Thermal denaturation curve	[21]
$\Delta G_{(H2O)}$	Conformational stability	Two-state unfolding	Denaturant unfolding curve	[21, 22]
T_S	Temperature of maximum stability	Two-state unfolding	Stability curve	[23]

Thermodynamic stability concerns the resistance of the folded protein conformation to denaturation (i.e., its Gibbs free energy of unfolding) while long-term stability measures the resistance to irreversible inactivation (i.e., persistence of biological activity under adverse conditions of temperature, pH, solvents, salt concentration, and so on). Both types can be represented in a single scheme [15, 16]:

$$\mathrm{N} \overset{K}{\leftrightarrow} \mathrm{U} \overset{k}{\rightarrow} \mathrm{I}$$

where N stands for the native, U for the unfolded (denatured), and I for the irreversibly inactivated forms of the protein. K is the equilibrium constant for the reversible N ↔ U transition while k is the rate constant for the irreversible U → I reaction. The N ↔ U transition corresponds to thermodynamic stability while the U → I reaction represents long-term (kinetic) stability. Table 1 sets out the main features of various indices of protein stability. Measurement of the Gibbs energy value is beyond the scope of this chapter, but *see* Table 1 (and its references) (*see* also **Note 1**).

Kinetic stability is distinct from (and needs not to correspond with) *thermodynamic* stability. It involves measuring the persistence of catalytic (or other biological) activity with time under adverse conditions of temperature, pH, solvents, salt concentration, and so on (or, to put it another way, the progressive loss of function). It can be represented by the scheme

$$\mathrm{N} \xrightarrow{k_{\mathrm{in}}} \mathrm{I}$$

where N is the native, functional protein, I is an irreversibly inactivated form, and k_{in} is the rate constant for the inactivation process. The equation $V_{\mathrm{in}} = -d[\mathrm{N}]/dt = k_{\mathrm{in}}[\mathrm{N}]$ describes the process mathematically, where V_{in} is the experimentally observed rate of disappearance of the native form [15]. Experimentally, determination of kinetic stability (usually equated with the molecule's long-term stability) [15] is fairly simple: *see* Subheading 3.2. If the loss of activity with time fits a single exponential function (i.e., if a plot of ln(activity) versus time is linear), then one may use the first-order rate constant k to calculate a true half-life (*see* **Note 2**).

1.3 Scope of Review

The following sections attempt to give a very selective overview of developments during recent years in the field of protein stability/stabilization (*see* **Note 3**). Emphasis is mainly on protein engineering strategies (i.e., the use of genetic manipulation techniques), but some protein stability enhancements achieved by chemical modification are also described. In addition, some other methods for protein stability measurement are outlined (*see* Table 2). Immobilization methods for enzyme stabilization have been reviewed in detail elsewhere [36].

1.4 A Stability-Activity Trade-Off?

There are many examples where proteins are "forced" to compromise between stability and optimal activity/function (e.g., [37]). Contrariwise, some reports describe improved function *and* stability obtained by directed evolution strategies (e.g., [38]). Why does this apparent stability-activity trade-off apply in some cases but not in others? The notion of polarity may provide an explanation.

First, we must distinguish two aspects to the evolvability of proteins, namely, robustness and innovability. Robustness refers to neutral mutations that do not affect the protein, while innovability concerns mutations that lead to new functions. Dellus-Gur et al. [39] propose the term *polarity* as an explanation for innovability. A protein shows polarity if it has a highly ordered framework or scaffold together with an active site made up of flexible, loosely packed loops. In studies of a TEM-1 beta-lactamase, Dellus-Gur et al. noted greater scaffold rigidity in stabilized variants—but no changes to the conformational plasticity of the active site loops. The stabilizing mutations had no adverse effect on TEM-1's original activity but did enable it to adapt to new substrates (i.e., they

Table 2
Additional techniques for protein stability measurement

Technique	Protein studied	Remarks	References
1D (one-dimensional) H-1 nuclear magnetic resonance (NMR) spectroscopy	Antibody therapeutics, in their final formulations	Uses H-1 nuclear magnetic resonance (NMR) spectroscopy for in situ study of both antibody (Ab) protein and small-molecule content and stability in high-concentration (100 mg/mL) formulations. Changes in 1D H-1 NMR signal intensity indicate protein degradation (aggregation or fragmentation). Apparent relaxation rates are specifically sensitive to Ab fragmentation. In addition, relaxation-filtered spectra respond to the presence and degradation of small-molecule excipients and to changes in solution properties (e.g., pH). Thus, H-1 NMR spectroscopy can give (i) an overview of biopharmaceutical formulation content and stability and (ii) an indication of degradation and (iii) enable triaging of additional analytical techniques	[24]
Direct in vivo monitoring of protein stability in cells	Human islet amyloid polypeptide and histone H3K4 methyltransferase	Describes an in vivo stability biosensor: a protein of interest (POI) is inserted into a microbial enzyme (CysGA) that catalyzes formation of endogenous fluorescent compounds, correlating POI stability to fluorescence readouts. The biosensor was used in (i) directed evolution to obtain stabilized variants of two POIs that were less prone to aggregation (including non-amyloidogenic variants of human islet amyloid polypeptide) and (ii) deep mutational scanning of the stability-related contributions of all residues of a histone H3K4 methyltransferase's catalytic domain	[25]
Mass spectroscopy-HPLC	Therapeutic PEGylated protein	Describes a novel ultrahigh performance liquid chromatography-high-resolution mass spectrometric (UHPLC-HRMS) assay to quantify a therapeutic PEGylated protein in monkey serum and to monitor its potential instability in vivo and methionine oxidation The method uses (i) a surrogate peptide, DCP(SS), with an intact disulfide linkage between two peptide sequences that are essential for drug function but may be prone to potential in vivo cleavages and (ii) a disulfide-free confirmatory peptide, CP. DCP(SS) and CP each have one Met and the method could also detect Met oxidation	[26]

Atomic force spectroscopy (AFS)	Immobilized proteins	Uses atomic force spectroscopy (AFS) to indirectly characterize proteins immobilized on porous materials and to observe the immobilization process in real time. Mechanical properties of porous agarose microbeads (for immobilizing proteins) could be monitored by indenting a colloidal probe (silica microparticle) into a single bead. AFS indicated altered stiffness of an agarose microbead's solid matrix upon protein binding. Irreversible and multivalent immobilizations that stiffened the microbeads also stabilized the immobilized proteins against heat	[27]
96-Well microtiter plates	Bovine and equine cytochrome c and bovine serum albumin	Uses microplate reader fluorimeter to monitor Trp fluorescence at 340 nm. High-throughput method uses serial additions of denaturant to a single well; more accurate medium-throughput method uses one well per denaturant concentration. Both methods give correct protein stability rank orders	[28]
Differential scanning fluorimetry	Hen egg lysozyme and pig heart citrate synthase	A dye compound shows increased fluorescence on binding to newly exposed hydrophobic regions of an unfolded protein. Thermal unfolding is conducted and monitored in a real-time PCR machine. A preferred dye is SYPRO Orange. It requires single-step transition but only small amounts of protein (75 $\mu g/mL^{-1}$). It allows screening of potentially stabilizing ligands. Detailed procedural instructions are given	[29]
Differential scanning fluorimetry	Y220C oncogenic mutant of p53 tumor suppressor protein	p53 is only marginally stable and many oncogenic mutants, including Y220C, are very unstable. This study tested the stabilizing effects of small ligands on the Y220C protein. SYPRO Orange dye was used to bind to 10 μM protein samples at scan rate 270 K/h due to irreversibility of the unfolding. Thermal unfolding was also followed by differential scanning calorimetry (scan rate 250 K/h) and unfolding kinetics by time-dependent Trp fluorescence	[30]
SPROX (stability of proteins from rates of oxidation)	Four model proteins (RNase, ubiquitin, cyclophilin A, carbonic anhydrase)	H_2O_2 (at constant concentration) oxidizes proteins, in presence of increasing concentrations of a chemical denaturant, for a given time before quenching. Extent of oxidation (irreversible, unlike H/D exchange) is determined by mass spectrometry. Underlying assumptions are set out. Oxidation rates are consistent with Met oxidation to sulfoxide. It allows analysis of proteins in complex mixtures. It is also applicable to protein-ligand complexes	[31]
High-throughput stability analysis using yeast surface two-hybrid system	Fibronectin type III domain	Combines yeast surface two-hybrid and fragment reconstruction methods to quantify protein stability. Can estimate tolerance of amino acid substitutions at a single position by analysis of a randomized library. Uses Trp fluorescence to probe denaturation by guanidine thiocyanate	[32]

(continued)

Table 2
(continued)

Technique	Protein studied	Remarks	References
Nuclear magnetic resonance	Nine test proteins. Software validated against five sample proteins	Defines and calculates an autocorrelation function $C(\omega)$ of the one-dimensional proton NMR spectrum. The value of $C(\omega)$ at 0.5 ppm differs between folded, partly folded, and random coil proteins: value is >0.5 for folded and <0.4 for partly or unfolded proteins. No isotope labeling is required	[33]
Pulse proteolysis	*E. coli* Rnase H (and variants I53A I53D) and ligand binding to maltose binding protein	Unfolded proteins are much more prone to proteolysis than folded proteins. Pulse proteolysis (using excess thermolysin over timescale of minutes) follows overnight incubation of target protein in various urea concentrations. Only the unfolded fraction is digested. Proteolysis is quenched with EDTA and normal SDS-PAGE is used for separations. ΔG values for unfolding agree with those obtained by other methods. May be a useful high-throughput method and can be used on crude lysates, although suitable pulse times and conditions need to be ascertained. 30–500 μg sample may suffice	[34]
Capillary isoelectric focusing with whole-column imaging detection (CIEF-WCID)	β-Lactoglobulin B (pI 5.2), trypsin inhibitor (pI 4.5), phosphorylase b (pI 6.3), and trypsinogen (pI 9.3), also with phosphatidyl choline (zwitterionic) and phosphatidyl serine (acidic)	Denaturation at 60 °C yields altered CIEF profile, with fast separations, for all four proteins. The two phospholipids interact with the proteins in different ways. Image of entire column is detected by a camera. The phospholipids form vesicles which can influence stability	[35]

improved its innovability). Thus, in TEM-1 at least, robustness and innovability are not in conflict, and one can improve both attributes. Polarity, therefore, means that stabilizing mutations need not to trade off with activity function. Instead, polarity can enable the active site portion(s) of the protein to evolve new activities while the stabilized scaffold can withstand mutational effects. TIM barrel proteins, for example, show polarity: they embody a variety of functions (divergence within active site sequences) on a consistent, rigid scaffold. Dihydrofolate reductase, on the other hand, does not allow such divergence: at least 60% of its active site residues are located on the folded scaffold [39].

A study on TEM-116 beta-lactamase inserted "rescue" mutations into shortened, destabilized polypeptide chains. A library of TEM-116 mutants, "clipped" by 15 N-terminal and 3 C-terminal residues (Bla-NΔ15CΔ3), underwent activity selection and DNA shuffling [40]. The best-performing clone to emerge from this process was less prone to guanidine-induced unfolding, had a 5.3 °C higher T_m value, and displayed notably greater activity at elevated temperature than any of the input proteins. This "best clone" contained eight mutations and its crystal structure gave insight into the stabilizing factors. Next, progressive reincorporation of the "missing" N- and C-termini successively increased stability to heat, denaturant, and proteolysis until a full-length enzyme was attained. Compared with wild-type TEM-116, the T_m value of this full-length multiple mutant was 5.3 °C higher while its half-denaturation concentration for guanidine had increased from 0.53 to 1.75 M. The strategy, which involves iterative cycles of truncation-optimization, shows that second-site suppressors which "rescue" the stability of a "perturbed" protein which can also benefit the stability of the corresponding full-length enzyme [40].

Kurahashi et al. examined the activity-stability relationship during random mutational drift of an esterase from *Sulfolobus tokodaii* (a hyperthermophilic archaeon) [41]. They noted that numerous variants with improved activity emerged from highly stable "parent" proteins—but a few highly active mutants came from less stable "parents." They put forward the idea of robustizability (or stabilizability), namely, the frequency of appearance of variants that are more stable than their parent protein. Robustizability shows a positive correlation with parental activity but a negative correlation with parental stability [41].

Another study of the protein evolution/stability relationship subjected the thermophilic bacterial esterase of *Alicyclobacillus acidocaldarius* (Aac-Est) to multiple rounds of random mutational drift at high and low temperatures [42]. In the first round of mutation, few Aac-Est variants showed increased activity at 65 °C, indicating that wild-type Aac-Est sits at the peak of a mountain in a fitness landscape for activity at high temperature. To gain higher

activity than wild type, Aac-Est must descend that mountain and climb another, higher mountain. In the second and third rounds of mutation from lower-active templates, evolvability (the proportion of variants with higher activity than their parent protein among all the variants of a given generation) depended on the template proteins' stability. Compared with wild type, the stability-maintaining template could recover more activity. Thus, a low--activity/high-stability variant can drift considerably in sequence space and reach the base of a higher mountain. Meanwhile, random mutations in stability-loss templates yielded several variants with greater activity at 40 °C than those from wild type, via cold adaptation [42].

Stimple et al. [43] focused on activity/stability trade-offs in enzymes and antibodies, two key categories of proteins with numerous applications. Ways of overcoming these activity/stability trade-offs, both experimental and computational, are also considered [43]. A detailed study of activity-stability trade-offs describes how these occurred and were overcome by rational approaches, in the laboratory evolution of a bacterial phosphotriesterase designed to act against different nerve agents [44].

Rivoire et al. [45] consider a possible link between evolvability and allostery in proteins—two very different properties. They propose that evolutionary selection promotes "exquisite discrimination," namely, the ability to bind to very similar ligands with different discriminations. Often, the capacity for protein conformational changes is needed to gain this discriminating ability, and this capacity depends on a group of sites that extend from the binding site. Long-range effects give the protein a latent potential for evolutionary adaptation and allosteric regulation [45].

In vivo, the role of chaperones in relieving the effects of destabilizing mutations (hence enabling sequence diversity) has been explored. In a study of poliovirus evolution, the Hsp90 chaperone protein offset evolutionary trade-offs between protein stability and aggregation. Hsp90 influenced the sequence landscape at both protein *and* RNA levels to reconcile competing constraints of protein stability, aggregation propensity, and translation rate on protein biosynthesis [46].

In a 2018 review, Finch and Kim [47] outline the promise of thermophilic proteins as useful scaffolds for protein engineering. Their high inherent thermostability gives these proteins notable mutational robustness. Studies on the evolvability of thermophilic versus mesophilic proteins strongly support the view that thermophilic proteins are the more evolvable, suggesting that thermophilic proteins could be the scaffold of choice for future protein engineering [47].

1.5 Protein Engineering for Enhanced Stability

1.5.1 Directed Evolution

The pivotal importance of directed evolution strategies in the development of novel, improved, and stabilized proteins was recognized by the award of the 2018 Nobel Prize in Chemistry to FH Arnold, GP Smith, and GP Winter for the directed evolution of enzymes and for the phage display of peptides and antibodies (https://www.nobelprize.org/prizes/chemistry/2018/summary/).

An experimental strategy for directed evolution of enzymes would often follow the following steps:

1. Identify a suitable starting enzyme for the chosen task.
2. Construct a DNA sequence library that will cover key subsets of protein sequence space.
3. Devise selection criteria that will enable detection of new/-enhanced functions and methods to select optimized enzyme variants.
4. Re-diversify the genes to create new DNA sequence libraries around the sequences from the first selection to cover new subsets of sequence space.
5. Establish selection criteria that are more rigorous, demanding, and stringent than those employed in the preceding round (**step 3**) and so on for as many rounds of in vitro evolution as are needed to reach the desired level of enzyme performance [48] (https://www.nobelprize.org/uploads/2018/10/advanced-chemistryprize-2018.pdf).

Useful reviews of the field of directed evolution focus on ultrahigh-throughput methods [49], fast screening methods [50], machine learning [51], and evolutionary development of enzymes for (i) biocatalysis [52] and (ii) synthesis of small-molecule pharmaceuticals (including chiral alcohols and amines) [53]. Trudeau and Tawfik review developments in bioengineering and molecular evolution and suggest pathways toward the best starting points in order to achieve efficient and robust enzymes (i.e., how to pick the best starting point(s) for a directed evolution campaign). It seems that the most "engineerable" enzymes are stable, broadly specific, and robust to mutations [54].

1.5.2 Ancestral Sequence Reconstruction

The inference and construction of ancestral protein sequences (i.e., presumed evolutionary intermediates and relatives, based on comparisons of present-day proteins, and termed ancestral sequence reconstruction or ASR) can give notable insights into the evolution of proteins, for example, factor VIII blood coagulation protein [55, 56]. Variable/enhanced stabilities, improved/novel activities, and other changes to key protein properties have been discovered in such studies (e.g., [57]). Although a detailed consideration of ASR

is beyond the scope of this chapter, the interested reader should note two informative recent reviews of this fascinating field [58, 59].

1.5.3 Inference and Prediction

Many computer-based bioinformatic approaches have been brought to bear on protein stability problems, especially to the prediction of the effects on stability of amino acid changes in engineered proteins. A detailed discussion is beyond the scope of this chapter but two recent developments are summarized below.

Goldenzweig et al. [60] developed a mutational prediction algorithm and applied it to human acetylcholinesterase (hAChE, an enzyme involved in synaptic transmission) that recombinantly expresses only from mammalian host cells. A designed 51-mutant hAChE variant was notably more thermostable, with no change in enzymatic properties—and expressed at 2000-fold higher levels than wild-type type from *E. coli* cells. Similar encouraging results were obtained for four other proteins tested. The algorithm is available at http://pross.weizmann.ac.il and needs only a three-dimensional structure plus numerous sequences of natural homologs for use [60].

Focusing on alpha-/beta-hydrolase-fold enzymes, Jones et al. [61] compared five mutational strategies, previously used on this type of protein, to identify stabilizing mutations in salicylic acid binding protein 2, a member of this group. All five strategies (random mutagenesis via error-prone PCR, computational design approaches using Rosetta and FoldX, mutation of flexible regions, addition of Pro at locations where it occurs in a more stable homologue, consensus mutation) identified stabilizing mutations, but consensus mutation yielded the best balance of success rate, degree of stabilization achieved, and ease of use [61].

1.5.4 Rational Mutations

Antibodies and their derivatives have huge value in diagnostics and therapeutics, but their applications are often limited by instability. Ma et al. [62] converted a single-chain variable fragment (scFv) antibody to a single-chain antibody fragment (scAb) with notably improved stability characteristics. This scAb retained antigen-binding activity at elevated temperature (up to 60 °C), in guanidine hydrochloride (up to 1 M), and on storage at 37 °C for 6 months. However, there was limited improvement when the original scFv was converted to a larger fragment antigen-binding (Fab) format. Certain Cys-to-Ala mutations in the third complementarity-determining region of the antibody heavy chain (CDReH3) also yielded stability improvements [62].

At present, antibody-based/antibody-derived therapeutic proteins are not given by mouth, as they cannot withstand passage through the stomach (due to the presence of HCl and pepsin). To overcome this drawback, Wicke et al. [63] developed

"gastrobodies" on a protein scaffold derived from Kunitz soybean trypsin inhibitor (SBTI). SBTI is very resistant to digestive proteases, low pH, and bile acids. Informed by computational methods, they randomized two loops in the protein scaffold so as to create a binding site. Phage display techniques enabled selection of gastrobodies that bound to the glucosyltransferase domain of *Clostridium difficile* toxin B (GTD) with nanomolar affinity and inhibited the enzyme. These anti-GTD gastrobodies maintained the parent scaffold's resistance to heat, acid, and digestive proteases. Gastrobodies could point the way to the development of protein therapeutics resistant to digestive tract conditions [63].

A protein may need to tolerate adverse factors other than those discussed thus far. Chemical or oxidative stability can be of great interest and importance. Valderrama et al. [64] achieved notable improvements in the resistance of iso-1-cytochrome *c* to oxidation by following a redox-based design strategy. This heme-containing protein can react with hydrogen peroxide but is vulnerable to oxidative inactivation by excess peroxide substrate. Introduction of five mutations (N52I, W59F, Y67F, K79A, F82G) endowed the protein with full stability against catalytic peroxide concentrations but with a total turnover number 15 times greater than the native protein. Their study clearly shows that rational changes to the intramolecular electron transfer network can prevent suicide inactivation of a heme peroxidase. Note that most of the changes involve the substitution of the chemically reactive side chains of Asn, Trp, Tyr, and Lys [64].

Stability at extremes of pH can be important in industrial situations, especially where cleaning-in-place of chromatography media must take place between purification runs. Protein G, a widely used affinity chromatography ligand for antibody purification, needs to withstand passage of caustic alkali (0.5 M NaOH) solutions. Palmer et al. [65] achieved an eightfold gain in alkaline stability of protein G by replacing three Asn residues. Electrostatic calculations indicated that deprotonation at high pH of Tyr, Lys, and Arg residues would be destabilizing. Incorporation of the further triple mutation Y3F/T16I/T18I gave a further stability gain (6.8 cal/mol), and the resulting protein G unfolded at around pH 13, 1.5 units higher than wild-type type.

Kinetic stability is particularly important for industrial enzymes such as lipase, a broadly specific (and, hence, very versatile) enzyme. Rodriguez-Larrea et al. [66] studied heat denaturation of wild-type *Thermomyces lanuginosa* lipase, of four single-site mutants and of two very stable multiple-site mutants. Denaturation was two-state, irreversible, and kinetically controlled in all cases. Mutations greatly affected activation enthalpy and entropy but not the kinetic urea *m*-value. Authors concluded that the mutations affected some structural feature of the transition state for irreversible denaturation that is not related to solvent accessibility changes. They went on to

propose that a solvation barrier (due to a time separation between the breaking of internal contacts and penetration by water) may contribute to the *T. lanuginosa* lipase's stability [66].

Membrane proteins are often challenging research targets due to their immersion in a hydrophobic lipid bilayer instead of in aqueous surroundings. Minetti et al. reviewed work on membrane protein folding and stability. Representative examples of α-helical and β-barrel structures, viral receptors, and pore-forming toxins are discussed, as are techniques used in the field [67].

1.6 Chemical Modification, Crosslinking, and Conjugation to Polymers

L-Asparaginase is used to treat acute lymphoblastic leukemia but it has side effects and a short half-life in the bloodstream. Chemical modification of L-asparaginase can relieve some of these drawbacks, and PEGylated forms are available. In an alternative approach, L-asparaginase was chemically modified with carboxymethyl dextran under mild conditions using a carbodiimide crosslinker. The "dextranized" form had higher specific activity and stability than the unmodified enzyme. Chemical and structural methods confirmed the chemical modification and indicated conformational changes in the modified form [68].

A commercial lipase from *Candida cylindracea* was "dextranized" by conjugating the s-amines in lysine residues with carbonyl groups, to hydrophilize the lipase's microenvironment in the presence of organic solvents. Following modification with dextran at pH 8.0, the lipase was more stable in 25% (v/v) dimethyl sulfoxide (DMSO), ethanol, 2-propanol, toluene, and isooctane [69].

Phospholipase C (PLC) has many potential applications, but it has poor thermal stability and is costly to produce. A PLC gene from *Bacillus cereus* HSL3 was overexpressed in *E. coli*, and the recombinant protein was modified with methoxypolyethylene glycol-succinimidyl succinate (SS-mPEG, MW 5000). The free enzyme showed maximum activity at 80 °C and was quite thermostable at 40–70 °C. SS-mPEG-PLC complex had greater thermal stability at 70–80 °C, and the catalytic efficiency (k_{cat}/K_m) increased 3.03-fold versus free PLC. Circular dichroism spectral analysis indicated structural changes in SS-mPEG-PLC. The presence of SS-mPEG chains on the enzyme surface altered substrate binding, leading to improved catalytic efficiency [70].

Ionic liquids (ILs) provide a nonaqueous medium that can be used for enzyme catalysis. Their use as additives and solvents in protein applications, and their effects on protein stability and refolding behavior, has been reviewed (e.g., [71]). Xu et al. applied ILs in a different way. Using carbonyldiimidazole, they chemically modified *Candida antarctica* lipase B (CALB) with four types of chiral amino acid ionic liquids (AAILs; containing N-acetylated Pro in D- or L-form). The degree of modification of available CALB free amino groups ranged 35–48%, and relative to native, hydrolytic activity increased by up to 89% in the modified forms. For all

modified CALBs, thermostability and tolerance of the organic solvents methanol and DMSO were improved. The best results were obtained with the IL [N-Ac-L-Pro] [Cl] [72].

The raw starch digesting type of amylase (RSDA) has potential advantages in terms of process cost and time versus regular amylases. RSDA from *Aspergillus carbonarius* was modified with phthalic anhydride (PA) and with chitosan. Retention of activity was 87.3% and 80.9%, respectively. PA modification shifted the pH optimum from 5 to 7. The half-life of native RSDA at 80 °C was 6.1 h; this increased to 25.7 h for the PA-modified and 138.6 h for the chitosan derivative. Specificity constants (V_{max}/K_m) were 73.2 for PA-modified, 63.1 for chitosan-modified, and 77.1 for native RSDA [73].

Various homo- and hetero-bifunctional reagents are available for the formation of intra- or intermolecular crosslinks in proteins (e.g., Table 5). In most cases, the spacer regions of these compounds are flexible, and the exact structures of the linked components are unpredictable. Jeong et al. [97] described the use of EY-CBS, a crosslinker that selectively targets Cys residues in alpha-helices of proteins. The use of EY-CBS enabled the formation of a single "fusion alpha helix" from two pre-existing helices. The presence of the fusion helix could be shown in crystal structures of the fusion proteins determined in the presence and absence of EY-CBS. The method is likely to be applicable beyond the necessarily limited sample of proteins used in the study [97].

Examples of site-directed chemical modification reactions, with particular focus on magnetic enzymes, have been reviewed by AM Shemsi et al. [98].

It can be difficult to estimate the degree and chemoselectivity of chemical modification of specific amino acids by specific chemicals (blocking or modifying agents). More et al. [99] designed, synthesized, and used fluorogenic substrates with trypsin to determine the extent and chemoselectivity of chemical modification of Lys or Arg. They used two fluorogenic tripeptide probes, Lys-specific MeRho-Lys-Gly-Leu(Ac) and Arg-specific MeRho-Arg-Gly-Leu(Ac). The half-maximal inhibitory concentrations (IC_{50}) indicated the extent of modification, while the ratios of IC_{50} values ($IC_{(50)Arg}/IC_{(50)Lys}$ and $IC_{(50)Lys}/IC_{(50)Arg}$) measured the chemoselectivity for amino acids Lys and Arg. This novel fluorogenic assay worked rapidly, precisely, and reproducibly in determinations of the extent and chemoselectivity of chemical modification [99].

1.7 Conclusion

Therapeutic proteins can be of immense value to patients and are becoming increasingly prominent in the pharmaceutical industry. These proteins must be produced to a very high specification and quality, and the processes involved can affect protein stability. A

detailed discussion of this topic is beyond the scope of this chapter, but Krause and Sahin discuss chemical and physical instabilities in the manufacture and storage of therapeutic proteins [100].

Aside from therapeutics, enzymes (and other proteins) find use across a wide spectrum of modern and traditional biotechnology-based industries. Enzymes are also ideal biocatalysts for green chemistry reactions. Stability issues are often important in these situations, as with therapeutic proteins. Immense progress has been made in stabilizing proteins (e.g., derivatives that can withstand boiling [101]). Protein stability will continue to attract much scientific interest, especially in light of its evolutionary implications [1–6]. It is hoped that the literature reviewed in this chapter (and particularly the 2014–2021 reports and reviews summarized in Tables 3 and 4, respectively), together with the methods set out in Subheading 3 below, will assist protein scientists in their work.

2 Materials

Required for Subheadings 3.1 and 3.2

1. Water bath with adjustable heater.
2. Thermometer.
3. Stopclock.
4. Test tube rack(s).
5. Test tubes (with good heat transfer characteristics and low heat capacity).
6. Adjustable-volume automatic pipette(s) with suitable disposable tips.
7. 96-Well transparent microtiter plates.
8. Ice bath.
9. Assay mix (to measure catalytic, or other biological, activity of the protein of interest).
10. Personal computer with spreadsheet and graphics application.

Most of the items above are required for Subheading 3.3 also.

Additional materials for Subheading 3.4:

11. Screw-cap bijou bottles are convenient for the preparation and mixing of small-volume chemical modification reaction mixtures.

Table 3
Examples of protein stability enhancement

Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
Immobilized biocatalyst	Enzyme	Alpha-amylase	C: Immobilization on olibanum-bovine serum albumin@zeolitic imidazolate framework nanocomposite (OLB/BSA@ZIF-8)	The pH and thermal stability of the OLB/ BSA@ZIF-8-alpha-amylase were notably enhanced versus the free enzyme	OLB/ BSA@ZIF-8-alpha-amylase had very good long-term storage stability: >90% of the initial activity persisted for 8 weeks. It also had high reusability, retaining >81% activity after 20 cycles	[102]
Potential anti-virulence tool: inactivates virulence-associated *Pseudomonad quorum*-sensing signal	Enzyme	Mycobacterial PQS dioxygenase AqdC	M: FRESCO-based protein engineering (computational library design to predict stabilizing amino acid replacements)	Although 57 of 91 tested single substitutions throughout the protein were stabilizing (as judged by increases in T_{appm} of >2 °C), they all impaired catalytic activity	Combining substitutions, AqdC-G40K-A134L-G220D-Y238W and AqdC-G40K-G220D-Y238W showed extended half-lives and the best trade-off between stability and activity, with T_{appm} increases of 11.8 and 6.1 °C, and relative activities of 22% and 72%, respectively, compared with AqdC	[103]
Cellulose breakdown and industrial bioconversions	Enzyme	Endoglucanase II (*Penicillium verruculosum*)	M: Mutations based on protein surface topography, plus multiple sequence alignment and ΔG calculations	Improved thermostability and stability in the ionic liquid 1-butyl-3-methylimidazolium chloride ([Bmim]Cl)	Thermal half-life was 1.3–1.6 times longer at 70 °C and 1.2–1.4 times at 80 °C. Residual activity after	[104]

(continued)

Table 3
(continued)

Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
					72 h of incubation in the presence of [Bmim]Cl (50 g/L, 50 °C, pH 4.5) was 1.7–1.9 times greater with the tailored enzyme. Yield of reducing sugars from enzymatic hydrolysis of aspen wood pretreated with [Bmim]Cl was 10–20% higher using the tailored enzyme	
Research tool; feed additive enabling phytic acid breakdown in poultry and non-ruminant animals	Enzyme	Phytase from *Yersinia mollaretii* (Ymphytase)	M: Consensus	Protein consensus-based surface engineering (ProCoS): a computer-assisted method for directed protein evolution. It comprises four main steps: (i) identification of conserved and highly variable regions, (ii) protein sequence design by substituting residues in the highly variable regions and gene synthesis, (iii) in vitro DNA	Screening 1050 clones from ProCoS mutant libraries yielded an enzyme with 34 amino acid substitutions. The surface-engineered Ymphytase had 3.8-fold higher pH stability (at pH 2.8 for 3 h) and retained 40% specific activity (400 U/mg) compared with the wild-type Ymphytase	[105]

				recombination of synthetic genes, and (iv) screening for active variants		
Research tool; feed additive enabling phytic acid breakdown in poultry and non-ruminant animals	Enzyme	Phytase from *Yersinia mollaretii* (Ymphytase)	M: Directed evolution	KeySIDE is an iterative *Key* residues interrogation of the wild type with *S*ubstitutions *I*dentified in *D*irected *E*volution Directed evolution discovered nine positions in Ymphytase which were combined iteratively to identify key positions	The best combination (M6: T77K, Q154H, G187S, K289Q) showed notably improved thermal resistance. Residual activity improved from 35% (wild type) to 89% (M6) at 58 °C over 20 min. M6 melting temperature increased by 3 °C with no loss of specific activity	[106]
Glucose sensors	Enzyme	FAD-dependent glucose dehydrogenase (*Mucor prainii*; MpGDH)	M: Constructed chimeric enzyme	MpGDH inactivates at 45 °C over 15 min. A chimeric MpGDH (Mr144-297) was constructed from glucose-specific MpGDH and very thermostable FAD-GDH from *Mucor* sp. RD056860 (MrdGDH)	The Mr144-297 chimera had better heat resistance and was stable at 55 °C. It also retained high affinity and substrate specificity for D-glucose. Eight mutation sites that enabled to improved thermal stability and increased productivity in *E. coli* were identified	[107]
Proteolytic enzyme biocatalyst for organic solvent use	Enzyme	*Salinivibrio proteolyticus* protease (SVP)	M: Site-directed mutagenesis:	Replaced five charged or polar residues in wild type by	Versus wild-type SVP, catalytic efficiency of Y23V and N248G	[108]

(continued)

Table 3
(continued)

Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
			hydrophobic residue substitutions	hydrophobic ones: T21V, Y23V, K30P, D25P, and N248G. There was a straight relationship between organic solvent hydrophobicity and irreversible thermoinactivation rate of the protease	increased about 1.8- and 2.6-fold in DMF and methanol and increased about 3.8- and 5.0-fold in isopropanol and n-propanol, respectively. $\Delta\Delta G^{\ddagger}$ values of Y23V and N248G increased about 6.5 and 9.5 kcal/mol in DMF and methanol, and by about 13.6 and 16.6 kcal/mol in isopropanol and n-propanol, respectively	
Breakdown of xylans/ hemicelluloses and production of prebiotics	Enzyme	Xylanase	M, C: Formation of crosslinked enzyme aggregates (CLEAs) from mutant xylanase containing four additional Lys	Four additional Lys were substituted for residues opposite the catalytic region. After optimizing the CLEA parameters, a stable crosslinked mutant xylanase with the addition of BSA (mXyn-CLEA-BSA) was obtained: it was 1.09-, 1.35-, and 1.77-fold more stable than	rXyn-CLEA can be reused up to five cycles, both rXyn-CLEA-BSA and mXyn-CLEA up to seven cycles, and mXyn-CLEA-BSA up to eight cycles until total loss of activity. The increased stability and reusability from this approach provides a biocatalyst with promise for producing	[109]

				the crosslinked recombinant xylanase with BSA (rXyn-CLEA-BSA), without BSA (rXyn-CLEA) and free enzyme (rXyn), respectively	prebiotics in the biomass industry.	
Pulping and bleaching processes	Enzyme	Xylanase (*B. circulans*, BCX) and maltodextrin binding protein (*Pyrococcus furiosus*, PfMBP)	M: Directed evolution	Aimed to evolve the inserted BCX domain for greater toward alkaline pH while maintaining enhanced thermal stability	T50R mutation enhanced xylanase activity of PfMBP-BCX165 toward alkaline pH without decreasing thermal stability. Compared with PfMBP-BCX165 (T50R), the unfused BCX mutant, BCXT50R, showed similar pH dependence of xylanase activity yet had limited thermal stability	[110]
Enzyme stabilization by protein fusion	Enzyme and carbohydrate binding protein	Xylanase (*B. circulans*, BCX), maltodextrin binding protein (*Pyrococcus furiosus*, PfMBP)	M: Fusion-insertion combinations	In the PfMBP-BCX161 and PfMBP-BCX165 fusions, aggregation of BCX was decreased and its termini showed reduced conformational flexibility (BCX protein sequence was not altered)	PfMBP-BCX161 and PfMBP-BCX165 fusions had notably improved kinetic stability at high temperatures versus unfused BCX and other controls	[111]
Has uses for lactobionic acid production, in biosensors for	Enzyme	Cellobiose dehydrogenase (CDH) from	M: Error-prone PCR with screening for activity against	Mutants with improved activity and specificity toward lactose and cellobiose were found,	Mutant S137N retained threefold more activity than wild-type enzyme after 15 min at 70 °C.	[112]

(continued)

Table 3
(continued)

Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
disaccharides and in wound healing		*Phanerochaete chrysosporium*	2,6-dichlorophenol indophenol	purified, and characterized	Mutants M65S and S137N also showed 1.6 and 1.5 times increased productivity of hydrogen peroxide versus wild type in presence of 30 mM lactose	
Therapeutic: thrombolytic	Enzyme: multidomain hydrolase	Tissue plasminogen activator (tPA)	Design of experiment (DoE) approach to evaluate how formulation excipients affect and influence tPA's thermal unfolding profile	Each excipient's effects were often selectively stabilizing or destabilizing to specific tPA domains and changed the stability of particular domains relative to others	Formulation excipient behaviors grouped according to their combined impact on T_m and Van't Hoff entropy for unfolding. Aggregation rates (ln ν) correlated well with T_m and Van't Hoff entropy	[113]
To decrease antigenicity of injected proteins	Enzyme	Lysozyme	C: Conjugation with non-PEG polymers	Conjugated lysozyme with poly(N-acryloylmorpholine) (PNAM) and poly (oligoethylene glycol methyl ether methacrylate) (POEGMA). Investigated different conjugation degrees for residual activity,	Shows the value of evaluating conjugates with discrete conjugation degrees, instead of at the batch level. Monovalent conjugates with quite low molar mass polymers had equal or higher activity than native protein. All	[114]

				aggregation behavior, and solubility, using a high-throughput screening approach	conjugates had improved solubility, but higher molar masses of PNAM and POEGMA were needed for solubility effect comparable to PEGs	
Biocatalyst	Enzyme	Lipases, including lipase B (*Candida antarctica*, CALB)	C: Immobilization on octyl agarose beads	CALB was more stable at less intense loadings onto solid phase, unlike other lipases	Suggests that a different procedure may be more appropriate for CALB versus other lipases	[115]
Agent for protein PEGylation	Enzyme	Transglutaminase	C: Immobilization of microbial transglutaminase N-terminal sequence on agarose	Altered PEGylation of alpha-lactalbumin (alpha-LA) and granulocyte colony-stimulating factor (G-CSF)	Greater selectivity toward one Gln of alpha-lactalbumin (alpha-LA). Avoidance of deamidation of granulocyte colony-stimulating factor (G-CSF)	[116]
Depolymerization of glycosaminoglycans to aid with spinal injuries	Enzyme	Chondroitinase ABC I	M: Site-directed mutagenesis	L679S and L679D	L679S: improved catalysis. L679D: improved storage stability	[117]
Catalyzes a variety of redox reactions	Enzyme	Versatile peroxidase (*Pleurotus eryngii*)	M: Grafting of structural motifs from other peroxidases	Incorporated sequence/structural motifs from CiP (*Coprinopsis cinerea* peroxidase) and PNP (peanut peroxidase). Four of six such variants were inactive	T_{50} increased by 3 °C for two variants, increased stability at pH 3.0 for one variant	[118]

(continued)

Table 3
(continued)

Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
Redox enzyme	Enzyme	Fungal peroxidase	C: Co-immobilization of peroxidase with cytochrome c on mesoporous silicates. Immobilization of peroxidase alone on silica microparticles	Fourfold increase in co-immobilized peroxidase oxidative stability	Fourfold increase in peroxidase oxidative stability. Thermal stability improvement on biogenic silica microparticles, but not on derivatized porous microsilicates	[119]
D-Psicose production from vegetable residues	Enzyme	D-Psicose 3-epimerase	M: N-terminal fusion with Smt-3 SUMO protein	Increased thermal and operational stability	Improved catalytic efficiency and D-psicose productivity	[120]
Hydrolysis of alpha-1,6 links in polysaccharides	Enzyme	Isoamylase (*B. lentus*)	M: Semi-rational mutation	Mutations R505P and R505E enhanced thermal and acidic stability, respectively. Mutant G608V showed a 33% increase in specific activity and a 49% increase in k_{cat}/K_m values	Increased thermal and acidic stabilities of mutant R505E. Gains in specific activity and catalytic effectiveness	[121]
Method with potential to stabilize administered bioactive proteins in vivo	Enzyme	Beta-glucosidase (beta-Glu)	C: Immobilization on magnetic nanoparticles (MNP-beta-Glu), plus PEGylation (MNP-beta-Glu-PEG)	Stability of immobilized versus free beta-Glu was lower in deionized water but higher in blood serum at 37 °C. Over 30 days at 4 °C, MNP-beta-Glu-PEG	Pharmacokinetic studies in rats showed that MNP-beta-Glu-PEG retained higher enzyme activity in vivo after 50 min (41.46%) than MNP-beta-Glu	[122]

				retained 77.9% of its initial activity but free enzyme retained only 58.2%	(0.03%) or beta-Glu group (0.37%). Unlike MNP-beta-Glu, enzyme activity was not fully synchronous with decreased Fe concentration in MNP-beta-Glu-PEG	
Method for crosslinking protein nanoparticles	Casein nanoparticles	Sodium caseinate	C: Tyrosinase-mediated crosslinking of sodium caseinate nanoparticles using catechol or chlorogenic acid	Effective crosslinking of protein nanoparticles	Workable alternative to glutaraldehyde crosslinking	[123]
Method to introduce a non-disulfide-stabilizing crosslink	Immunoglobulin-like protein	Minor pilin FctB (*Streptococcus pyogenes*)	M: Insertion of de novo Lys-Asp/Asn isopeptide bond crosslink	Insertion of Lys, Asn, and Glu residues for the bond-forming reaction, plus a fourth Val/Phe mutation to help steer the Lys side chain into position	Thermal stability increased by 10 °C versus wild type	[124]
Method for stabilizing antibodies	Antibody (IgG)	Antibody (IgG)	C: Orientated immobilization onto protein film	Crosslinked albumin solution was added to the substrate, and then antibodies were attached in uniform orientation. This was followed by drying and detachment of the formed film from the substrate by dry heating at 120 °C	Antibodies in the film retained high antigen-binding capacity, comparable to oriented immobilized antibody achieved using protein G. Antibodies in the film were very stable: they retained complete antigen-binding capacity even after dry storage at 40 °C for 1 month	[125]

(continued)

Table 3
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Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
Fluorescent indicator protein and research tool	Green fluorescent protein (GFP)	Green fluorescent protein (GFP)	C: Site-specific incorporation of L-3,4-dihydroxy phenylalanine (DOPA) and residue-specific incorporation of (2S, 4S)-4-fluoro proline (4S-FP) or L-homopropargyl glycine (hpg)	Improved folding, thermal stability, and scope for conjugation reactions (depending on the precise modification)	Modification of GFPhpg and GFPdphpg with PEG (via a Cu(I)-catalyzed click reaction) increased thermal stability by about twofold versus wild-type GFP	[126]
Research tool. See row below	Enzyme	Esterase (*Alicyclobacillus acidocaldarius*, Aac-Est)	M: Random mutational drift of a thermophilic bacterial protein	In the first random mutations, few proteins showed increased activity at 65 °C. In the second and third generations from lower-active templates, evolvability of higher activity depended on the template proteins' stability. Random mutations in stability-loss templates produced variants with higher activity at 40 °C than those produced by WT, via cold adaptation	Results indicate that maintaining stability enables a protein to search sequence space and evolve in the original environment, while proteins with lost stability use a cold adaptation path.	[42]

Research tool. See row above	Enzyme	Esterase (*Sulfolobus tokodaii*)	M: Random mutation	Approximately 20% of variants showed higher activity than wild type (i.e., 20% evolvability). Evolvability depended on the stability of template proteins, suggesting that protein evolution may be governed by protein stability	Decreased activity could be recovered during evolution by maintaining variants' stability. Results suggest that protein evolutionary sequence space can expand during nearly neutral evolution, where mutations are slightly compromise activity but are rarely fatal for stability. These findings are relevant to protein engineering	[3]
Research tool	Various: protein G	Various: protein G	Software: stability prediction algorithms	Results indicate that (i) most single mutants have a neutral effect on stability, (ii) mutational sensitivity is largely governed by residue burial, and (iii) hydrophobic amino acids are the best tolerated. Comparison of lab data with the outputs of various stability-prediction algorithms shows that many (a) perform better on boundary and surface positions than for core positions and (b) better predict large-to-small mutations than small-to-large ones	In the single-mutant landscape, the most stable variants are better identified using two prediction algorithms in combination. Inclusion of more algorithms gave diminishing returns. Strategies to extract stabilities from high-throughput fitness data (e.g., deep mutational scanning) seem promising and data provided by these methods may be useful for training future stability-prediction tools	[127]

(continued)

Table 3
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Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
Research tool	Histone-like HU bacterial proteins	Histone-like HUs	M: Consensus-based mutations based on sequences from 40 mesostable, thermostable, and hyperthermostable HUs. The consensus-derived protein was named "HUBest"	HUBest's correct folding and thermodynamic stability, plus its ability to interact with plasmid DNA, were characterized.	HUBest's stability at high temperatures increased substantially, and its biological performance at ambient temperature significantly improved, with very low K-d values for binding plasmid DNA (as indicated from the Gibbs energy profile)	[128]
Research tool for investigation of G protein-coupled receptors (GPCRs)	G protein-coupled receptors (GPCRs)	Human oxytocin receptor	M: Combination of three directed evolution strategies	This combination approach enabled successful selection for improved and crystallizable variants of the human oxytocin receptor, a GPCR with especially low intrinsic production levels	Development of a next-generation sequencing (NGS) strategy that combines long reads, covering the whole receptor, with very low error rates. This tool provides a general methodology for highly accurate analysis of libraries of point mutants during directed evolution	[129]
Prediction tool for protein modification sites	Enzyme (as target for investigation)	TEM-1 beta-lactamase	Computational screening of all surface-accessible functionalization sites on TEM-1, so as to evaluate simultaneously the effects of tethering and	Functionalization site effectiveness seems to be a function of super-secondary and tertiary structures, rather than primary structure. Sites	The aim is to select modification sites that will result in the best protein function. This study shows the usefulness of molecular	[130]

			PEGylation with a view to creating a more accurate screen	in the middle of secondary structure elements, and not just those in loop regions, are good options for functionalization	simulation to screen potential attachment sites for surface tethering or PEGylation and to facilitate rational design of protein devices	
Potential treatment for inflammatory diseases	Immunoregulatory cytokine	Interleukin-10 (IL-10)	M: Prepared a novel IL-10, comprising two IL-10 monomer subunits linked head-to-tail by a flexible linker	The new IL-10 had much-improved temperature- and pH-dependent biological stability versus natural IL-10. The stable IL-10 molecule was more active than natural IL-10, both in vitro and in vivo	The new IL-10 dimer binds to the IL-10 receptor similarly to natural IL-10 (shown by antibody blocking and through genetic modifications of one monomer in the IL-10 dimer specifically at the IL-10 receptor binding site). Stable IL-10 is more effective at suppressing LPS-induced inflammation in vivo compared with natural IL-10. This novel stable IL-10 dimer could aid in the development of targeted anti-inflammatory drugs	[131]
Filgrastim (granulocyte-stimulating factor (G-CSF))	Biotherapeutic cytokine	Granulocyte colony-stimulating factor (G-CSF, Filgrastim)	C: PEGylation	Attachment of PEG to G-CSF	PEG-GCSF was shown to have improved resistance to heat-induced aggregation	[132]

(continued)

Table 3
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Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
Biotherapeutic cytokine						
Prospective HIV-1 vaccine candidates	Potential vaccine antigen	Soluble envelope glycoprotein (Env) trimers (SOSIP.664 gp140)	M: Shotgun mutagenesis to identify trimer variants with improved antigenicity and stability. Created 341 Cys pairs at predicted stabilization points throughout gp140, 149 Pro substitutions in the gp41 ectodomain, and 362 space-filling substitutions at every hydrophobic/aromatic residue in gp140. Each of 852 mutants was screened for antigenicity using four different monoclonal antibodies (MAbs)	Identified 29 trimer variants with antigenic improvements derived from each of the three mutagenesis strategies. Selected four variants (Q203F, T538F, 1548F, and M629P) for deeper study	T538F substitution showed the greatest overall benefit, including restored epitope recognition by the key broadly neutralizing antibody PGT151. These strategies may help identify further antigenically and structurally improved soluble trimers based on multiple genotypes toward the creation of a broadly protective HIV-1 vaccine	[133]
Potential low calorie sweetener	Sweetness protein	Monellin	M: Site-directed mutagenesis	Alanine substitutions at wild-type positions Glu23 and Cys41	Increased thermal stability (E23A) and sweetness (C41A)	[134]
Biological pest control	Toxin: Midgut disruptor in Spodoptera	Vip3A proteins	M: Vip3Ab1 chimera with a modified C-terminus	Native Vip3Ab1 controls *Spodoptera frugiperda* (fall armyworm) but is not effective against	Increased Vip3Ab1-740 stability in the spodopteran midgut, due to C-terminal	[135]

				S. eridania (southern armyworm)	modification, led to gain of toxicity against the soybean crop pest *S. eridania*	
Collagen alteration toward possible edible food packaging	Structural protein	Collagen	C: Transglutaminase-mediated crosslinking with the thermostable proteins casein, keratin, and soy protein isolate	Notably improved thermostability of protein complexes, especially that of the collagen-casein complex	Improved mechanical properties of the combined films; specifically tensile strength (TS) and elongation-at-break (EAB)	[136]
Potential packaging film	Structural protein	Collagen	C: Crosslinking with other proteins (casein, keratin, and soy protein isolate (SPI)) using transglutaminase (Tgase)	Enhanced mechanical properties of collagen fiber films: tensile strength (TS) and elongation at break (EAB). Improved EAB at wet and heated state when collagen fiber was crosslinked with 50% keratin or 50% casein, respectively	Increased thermal stability of collagen fiber films, especially when collagen fiber was crosslinked with 50% casein composite film	[137]
Catalyst	Enzyme	Laccase (*Bacillus* sp. HR03)	M: Site-specific mutagenesis	Substitutions of position E188 (located in a negatively charged segment of an inter-domain region) by A, I, L, or V; or by K or R	Increased stabilities to MeOH, EtOH, and 1-propanol. With non-polar amino acids, thermal stability also increased	[138]
Catalyst	Enzyme	Ligninolytic versatile peroxidase (*Pleurotus eryngii*)	M: Rational, site-specific mutagenesis	Substitutions of oxidation-prone Met residues and of residues near the heme and the peroxide binding pocket	Increased resistance to peroxide/oxidation	[139]

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Table 3
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Function/application	Protein type	Protein name	Strategy used M: mutational; C: chemical	Change	Benefit	Ref.
Model for non-two-state proteins	Electron transfer protein	Apo-flavodoxin	M: Structure-based rational mutagenesis	Insertion of rationally designed mutations into less stable regions of this non-fully cooperative protein	Variants with T_m 32 °C above wild-type value—with fully cooperative thermal unfolding	[140]
Detergent ingredient	Enzyme	Lipase A (*Bacillus subtilis*)	M: Site-saturation mutagenesis	Prepared 3439-variant library of the 181-residue enzyme and tested these against four detergents	Identified amino acid residues that influence tolerance of each detergent and indicated surface remodeling as an effective strategy to enhance detergent tolerance	[141]
Biodiesel production	Enzyme	Lipase T6 (*Geobacillus stearothermophilus*)	M: Random and structure-guided consensus mutagenesis in parallel	Best mutant found was H86Y/A269T	66-fold increased stability to 70% methanol, with improved thermostability and productivity	[142]
Model proteins for study	Receptor protein and marker protein	G-protein-coupled receptors (GPCR) and green fluorescent protein (GFP)	M: Directed evolution	Describes CHESS (cellular high-throughput encapsulation solubilization and screening) methodology, based on encapsulation within robust detergent-resistant polymers	Permits isolation of detergent-tolerant proteins. (GFP was also thermostabilized.) Capsules have 70 kDa lower molecular weight limit. Method requires fluorescent reporter of protein stability for selection by flow cytometry	[143]

Model for fusion to therapeutic proteins	46-residue albumin binding domain from Protein G	Albumin binding domain (ABD)	C: Crosslinking	Insertion of intramolecular thioether bridge	Greatly increased resistance to proteases and enhanced heat stability	[144]
Enzyme-mediated crosslinking	Structural protein: collagen	Collagen	C: Crosslinking	Aldehyde-forming enzyme (AFE) changes collagen hydroxymethyl functions to formyl groups, leading to peptidyl aldehydes and to formation of conjugated double bonds within the collagen	Increased resistance to heat and to collagenolysis. Achieves crosslinking with nontoxic reagents. Possible applications in tissue engineering	[145]
Model for PEGylation study	Domain of larger protein	WW domain of human Pin1	C: Conjugation with polyethylene glycol (PEGylation)	PEG-stabilized WW shows enhanced resistance to proteolysis	Method predicts best sites in target protein for attachment of PEG (by site-specific techniques) in order to increase stability	[146]
Model for PEGylation study	Model alpha-helical coiled coil	Alpha-helical coiled coil and villin headpiece domain	C: PEGylation	Maleimide-mediated PEGylation of solvent-exposed Cys	Stability gain results where Lys residues occupy the +3 and +4 sites from the PEGylated Cys site	[147]

Summaries of a selection of reports published 2015–2021, showing categories of protein name, type and function, manipulations performed, change(s) made, and benefits obtained

Table 4
A selection of reviews published 2014–2021 on the theme of protein stability or stabilization

Description	Keywords	References
Reviews recent publications concerning degradative reactions occurring in protein-based drugs during manufacture, storage, and clinical use. Notable hazards include oxidation, deamidation, and aggregation. Ways of preventing or minimizing these adverse events are discussed. Contains 61 references	Therapeutic proteins, chemical instability, physical instability, protein drug manufacturing, and storage of protein drugs	[100]
Reviews antibody stability under five headings: (i) measurement techniques, (ii) stability issues in expression and production (expression, proteolysis, aggregation), (iii) effects of antibody format and engineering on stability, and (iv) formulation, drying, and storage conditions. Considers >100 sources, including patents, and concludes with (v) recommendations to promote antibody stability	Antibody stability, protein stability measurement, protein expression, antibody engineering, and formulation	[148]
Enzyme instability remains a limitation in the development of biocatalysis, despite notable successes. Reviews strategies for protein engineering of stable enzymes, including multidisciplinary techniques (such as combined computational design and molecular evolution). Includes pharmaceutically and industrially relevant examples	Enzyme stabilization, protein engineering, rational design, directed evolution, and industrial application	[149]
Enzyme immobilization enables the recovery and reuse of protein catalysts in industrial processes and may improve enzyme stability. Stabilization effects may arise from (i) the fact of being inside a solid particle, (ii) favorable enzyme environments, (iii) prevention of subunit dissociation in multimeric enzymes, (iv) promotion of more stable enzyme conformations, or (v) enzyme stiffening through multipoint covalent attachments. Hints are given toward an "ideal" immobilization protocol. That could maximize the degree of enzyme-support interaction. Also it considers cases where the number of enzyme-support bonds does not yield greater stabilization and the prospects of coupling site-directed mutagenesis or chemical modification to intensify multipoint covalent immobilization	Enzyme stabilization, enzyme-support interactions, lipase interfacial activation, multipoint covalent immobilization, multimeric enzymes, glutaraldehyde, glyoxyl epoxides, and vinyl sulfone	[36]

(continued)

Table 4
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Description	Keywords	References
Experimental report that examines the preparation of saturation mutagenesis libraries from an economic perspective. Proposes improved control of library quality based on (i) cheaper and faster "Quick Quality Control" (QQC), (ii) input primer purity, (iii) use of primers from different suppliers, (iv) use of different randomization schemes (redundant and non-redundant), and (v) analysis of bias favoring the wild-type codon and its implications for library diversity. Facilitates choice of the most cost-effective randomization scheme so as to reduce the cost of experiments	Saturation mutagenesis, mutagenesis library generation, mutagenesis library diversity, experimental design, economic analysis.	[150]
Focuses on proteins in solid form. Reviews metrics to predict resistance of biopharmaceuticals to aggregation and other adverse processes. Outlines principles to achieve stable solid-form proteins	Dynamic stabilization, water substitution, aggregation, protein stability, and lyophilization	[151]
Systematically reviews basic thermodynamic principles that influence structural transitions of proteins and how they interact with their (micro) environment	Structural and functional stabilization, protein stability, biopharmaceutical applications, bacteriophages, immobilization, and post-immobilization	[152]
Analyzes bioinformatic tools that aim to improve protein stability. Bioinformatics can indicate key sites for mutation so as to generate proteins and enzymes with improved stability and functional diversity	Biocatalytic processes, bioinformatics, computational biology, protein stability, and structure-function relationship	[153]
Considers techniques for measurement of the in vivo and in vitro stabilities of therapeutic fusion proteins against proteolysis	Binding mass spectrometry, limited proteolysis, pulse proteolysis, and degradation products	[154]
Short review of the effects of process-generated impurities and contaminants on recombinant protein stability. Also considers possible means of lessening these effects	Formulation, protein aggregation, oxidation, deamidation, and biotechnology	[155]
Enables design or choice of room temperature ionic liquid for enzymatic or protein-based applications. Considers ionic liquids' effects on stability and activity	Ionic liquid, enzymes, protein stability, protein solubility, and self-aggregation	[156]

(continued)

Table 4
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Description	Keywords	References
Short review of (i) physical degradation pathways that occur in protein drugs and (ii) the use of high-throughput biophysical techniques to enable the development of stability-improving formulations. Includes case studies of a monoclonal antibody, an albumin fusion protein, a recombinant multimeric glycoprotein, and a Fab antibody fragment	Aggregation, biophysical, conformation, formulation, mini-review, monoclonal antibody, protein, stability, and structure	[157]

3 Methods

Take care to avoid burns or scalds when working with samples, water baths or other apparatus at elevated temperatures.

3.1 Thermal Profile

One can determine a thermal profile for an enzyme by placing samples for a fixed, short period at successively increasing temperatures and then assaying the samples' residual activities. This procedure also allows one to select a suitable temperature for a thermal inactivation experiment (Subheading 3.2 below):

1. Incubate a sample of the protein in question, plus a sample of native or untreated protein, at one of a range of increasing temperatures for a fixed period. Suitable incubation periods are 10–15 min while 10 °C increments will do for a first run (*see* **Notes 4**–**6**).
2. When the incubation period at each temperature has elapsed, remove the samples onto ice in order to cool them rapidly (*see* **Notes 7** and **8**).
3. Upon completion of all of the temperature incubations, rewarm the entire complement of samples to room (or assay) temperature, and assay the remaining biological activity by your usual method.
4. Defining the activity at the "normal" temperature of assay as 100%, express the remaining activity at each of the progressively rising temperatures as a percentage of this. Plot percent activity remaining against temperature and observe the resulting graph. There will likely be a sharp fall in activity over a narrow temperature range. Inspection of the plot will reveal the temperature where approximately 50% of the original activity remains, that is, the temperature of half-inactivation, T_{50}, analogous to T_m (Table 1). Be aware that this T_{50} is a purely

empirical value, and the conditions under which it is obtained must be specified for each case (*see* **Note 9**).

5. For a more accurate determination, perform further experiments over narrower increments of 5 °C (or even 2 °C over the steepest part of the plot) (*see* **Note 10**). Possibly, activation effects will occur, where the activity increases with temperature (i.e., to values >100%) until one reaches a threshold temperature where a sharp decline in activity is observed (*see* **Note 10**).

3.2 Thermal Inactivation

Once a suitable fixed inactivation temperature has been ascertained (i.e., one where activity loss is neither too rapid nor inconveniently slow, typically the T_{50}—*see* Subheading 3.1 above), one can perform a thermal inactivation over time (*see* **Notes 4**–**7** and **11**).

1. When comparing different treatments, include an untreated (control) protein sample in each experiment. This can provide an internal validation and a qualitative result even in cases of experimental variation, of one (or a few) missed time points, or of data that fit poorly to model equations.
2. Place the samples in the water bath or heating block, starting a stop clock at the same time (*see* **Note 6**). Immediately remove a "time zero" sample (= 100% activity) onto ice. Withdraw further samples onto ice at short intervals; as the experiment progresses, one can extend the intervals between samplings (*see* **Note 12**).
3. At the end of the experiment, rewarm all samples and assay under optimal conditions by the customary method.
4. If activity at time zero is defined as 100%, a plot of percent activity remaining against time will allow one to estimate and compare stabilities. Frequently, but not always, the loss of activity will follow a first-order decay to yield an exponential plot of percent remaining activity versus time. Such a graph declines steeply at first before tracking a slower rate of decline.
5. Fit the experimental data to a first-order decay equation using a computer with suitable statistical software, paying special attention to goodness-of-fit, confidence limits, and other appropriate quality indices. If the fit is good, a semi-log plot of ln or log (% activity) versus time will yield a straight line of slope k, and one may calculate a true half-life from the first-order rate constant k (*see* **Note 13**). More complex decay functions will not yield a straight line in semi-log form. Nevertheless, even in these cases, empirical comparisons of stability can be made from plots of percent activity versus time (*see* also **Note 11**).

3.3 Measurement of Oxidative Stability

Hydrogen peroxide, H_2O_2, a powerful oxidizing chemical, may be used to assay the oxidative stability of a test protein. It is often supplied as a 30% w/v solution, but for accuracy, one should ascertain the stock's exact molar concentration spectrophotometrically at 240 nm using 43.6 $M^{-1}.cm^{-1}$ as the extinction coefficient [158].

Two convenient protocols are as follows:

1. The protein of interest, at a known molar concentration in a suitable buffer, is incubated with increasing concentrations of H_2O_2 (e.g., 0–500 mM, the exact range will depend on the protein(s) concerned) for 30 min at a suitable assay temperature in a water bath [159]. Following this exposure, aliquots are withdrawn, and remaining catalytic (or other biological) activity is assayed using a standard assay method (choose the volume of aliquots removed for testing with care). It is important that H_2O_2 amounts carried over from the "challenge" into the assay mix are not themselves inhibitory and are diluted into a "catalytic" range. For example, if 10 μL is removed from a 100 mM H_2O_2 "challenge" mix and added into 990 μL of assay mix, the concentration of H_2O_2 in the latter will be at least 1 mM. Plot percent remaining activity (i.e., where 0 mM H_2O_2 = 100%) versus molar H_2O_2 concentration. One can use the C_{50} value (mM H_2O_2 where 50% of maximal activity remains) to compare H_2O_2/oxidative stabilities.
2. Alternatively, a plot of percent remaining activity versus H_2O_2/enzyme ratio (each in molar units) may be generated. The incubation period may be increased to 4 h, while the protein's molar concentration is fixed and the H_2O_2 concentration is varied between 0 and 0.5 M [160]. As in (**step 1**) above, aliquots are withdrawn at the end of the incubation period and remaining activity is assayed.

3.4 Chemical Modification of a Protein

1. Assemble as much information as possible concerning the protein to be modified: functionally/biologically essential residues (to be avoided or protected); number of Lys, Cys, (Asp + Glu) or other modifiable residues per polypeptide molecule or subunit; molecular mass (to calculate molarity); suitable/unsuitable buffers and storage conditions; and assay method(s). Literature searches will be helpful here.
2. Decide which type of residue(s) to modify and which chemical reagent(s) to use: type of chemistry, single-step or multistep reaction, monofunctional modifier or bifunctional crosslinker (what length? homo- or hetero-bifunctional?), and suitable protocol and scale (*see* Table 5) [90, 94].
3. Place the protein of interest in a buffered reaction mixture that is suitable both for it and for the chemical reaction envisaged.

Table 5
Amino acid side chains and reagents for their modification

Amino acid	Side chain	Reagents	Reaction	References
Cysteine	Thiol	5,5′-Dithiobis(2-nitrobenzoic acid)[a] Maleimido compounds *p*-Mercuribenzoate[a]	Disulfide formation Alkylation Addition	[74][a], [75][a] [76] [75][a], [76]
Lysine	Amino	Trinitrobenzene sulphonate[a] Imidates Acid anhydrides (Cyano)borohydride + aldehyde	Addition Amidination Acylation Reductive alkylation	[75][a], [77][a] [78] [79] [80]
Arginine	Guanidino	Dicarbonyls 9,10-Phenanthrenequinone[a] Camphorquinone-10-sulfonic acid	Not fully known	[75], [81] [75][a] [82]
Histidine	Imidazole	Diethylpyrocarbonate[a]	Addition	[75][a], [83]
Aspartic acid and glutamic acid	Carboxyl	Carbodiimides Trialkoxonium salts	Amidation Esterification	[84] [85]
Tyrosine	Phenol	Tetranitromethane Iodine	Nitration Iodination	[86] [87]
Tryptophan	Indole	N-Bromosuccinimide	Oxidation	[88]
Methionine	Thioether	Hydrogen peroxide	Oxidation	[89]

[a]These compounds may be used for the spectrophotometric (or, in the case of Arg, fluorimetric) quantitation of the content of the reactive R-group in a protein: *see* respective references
This table presents only some of the more common reagents for protein R-group modification; it is by no means exhaustive
Reaction of Arg with the dicarbonyl 2,3-butanedione should be carried out in the dark [75, 80]
Volumes by RL Lundblad, notably [90], long Chapter 1 of [91] (Chapter 10 is also of interest) and Chapter 2 of [92], give detailed descriptions of the types of reagents that target particular functional groups of amino acids and proteins. The review articles by Wong and Wong [93] and Means and Feeney [94] and certain volumes of Methods in Enzymology [95, 96] remain useful, despite the passage of time

In parallel, run a protein sample that is not exposed to the chemical modifier but is otherwise treated similarly to the test sample(s).

4. Add the modifying reagent in considerable molar excess over the number of target groups on each protein molecule (e.g., if a 1 mg/mL protein solution represents a 50 μM concentration of the protein to be modified and there are 10 Lys residues per protein molecule, the molar concentration of Lys (the residue to be modified, and assuming that all are available for reaction) is 500 μM. The modifying reagent should be used in excess: here, a final modifier concentration of 5 mM in the reaction mixture represents a tenfold excess of reagent over target residue (*see* **Note 14**).
5. Ensure that any elevated temperatures used will not inactivate the protein.

6. Terminate the reaction after a set time by means of a chemical step or a rapid separation technique such as centrifugal gel filtration (dialysis is also effective but requires more time).
7. Test the catalytic or biological activity of the treated and untreated (activity = 100%) protein samples. Estimate any loss of activity arising from exposure to the chemical (s) concerned.
8. If possible, estimate the number of modified/unmodified target residues using a suitable diagnostic chemical reaction (e.g., 2,4,6-trinitrobenzenesulfonic acid (TNBS) for Lys residues, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for Cys; *see* Table 5 and references therein) or other techniques.
9. Compare the stabilities of the treated and untreated samples as described in Subheadings 3.1 and 3.2.

4 Notes

1. T_m (the melting, or unfolding, temperature) is a robust index of folding stability: it is purely empirical and involves no underlying assumptions. Measurement of a protein's T_m is often carried out spectrophotometrically at a single diagnostic wavelength, using a thermal programmer which gradually raises the cell temperature over a set range. Folding stability can also be measured by a variety of biophysical techniques, but many of these methods (circular dichroism, hydrogen-deuterium exchange, fluorimetry, differential scanning calorimetry) require the use of specialized (and sometimes quite expensive) equipment. RT-PCR machines have become commonplace and can be used for differential scanning fluorimetry [29], while pulse proteolysis [34] needs no specialized apparatus; *see* Table 2. Calculation of Gibbs energies may be performed on thermal data of this sort or on unfolding studies using urea or guanidine hydrochloride but requires care. Pace [21, 22] has described the experimental and data analysis requirements for reliable Gibbs energy estimations.
2. *k*-Values can also be informative when correlated with other data such as the number of attachment sites to a solid phase (immobilized enzyme) or changes in protein hydrophilicity [15]. The activity loss will often be first order, although more complex inactivation patterns are well documented [161]. It is possible, however, that an apparently unimolecular first-order time course of inactivation may mask a more complex set of inactivating molecular events [161].

3. A search undertaken on 8 July 2021 on the ISI Web of Science for the terms "protein and stabili*" in the *titles* of papers published between 1 January 2016 and 30 June 2021 revealed 3577 publications, including 116 reviews. These search results, however, extend beyond in vitro proteins: they include reports of, for example, effects on in vivo protein stability. Although further refinements would be needed to filter out publications that are of little relevance to this chapter, it is nonetheless clear that protein stability/stabilization is a field of great interest.

   The following terms were used separately to filter the initial 3577 "hits," with the number of published papers shown in brackets: additive (7), excipient (16), chemical modif* (1), OR pegylat* (11), OR crosslink* (10), evolution (25), fitness (5), immobili* (19), protein engineering (54), consensus (5), directed evolution (5), shuffling (0), site-directed OR site-specific (7), chimer* (2), and fusion (52).

4. All samples to be tested should be at uniform protein concentration in an identical buffer composition. Certain ions can stabilize or destabilize proteins, for example, calcium has stabilizing effects on amylase, peroxidase, and some proteolytic enzymes. Variations in protein concentration can also influence stability.

5. Place a thermometer as close as possible to the samples being incubated, so that the temperature indicated accurately reflects that of the samples. Conditions may not be uniform throughout the water bath.

6. When testing multiple samples, stagger the insertion of each one into the water bath by 10–15 s; removal of samples in a similar sequence will ensure the exposure of each one to high temperature for the exact period required.

7. Removal of aliquots at various time points from different samples can lead to a considerable number of samples for assay. We find it very convenient to dispense individual aliquots/time points into the wells of a 96-well microtiter plate that is held on ice until the end of the thermoinactivation experiment. The plate's 12 × 8 grid allows one to arrange the samples in a pre-planned fashion, to rewarm them uniformly to assay temperature, and, in many cases, to assay in situ if a micro-assay protocol is possible.

8. One may obtain different values for T_{50} depending on the exact protocol followed: subjecting different aliquots of a protein sample to a single high-temperature incubation (followed by their withdrawal onto ice) is not equivalent to subjecting a single aliquot/sample to progressively increasing temperatures and withdrawing portions of this onto ice after the fixed time has elapsed at each temperature of measurement. We prefer the

former procedure. Whichever way one decides to conduct the experiment, use that procedure consistently.

9. The T_{50} and T_m values may well be equivalent in the case of a simple monomeric protein but one should be aware that they need not agree, especially with oligomeric proteins or proteins containing prosthetic groups.
10. In the case of a thermophilic protein, there may be little or no decline in activity even approaching 100 °C. In such a case, the use of moderate concentrations of a denaturant, of a reducing and/or chelating agent, or a combination of these, can reduce the T_{50} to a suitable, measurable value.
11. We have always held inactivation samples on ice prior to simultaneous assay of all samples, but others sometimes assay each inactivation sample immediately following removal from elevated temperature. Since proteins inactivate according to the model N ↔ U → I, where N is the native (and only active) form, U is reversibly unfolded, and I is irreversibly inactivated [15, 16], it is possible that some refolding may occur during the ice storage stage, so that the measured residual activities may represent (N + refolded U) and not N alone. If one observes no difference between data obtained from immediate or delayed assay of time samples in a control thermoinactivation experiment, refolding is unlikely, and the observed activities are probably due to N alone.
12. To obtain a good statistical fit of experimental data, it is particularly important to sample as frequently as possible during the initial steep decline to approximately 40–50% of the starting (time zero) activity. After this period, the curve will be flatter; it is this feature that allows one to extend the intervals between samplings. Practice and familiarity will allow one to refine the sampling intervals and the duration of the experiment.
13. Recall that the equation for a first-order [single] exponential decay is $A_t = A_0\exp[-kt]$, where A_0 and A_t represent the activities at times zero and t, respectively, and k is the first-order rate constant. Half-life is defined as $0.693/k$.
14. Protein crosslinks accomplished with bifunctional reagents may be *intra*molecular (within the same protein molecule) or *inter*molecular (between different protein molecules/subunits). The length or span of the crosslinking reagent, the properties of the protein itself, and the reaction conditions will each influence which type of crosslink forms. With respect to the experimental conditions, low concentrations of highly charged protein (influenced by the buffer pH) with excess amounts of crosslinker will favor the formation of an intramolecular crosslink, while greater concentrations of minimally charged protein (buffer pH close to the protein's pI) will tend to favor

intermolecular links [162]. SDS-polyacrylamide gel electrophoresis will allow one to distinguish between these two possibilities in a stabilized (or otherwise altered) protein that has been treated with a crosslinker: an intra-polypeptide crosslink will not alter the electrophoretic mobility (apparent molecular mass), whereas an inter-polypeptide will lead to decreased migration (due to increased molecular mass/radius).

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Chapter 19

Storage and Lyophilization of Pure Proteins

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Abstract

This chapter outlines empirical procedures for the storage of pure proteins with preservation of high levels of biological activity. It describes simple and workable means of preventing microbial contamination and proteolytic degradation and the use of various types of stabilizing additives. It sets out the principles of lyophilization (a complex process comprising freezing, primary drying, and secondary drying stages, otherwise known as freeze-drying). There follows a general procedure for the use of lyophilizer apparatus with emphasis on best practice and on pitfalls to avoid. The use of modulated differential scanning calorimetry to measure the glass transition temperature, a key parameter in the design and successful operation of lyophilization processes, is described. This chapter concludes with brief summaries of interesting recent work in the field.

Key words Protein stability, Storage of pure proteins, Stabilizing additives, Formulation excipients, Freezing, Freeze-drying, Lyophilization, Modulated differential scanning calorimetry, Glass transition, Cake appearance, Micro-collapse

1 Introduction

There is often a need to store an isolated or purified protein for varying periods of time. It is vital, therefore, that the protein retains as much as possible of its original, post-purification, biological (or functional) activity throughout an extended storage period ("shelf life") that may exceed 1 year. Shelf life can depend on the nature of the protein, on the chosen formulation excipients, and on the storage conditions. This chapter outlines the means by which activity losses occur on storage and discusses a range of measures to prevent or lessen these protein-inactivating events.

Extremes of temperature and pH will, naturally, be avoided as conditions for routine or long-term storage. Various other factors, however, can lead to loss or deterioration of a protein's biological activity. Proteins can undergo conformational changes, especially during lyophilization, and different conformations can have different stability characteristics [1, 2].

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Typical *physical* phenomena are aggregation [3–5] and precipitation. Adsorption to surfaces may also lead to inactivation [3, 4]. Biological deterioration can result from the loss of an essential cofactor, from the dissociation of subunits (in the case of an oligomeric protein), or from the action of proteolytic enzymes (either endogenous or arising from microbial contamination) [3].

The purely *chemical* reactions are few and well defined. Deamidation of glutamine and asparagine can occur at neutral-to-alkaline pH values while peptide bonds involving aspartic acid undergo cleavage under acidic conditions. Cysteine is prone to oxidation, as are tryptophan and methionine. Alkaline conditions lead to reduction of disulfide bonds, and this is often followed by β-elimination or thiol-disulfide exchange reactions [3, 5, 6]. Where reducing sugars are present with free protein amino groups (N-termini or lysine residues), there may be destructive glycation of amino functional groups by the reactive aldehyde or keto groups of the sugar (the Maillard reaction) [7].

Aggregation and deleterious chemical reactions can occur even at moderate temperatures, for example, aggregation of lyophilized proteins as reported by Liu et al. [5] (*see* also **Note 1**).

These various phenomena underline how important it is to ascertain correct storage conditions for the protein of interest.

Storage concerns a protein's *long-term* or *kinetic* protein stability, which is distinct from (and need not correspond with) *thermodynamic* (*folding* or *conformational*) stability. Kinetic stability measures the persistence of activity with time (or, put another way, the progressive loss of function). It can be represented by the scheme

$$N \xrightarrow{k_{\text{in}}} I$$

where N is the native, functional protein, I is an irreversibly inactivated form, and k_{in} is the rate constant for the inactivation process. The equation $V_{\text{in}} = -d[N]/dt = k_{\text{in}}[N]$ describes the process mathematically, where V_{in} is the experimentally observed rate of disappearance of the native form [3]. Often the activity loss will be first order, although more complex inactivation patterns are well documented [8]. It should be noted that an apparently unimolecular first-order time course of inactivation may mask a more complex set of inactivating molecular events [8]. There is, however, an important distinction between pharmaceutical stability and thermodynamic stability. Irreversible degradation pathways are usually referred to as pharmaceutical instability, while thermodynamic instability refers to reversible protein unfolding [9].

Water participates directly in many of the above deleterious chemical reactions (including proteolysis) and also provides a medium for molecular movement and interactions. Thus, removal

of water can effectively prevent deterioration of the protein. Lyophilization is used within the pharmaceutical industry to stabilize biological products in order to achieve a longer shelf life ([1]; *see* also Subheadings 3.7, 3.8, 3.9, 3.10, and 3.11 and **Note 2**) [4]. Most biologics are delivered parenterally: intravenously (injection into a vein), subcutaneously (injection under the skin) or intramuscularly (injection into a muscle). The products are usually formulated as a liquid, and because of this, they have limited stability. Therefore, lyophilization is used to stabilize the biologic by converting solutions of unstable materials into solid form by removing water, thus improving long-term storage stability [2, 10]. The dried product is then reconstituted using water or a saline solution before parenteral administration. Effective freeze-drying cannot be hurried. Process times of 72 h or longer are not unusual, depending on the nature of the product formulation and the properties of its constituents. Complex lyophilizer equipment may have high capital and running costs. Freeze-drying is usually reserved, therefore, for high-value products and pharmaceuticals.

A complex interplay of chemical and physical phenomena takes place during the freeze-drying process. The product yield (i.e., the percentage recovery of the initial active protein) depends on the formulation in which the protein is placed prior to lyophilization [11, 12] while its ease of rehydration (reconstitution) and its stability on long-term storage (or "shelf life") are influenced by the processing regime [13–15]. Subheading 3.6 describes, in broad terms, the operation of a typical freeze dryer apparatus. Some critical factors concerning the operations of freezing and primary and secondary drying are outlined in Subheadings 3.9, 3.10, and 3.11.

Good manufacturing practice, ISO 9000 disciplines, and regulatory concerns are beyond the scope of this chapter. The interested reader should consult (for instance) http://www.ich.org/home.html or the linked Quality Guidelines webpage http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.

2 Materials and Equipment

Mention of suppliers' names does not imply endorsement of particular product(s).

1. *Buffers and Chemicals*

   The choice and preparation of an appropriate buffer system (composition, pH, molarity, ionic strength) for a given protein will vary with that protein's characteristics, so it is not possible to give precise directions in a chapter such as this. Consult reference works and/or the scientific literature for guidance

regarding a protein of interest (*see* also Subheading 3.7). Useful chemicals and additives are discussed in Subheadings 3.1, 3.2, and especially 3.3. Highly purified forms of chemicals are preferable.

2. *Containers*

   All containers used for storage of pure proteins should be of good quality and should tolerate temperatures as low as −20 °C or even −80 °C if freezer storage is desired or necessary. A number of manufacturers (such as Sarstedt or Schott, Germany, or Nunc, Denmark; there are many others) supply pre-sterilized screw cap plasticware with good mechanical and low-temperature properties. Clean glassware items exhaustively and sterilize them by dry heat. Autoclave screw caps or rubber stoppers that cannot withstand dry heat (*see* **Note 3**). Ensure that vials (usually glass) or any other vessels (e.g., round-bottomed flasks) used for lyophilization are of sufficient quality to withstand the temperatures and pressures associated with freeze-drying. Vial characteristics are important: *see* **Note 4**. Use the same model of vial consistently [11]. Also choose stoppers with care (*see* **Note 3**). Novel container systems such as syringes, Lyoguard trays, ampoules, and 96-well plates are also available [10, 16].

   Products for lyophilization are usually filled in to vials, ranging in fill volumes from 2–100 mL. Vials for lyophilization purposes are manufactured using Type 1 borosilicate glass to ISO 9001 and ISO 15378 standards and comply with European Pharmacopoeia, US Pharmacopoeia, and Japanese Pharmacopoeia international standards [1, 17].

3. *Filters*

   Membrane or cartridge filters, of pore size 0.22 μm for sterile filtration, are available from companies such as Sartorius, PALL, or Merck Millipore with or without a Luer lock for extra secure attachment to a handheld syringe.

4. *Low-Temperature Storage*

   Use an ordinary domestic refrigerator for storage at temperatures of 4–6 °C. Modern fridge-freezers can maintain temperatures as low as −18 to −20 °C. Storage at −70 °C or below will require a specialized low-temperature freezer.

5. *Lyophilizer Equipment*

   Freeze dryers are manufactured in a range of sizes and are supplied by several manufacturers such as the IMA Life, SP Scientific, Millrock, and Labconco. A laboratory freeze dryer can have a shelf area from 0.1 m^2 to 1 m^2, with a condenser size of up to 30 L, whereas a pilot-scale or commercial-scale unit can have shelf areas from 1 m^2 to over 40 m^2, with condenser sizes up to 120 L. Regardless of size, the internal anatomy is the same for all freeze dryers. A typical freeze dryer comprises a

product chamber, shelves, a condenser, a vacuum pump, and a refrigeration system. The chamber is constructed of 316 L stainless steel and houses temperature-controlled shelves, onto which partially stoppered vials filled with product are frozen. The shelves are hollow, containing heat transfer fluid (HTF), and are generally capable of heating from −70 to 65 °C. The shelves supply the energy via the HTF which allows water to freeze and provides heat energy for drying. The function of the condenser is to collect the water vapor evolved during primary and secondary drying, that is, it acts as a "cold trap." This explains why the condenser must be colder than the chamber (*see* **Note 5** for further details). The condenser is maintained at a temperature below −40 °C to ensure that the vapor pressure of the solvent collected on the condenser is lower than the vapor pressure of the solvent in the chamber. Once at the condenser, the water vapor is converted to ice. The vapor pressure is the pressure exerted by a gas in equilibrium with the same substance in liquid or solid form [15, 18]. The vacuum pump removes atmospheric gases from the chamber, reducing the pressure in the chamber. The refrigeration system provides cooling to the shelves through heat exchangers within the shelf HTF system. There are several different types of refrigeration systems available, with the type of configuration depending on a number of factors such as the load capacity, type of refrigerant, and size of the freeze dryer.

Regardless of the type of freeze dryer, one must use it at all times in accordance with the manufacturer's instructions. Subheading 3.6 assumes that the reader studies this chapter together with the pertinent user's handbook.

3 General Stabilization Methods

3.1 Prevention of Bacterial Contamination

3.1.1 Antimicrobials

Microbial contamination can lead to significant losses of a pure protein by proteolysis. Even if one can eliminate or remove contaminating microorganisms, the protein of interest may already have lost some activity or may have deteriorated in ways that are difficult to detect. Where permissible and feasible, add an antimicrobial compound such as sodium azide (to a final concentration of 0.1% w/v) or thiomersal (sodium merthiolate, a mercury-containing compound, to a final concentration of 0.01% w/v) in order to prevent microbial growth (*both of these compounds are poisonous: handle them with care; see* also **Note 6**). Note that azide will inactivate haem-containing proteins such as peroxidase. Phenol is used as an antimicrobial in some parenteral preparations [19].

3.1.2 Filtration

1. It is good practice to perform filtration operations in a Class 2 laminar flow microbiological safety cabinet, the design of which prevents contamination of the sample. Following the manufacturer's instructions closely, turn on the cabinet's fans and allow to run for at least 10 min to allow adequate filtration of cabinet air. Open and remove the front door. Swab the cabinet's internal surfaces, and the outer surfaces of storage containers brought inside the cabinet, with 70% v/v alcohol (isopropyl alcohol), and allow it to evaporate.
2. Sterilize labile materials, which cannot be autoclaved or irradiated, by filtration. A filter of pore size 0.22 μm will exclude all bacteria. Disposable filter cartridges are widely available in various configurations, often with low protein-binding capacities.
3. Draw the solution to be sterilized into a syringe and then remove the needle or tube.
4. Connect the filter to the syringe nozzle, ensuring it is firmly mounted. Uncap a suitable sterile storage container directly beneath the filter outlet (using standard aseptic manipulations to avoid contamination of container or cap), and depress the syringe plunger to force the protein solution through the sterilizing filter into the container. Recap immediately. It is not always possible to use a filter as fine as 0.22 μm directly (*see* **Note 7**); neither is it possible to "flame" plastic containers in a Bunsen burner as part of aseptic technique.
5. Upon completion of the filtration manoeuvers, remove the storage containers and dispose of waste materials appropriately. Swab the internal surfaces of the cabinet with alcohol once again, replace the front cover, and allow to run for 10 min (or according to the user's handbook) before shutting down.

3.2 Avoidance of Proteolysis

It can be difficult to remove proteases completely during protein purification. Unless the target protein is completely pure (homogeneous), even tiny amounts of contaminating proteolytic enzymes can cause serious losses of activity during extended storage periods. The molecular diversity of proteases complicates matters: there are exo-acting (which remove amino acid residues from the N- or C-termini) and endo-acting (which cleave internal peptide bonds) serine, cysteine (or thiol), acid, and metalloproteases [20]. Ensure before addition that none of the following compounds will adversely affect the protein of interest (*see* **Note 8**). Use EDTA in the concentration range 2–5 mM to complex the divalent metal ions essential for metalloprotease action. Pepstatin A is a potent but reversible inhibitor of acid proteases; use at concentrations of around 0.1 μM, as with similar protease inhibitors. Phenylmethylsulfonyl fluoride (PMSF) reacts irreversibly with the essential serine in the active site of serine proteases, inactivating them (it can also

act on some thiol proteases). A stock solution (>10 mg/mL) in pure isopropanol will be stable for months, but it has a very short half-life when diluted into aqueous solution [21]. Use it at a final concentration of 0.5–1 mM.

If the protein of interest is itself a proteolytic enzyme, one may need to store it in dried form (Subheading 3.5) or as a freeze-dried preparation (Subheadings 3.6, 3.7, 3.8, and 3.9).

3.3 Use of Stabilizing Additives

Timasheff and colleagues have shown that substances such as glycerol or sucrose are preferentially excluded from the vicinity of protein molecules: their binding to protein is thermodynamically unfavorable and the protein becomes preferentially hydrated [22]. This preferential hydration of the protein molecule arises from a polyol-induced increase in the surface tension of the solvent water [23]. Naturally occurring stabilizing additives increase a protein's T_m (the temperature at which 50% of the protein molecules are unfolded) but do not affect the protein's denaturation Gibbs energy (ΔG_D^o) [24]. The intrinsic conformational stability of the protein molecule itself is not increased—but its unfolding is greatly disfavored by virtue of the stabilizing additives in the medium.

Note that the additives discussed below are *generally* applicable for stabilizing proteins, but a given substance may not be effective for a particular protein.

3.3.1 Addition of Salts

Certain salts can significantly stabilize proteins in solution. The effect varies with the constituent ions' positions in the Hofmeister lyotropic series, which relates to ionic effects on protein solubility. This series ranks both cations and anions in order of their stabilizing effects. Below, the most stabilizing ions are on the left while those on the right are destabilizing:

$$(CH_3)_4N^+ > NH_4^+ > K^+, Na^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$

$$SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

The "stabilizing" ions force protein molecules to adopt a tightly packed, compact structure by "salting out" hydrophobic residues. This prevents the initial unfolding event of any protein deterioration process. At low concentrations (<0.1 M), protein stability and enzyme activity are influenced mainly by electrostatic interactions [25–27]. Conversely at higher salt concentrations (>0.1–0.3 M), ionic dispersion forces (Hofmeister lyotropic series) are the dominant mechanism [25–27]. Ammonium sulfate, widely used as a stabilizing additive and as a non-inactivating precipitant, comprises the two most stabilizing ions in the above list, the NH_4^+ cation and the SO_4^- anion. To stabilize proteins in solution while avoiding precipitation, add ammonium sulfate to a final concentration in the range 20–400 mM [28]. Do this by adding a minimal

volume of a stock solution of ammonium sulfate of known molarity or by carefully adding solid ammonium sulfate. Sprinkle the solid salt, a few grains at a time, into the protein solution. Ensure that each portion of ammonium sulfate added dissolves fully before addition of the next lot. This will prevent accumulation of undesirable high local salt concentrations (ensure that the protein-containing buffer is of sufficient molarity to resist a possible pH decrease upon addition of ammonium sulfate, the salt of a strong acid and a weak base). Salts containing citrate, sulfate, acetate, phosphate, and quaternary ammonium ions are also useful [28]. Note that the nature of the counterion will influence the overall effect on protein stability [22].

Polyethyleneimine is a cationic polymer with numerous uses, including protein stabilization [29]. Both high- and low-molecular-weight fractions of polyethyleneimine, at 0.01–1% (w/v) concentrations, greatly increased the shelf lives of dehydrogenases and hydrolases stored at 36 °C. The effect seems to be kinetic rather than thermodynamic, as the denaturation temperature of lactate dehydrogenase was unaffected by the presence of polyethyleneimine [29] (*see* **Note 9**). The cationic surfactant benzalkonium chloride (0.01% or 0.1% w/v, 0.3 or 3 mM) maintained the activity of bovine lactoperoxidase stored at 37 °C, pH 7, for much longer than that of a control sample (but not at pH 6) [30].

It is assumed that ions (or other substances) used/added are not substrates, activators, or inhibitors of the enzyme/protein under study (ammonium ion, for instance, is a substrate for glutamate dehydrogenase) and that added ions do not interfere with, or precipitate, essential ions already in solution.

3.3.2 Use of Osmolytes

Osmolytes are a diverse group of substances comprising such compounds as polyols (e.g., glycerol, xylitol), mono- (e.g., glucose), oligo- (e.g., sucrose, trehalose), and poly-saccharides, neutral polymers (such as polyethylene glycol, PEG), amino acids (and their derivatives), and methylamines (such as sarcosine and trimethylamine N-oxide or TMAO) [23]. They are not strongly charged and have little effect on enzyme activity below 1 M concentration. In general, they affect water's bulk solution properties and do not interact directly with the protein [28] (*see* also Table 2).

Use polyols and sugars at high final concentrations: typical figures range from 10% to 40% (w/v) [28]. Sugars are reckoned to be the best stabilizers (but reducing sugars can react with protein amino groups, leading to inactivation [7]; this can be avoided by using nonreducing sugars or sugar alcohols). Glycerol is a widely used low-molecular-weight polyol. It is easily removed by dialysis and it does not interfere with ion exchange chromatography [28]. Glycerol has two significant disadvantages, however: it is a good bacterial substrate [28] and it greatly lowers the glass transition temperature (T_g') of materials to be preserved by

lyophilization (*see* Subheadings 3.7, 3.8, 3.9, 3.10, and 3.11) or drying (*see* Subheading 3.5). Xylitol, a 5-carbon sugar alcohol, can often replace glycerol, can be recycled from buffers, and is not easily degraded by bacteria [28].

Polymers such as PEG are generally added to a final concentration of 1–15% (w/v). They increase the viscosity of the single-phase solvent system and so help prevent aggregation. Higher polymer concentrations, however, will promote the development of a two-phase system. The protein may concentrate in one of these phases and, possibly, aggregate [28].

Amino acids with no net charge, notably glycine and alanine, can be stabilizing in the range 20–500 mM, as can related compounds, such as γ[gamma]-amino butyric acid (GABA) and trimethylamine N-oxide (TMAO), at similar concentrations [28].

3.3.3 Substrates and Specific Ligands

Addition of specific substrates, cofactors, or competitive (reversible) inhibitors to purified proteins can be very stabilizing (indeed, they may be necessary where an essential metal ion or coenzyme is only loosely bound to the apoprotein). Occupation of the protein's binding/active site(s) leads to minor but significant conformational changes in the polypeptide backbone. The protein adopts a more tightly folded conformation, reducing any tendency to unfold [31] and (sometimes) rendering it less prone to proteolytic degradation. Occlusion of the protein's active site(s) by a bound substrate molecule or reversible inhibitor will protect those amino acid side chains which are critical for function.

Note that dialysis (or some other procedure for the removal of low molecular mass substances) may be necessary to avoid carryover effects of the substrate or inhibitor when the protein is removed from storage for use in a particular situation where maximal activity is desired.

3.3.4 Use of Reducing Agents and Prevention of Oxidation Reactions

Cysteine's thiol group is prone to destructive oxidative reactions. One can prevent or minimize these by using reducing agents such as 2-mercaptoethanol (a liquid with an unpleasant smell) or dithiothreitol ("Cleland's reagent," or DTT, a solid with little odor). Add 2-mercaptoethanol to reach a final concentration of 5–20 mM, and then keep the solution under anaerobic conditions, achieved by gently bubbling an inert gas such as nitrogen through the solution before adding the reducing agent. Fill the solution to the brim of a screw cap container to minimize headspace and the chances of gaseous exchange. DTT is effective at lower concentrations: usually 0.5–1 mM will suffice [32]. The DTT concentration should not exceed 1 mM, as it can act as a denaturant at higher temperatures and is not very soluble in high salt [28] (note that reducing agents are themselves prone to oxidation: solutions containing them must be stored so as to eliminate, or minimize, contact with air). DTT oxidizes to form an internal disulfide which is no longer effective

but which will not interfere with protein molecules [32]. On the other hand, 2-mercaptoethanol participates in intermolecular reactions and can form disulfides with protein thiol groups [32]. Such thiol-disulfide exchanges are highly undesirable and may actually lead to inactivation or aggregation. It is probably best to use reducing agents only where they are known (or demonstrated) to be beneficial.

Oxidation of thiol groups may be mediated by divalent metal ions which activate molecular oxygen. Complexation of free metal ions (where they are not essential for activity) can prevent destructive oxidation of thiols. Polyethyleneimine at 1% (w/v) concentration protected the -SH groups of lactate dehydrogenase against oxidation and prevented the consequential aggregation of the protein, even in the presence of Cu^{2+} ions; the protecting effect was ascribed to metal chelation by polyethyleneimine [29] (see Subheading 3.2 regarding the use of EDTA to complex metal ions).

3.3.5 Extremely Dilute Solutions

Very dilute protein solutions are highly prone to inactivation. This is especially true of oligomeric proteins, where dissociation of subunits can occur at low protein concentration. Protein solutions of concentration less than 1–2 $mg.mL^{-1}$ should be concentrated as rapidly as possible [32] by ultrafiltration (*see* **Note 10**) or sucrose concentration (*see* **Note 11**).

Where rapid concentration is not possible, inactivation may be prevented by addition of an exogenous protein such as bovine serum albumin (BSA), typically to a final concentration of 1 $mg.mL^{-1}$. Scopes has discussed possible reasons for the undoubted benefits of BSA addition [32]. It may seem foolish to deliberately add an exogenous, contaminating protein such as BSA to a pure protein preparation, but occasionally, this may be a price to pay in order to avoid inactivation.

3.4 Low-Temperature Storage

Refrigeration at 4–6 °C often suffices to preserve a protein provided the hints in Subheadings 3.1, 3.2, and 3.3 of this chapter are followed. Many proteins are supplied commercially in 50% (v/v) glycerol or as slurries in approximately 3 M ammonium sulfate. Freezing of such preparations is not necessary and should be avoided.

Some proteins can deteriorate at "refrigerator" temperatures and require storage at temperatures <0 °C. Usually, temperatures between −18 and −20 °C (domestic freezer) will permit stable storage (*see* **Note 12**). Sometimes, however, a low-temperature laboratory freezer, designed to maintain temperatures in the range −70 to −80 °C, may be needed (*see* **Note 13**).

Most protein solutions will freeze to a solid at temperatures <0 °C (mixtures containing high concentrations of glycerol will remain liquid at −20 °C: *see* Subheadings 3.3.2, 3.7, 3.8, and 3.9). Much more complex events occur on freezing of a protein-

containing mixture or biological system than the simple phase change would suggest. Differential freezing of particular components of the mixture can lead to enormous concentration effects and to dramatic changes of pH at low temperatures. These processes can lead, in turn, to protein inactivation. Freezing damage and its avoidance are discussed in Subheadings 3.7, 3.8, and 3.9. This problem can often be minimized by careful choice of stabilizing additives (*see* Subheading 3.3).

Prevention of freezing will, of course, avoid freezing damage. It is possible to undercool liquids without freezing by preventing the nucleation of ice crystals. This means that proteins can be stored well below 0 °C in the liquid phase. The preparation of protein-containing aqueous-organic emulsions which can maintain complete biological activity in the liquid state over extended periods at −20 °C has been described [33]. The method is very useful for small volumes of valuable proteins, avoids the need to use additives, and is more economical than freeze-drying. The same process is used for many different proteins, and one can remove portions of a sample without effect on the activity of the remainder. The actual storage temperature matters little, provided the upper temperature is less than 4 °C and the lower temperature remains above −40 °C, the nucleation temperature for ice crystal formation [34].

3.5 Drying for Stable Storage

The advantages of water removal as a protein storage/stabilization strategy have been set out in Subheading 1. Lyophilization can remove more than 95% of water from a protein preparation, but there is the risk of freezing damage (Subheadings 3.7, 3.8, and 3.9). One can design protein-compatible formulations with glass transition temperatures (T_g') typically as high as 37 °C [35]. With these high T_g values, controlled evaporative drying can be used in place of lyophilization to stabilize proteins in the solid state. Worthwhile evaporation rates will occur below these high T_g values at reduced pressure. Evaporation is faster, less costly, and more easily controlled than freeze-drying [34, 35]. The high T_g values also mean that one can sometimes store the resulting dried product at ambient temperature: as long as room temperature does not exceed the glass transition temperature (*see* **Note 2**), the protein formulation will not undergo a glass/rubber transition during storage at room temperature. The glass-forming compounds are typically carbohydrates; maltose and maltohexaose are particularly valuable [35]. Reconstitution of the solid protein preparation is accomplished simply by rehydration with added water or buffer [36]. References [37] and [38] describe additional drying techniques.

3.6 Lyophilization: Operation of Freeze Dryer Apparatus

The directions below for freeze-drying are very general. Exact details will depend on the material and apparatus in question and on the user's requirements. Useful practical guidelines are given elsewhere [39, 25], and fuller treatments of lyophilization, with emphasis on instability, stabilization, and formulation of protein preparations, are also available [39, 40].

3.6.1 Start-Up

Modern units have advanced PLC (programmable logic controller) systems as standard installation. These PLC systems allow for automatic control of vacuum pressure, shelf temperature, and condenser temperature. The software also allows the end user to program a "recipe," which is a series of sequential steps that will be followed during the lyophilization process. The end user inputs the freezing rate, temperature, and duration, plus the shelf temperature ramp rates, holding step temperatures, and durations and pressures for primary and secondary drying steps.

1. In older units, it is important to start the vacuum pump first so as to reach a steady-state high vacuum long before evacuation of the main chamber.
2. Ensure that the butterfly valve connecting the drying chamber to the condenser is closed.
3. Start the vacuum pump and allow it to evacuate. Observe the decrease in pressure on the vacuum indicator. It is also important that the pump warms up thoroughly before the condenser or shelves are cooled: water in any form reaching the pump can cause damage. A 30 min warm-up time will usually suffice.
4. Close the condenser drain outlet, which should always be left open when the freeze dryer is not in use (if not, open it, allow any water to drain completely, and then close tightly once again).
5. Switch on the condenser and allow it to cool to −60 °C.

3.6.2 Filling and Loading

Vials should be filled in a biological safety cabinet (BSC) or under a laminar flow hood. Lyophilization cycles in the laboratory are often shorter than those in grade C manufacturing environments, as there are lower amounts of particulates in grade C manufacturing environments. This means there are fewer nucleating sites, which leads to higher degrees of supercooling [41] (also *see* **Note 14**).

Vials are placed on a metal tray and packed in a hexagonal pattern from left to right (*see* Fig. 1). Vials should be filled in the BSC to prevent ingress of particles. Bottomless trays with fences are recommended, as they allow the vials to sit directly onto the lyophilizer shelf, promoting efficient heat transfer. The fences are used to keep the vials in a tight pack during loading and unloading. Fill only minimal amounts of material into each sample container to ensure a high ratio of surface area to volume: this will aid effective freeze-

Fig. 1 Hexagonal packing of vials on lyophilizer tray

drying. Fill depths should not exceed 20 mm [42]. Irrespective of vial size, it should not be filled to over 50% of its capacity. This avoids unnecessarily long drying times and minimizes the potential for vial breakage. The product matrix will tend to inhibit sublimation of water vapor from the surface of the ice crystal. This resistance depends on the depth of liquid and on the solid content of the product [12]. A solid content of about 10% w/w is usually best [42] (*see* **Note 15**). Also, a good head space in the vial or ampoule will allow easier and better gaseous movement. After filling, the vials are partially stoppered while maintaining a sufficient gap to allow water vapor to escape during lyophilization. Vials have narrow necks into which rubber stoppers are placed. Take care to ensure that the stopper is placed correctly. Failure to do so could lead to the stopper falling into the neck of the vial, restricting mass flow and leading to cake defects. At the end of the lyophilization cycle, vials are stoppered automatically in the freeze dryer. Before freeze-drying, it is a good practice to wash and/or autoclave the stoppers and dry them before use and to wear gloves when handling them. At a minimum, the stoppers should be washed in deionized water and then dried in an oven at 110 °C for 3 h post washing (or autoclaving) and allowed to cool to ambient temperature before use.

If the freeze dryer has ports for thermocouples, they should be utilized (*see* **Note 16**). It is essential to measure the product temperature of the protein formulation throughout the lyophilization process. Type T thermocouples should be used for lyophilization experiments. A temperature record of the *actual* solution (instead of the shelf underneath it) is well worth the loss of a small amount of product.

3.6.3 The Freeze-Drying Operation

1. Check that the door of the drying chamber is properly closed and sealed (or, for simpler devices, attach the flasks of previously frozen material to the instrument manifold, checking for a good seal). Failure to ensure that the chamber is sealed may result in the unit being unable to reach and hold the desired vacuum level.
2. Load vials with shelf temperature at 20 °C.
3. Ensure that all inputted lyophilization cycle recipe parameters are correct (it is very easy to make errors at this point, so it is advisable to ask a second analyst to review the recipe parameters). **Steps 4–16** outline a generic lyophilization recipe. In freeze-drying, there is no such thing as "one recipe fits all," that is, a recipe that is suitable for one particular formulation may not be at all appropriate for another, because each formulation has different thermal properties. Experimentation with temperatures, pressures, and durations is advised.
4. Hold at 20 °C for 5 min.
5. Ramp from 20 to −50 °C in 70 min (i.e., at 1 °C/min).
6. Hold at −50 °C for 1.5 h.
7. If mannitol or glycine were used in the formulation, it is necessary to crystallize those components by annealing as follows:
8. Ramp from −50 to −15 °C in 70 min.
9. Hold at −15 °C for 3 h. *Note:* Annealing temperature and hold times will vary across different formulations: use mDSC| (Subheading 3.13) to determine appropriate annealing temperature and duration (*see* Subheading 3.11).
10. Ramp from −15 to −50 °C in 1 h.
11. Hold at −50 °C for 1 h.
12. Once freezing is completed, primary and secondary drying steps can be programmed.
13. Ramp from −50 to 0 °C in 60 min at 100 mT.
14. Hold at 0 °C for 12 h at 100 mT (primary drying).
15. Ramp from 0 to 20 °C in 12 h at 100 mT.
16. Hold at 20 °C for 12 h at 100 mT (secondary drying).

During lyophilization, the pressure gradient between the product and the drying chamber, together with the temperature gradient between the shelf and the condenser, is the driving force for sublimation. The sublimed water will collect as ice on the condenser. Sublimation, or primary drying, removes mostly the bulk water (in the form of vapor, sublimed directly from ice) from the system. The shelf temperature for primary drying is insufficient to remove the "bound" or "unfrozen" water closely associated with the protein molecules, unless primary and secondary drying temperatures are the same. Bound water is a water that did not freeze during the freezing step but rather has been adsorbed onto the surface of the protein and/or excipients. Secondary drying (desorption of the bound water) is performed at a higher temperature than primary drying. One can program the shelves to heat to a particular temperature at a defined rate appropriate for the product in question. One must select the primary and secondary heating regimes with particular care (*see* Subheadings 3.8 and 3.9).

3.6.4 Termination of Run and Removal of Product

1. Use the Pirani vacuum gauge (PVG) to determine the end point of primary drying. When the PVG reading converges with the capacitance manometer (CM) reading, primary drying is essentially complete—allowing secondary drying to commence (if at the end of primary drying the PVG has not converged with the CM, simply amend the recipe to extend primary drying time until such time as the PVG and CM converge).
2. At the end of lyophilization cycle, the partially stoppered vials can be sealed inside the freeze dryer. Stoppering requires the shelves in a freeze dryer to be able to move up and down. The shelves are compressed together to provide the force for stoppering. This operation will insert the stoppers fully before releasing the vacuum. Before stoppering, nitrogen gas can be introduced to the chamber in order to provide an inert blanket over the product which will assure maximum stability during storage. Failure to fully stopper vials can allow an ingress of air into the product vial when the chamber vacuum is released, and this may impact the long-term stability of the product. When sampling a lyophilized product, the stopper is removed in order to gain access to the lyophilized cake. If a vial was not sealed correctly, or where the seal has broken down, the sound of inrushing air will be absent from a defective vial when the stopper is removed. In this way, the user will immediately know of the defect and be aware that the vial contents may have deteriorated [43]. Remember to return the shelves back to their starting position before breaking the vacuum.

3. Once stoppering is completed, the vacuum can be released. Modern freeze dryers allow the user to simply click a button in order to release the vacuum. The vacuum/pressure gauge will show a rise in pressure; this will eventually equalize to atmospheric and one can then open the chamber door. After vacuum is broken, shelves can be returned to their initial position. Vials of freeze-dried product can be removed for storage (preferably at refrigerated temperatures). It is a good practice to inspect the product vials for any defects or abnormalities once removed from the freeze dryer.

3.6.5 Shutting Down

If the freeze dryer is to be reused immediately, one must be certain that the condenser's ice capacity will withstand the accumulation of ice from two runs (*see* **Note 15**). If there is no more material for lyophilization, shut down the apparatus carefully. Remove any material spilled in the drying chamber and clean the apparatus carefully according to the manufacturer's instructions. Leave the chamber door slightly ajar to allow circulation of air and to prevent sticking and compression of the door seals. Switch off the condenser and open its drain outlet (the drain should remain open until the freeze dryer is next used). Over a period of hours, the ice on the condenser surfaces will melt and drain away. To maintain good vacuum pump performance, regularly inspect the quality of the oil and change when necessary. If upon inspection the oil is cloudy, it needs to be changed. Follow the manufacturer's instructions when changing the oil.

3.6.6 Using Simpler Freeze Dryers

The use of simpler apparatus with manifold or centrifuge accessories is carried out in much the same stepwise fashion as above. In these cases, aliquots of the product are frozen in individual open-necked flasks or tubes. Switch on the vacuum pump and condenser, and allow to run as noted above. Freeze the flask contents by immersion in an alcohol cooling bath (follow the normal precautions). Swirl or rotate the flask during the freezing step to effect even distribution of the product over the widest possible surface area. This will minimize the depth of material through which water loss must occur: *see* Subheading 3.6.2 and [12]. Connect the frozen material directly to the manifold assembly (or load into the centrifugal tube dryer), and draw a vacuum in the chamber as quickly as possible so as to minimize back-melting of ice while the sample is still under atmospheric pressure. The rapid reduction of pressure by the vacuum pump will aid sublimation and help minimize melting of frozen solution. While control of temperature and heating rates are more problematic with such accessories, visual inspection of the material and of the dried cake is much easier than in an enclosed chamber.

3.7 Lyophilization: Formulation

The goal of formulation is to create a multicomponent system, which can be freeze-dried in a suitable time frame, while at the same time ensuring stability during the product's life cycle [18, 39]. When a formulation is cooled during the initial freeze segment of a lyophilization cycle, the structure that is formed may exhibit crystalline or amorphous properties and in some cases a mixture of both. Excipients may be classed as, but are not limited to, bulking agents, tonicity modifiers, buffers, and cryoprotectants/lyoprotectants [17, 40]. Each excipient added to a formulation should have a specific function, for example, amorphous sucrose or trehalose can be used as stabilizers and/or cryoprotectants [44, 45] whereas crystalline excipients such as mannitol and glycine are used as bulking agents to improve the elegance of the freeze-dried cake [39, 46]. There are two major hypotheses to describe the stabilization of proteins by sugars. The first one is the "glassy dynamics" hypothesis. This theory proposes that the sugar provides a rigid glassy matrix in which the protein is dispersed, and the limited mobility in the glassy matrix reduces molecular motion, thereby preventing inactivation reactions [45, 47]. The second mechanism is the "water substitute" hypothesis. This theory proposes that the sugar will form hydrogen bonds with polar sites on the protein's surface, stabilizing the native structure of the protein during lyophilization [46–49]. Lyophilized formulations typically contain excipients in both amorphous and crystalline forms. Formulations consisting solely of crystalline excipients by themselves are not commonly used for lyophilization, as the potential for crystallization during storage can remove the stabilizing effects of those excipients [48, 50–52]. Therefore, the physical state of the freeze-dried cake is usually partially crystalline (amorphous protein and crystalline excipients) or amorphous (amorphous protein and amorphous components) [18, 39]. The portion of the excipient matrix added to stabilize the protein should be in the same amorphous phase as the protein for effective stabilization [18, 39]. A comprehensive review of formulation for lyophilization can be found in the literature [1, 25, 49, 52].

Suitable excipients/protective substances can lessen or overcome some of these damaging effects: especially, they can influence melting or collapse temperatures [40]. Excipients may be classed as bulking agents, tonicity modifiers, buffers, and cryoprotectants/lyoprotectants [40]. Bulking agents help to ensure the development of a plug of dried material and are used where the product's solids content is low. Bulking agents can also prevent blowout (loss of the dried product from the container along with the vapor) [40, 53]. Typical bulking agents include polyols and certain sugars, mostly nonreducing.

Buffers must be chosen with great care, considering possible pK_a variations with temperature, solubility, and compatibility with the protein(s) and other constituents. Useful information can be

found in [54] and in monographs and articles dealing with the handling of proteins. Note that the most stable pH for a protein in solution may not be appropriate for the solid state [40].

It is often necessary to formulate the protein with other excipients in order to ensure that the protein is protected during the lyophilization process and during storage in the dried state. Buffers are often employed to control pH during the lyophilization process. Examples of most common buffers employed are sodium citrate, acetate, histidine, Tris–HCl, and glutamate. Sodium phosphate and potassium phosphate buffers can undergo pH changes during freezing and annealing and should generally be avoided. Care should be taken when selecting excipients, and it is important to understand the function of each excipient added to the formulation. The reader should consult references [1, 53] for additional information on formulation for lyophilization.

Cryoprotectants and lyoprotectants stabilize a protein against the effects of freezing and of lyophilization/storage, respectively. These protecting additives are preferentially excluded from the protein surface, strengthening of the water "shell" surrounding the protein [22]. Sugars, certain salts, and polyols can each be beneficial (although PEG-dextran mixtures may give undesirable and damaging phase separations at low temperatures) [55]. In general, nonreducing sugars are preferable since these cannot participate in Maillard reactions. Xylitol's T_g' is −46.5 °C in a freeze concentrate of 42.9 weight % water while T_g' values for sorbitol, sucrose, and trehalose are −43.5, −32, and −29.5 °C at water weight % values of 18.7, 35.9, and 16.7, respectively [11]; *see also* [40]. Mannitol and lactose may separate as crystals from a frozen solution under certain conditions [25, 40, 42], so these compounds should be used with caution. Glycerol is not an ideal lyophilization excipient: it has a notably low T_g' value of −65 °C at 46 weight % water [11]—meaning that glycerol-containing formulations become glassy only at very low temperatures. Volatile excipient compounds, such as ammonium bicarbonate, will be removed with the subliming ice and therefore will not occur within the final product [42]. Sucrose or glycine in combination can be a useful starting point for the formulation of a solid protein product [40]. T_g of protein formulations increases with the protein/excipient ratio [40]. Useful discussions and examples of cryoprotectants occur in [22, 28, 40]; *see also* **Notes 17** and **18** and Subheading 3.8.

3.8 Lyophilization: Glass Transition and Collapse Phenomena

Determination of the critical temperature of a product prior to lyophilization is vital in order to design an efficient and safe cycle for that product. To achieve this, it is essential that the formulation be characterized in order to determine the temperature above which desirable properties of a freeze-dried product are lost [9, 56] (*see* Subheading 3.11 and **Note 19**). For amorphous

formulations, the critical temperatures are as follows: (i) the glass transition temperature (T_g' and T_g, *see* **Note 2**) and (ii) the collapse temperature (T_c). For crystalline formulations, the critical temperature is the eutectic melt temperature (T_{eut}). A eutectic mixture is a mixture of two or more crystalline materials that are in such close contact that they melt like a pure substance. Eutectic melting of crystalline excipients can lead to a defect called meltback. During the freezing stage, water is converted to a crystalline ice phase. As the temperature drops further, the excipients and protein molecules lose translational mobility and do not have enough energy to form a crystal lattice. Instead, they arrange themselves between the ice crystals in a disorderly amorphous configuration and are described as glasses [56, 57]. Therefore, it is necessary during freezing that the formulation is frozen at a temperature low enough to ensure that all of the formulation components are immobile, that is, the formulation must be frozen below its glass transition temperature, T_g'. The glass transition temperature can be defined as the temperature at which the dynamics of an amorphous system changes from a more mobile phase "rubbery" state to a less mobile "solid-like" glassy state [40, 58]. The temperature at which maximum freeze concentration occurs is defined as T_g'. Below T_g', a rigid glass with high viscosity and low mobility is formed [2, 57]. *Amorphous* liquids undergo a *glass transition* where viscosity increases dramatically with cooling and the solution takes on the macroscopic properties of a solid, even though it has not crystallized. At the glass transition temperature, the viscosity of the maximally freeze-concentrated solution can increase to $10^{13–14}$ P orders of magnitude [13, 59–61]. Below the glass transition temperature (T_g'), virtually no adverse chemical or biological reaction can take place. It is well established in the lyophilization literature that the collapse temperature, T_c, is generally a few degrees higher than the glass transition temperature. The collapse temperature is measured using freeze-drying microscopy (FDM). This instrument comprises a light microscope with a camera on top and attached is a vacuum pump, a PVG, and a Dewar flask containing liquid nitrogen fixed to the microscope stage. It is essentially a mini freeze dryer, and it allows the user to program a recipe and then visually identify a temperature at which various thermal events such as collapse, onset of crystallization, and melting occur. T_c is usually within 1–2 °C of T_g' and both are often used interchangeably [60, 62]. However, for higher concentration protein formulations, the difference between T_g' and T_c may be larger as protein concentration increases [61, 63]. There are several definitions of the collapse temperature. One defines it as the disappearance or annihilation of the freezing pattern with the passage of the sublimating interface [62, 64]. Another is defined as a loss of structure, a reduction in pore size, and a volumetric shrinkage of dried materials [63, 65]. During primary drying, when the product temperature

exceeds the T_g' of the maximally freeze-concentrated solution, it can result in a loss of the microstructure established by the freezing process [60, 62]. When a product collapses, it can result in the clogging of the pores formed as a result of sublimation of ice, and this can significantly reduce the rate of sublimation [64, 66]. It has been reported in numerous papers that freeze-drying above collapse should be avoided as it can lead to product with loss of pharmaceutical elegance and higher moisture content [50, 65–75]. Collapse is often referred to as "total" or "gross collapse," and almost certainly product with a collapsed appearance would be rejected from a commercial process. A product that is freeze-dried above the collapse temperature may have (i) a product appearance that is heterogeneous and relatively poor, (ii) longer reconstitution time, and (iii) higher residual moisture. Besides unacceptable appearance, various undesirable properties result from the collapse during freeze-drying. For example, collapse can significantly reduce the rate of sublimation [67] by clogging the paths through which water can escape. As a result, the final product tends to retain higher moisture content than one dried below collapse, and the residual moisture may be distributed unevenly through the sample [68]. Thus, accurate measurement of T_g' is of great importance. In most cases (where the protein of interest is in dilute solution), T_g' depends on the types and proportions of the excipients and salts in the product formulation [11, 40].

It has also been demonstrated that proteins lyophilized above the collapse temperature have exhibited aggregation and loss of activity on stability [50, 51, 67, 69]. There is another type of collapse phenomenon to consider: micro-collapse. It has been demonstrated that when amorphous formulations are lyophilized above the collapse temperature, complete collapse does not occur but rather small holes can appear in the cake, and these additional pathways for vapor flow result in a significant decrease in cake resistance [51, 69–71]. Lyophilization in this manner has been found to increase the size of pores in the dry layer and reduce their tortuosity, resulting in low resistance to the flow of water vapor which does not increase with dry layer thickness [14, 70]. An advantage of drying near the microscopic collapse temperature is that the reduced resistance can result in shorter primary drying times. However, concerns have been raised that exposing proteins to the micro-collapsed state could affect long-term stability [16, 71].

Understanding these low-temperature features of liquids is important for effective freeze-drying (*see* **Note 2**). Eutectic and glass transitions will greatly influence (i) the freeze-drying protocol (the way the freeze dryer is run) and (ii) the choice of substances used as preservatives or excipients in the product formulation subjected to lyophilization. The process of freeze-drying has been described in detail [13, 61], as are the effects of additives

[53]. Reference [35] contains a useful treatment which touches on the underlying theory. Franks outlines principles for process design [42], while Oetjen has published detailed monographs [72, 73]. Bhatnagar et al. provide a detailed review of lyophilization literature [39].

3.9 Lyophilization: Freezing and Cold-Related Phenomena

3.9.1 Freezing

The objective of freezing is to create an optimum ice crystal structure to enable the removal of water vapor from the cake. The freezing step influences the performance of the subsequent drying steps, and the quality attributes of the final drug product depend on the way in which the liquid was frozen [10, 58]. The first step of the freezing process involves loading vials containing the product onto the shelf of the freeze dryer and normally freezing below −40 °C with the aim of converting water (solvent) to a crystalline ice phase. This ice phase is removed later during the primary drying stage of the cycle. The remaining product and excipients (solutes) remain mainly in an amorphous phase [2, 10]. The ice nucleation temperature, which is stochastic in nature, is the temperature at which ice crystals first appear. The degree of supercooling is the temperature difference between the thermodynamic ice formation temperature and the actual temperature at which ice begins to form, usually around 10–25 °C lower [25, 52]. It governs the rate of nucleation and determines the number of ice crystals formed; this in turn affects the porosity of the freeze-dried cake [27, 74]. The pores in the cake, remaining after the sublimation of ice, are a direct reflection of the size and geometry of the ice crystals formed during freezing [27, 74]. As described by Patel and co-workers, a higher degree of supercooling leads to smaller ice crystals being formed, resulting in a smaller pore size. This in turn leads to greater resistance to vapor flow and longer primary drying times [74, 75]. In a manufacturing environment, there are fewer nucleation sites available for freezing, due to the low particulate environment; therefore, the degree of supercooling is higher. This is why manufacturing lyophilization cycles are longer than those in the laboratory. Proteins are sensitive to extremes in temperature and are stable only in a defined temperature range [18, 39].

The freezing step can have a direct influence on the tendency of a protein to denature [75–77]. Similar to denaturation caused by heating, a decrease in temperature can also result in protein unfolding, by a process called cold denaturation [77–79].

Certain proteins are more stable at room temperature than in the refrigerator and are said to be *cold labile*. This cold denaturation has been well characterized for yeast frataxin [80, 81], myoglobin, and numerous other proteins [82]. It is a property of the protein itself and is distinct from freezing inactivation. This phenomenon arises from the fact that it is thermodynamically possible for a protein to unfold at low as well as at high temperatures (*see* [34] for a summary of the notable features of cold denaturation, which can be reversible in many cases [82, 34]).

Another potential stress a protein can encounter is the freeze concentration of solutes. During freezing, water is converted to crystalline ice, and this results in the solutes in the amorphous region become more concentrated [55, 79]. Buffer salts may concentrate, causing undesirable effects on protein structure [55, 83]. The freezing process can also influence the primary drying rate due to difference in ice nucleation temperature [83, 84]. In the case of protein-saccharide systems, phase separation into protein-rich and saccharide-rich phases can result in protein instability and can also facilitate the crystallization of other components [84, 85]. In addition, the freezing step has also been shown to influence cake appearance [85, 86] and residual moisture content [86]. A detailed discussion of the stresses involved during the freezing of proteins can be found in [87].

3.9.2 Annealing

When mannitol and glycine are used as excipients in lyophilized products (mainly as bulking agents, *see* Subheading 3.7), it is necessary to crystallize them prior to commencing primary drying. Failure to do this can result in (i) collapse of the product matrix during primary drying and/or (ii) crystallization of mannitol or glycine during storage (which can compromise the stability of the product). In order to crystallize these excipients, after the minimum one-hour freezing-hold step at −50 °C, raise the shelf temperature above T_g' of the formulation (as determined using modulated DSC; *see* Subheading 3.11 and **Note 19**) and hold for a predetermined time. This process is called annealing, and it can (i) significantly increase the primary drying rate and (ii) improve cake appearance [88]. Annealing is also used to minimize heterogeneity of a batch by allowing large ice crystals to grow at the expense of smaller crystals (Ostwald ripening) [89]. Once annealing is completed, lower the shelf temperature back down to −50 °C and hold for 60 min. Depending on the formulation, it may be necessary to maintain product temperature below T_g' (T_g', for amorphous substances; *see* **Notes 17** and **19**) or the collapse temperature, T_{col}, during primary drying. If the temperature rises above this value, the material can undergo viscous flow and become rubbery; it is then very prone to deleterious reactions, with resulting losses of activity.

Other changes, many of which can lead to significant protein denaturation, may occur in the product during freezing [87]. Altered secondary structures have been detected in lyophilized proteins [55, 87]. Useful methods to characterize protein secondary structure in the solid state include Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, and near-infrared spectroscopy [87, 90]; *see* also Table 2 and Fig. 2, respectively. The protein may undergo inactivation, as indicated by poor recovery of pre-lyophilization activity. As the bulk water freezes to ice, the amount of liquid water remaining naturally decreases. This

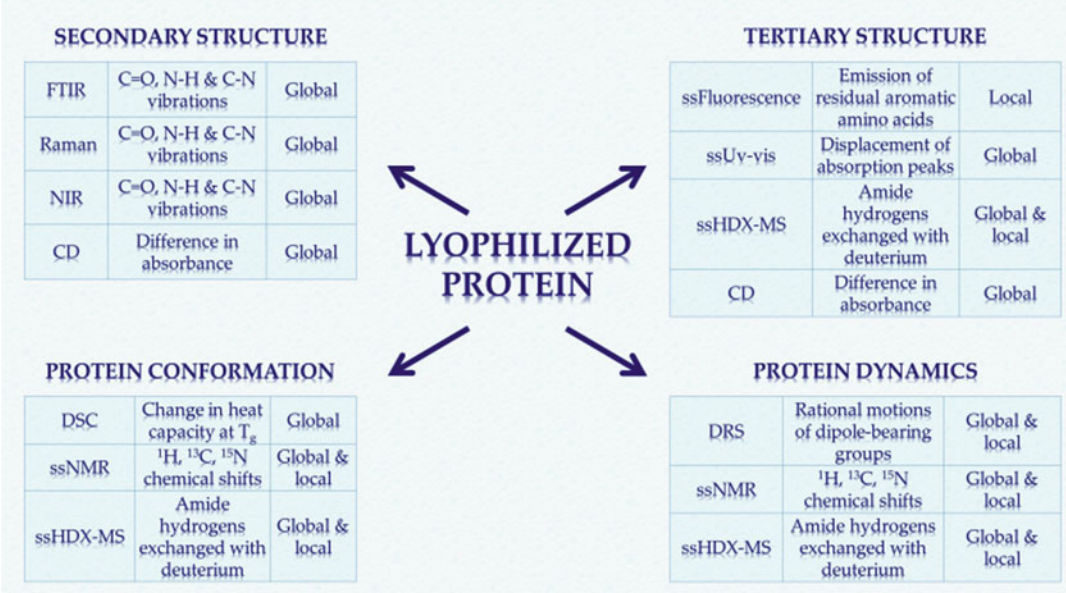

Fig. 2 The most common analytical techniques for the structural characterization of proteins in solid pharmaceutical forms are presented with corresponding type of measurements. Changes in secondary/tertiary structure and conformation can be studied on a global and local scale. Protein dynamics can also be traced using some of the above methods. FTIR Fourier transform infrared, NIR near-infrared, CD circular dichroism, ss solid-state, HDX-MS hydrogen-deuterium exchange mass spectrometry, DSC differential scanning calorimetry, NMR nuclear magnetic resonance, DRS dielectric relaxation spectroscopy. (Reproduced from [Pharmaceutics 2021, 13: 534], with permission from the authors and copyright holders: Bolje A and Gobec S. Analytical Techniques for Structural Characterization of Proteins in Solid Pharmaceutical Forms: An Overview. [DOI: 10.3390/pharmaceutics13040534] Published by MDPI, Switzerland [90])

leads to great freeze concentration of solutes such as salts, perhaps with far-reaching effects (*see* **Note 18**). Concentration increases the rates of unwanted chemical reactions such as oxidations. Buffer components may crystallize differentially, leading to pronounced pH shifts; also, pK_a values are temperature dependent. For phosphate buffers, one should use potassium or mixed salts in preference to sodium salts [11, 91]. For all of these reasons, one should accomplish the freezing steps (crystallization of bulk water and cooling below the eutectic melt or T_g' temperatures) in any lyophilization process as quickly as possible.

3.10 Lyophilization: Primary Drying

Primary drying is the sublimation under vacuum of bulk ice from the product to the much colder condenser typically held at −60 °C. Primary drying is the longest part of a lyophilization cycle and is most susceptible to chamber pressure and shelf temperature deviations. To facilitate sublimation during primary drying, the pressure is kept below the vapor pressure of ice.

Sublimation will be faster at higher temperatures, so if possible, heat the samples to a few degrees below T_g' to quicken the process (*see* **Note 17**). A usual safety margin is 2–5 °C below the eutectic or collapse temperature [12, 25, 40] (however, it is possible to lyophilize products above their T_g' without compromising their quality). Drying at these conditions could result in micro-collapse, but it could be acceptable. The sublimation rate depends on a number of factors, such as the formulation excipients, the chamber pressure, the shelf and condenser temperatures, and also depends on the characteristics of the lyophilizer [10, 11]. It is important to monitor temperature continuously in order to ensure that the temperature of the actual product remains below the critical temperature (often, this means the collapse temperature) throughout the primary drying operation. Drying often becomes easier as the temperature approaches T_g' or the eutectic temperature: the sublimation rate can increase by about 13% for each 1 °C rise in temperature [12]. Drying time also decreases with decreasing product thickness (i.e., filling height) and with increasing vial diameter (which influences the area of the drying surface) [12]. The sublimation rate will decrease as primary drying proceeds, and therefore, the degree of product cooling due to sublimation will decrease also. Be sure to adjust any heat input to the vials (or other containers) as the product dries in order to prevent a net rise in product temperature; an uncontrolled increase could lead to collapse. Even under uniform conditions, primary drying times may vary by up to 10%. Make sure to include a delay period or "soak time" at the end of the primary drying (use the PVG and CM convergence readings to identity the end of primary drying). The purpose of this "soak time" is to ensure that all ice has sublimed before commencing the temperature ramp to secondary drying (failure to ensure complete sublimation can result in meltback or collapse; *see* Subheading 3.8). If the freeze dryer does not have a PVG, one can estimate the end of primary drying when the product temperature in the vial (as measured by the thermocouple) reaches a steady temperature and is closely aligned with the shelf temperature (however, installation of a PVG is strongly recommended, as it is an invaluable tool and is relatively cheap).

3.11 Lyophilization: Secondary Drying

After ice is removed during primary drying (sublimation), unfrozen bound water (adsorbed on to the surface of the dried cake) is removed during secondary drying (desorption). The purpose of secondary drying is to reduce the level of moisture to assure maximum stability during storage [25]. At the end of primary drying, the shelf temperature is increased over a number of hours to a higher temperature (experimentation is advised both for temperature and time). After primary drying, approximately 5–20% of the remaining water resides in the amorphous phase, and this water must be removed [92]. The unfrozen water may be adsorbed on

the surface of the crystalline product or is in the solute phase and a crystalline hydrate or dissolved in an amorphous solid to form a solid solution [68]. Once ice has been removed during primary drying, the shelf temperature is raised to remove this unfrozen water [52]. The optimal level of moisture in a product is highly formulation and product specific [39] but is usually less than 1% w/w for proteins and varies between 2% and 3% for vaccines [58]. When the optimum moisture level required is unknown (which is common during cycle development), a slow heating rate (0.1–0.2 °C/min) from the end of primary drying to the secondary drying temperature should be used to minimize risk of collapse and shrinkage [58, 25]. A secondary drying shelf temperature of 25–30 °C for 3–4 h is a good starting point [58]. As mentioned above, 5–20% of residual water will remain at the end of primary drying, and the T_g/collapse temperature of the product will be quite low; thus, fast ramping to the elevated temperature of secondary drying carries the risk of product damage in terms of both quality and stability [58]. In the past, the pressure during secondary drying was carried out as low as possible. It was demonstrated with both amorphous and crystalline formulations that the rate of secondary drying does not depend on the chamber pressure in the range of pressures typically encountered in the freeze-drying of pharmaceuticals, 0–200 mT [68]. In the same study, it was recommended that a combination of a higher drying temperature and a shorter drying time be used as opposed to a low drying temperature for a longer time. In general, for most lyophilization cycles, pressure has no impact on secondary drying and should remain the same as that used during primary drying [68].

Care should be taken not to increase shelf temperature so fast as to collapse the product. Secondary drying should only commence once primary drying is complete. Therefore, it is essential to have a method to determine the end of primary drying, that is, convergence of the PVG with that of the CM. This is to prevent collapse in any vials which have not quite finished sublimation ([12, 25,40] and Subheading 3.8). The partial pressure of water within the drying chamber drops at the end of primary drying as the last of the ice sublimes. If it can be monitored, this drop in water partial pressure can be a good indicator of the completion of primary drying. Even during secondary drying, with much of the original water removed, the preparation's temperature should never rise above T_g [11]. T_g, however, rises as the residual water content drops [12]. One can, therefore, increase the temperature (within limits) during secondary drying (overheating during primary and/or secondary drying will likely be deleterious, however).

The final residual moisture content of the product and its subsequent stability profile are a function of a successful secondary drying step.

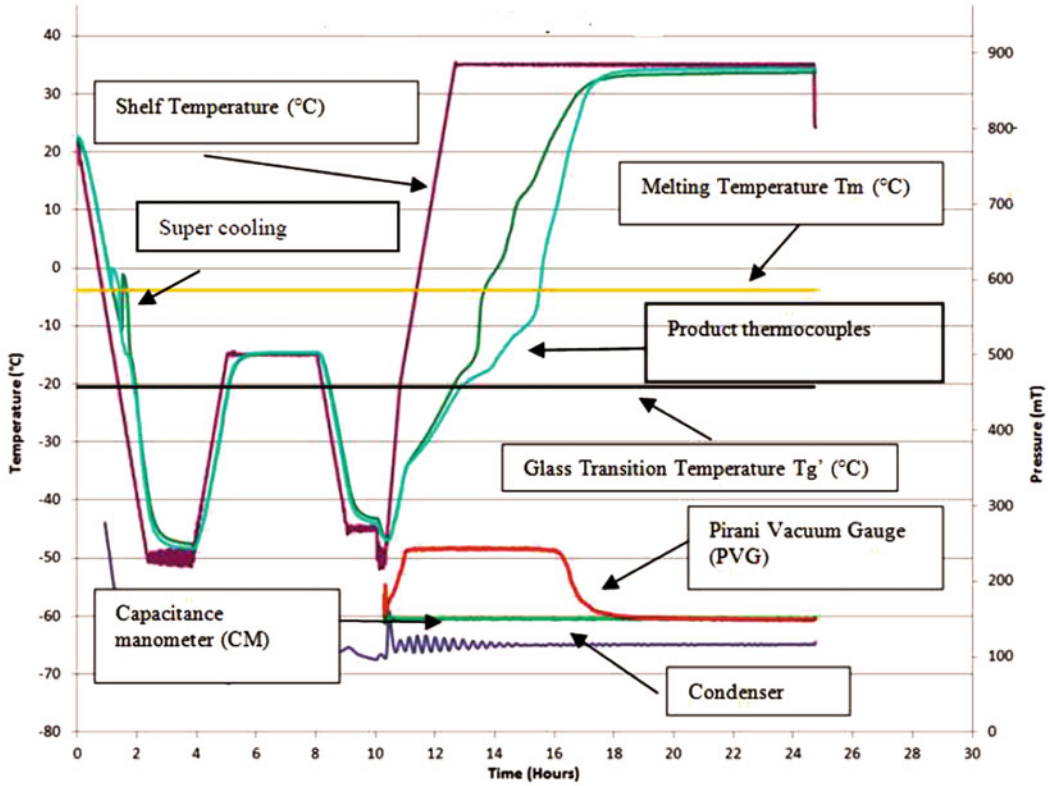

Fig. 3 Excel output chart from lyophilizer

3.12 Lyophilization: Quality Indices

Once a cycle is completed, it is a good practice to analyze the data collected during a run. Readings such as shelf temperature, condenser temperature, vacuum pressure (PVG and CM), and product temperature (from the thermocouple) can be exported from the system to Microsoft Excel allowing cycle information to be visually represented; *see* Fig. 3. Note the cake shape and texture and any variations between vials, especially those located at different positions within the chamber. Each vial should be inspected. A library of lyophilized cakes should also be curated, and a link (if one exists) should be established between cake appearance and product quality. Indeed, the cake appearance may or may not be indicative of overall product quality. It is important to establish if product quality attributes, for example (but not limited to), reconstitution time, pH, potency, and molecular weight, have been impacted by the lyophilization process. Reference [93] describes a science- and risk-based approach for establishing acceptance criteria for cake appearance and provides guidance on overcoming the challenges related to visual appearance of lyophilized products. It also states that it is a waste of product to discard a batch of vials based solely on cake appearance, if other quality attributes are within specification [90, 93]. The present authors agree with this advice!

Choose a representative number of vials from different chamber locations (front row, middle row, and back row) for scrupulous moisture determination (*see* **Note 20**). Measure the yield or recovery (%) of the initial biological activity by appropriate assay following rehydration of a representative number of vials. While waiting to assay, note the time required for complete product rehydration/reconstitution. Also note whether any turbidity remains on rehydration or after what interval turbidity appears in a clear sample [11]. The reconstituted product should be free of visible solid matter. Note that the formulation used greatly influences yield while the process parameters affect ease of rehydration and shelf life [11]. Persistence of the rehydrated biological activity can be measured at suitable or convenient time intervals. Accelerated degradation methods can predict shelf lives of the lyophilized preparation at temperatures of interest for long-term storage (Subheading 3.12).

3.13 Modulated Differential Scanning Calorimetry (mDSC) as an Aid to Lyophilization

Modulated differential scanning calorimetry (mDSC) can be used to determine the glass transition temperature and other transitions such as crystallization and melting [17, 40]. It measures the difference in heat flow to a sample compared to a reference and detects both (i) first-order irreversible/kinetic thermal events such as crystallization and eutectic melt (exotherms or endotherms) and (ii) second-order reversible events such as glass transitions [10, 58]. Hence, mDSC can enable assessment of the thermal properties of both liquid (pre-lyophilized) and lyophilized (powder) (*see* **Note 19**).

For dry powder analysis (lyophilized samples), vials should only be opened in a glove box purged with nitrogen; relative humidity (RH) in the glove box should be less than 2% (if the lyophilized powder absorbs water vapor, this can lower T_g. A lower T_g may impact the stability of the product during storage). The lyophilized powder should be ground down to a fine powder using a mortar and pestle to ensure that there are no thermal gradients across the sample during thermal analysis. Between 3 mg and 6 mg should be added to aluminum sample pans.

Example of DSC recipe for bulking agent containing formulations:

Liquid thermal method
1: Equilibrate at 20 °C
2: Modulate +/− 0.50 °C every 100 s
3: Mark end of cycle 1
4: Ramp 0.50 °C/min to −50 °C
5: Mark end of cycle 2
6: Isothermal for 90 min

(continued)

Liquid thermal method
7: Mark end of cycle 3
8: Ramp 0.50 °C/min to −30 °C (annealing step to crystallize mannitol or glycine)
9: Mark end of cycle 4
10: Isothermal for 180 min
11: Mark end of cycle 5
12: Ramp 2 °C/min to −50 °C
13: Mark end of cycle 6
14: Isothermal for 5 min
15: Mark end of cycle 7
16: Ramp 2 °C/min to 25 °C

The glass transition, T_g', can be detected by analyzing the reversing heat flow signal. Glass transitions are always found in the *reversing* heat flow signal, while crystallization events occur in the *nonreversing* and *total* heat flow signals. Glass transition values are normally calculated as a range: onset, midpoint, and offset. The midpoint is the most important value, as it is more reproducible. The T_g' is seen as a step in the heat flow due to the increase in the heat capacity of the sample through the glass transition.

Example of DSC recipe for lyophilized powder:

Powder thermal method
1: Equilibrate at −20 °C
2: Mark end of cycle 1
3: Modulate +/− 0.50 °C every 100 s
4: Mark end of cycle 2
5: Ramp 2 °C/min to 250 °C
6: Mark end of cycle 3

3.14 Stability Analysis and Accelerated Degradation Testing

Kinetic stability, defined in Subheading 1, is usually measured at elevated temperatures [3], but the inactivating event(s) at high temperatures may not mirror that/those at the much lower temperatures used for storage. It is not feasible, however, to monitor stability in real time at the actual storage temperature: the experiment would take too long. Inaccuracy may result over shorter intervals, since only minimal losses, scarcely distinguishable from the starting activity, would be apparent.

Accelerated degradation (accelerated storage) protocols can be of value in these situations *provided* the activity decay is first order at each of the temperatures tested and all data are scrupulously accurate and precise. Accelerated degradation involves the periodic assay of samples incubated at different temperatures and use of the Arrhenius equation ($\ln k = -E_a/R.T + \ln A$, where k is the first-order activity decay constant, E_a the activation energy, R the gas constant, and T the temperature in Kelvins) to predict "shelf lives" at temperatures of interest. Extrapolation of the Arrhenius plot ($\ln k$ vs. $1/T$, slope $-E_a/R$) can give the rate constant (and hence the useful life) at a particular temperature. Kirkwood [94] gives guidelines for the proper use of accelerated methods. Experimental data undergo transformations before use in the Arrhenius plot (conversion to natural log or reciprocal values), perhaps affecting error relationships. To minimize such errors, always use a computer for statistical fitting of data. The use of good quality replicate results is very important (*see* **Note 21**). Accelerated storage testing has been used as a practical means of quality assurance for biological standards [88, 95] and has been employed in some scientific investigations [96].

3.15 Conclusion

Despite the emergence of alternative drying techniques (e.g., [36–38, 98]), lyophilization (or freeze-drying) will remain a key technology for the preservation of important proteins. Subheadings 3.6, 3.7, 3.8, 3.9, 3.10, and 3.11 have described methodologies based on tried and trusted methods and on the collective experience of those working in the field over many years. The field is far from static, however: **Note 22** lists some interesting work from the recent past, while Tables 1 and 2 summarize a selection of 2016–2021 patent disclosures and publications, respectively.

4 Notes

1. Loss or decrease of the protein's biological or functional activity will be the main and most important index of deterioration. Often, however, the degree or time course of activity loss will not give any indication of the underlying molecular cause (although aggregation may be readily visible). Reference [3] provides a useful table of methods to identify the molecular changes leading to inactivation of the protein.
2. The glass transition temperature of a lyophilized amorphous material determines its chemical stability, physical stability, and viscoelastic properties. The glass transition temperature (T_g) can be defined as the temperature at which the dynamics of an amorphous system changes from the "solid-like" glassy state (less mobile) to a supercooled liquid (a more mobile phase, which is also described as the "rubbery" state in the polymer

Table 1
Some freeze-drying-related patents or patent applications published between 1 January 2016 and 31 July 2021

Title and number	Brief description	Advantages/benefits	References
Freeze-drying methods and related products. US 2021 0180865 A9	Describes a system that continuously moves liquid-containing vessels through different modules so as to promote stepwise freezing and/or drying of the liquid composition. The vessels' design and deployment promote heat transfer between their exterior surrounding and their interior space. Wireless thermocouples and a control system enable control of the liquid composition's temperature. Vessels may be conveyed between modules for conditioning, freezing, primary drying, secondary drying, prestorage, filling with inert gas, and final closure. Shorter cycle times are possible compared with conventional batch freeze-drying.	Achieves a continuous flow-through freeze-drying process with reduced handling and shorter cycle times.	[114]
Method and apparatus for freeze-drying. EP 3 500 811 B1	Uses thermal infrared cameras and image processing to measure temperature at a number of points on a freeze-drying container and so to calculate the maximum product temperature in that container. Heat supply to the container may be controlled, based on this maximum product temperature and on a dynamically calculated temperature safety margin.	Temperature data are captured without physical contact with the product, reducing the risk of contamination. Improved control of the process and improved quality parameters.	[115]
Room temperature stable lyophilized protein. WO 2018/ 068012 Al	This approach can stabilize proteins (up to 200 mg/mL concentration) for up to 2 years at room temperature. Residual moisture contents may be higher than "normal"—in some embodiments up to 10% by weight. The water may act as a plasticizer and may permit annealing at relatively high temperatures (but below T_g) for some hours (annealing enables the protein to relax to a lower energy	Good for concentrated protein formulations. Permits extended shelf life. Describes numerous combinations of proteins and excipients.	[116]

(continued)

Table 1 (continued)

Title and number	Brief description	Advantages/benefits	References
	state that improves its stability). Histidine may be used as an excipient, as may be various other stabilizing substances (polyols, sugars, amino acids, salts, or combinations of these).		
Lyophilization container and method of using same. WO 2019 074886 A1	Describes a flexible pouch-type container for freeze-drying a fluid (e.g., blood or blood components). The container has non-permeable and semipermeable sections. The non-permeable section is for liquid (or solid, or gas, or a combination) and has a port. The semipermeable section is for gas only and has a membrane and an occlusion zone that forms a boundary between the two sections. With the occlusion zone clamped, liquid is introduced into the non-permeable section and is frozen. Removal of the clamp (temporary seal) enables vapor transport between the two sections, and heat is applied under vacuum. When freeze-drying is complete, re-occlusion may take place, creating a permanent seam. The semipermeable section may then be removed and discarded.	Could avoid the need for cold chain maintenance of blood-derived products such as plasma. Sterile pouch containers may enable processing without need for a clean room.	[117]
Energy recovery in a freeze-drying system. US 010782070 B2	Describes a means of energy recovery in the freeze-drying system. Waste heat from the refrigeration condenser can be used to heat the product shelves of the freeze-drying chamber. This transfer of heat is controlled according to the temperature of the shelves within a defined range. The system includes a compressor and a heat exchanger and can be adapted to enable cleaning in place (CIP) or steam sterilization cycles.	Improves energy recovery and use, reducing costs.	[118]

(continued)

Table 1 (continued)

Title and number	Brief description	Advantages/benefits	References
Low-temperature quick-freezing freeze-drying system. US 10900713 B2	System for low-temperature quick-freezing freeze-drying. Comprises circulation loops for refrigeration, quick-freezing/freeze-drying, and desorption drying. Uses heat exchangers in a design that saves time and costs in comparison with conventional freeze dryers.	Faster processing times and reduced energy costs.	[119]

Search conducted on Google Patents on 18 August 2021 using terms (((lyoph* OR freeze dr*))) before: priority:20210731 after: priority:20160101

literature) [59, 98]. T_g' (pronounced as T_g prime) is used within the pharmaceutical industry to denote the glass transition of a frozen solution, whereas T_g denotes the glass transition of the final lyophilized product. It is critical to ensure that the product temperature in the dry state does not exceed the T_g during storage in order to assure maximum stability [99].

3. The type of stopper used can influence the residual moisture contents of freeze-dried materials. Such effects can arise from the nature of the stopper material itself and also from its prehistory [72]. Such considerations will likely apply also to dry preparations for storage, so stoppers should be chosen with care. It is important to ensure that stoppers are sufficiently dried before use because over the shelf life of the product, moisture can transfer from the stopper to the lyophilized cake, leading to an increase in moisture content and stability issues.
4. The vial diameter, glass type, and bottom shape and thickness will all affect the rate of heat transfer from the shelves to product. The vials should withstand freezing and pressure changes and should be uniform with respect to internal diameter and bottom thickness. Ideally, the vial bottoms should be completely flat to make good contact with the shelves [11]. Vials for lyophilization purposes are manufactured using Type 1 borosilicate glass to ISO 9001 and ISO 15378 and comply with European Pharmacopoeia, US Pharmacopoeia, and Japanese Pharmacopoeia international standards. Vials can be purchased, prewashed, and depyrogenated from a number of suppliers, although pretreated vials are more expensive. Alternatively, vials can be purchased "off the shelf," requiring the end user to wash them before use. Vials can be washed in deionized water, placed on a tray in blocks 20 cm × 30 cm

Table 2
A Selection of some lyophilization-themed research papers published in 2016–2021

Publication title	Brief description	Key themes /focus	References
A Kinetic Model for Spray-Freezing of Pharmaceuticals	Spray freeze-drying (SFD) has potential as an alternative process to conventional freeze-drying in vials. SFD process development has, to date, relied on empirical approaches, and predictive models have been lacking. Authors propose and validate a model that describes (i) the fast freezing (<1 s) of sub-millimeter droplets during a SFD process and (ii) accounts for ice growth kinetics and for droplet heat transfer mechanisms. The model is refined and validated using computational fluid dynamics (CFD) simulations together with data from bulk spray-drying experiments and pharmaceutical excipients. This compact model allows estimation of process parameters that are not measurable in real time and can assist in the choice of optimal spraying conditions so as to produce free-flowing, high-quality frozen droplets that match the target product profile.	Spray freeze-drying, process modeling, and validation	[120]
Effects of Drying Method and Excipient on the Structure and Physical Stability of Protein Solids: Freeze-Drying Versus Spray Freeze-Drying	Analyzed protein conformation and physical stability of formulations prepared by (i) spray freeze-drying and (ii) freeze-drying. The model proteins myoglobin and lysozyme were prepared in the presence of sucrose, mannitol, or no excipient. The protein powders were examined by various techniques (solid-state Fourier transform infrared spectroscopy (ssFTIR), differential scanning calorimetry (DSC), circular dichroism spectrometry (CD), size exclusion chromatography (SEC), BET	Spray freeze-drying, freeze-drying, and analytical methods	[121]

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	surface area measurements, and solid-state hydrogen-deuterium exchange with mass spectrometry (ssHDX-MS). With ssFTIR and CD, little/no difference in structure of the proteins in the formulation could be identified. ssHDX-MS was able to identify population heterogeneity, unlike ssFTIR and CD. ssHDX-MS metrics (D-max [maximum degree of deuterium exchange], peak area) correlated well with lysozyme physical instability (loss of the monomeric peak area by size exclusion chromatography) in a 90-day stability studies at 40 °C. Higher specific surface area was associated with greater loss of monomer content for myoglobin-mannitol versus myoglobin-only formulations. Spray freeze-drying may be a viable manufacturing technique for protein solids where there is appropriate optimization of formulations.		
In Situ Characterization of the Microstructural Evolution of Biopharmaceutical Solid-State Formulations with Implications for Protein Stability	Small-angle neutron scattering (SANS) is used with a humidity-control vapor cell environment to examine changes to protein-scale microstructures in solid-state formulations, in situ, during humidifying/drying cycles. Introduction of deuterated water vapor to the vapor cell enables signal contrast between excipients and the protein structure. Spray-dried and freeze-dried formulations showed similar protein-scale microstructures (at a sugar-protein ratio of 1).	Analytical method, protein aggregation, and freeze-drying and spray-drying	[122]

	In stressed formulations, results indicated that irreversible protein aggregates do not form within the solid-state but upon reconstitution. Following exposure to humidity, the formation of reversibly self-associating aggregates can be detected in the solid-state matrix in situ.		
Effects of Drying Method and Excipient on Structure and Stability of Protein Solids Using Solid-State Hydrogen/Deuterium Exchange Mass Spectrometry (ssHDX-MS)	Four model proteins (myoglobin, bovine serum albumin, lysozyme, beta-lactoglobulin) were formulated with one of sucrose, trehalose, or mannitol and dried to powders using freeze- or spray-drying. The various powders were analyzed by solid-state Fourier transform infrared spectroscopy (ssFTIR), solid-state fluorescence spectroscopy (FS), differential scanning calorimetry (DSC), and solid-state hydrogen/deuterium exchange mass spectrometry (ssHDX-MS). For some proteins, ssFTIR and FS identified minor structural differences among powders with different excipients and drying methods. With ssHDX-MS, protein structure differences were observed among formulations containing sucrose or trehalose and mannitol, with varying processing conditions. This included beta-lactoglobulin, which showed no differences in standard characterization techniques. Spray-dried proteins usually showed greater heterogeneity by ssHDX-MS than those lyophilized; ssFTIR or FS did not detect these differences. ssHDX-MS metrics better correlated better with protein physical instability measured by size exclusion chromatography in 90-day stability studies (40 °C, 33% RH) than with the results of DSC, ssFTIR, or FS.	Analytical method; Protein aggregation and freeze-drying and spray-drying	[123]

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Understanding the Impact of Protein-Excipient Interactions on Physical Stability of Spray-Dried Protein Solids	Effects of the widely used excipients mannitol, leucine, and trehalose on spray-dried preparations of bovine serum albumin (BSA) were examined using scanning electron microscopy (SEM), powder X-ray diffraction (XRD), ssFTIR, ssHDX, and solid-state nuclear magnetic resonance (ssNMR). Following spray-drying, trehalose remained amorphous and was best at retaining BSA as a monomer. Mannitol showed both crystalline and amorphous forms in the spray-dried solid and partially stabilized BSA. Leucine was not stabilizing and was the most prone to crystal formation, which led to immiscibility and phase separation. ssNMR was valuable for investigating protein conformation and protein-excipient interactions in dry formulations.	Analytical methods, excipients, additives, and spray-drying	[124]
Effects of Excipient Interactions on the State of the Freeze Concentrate and Protein Stability	This study used differential scanning calorimetry (DSC), X-ray diffractometry, and FTIR spectroscopy to investigate the crystallinity of mannitol and trehalose in frozen solutions. Secondary structure of bovine serum albumin (BSA) was monitored in frozen and thawed solutions by FTIR and circular dichroism, respectively. Crystallization of trehalose coincided with BSA unfolding. Presence of BSA delayed and reduced the degree of mannitol and trehalose crystallization. In presence of D_2O (>=5% w/w) and low	Analytical methods, excipients, additives, and frozen state	[125]

	polysorbate 20 concentrations (<= 0.2% w/w), BSA retained its native conformation. Where the mass ratio of BSA to trehalose was high, the protein could stabilize itself in the frozen state but unfolded on thawing. Concentrations of the API and other excipients exert an influence on the physical state of the freeze concentrate, as well as on the API's stability.		
Raman Spectroscopy for In Situ, Real Time Monitoring of Protein Aggregation in Lyophilized Biotherapeutic Products	Uses Raman spectroscopy at 785 nm for in-vial detection of aggregation (critical quality attribute, CQA) in lyophilized therapeutic protein products. Erythropoietin (EPO) and human growth hormone (HGH) were studied as examples. The Raman spectra (100–1800/cm) were analyzed by partial least squares regression (range 300–1800/cm) and a model was devised to determine aggregation quantitatively. Size exclusion chromatography was also used to detect aggregation. In vial samples were analyzed between 100 and 1250/cm through a 20× objective. The method is noninvasive and quick and requires no sample preparation.	Analytical method and protein aggregation	[126]
A Kinetic Model for Spray-Freezing of Pharmaceuticals	Uses ^{1}H nuclear magnetic resonance (NMR) spectroscopy for in situ, no-dilution study of both antibody (Ab) protein and small molecule content and stability in high-concentration (100 mg/mL) formulations. Size exclusion chromatography was used as a comparative orthogonal method. Changes in 1D ^{1}H NMR signal intensity indicate protein degradation (aggregation or fragmentation). Apparent relaxation rates are specifically sensitive to Ab fragmentation. In addition,	Analytical method, antibodies, and formulation additives	[127]

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	relaxation-filtered spectra respond to the presence and degradation of small-molecule excipients and to changes in solution properties (e.g., pH). Thus, H-1 NMR spectroscopy can give (i) an overview of biopharmaceutical formulation content and stability and (ii) an indication of degradation and (iii) enable triaging of additional analytical techniques.		
Bilateral Effects of Excipients on Protein Stability: Preferential Interaction Type of Excipient and Surface Aromatic Hydrophobicity of Protein	A study of the effects of preferential interaction type of excipient and a protein's surface aromatic hydrophobicity on protein solution stability. Solubility and fluorescence studies of amino acid derivatives (N-acetyl amides of Trp, Tyr, and Phe) were performed in excipient solutions. Conformational, colloidal, and mechanical stabilities of model proteins with different surface aromatic hydrophobicities were examined. Bovine serum albumin (BSA) and ovalbumin (OVA) were included. A three-index empirical phase diagram was used to visualize stability data. Preferentially excluded excipients (trehalose, sucrose, and sorbitol) protected protein conformation against damage but could accelerate aggregation due to mechanical stress. Preferentially bound excipients (propanediol and arginine) suppressed BSA aggregation, but arginine did not inhibit OVA aggregation. This could be due to different	Excipients, additives, analytical methods, preferential interaction, and preferential exclusion	[128]

	conformational perturbing effects of arginine on aromatic hydrophobic regions of BSA and OVA. Choice of excipient should take account of different preferential interaction behaviors of excipients with proteins, especially with their aromatic hydrophobic regions.		
Protein-Excipient Interactions Evaluated via Nuclear Magnetic Resonance Studies in Polysorbate-Based Multidose Protein Formulations: Influence on Antimicrobial Efficacy and Potential Study Approach	Phenol is used in parenteral preparations as an antimicrobial preservative excipient. It can, however, interact with nonionic surfactants (e.g., polysorbate) and potentially with the active pharmaceutical ingredient (API). This study used diffusion ordered nuclear magnetic resonance (NMR) spectroscopy to measure the binding of phenol to a model API (a fusion protein) and to polysorbate 20. Binding of phenol to the surfactant was negligible at pharmaceutically relevant concentrations, but its binding to the model API was relevant and was concentration-dependent, leading to decreased antimicrobial efficacy. NMR analysis is proposed as a material-saving method for use together with antimicrobial activity tests described in Pharmacopeias.	Excipients, additives, antimicrobials, phenol, and analytical methods	[19]
Control of Globular Protein Thermal Stability in Aqueous Formulations by the Positively Charged Amino Acid Excipients	Focuses on the positively charged amino acids (Lys, His, and Arg) as excipients for stabilizing therapeutic proteins. These influence protein stability in dissimilar ways. For three tested proteins (bovine serum albumin, lysozyme, and myoglobin), Lys and His affected thermal stability by different mechanisms at low versus high concentrations. Each amino acid seems to act as a combination of its constituent parts (Gly portion + side chain). At >100 mM concentrations, Lys and His were less	Excipients, additives, lysine, histidine, and differential scanning calorimetry	[129]

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	destabilizing of the three tested proteins than was Arg.		
Dynamic Properties of Novel Excipient Suggest Mechanism for Improved Performance in Liquid Stabilization of Protein Biologics	Buffers, salts, sugars, and surfactants are used as excipients to stabilize liquid formulations of protein drugs. Surfactants (often a polysorbate or poloxamer) can help to protect the protein drug against destabilizing surface interactions, but aggregation induced by agitation remains a hazard. Novel surfactants that possess an alkyl chain, an amino acid, and a polyetheramine include FM1000. The dynamics of FM1000 at various water/hydrophobic interfaces, measured by dynamic surface tension, were notably faster (10–20-fold) than those of polysorbate 20, polysorbate 80, and poloxamer 188. These faster dynamics led to improved resistance of model protein biologics (IgG and abatacept) to agitation-induced aggregation.	Excipients, additives, surfactants, and aggregation.	[130]
General Mechanism of Osmolytes' Influence on Protein Stability Irrespective of the Type of Osmolyte Cosolvent	The stabilizing or destabilizing properties of osmolytes do not correlate with their structure-making or structure-breaking influence on water. Also, the osmolyte' effects on protein stability do not depend on their accumulation at, or exclusion from, the protein surface. Water's infrared spectral characteristics in an osmolyte solution enabled the development of practical criteria to classify solutes as stabilizing or destabilizing.	Additives, osmolytes, analytical methods, hydration sphere, hydrogen bonding, and lysozyme	[131]

Characterizing Osmolyte Chemical Class Hierarchies and Functional group Requirements for Thermal Stabilization of Proteins	Studied the stabilizing effects of protective osmolytes (from three main classes: small carbohydrates/polyols, amino acids and derivatives, and methylamines) on two model protein systems (C-reactive protein and tumor necrosis factor alpha) using differential scanning fluorimetry. Among these osmolytes, there was no strict chemical class hierarchy with regard to thermal stabilization, and the proteins showed different responses to certain osmolytes. Stabilizing effects of amino acid osmolytes on myoglobin and C-reactive protein were also investigated. To stabilize myoglobin, the complete amino acid zwitterion was required, but in the case of C-reactive protein, the osmolyte's amino group was not needed for stabilizing effects. These various responses of proteins to osmolytes agree with previous findings that osmolytes' effects on stability are protein-specific.	Additives, osmolytes, and protein-specific effects	[132]
The Osmolyte TMAO Modulates Protein Folding Cooperativity by Altering Global Protein Stability	Examined the effect of the stabilizing osmolyte trimethylamine N-oxide (TMAO) on the SH3 domain of the PI3 kinase using hydrogen exchange coupled with mass spectrometry (HX-MS). HX was shown to occur despite the presence of 2 M TMAO, and this enabled monitoring of the evolution over time of populations of different protein conformations that were present together. The cooperativity of protein folding was strongly linked with protein stability. As stability increased, the free-energy landscape became increasingly rugged, while cooperativity decreased. As TMAO	Additives, osmolytes, trimethylamine N-oxide (TMAO), and protein folding cooperativity	[133]

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	concentration increased, fewer amide sites opened up their structure simultaneously. An intrinsic dynamics study of TMAO's and urea's effects on the PI3K SH3 domain indicated that TMAO counteracts urea's effects on both protein stability and on protein folding cooperativity.		
Selective Stabilization and Destabilization of Protein Domains in Tissue-Type Plasminogen Activator Using Formulation Excipients	Used tissue-type plasminogen activator (tPA) as a model multidomain protein to study relationships between the various parameters of thermal unfolding and aggregation. Changes in tPA's thermal unfolding profile were parameterized by the overall thermal midpoint transition temperature, T_m, and the Van't Hoff entropy for unfolding, ΔS_{vh}, an index of unfolding cooperativity. Kinetics of degradation at 45 °C, leading to aggregation, were measured as rates of monomer and activity loss. These two rates coincided at all pH values. A design of experiments approach (DoE, 19 factors) enabled evaluation of how formulation excipients impact and control tPA's thermal unfolding profile and how excipients and pH could optimize T_m and ΔS_{vh}. Excipients' effects grouped according to their combined impact on T_m and ΔS_{vh}. Each excipient's effects often selectively stabilized or destabilized specific tPA domains.	Design of experiments, thermal unfolding, aggregation, excipients, and tissue plasminogen activator	[134]

Heightened Cold-Denaturation of Proteins at the Ice-Water Interface	Formation of an ice-water interface during freezing is a critically destabilizing factor for proteins. Molecular dynamic simulations of a 62-mer Protein L domain showed that the presence of an ice-water interface lowers the free-energy barrier for unfolding, leading to rapid protein unfolding. The protein needs not to adsorb directly to the surface; rather unfolding is mediated by nearby liquid molecules that show a greater tendency to hydrate nonpolar groups. This enhancement of the cold denaturation process upon ice formation can be relieved by adding glucose, which is preferentially excluded from protein side chains and so functions as a cryoprotectant.	Cold denaturation, molecular dynamics simulations, glucose, and cryoprotectant	[135]
The cold Denaturation of IscU Highlights Structure-Function Dualism in Marginally Stable Proteins	Studies the cold denaturation (above 0 °C, in absence of denaturants) of an ancient bacterial iron cluster protein, IscU. It has marginal stability, net negative charge, and cold denatures in the absence of iron-sulfur clusters or zinc and at low ionic strength. Provides an improved understanding of cold denaturation and enables generation of a model and general rules to induce and predict protein cold denaturation. Notes that clusters of like charges on a protein's surface can reduce its stability range under low-salt conditions. Observed conflicts between ligand binding and stability indicates that structure-function dualism may be an important factor in protein evolution.	Cold denaturation, IscU scaffold protein, circular dichroism, and modeling and prediction	[80]

followed by wrapping twice in aluminum foil. The tray with the vials can then be placed in an oven for 2 h at ~250 °C. When ready to use, the vials should be removed from the oven and left to equilibrate to room temperature.

5. The most basic lyophilizer equipment (IMA, SP) will have a unit housing a condenser and vacuum pump, to which one may attach a centrifugal test tube holder or a manifold for the drying of multiple product containers (often round-bottomed flasks). Freezing is accomplished in a separate cooling bath, usually filled with an alcohol. Such equipment can be used successfully for small-volume samples, but fine and reproducible control of the overall process may not be possible. Higher-grade equipment, with temperature-programmable shelves and a number of temperature probes, is preferable. Shelf-equipped freeze dryers are especially suitable for use with rubber-capped pharmaceutical vials. An externally operated screw press may be present, allowing one to seal vials (partially closed with rubber stoppers so as not to restrict gaseous movement) under vacuum before releasing air into the chamber.
6. Thiomersal and azide are totally unacceptable in any product for internal administration. Do not discard azide compounds or azide-containing solutions down laboratory sinks. Not only is azide toxic but it can accumulate in old lead piping, leading to the formation of potentially explosive compounds.
7. Some biological matrices, particularly sera, will not filter effectively through a 0.22 μm filter alone. One may need to prefilter the material initially through a coarser 0.45 μm filter to which the desired 0.22 μm filter is connected in series. Alternatively, one can accomplish the finer filtration as a separate operation. One can best filter larger volumes (hundreds of milliliters or liters) using a stack of filters clamped in a special filtration unit. A filter as coarse as 1 μm may be used directly in contact with the solution of interest, the stack comprising progressively finer filters until the sterilizing 0.22 μm filter is encountered at the bottom of the stack. Technical representatives of filtration manufacturers can give specialist advice for individual cases.
8. Numerous suppliers offer specific inhibitors of proteases or classes of protease. These inhibitors are often peptides or proteins, for example, aprotinin and soybean trypsin inhibitor. Cocktails of protease inhibitors are available from the Roche Life Science, Abcam, Promega, Merck Life Sciences (formerly Sigma-Aldrich), and other suppliers. Many of these products can give effective inhibition of serine, cysteine, and metalloproteases during protein extractions from a variety of tissues and sources.

9. Curiously, however, lactate dehydrogenase activity levels at pH 5 decreased with increasing concentrations of polyethyleneimine. In contrast, the polymer stimulated activity at pH 7.2 and 9 [29].
10. Many different vessels and membranes for laboratory-scale ultrafiltration, with a range of defined molecular weight cut-offs, are commercially available. These may comprise permanent stirred pressure cells with replaceable membranes (for volumes in the range 10–500 mL) or disposable centrifugal concentrators (for volumes up to 10 mL) [28]. Schein gives some useful observations on ultrafiltration and suggests some other means of achieving protein concentration [28].
11. Sucrose concentration is an effective and rapid means of concentrating a dilute protein solution. Place the solution of interest into a suitably treated, softened dialysis tube and secure the ends tightly. Record the volume added to each tube, so that concentration post dialysis can be reconciled. Tear off a piece of aluminum foil such that the dialysis tube will rest on the foil with roughly 5–6 cm to spare all round. Shake some solid sucrose onto the foil, and then rest the dialysis tube on top of the sucrose. Shake more sucrose on top of the dialysis tube, wrap the foil around the sucrose and dialysis tube to form a parcel, and place in the refrigerator. Water from the dilute protein solution will move by osmosis through the pores of the dialysis tube to the surrounding solid sucrose, leading to concentration of the protein. Examine the dialysis tubing every 15–20 min. The dialysis tubing surrounding the sucrose will gradually form a viscous liquid which can be removed periodically and replaced with fresh solid material. Volume reduction can take place quite quickly. The method has the drawback that sucrose will enter the dialysis tube in amounts not readily calculable (the sucrose will likely help to stabilize the protein, of course). If the presence of sucrose is undesirable, gently pull the dialysis tube between the index finger and the thumb to force its contents into one end. Knot or clamp the dialysis tube tightly as close as possible to the concentrated solution, and then dialyze the shortened dialysis tube against a suitable buffer to remove the sucrose. Note that the dialysis tube will swell in dilute buffer as water moves by osmosis into the protein solution which will have a high sucrose concentration. The dialysis tube must be clamped very tightly and as short as possible to prevent undue "re-dilution" of the sucrose-concentrated protein solution. It is also advisable to determine by experiment whether the dialysis step should be performed at 2–8 °C or at ambient temperature. Anecdotal evidence suggests that the dialysis temperature and/or the buffer medium (e.g., water or 0.9% NaCl) can influence product quality.

12. It can be a good idea to place a maximum/minimum thermometer inside the refrigerator, freezer, or incubator(s) close to the containers of interest in order to record any significant variations of temperature which may occur over an extended period (ensure first that the thermometer will withstand the low or high temperatures).
13. Low-temperature freezers typically function at −70 to −80 °C. These temperatures are extremely cold and can inflict a "cold burn" on the exposed skin. Always wear insulating or autoclave gloves when handling low-temperature items: latex or nitrile laboratory gloves are not sufficient. Cryogenic gloves from the Shenzhen Inf-way Technology Co., Ltd., are recommended.
14. The degree of supercooling is the difference between the equilibrium freezing point and the temperature at which ice crystals first form in the sample. The degree of supercooling governs the rate of nucleation and determines the number of ice crystals formed, which in turn affects the porosity of the freeze-dried cake [74]. A higher degree of supercooling means higher product resistance, and this leads to longer primary drying times in manufacturing [89]. In the laboratory, it is very important to ensure that conditions are kept as sterile as possible during filling in order to limit the ingress of particles which may impact freezing (ice structure) and subsequent primary drying step.
15. The volumes of bulk liquid subjected to freeze-drying must never exceed the manufacturer's recommendations. If the condenser's ice capacity is reached or exceeded, the degree of product drying will be insufficient and many problems can result.
16. Type T thermocouples are recommended for lyophilization experiments. One can purchase 13 mm and 20 mm stoppers along with guide tubes. These stoppers can be used across a wide range of vial sizes. Carefully clean all the thermocouples using *iso*propyl alcohol (IPA) and lint-free wipes. Thermocouples should be placed in center vials, as these center vials take the longest time to dry. Conditions will not be homogeneous across all vials, and monitoring should be as complete as equipment will allow, so it is a good practice to place thermocouples in vials across different shelf locations, for example, front, side edge, center, and back (*see* Fig. 1). Note that vials with thermocouples are not representative of the entire batch: the thermocouple itself can act as a nucleating site, leading to a lower degree of supercooling and to lower resistance during drying. These vials should be used only for temperature measurement and not for analytical testing post-lyophilization.

    When placing the thermocouple into the vial, place the 13 mm or 20 mm stoppers into the mouth of the vial and

thread the thermocouple through the small hole in the center of the stopper. The thermocouple should not touch the bottom of the vial but should be placed as low as possible to ensure that the tip of the thermocouple is in contact with the product (but not with the glass at the base of the vial).

It is important to calibrate the thermocouples (i.e., to verify their accuracy) before use. A thermocouple calibration function, included in most lyophilizer software, should be utilized. Alternatively verify the accuracy of the thermocouples using a temperature sensor simulator.

17. Inclusion of excipients (additives) with high T_g' values in the protein formulation to be freeze-dried can be very useful. The mixture will form a glass at relatively low temperatures, minimizing freezing damage. A high T_g' will also allow the use of higher temperatures during primary drying with less danger of product collapse. Any constituent that will lower the unbound water content of the freeze concentrate will help shorten the secondary drying operation [11], but uncrystallized salts will decrease T_g', since any salt will bring about a depression of freezing point. Thus, the salt content of the product formulation should be as low as practicable [11]. The optimum temperature for freezing and primary drying depends on the ratio (protein/protectant additive/salt) in the freeze concentrate rather than in the initial solution [11, 53]. The ratio protein: other solids in the freeze concentrate influences T_g' [11].
18. There have been reports of protein damage due to mechanical stresses at the interfaces of separated liquid phases arising from freeze concentration effects ([55] *and references therein*). Sucrose and trehalose exerted little protection against this phenomenon, despite being good glass formers. Rapid cooling below the glass transition temperature appeared to minimize damage from this cause, since the protein spends less time in a freeze-concentrated solution before attainment of the glassy state [55].
19. Modulated differential scanning calorimetry (mDSC) can enable assessment of the thermal properties of both liquid (pre-lyophilized) and lyophilized (powder). One can use mDSC to investigate the impact of a range of supercooling temperatures, isothermal hold times, and annealing temperatures on the glass transition temperature and crystallization temperature. The freezing rate in mDSC experiments can be used to determine the optimum freezing protocol to employ for a given lyophilization cycle. One can verify the functionality of the mDSC instrument by running an indium metal standard (T_m: 156.60 ± 0.1 °C) and n-decane (T_m: −30 °C) in hermetically sealed crimped aluminum pans. For liquid samples, add ~15 mg of the formulation of interest to an aluminum sample pan and crimp using a sample press, for example, a *T*zero press.

20. The residual moisture content of the lyophilized preparation, and its distribution throughout that preparation, will dictate its long-term stability. The stability of amorphous materials (both physical and chemical) is related to molecular mobility which in turn is strongly influenced by temperature [99] and moisture content. Residual or absorbed water can promote physical changes in an amorphous material through its role as a plasticizer. As a plasticizer, water acts as a physical diluent that increases free volume and molecular mobility [99]. The higher the levels of water present in a lyophilized product, the lower the glass transition temperature. Molecular mobility is usually relevant to physical and/or chemical stability in pharmaceutical systems. This is because the rates of most degradation processes are limited by the diffusion of reactants and products [59]. Therefore, it is critical to control the level of residual water content and storage temperature of a lyophilized biopharmaceutical product. Amorphous pharmaceutical materials are often more reactive (compared to their crystalline counterparts) and unstable to thermal and mechanical stresses above their T_g. This may result in significant variation in some of their key physicochemical properties [100]. It is generally understood that low mobility of a material in the glassy state makes chemical reactions improbable [101]. Since molecular mobility decreases with a decrease in the temperature below the T_g, it is considered a good practice to store a lyophilized material at least 50 °C below the glass transition temperature [102].

    Uneven moisture distribution between vials often leads to biphasic activity loss profiles on extended storage [11]. Each 1% of moisture can depress T_g' by more than 10 °C [35]. Significant aggregation of lyophilized recombinant human serum albumin occurred within hours upon incubation at 37 °C and 96% relative humidity [6], indicating just how critical the residual moisture content can be.

21. Amorphous solid preparations will follow Arrhenius kinetics provided they remain in the glassy state. However, if any of the elevated temperatures used exceeds the glass transition temperature (T_g'), the product will become rubbery and will no longer obey the Arrhenius equation. Other situations in which deviations from Arrhenius kinetics may occur are outlined in reference [103]; *see also* **Notes 17, 20** and reference [40].

22. Reference [104] outlines the application of process analytical technology (PAT) to lyophilization while [105] deals with process design space. An optical fiber system [106] and micro-Raman spectroscopy [107] have been used to monitor the lyophilization process. Recent advances in our understanding of the freezing step, primary drying, and scale-up issues receive attention in references [108–110] and [111],

respectively. Co-encapsulation of insulin with lyoprotectants in nanoparticles led to improved characteristics following lyophilization [112]. The tendency of a given protein to aggregate upon lyophilization has been shown to correlate with a limited number of protein structural descriptors [113].

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Chapter 20

Strategies for the Purification of Membrane Proteins

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Abstract

Membrane proteins account for approximately 30% of the coding regions of all sequenced genomes, and they play crucial roles in many fundamental cell processes. However, there are relatively few membrane proteins with known three-dimensional structures. This is likely due to technical challenges associated with membrane protein extraction, solubilization, and purification. Membrane proteins are classified based on the level of interaction with membrane lipid bilayers, with peripheral membrane proteins associating non-covalently with the membrane, and integral membrane proteins associating more strongly by means of hydrophobic interactions. Generally speaking, peripheral membrane proteins can be purified by milder techniques than integral membrane proteins, with the latter's extraction requiring phospholipid bilayer disruption using detergents or organic solvents. In this chapter, important considerations for membrane protein purification are addressed, with a focus on the initial stages of membrane protein solubilization, where problems are most frequently encountered. Protocols are outlined for the extraction of peripheral membrane proteins, solubilization of integral membrane proteins, and sample clean-up and concentration.

Key words Peripheral membrane protein, Integral membrane protein, Detergent, Protein purification, Protein solubilization, Protein concentration

1 Introduction

Membrane proteins are associated with the membrane of a cell or particular organelle and are generally more problematic to purify than water-soluble proteins. Membrane proteins represent up to 30% of the open-reading frames of an organism's genome [1–5], and they play crucial roles in basic cell functions including signal transduction, energy production, nutrient uptake, and cell-cell communication. It is currently estimated that over 50% of marketed therapeutics target membrane proteins [2, 3, 6–9]. However, membrane proteins represent only about 2% of all protein structures deposited in the Protein Data Bank [10], as the purification of membrane proteins remains a challenge due to the physicochemical properties that affect their solubilization and purification in sufficient quantities for crystallization.

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Membrane proteins are classified into peripheral and integral membrane proteins, which are associated with varying degrees with the phospholipid bilayer [11, 12]. Peripheral or extrinsic membrane proteins interact with the membrane surface non-covalently by means of electrostatic and hydrogen bonds. Peripheral membrane proteins can be recruited to the membrane during signaling events or are constitutively localized to the membrane. Integral or intrinsic membrane proteins are more strongly associated with the membrane and interact with hydrophobic moieties in the phospholipid bilayer. They contain one or more characteristic runs of apolar amino acids that span the lipid bilayer [11]. Integral membrane proteins are further classified into Type I, which are positioned so that their COOH-terminus is embedded in the cytosol, or Type II, which are positioned with the NH_2-terminus in the cytosol. Although there is no single protocol for the purification of membrane proteins, it is the initial stages of membrane protein solubilization where problems are most frequently encountered. This chapter discusses important criteria for membrane protein extraction and solubilization. The methods section describes protocols for the extraction of peripheral membrane proteins, solubilization of integral membrane proteins, and methods for detergent removal and concentration of the protein sample, which are important for the efficiency of downstream purification and analytical techniques.

1.1 Considerations for Membrane Protein Purification

The analysis of membrane proteins represents a significant technical challenge in the field of proteomics, and there are several reasons why the purification of membrane proteins is more difficult than that of water-soluble proteins. First, endogenous expression of membrane proteins is relatively low, and usually quite large quantities of protein are required for structural investigations. Increased protein yield may be obtained by overexpressing a recombinant membrane protein of interest in bacterial, yeast, mammalian, or various cell-free expression systems [4, 13, 14]. However, differences in posttranslational modifications such as glycosylation, phosphorylation, and acylation can result in decreased specific activity of the recombinant protein compared to the native form. This may be overcome by using site-directed mutagenesis of the amino acids that form such modifications or by adopting changes in the conditions of the protein expression system [6].

Second, integral membrane proteins are extremely hydrophobic and often require high concentrations of detergents for solubilization. Additionally, membrane proteins have the tendency to form aggregates, even in the presence of detergents, resulting in a reduction of the efficiency of subsequent separation techniques [15]. The choice of detergent may also affect the efficiency of downstream protein purification procedures. In such cases, detergents can be removed (*see* Subheading 1.4). Furthermore, many signaling proteins are contained within lipid rafts, which are micro-

domains of detergent-resistant sphingolipid and cholesterol within the plasma membrane. In such cases, cholesterol-disrupting chemicals, such as nystatin of filipin, should be included in the protein purification protocol either before or during the harvesting process [16].

Once solubilized, membrane proteins are often more susceptible to degradation by proteases. Thus, addition of protease inhibitors such as ethylenediamine tetraacetic acid (EDTA), which inactivates metalloproteases, or phenylmethyl sulfonyl fluoride (PMSF), which inhibits serine proteases, needs to be considered. A variety of protease inhibitor cocktails are commercially available and protect protein preparations from a number of protease inhibitors, including aminopeptidases, metalloproteases, and serine, cysteine, and aspartic acid proteases (*see* Chapter 6).

It is worthwhile considering the availability of efficient functional assays to detect the integrity of the protein of interest at different stages during the purification process, for example, measurement of enzymatic activity and immuno- or ligand-binding assays. Given the unique properties of individual proteins, it is usually necessary to determine appropriate assays on a case-by-case basis [15]. There is no single protocol for membrane protein purification; it is more likely that a series of methods are needed, depending on the particular needs of the investigator and the aims of subsequent downstream analyses.

1.2 Peripheral Membrane Protein Extraction

Peripheral membrane proteins can be dissociated using relatively mild techniques that break the electrostatic or hydrogen bonds between the peripheral proteins and the membrane, without total membrane disruption. Common dissociating reagents for the extraction of peripheral membrane proteins are listed in Table 1. Extractions using buffers containing high salts are useful as they decrease electrostatic interactions between proteins and charged lipids [11]. Chaotropic ions disrupt hydrophobic bonds present in the membrane surface and promote the transfer of hydrophobic

Table 1
Treatments for the extraction of peripheral membrane proteins

Treatment type	Example
Acidic buffers	pH 3.0–5.0
Alkaline buffers	pH 8.0–12.0 (e.g., 100 mM Na_2CO_3, pH 11.3, *see* Subheading 3.1)
Chaotropic ions	I^-, ClO_4^-, and SCN^-
Denaturing agents	8 M urea or 6 M guanidine hydrochloride
Metal chelators	10 mM EDTA or EGTA
Salt solutions/high ionic strength	1 M NaCl or KCl

groups from the non-polar environment to the aqueous phase [11]. Usually extraction procedures employing high ionic strength NaCl and KCl, alkaline or acidic buffers, and metal chelators result in a relatively distinct separation between solubilized peripheral proteins and membrane-associated integral membrane proteins [12]. High pH causes the fractionation of peripheral membrane proteins from integral membrane proteins by disrupting sealed membrane structures without denaturing the lipid bilayer or extracting integral membrane proteins [17]. The high pH method for extraction of peripheral membrane proteins is described in Subheading 3.1 of this chapter. It is worthwhile determining the effect of the buffer on any enzymatic activity the protein of interest may have and potential interactions the buffer may have with any column matrix that will be used at later stages in the purification process. Additionally, buffer cost may need to be considered if large-scale preparations are to be carried out.

Peripheral membrane protein purification protocols usually involve extraction (i.e., breaking of electrostatic and hydrogen bonds between peripheral protein and the membrane) in the chosen buffer for 10–30 min. The remaining membrane bilayer and its associated integral proteins are then separated by centrifugation (30–60 min, 100,000 × *g*), and the released peripheral membrane proteins are recovered in the supernatant [12, 18].

1.3 Integral Membrane Protein Extraction

In order to solubilize integral membrane proteins, it is necessary to disrupt the lipid bilayer, which may be achieved with organic solvents but is more commonly accomplished using detergents. Extraction using the organic solvent N-butanol (*see* Subheading 3.2) uses a biphasic system for solubilizing proteins from membranes into dilute aqueous buffers. The low solubility of N-butanol in water, combined with its lipophilicity, minimally denatures proteins [17].

Detergents are amphipathic molecules that contain both hydrophobic and hydrophilic moieties and form micelles in water. A micelle is a cluster of detergent molecules in which the hydrophilic head moieties face outward. Detergents solubilize proteins by binding to the hydrophobic parts of the protein on one side and interacting with the aqueous parts on the other side [19]. The detergent of choice should sufficiently solubilize the membrane protein without irreversibly denaturing it. Detergents can be ionic, nonionic, or zwitterionic. A list of commonly used detergents for extraction of integral membrane proteins is shown in Table 2. Selection of a particular detergent depends on the properties of the protein of interest and the given aims of subsequent experiments involving the purified protein. If there is little information in the literature on the purification of similar proteins, or if one is purifying a particular protein for the first time, it is often necessary to screen a number of detergents in order to optimize protein

Table 2
Detergents used for extracting integral membrane proteins

Detergent type	Name	Alternative chemical name	CMC[a] (m*M*)
Ionic	CTAB	Cetyltrimethylammonium bromide	1.0
	Sodium cholate		~10
	Sodium deoxycholate		~2
Nonionic	Big Chap	*N*,*N*-bis(3-D-gluconamidopropyl)cholamide	3.4
	$C_{12}E_8$	Octaethylene glycol monododecyl ether	<0.1
	Triton X-100	Nonaethylene glycol octylphenol ether	0.2
	Triton X-114	Tert-octylphenoxypoly (ethoxyethanol)	0.2
Zwitterionic	CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate	3–10
	CHAPSO	3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxypropane-1-sulfonate	4–8
	LDAO	Dodecyldimethylamine oxide	~1

[a] *CMC* critical micelle concentration

solubilization. Membrane aliquots should be incubated with various concentrations of commonly used detergents, and the incubation time, buffer concentration, salt solutions, and temperature conditions necessary for optimal solubilization should be determined. A protocol describing detergent screening for membrane protein purification for analysis by mass spectroscopy has recently been described [3].

When screening potential detergents, it is important to be aware of the unique critical micelle concentration (CMC), which is the concentration of free detergent at which the transition from disperse detergent molecules to a micellar structure occurs [18]. Since solubilization corresponds to the removal of the protein from the membrane into the detergent micelle, the CMC is the minimal concentration of detergent necessary to form the required micellar structure for protein extraction [18]. CMC values, some of which are listed in Table 2, vary between different detergents but are usually available from the detergent manufacturer.

Additional considerations when choosing detergents include evaluating the effects of a given detergent on the structural and functional properties of the protein of interest. The effects of detergents on the protein stability may be checked during preliminary screens using different detergents. More recently developed detergents, such as maltose-neopentyl glycol (MNG) amphiphiles

[20], nonionic amphipols (NAPols) [21], and steroid-based facial amphiphiles [22], have shown advantages over conventional detergents in terms of protein stability and enhancing the likelihood of obtaining crystals. The compatibility of the chosen detergent with subsequent purification steps should also be considered as certain detergents may affect the efficiency of the downstream chromatographic technique. For example, charged detergents may cause problems with purification operations based on charge difference, such as ion exchange chromatography, and lectin chromatography, which may be used to affinity purify subsets of glycoproteins, and are especially sensitive to high concentrations of a variety of detergents [8, 19]. It is often necessary to remove or replace detergents to overcome these problems; thus, the ease at which excess detergent can be removed from the solubilized protein fraction should be considered (*see* Subheading 1.4).

When solubilizing integral membrane proteins, buffered stock solutions at a physiological pH environment should be prepared containing the membrane preparation, detergent, and protease inhibitors [18]. Membrane preparations are used at a final protein concentration of 1–5 mg/mL and are solubilized by detergent concentrations of 0.1–5% (v/v) [12, 18]. The mixture should be stirred gently for 30–60 min at 4 °C, followed by centrifugation for up to 1 hour at 100,000 × *g* at 4 °C. Generally speaking, retention of a membrane protein in the supernatant following centrifugation for 60 min at 100,000 × *g* after solubilization defines the protein as soluble [12]. The pellet may subsequently be washed to remove residual detergent and finally resuspended in the appropriate buffer [18]. Protein recovery and activity should be investigated in both the pellet and supernatant at this stage. The procedure for solubilizing membrane proteins using the nonionic detergent Triton X-100 is outlined in Subheading 3.3.

1.4 Buffer Exchange, Detergent Removal, and Concentration of Membrane Protein Fractions

The composition of the buffer and/or high detergent concentrations that are often required during membrane protein extraction could potentially affect the stability and subsequent analysis of the isolated proteins. Frequently, methods such as mass spectrometry, isothermal calorimetry, or surface plasmon resonance suffer greatly with particular solvents, detergents, or high concentration salt buffers. Examples of methods used to remove or exchange buffers and detergents are listed in Table 3. The choice of technique depends on the unique properties of the buffer or detergent used and the concentration range of the protein fraction.

Successful detergent exchange or removal can be achieved using various chromatographic supports, followed by extensive washing with the desired buffer, containing a new detergent if necessary [11]. Alternatively, dialysis can be carried out to facilitate buffer and detergent exchange or removal. In the case of detergents, the efficiency of dialysis depends on the CMC and micelle molecular weight, which is determined by the aggregation number

Table 3
Commonly used techniques for buffer and detergent removal and exchange

Technique	Reagent
Affinity chromatography	Ligand immobilized sepharose
Equilibrium dialysis	Appropriate buffer or water (*see* Subheading 3.4)
Gel permeation chromatography	Sephadex G-25 (GE Healthcare)
Hydrophobic interaction chromatography	Bio-Beads SM-2 (Bio-Rad) (*see* Subheading 3.5)
Ion-exchange chromatography	Dowex 1-X2 (Sigma-Aldrich)
Precipitation	Acetone
Ultrafiltration	High molecular weight cut-off membrane (*see* Subheading 3.6)

of detergent molecules [23]. Most detergents with linear alkyl hydrophobic groups (e.g., Triton X-100) have a high micelle molecular weight value and do not pass through dialysis membranes [11]. Detergents with a low micelle molecular weight and high CMC (e.g., bile acids and their derivatives) can be removed by dialysis [11]. A protocol for dialysis is described in Subheading 3.4 of this chapter. Detergent removal by means of chromatographic supports (*see* Subheading 3.5) is relatively work-intensive but is a more rapid procedure than dialysis so can be advantageous in cases where protein stability is an issue.

Another problem many researchers encounter when purifying membrane proteins is the low yield that can be observed when compared to other protein purification protocols. To overcome this, it is possible to enrich or concentrate the membrane protein preparation by ultrafiltration using molecular weight cut-off spin filter devices, which can be used to both remove and replace the undesirable buffer, detergent, or solvent while simultaneously concentrating the protein (*see* Subheading 3.6).

Following initial extraction of membrane proteins, solubilization using detergent, and detergent removal or exchange, membrane proteins can then be purified to homogeneity using a variety of protein purification techniques, depending on the particular needs of the investigator and the given properties and abundance of the protein of interest. Because there is no single procedure to characterize membrane proteins, the key importance of membrane purification lies with the initial extraction and solubilization steps, in order to generate a high yield of pure protein in its native biologically active state. The methods section of this chapter outlines examples of techniques used during the important initial stages of membrane protein purification.

2 Materials

2.1 Fractionation of Peripheral and Integral Membrane Proteins Using High pH

1. High pH buffer: 100 mM Na_2CO_3, pH 11.3.
2. Dounce homogenizer, for example, Potter-Elvehjem PTFE pestle and glass tube (Sigma-Aldrich).
3. Ultracentrifuge, for example, Thermo Scientific Sorvall WX.

2.2 Extraction of Integral Membrane Proteins Using Butanol

1. N-butanol.
2. Cooled bench top centrifuge, for example, Eppendorf centrifuge 5417R.

2.3 Extraction of Integral Membrane Proteins Using Triton X-100

1. TE buffer: 10 mM Tris–HCl, 2 m*M* EDTA.
2. Swinging bucket benchtop centrifuge.
3. 20% Triton X-100 in phosphate-buffered saline (PBS) (*see* **Notes 1** and **2**).
4. Ultracentrifuge, for example, Thermo Scientific Sorvall WX.

2.4 Dialysis

1. Dialysis tubing with a molecular weight cut-off of approximately 10,000 Da.
2. Wash buffer: 100 mM $NaHCO_3$, 50 mM EDTA.
3. Distilled H_2O.
4. Dialysis buffer: 20 mM Tris–HCl, pH 7.4, and 0.15 M NaCl.

2.5 Removal of Nonionic Detergents by Chromatography

1. Columns with a bed volume of approximately 5 mL (e.g., Econo-column, Bio-Rad).
2. Commercially available detergent absorption matrix (e.g., Bio-Beads SM-2, Bio-Rad, *see* **Note 3**).
3. Distilled H_2O.
4. Blocking buffer: 0.1% (w/v) bovine serum albumin in 50 mM Tris–HCl, pH 7.4, and 0.15 M NaCl (s*ee* **Note 4**).
5. Washing buffer: 50 mM Tris–HCl, pH 7.4, and 0.15 M NaCl.

2.6 Membrane Protein Concentration and/or Buffer Exchange by Ultrafiltration Using Spin Filter Devices

1. Spin filters with an appropriate molecular weight cut-off (*see* **Note 5**).
2. Filtrate collection tube.
3. Concentrate collection tube.
4. Benchtop centrifuge capable of the following spin conditions: swinging bucket rotor, 4000 × *g* or 35° fixed angle rotor, 7500 × *g*.
5. Desired reconstitution buffer (*see* **Note 6**).

3 Methods

3.1 Fractionation of Peripheral and Integral Membrane Proteins Using High pH

1. Resuspend the membrane fraction (*see* **Note 7**) at a concentration of <2 mg/mL in high pH buffer (*see* **Notes 8** and **9**).
2. Homogenize the suspension in a Dounce homogenizer using 6–8 strokes.
3. Maintain at 4 °C for 30 min. Mix by vortexing three times during this period.
4. Pellet the membrane fraction containing integral membrane proteins by centrifugation for 60 min at 100,000 × *g* at 4 °C, and transfer the supernatant, which contains the peripheral membrane proteins, into a fresh tube and assay for protein (*see* **Note 10**).

3.2 Extraction of Integral Membrane Proteins Using Butanol

1. Add an equal volume of N-butanol to the membrane fraction (*see* **Note 7**), and maintain at 4 °C.
2. Centrifuge at 500 × *g* at 4 °C for 10 min to separate the mixture into an upper phase containing butanol and membrane lipids and a lower aqueous phase containing solubilized integral membrane proteins. Lipid-rich material is localized to the interface.
3. Separate the upper and lower aqueous phases into separate tubes.
4. Dialyze the lower aqueous phase against a large volume of water or suitable buffer (*see* Subheading 3.4).
5. Assay the dialyzed aqueous phase for protein (*see* **Notes 10** and **11**).

3.3 Extraction of Integral Membrane Proteins Using Triton X-100

The following is an example of a protocol for integral membrane protein extraction from mammalian cells.

1. Resuspend cells in TE buffer at a concentration of 1×10^7 cells/mL.
2. Centrifuge the cells at 2000 × g for 10 min in a swinging bucket benchtop centrifuge. Remove the supernatant and add fresh TE.
3. Centrifuge the cells at 2000 × g for 10 min and remove the supernatant. Resuspend the cells in fresh TE.
4. Add 20% Triton X-100 dropwise to the cell suspension while mixing gently for a final volume of 1% (*see* **Note 12**).
5. Allow to solubilize for 30 min at 4 °C. Mix by vortexing three times during this period.
6. Centrifuge at 100,000 × g for 60 min at 4 °C.
7. Transfer the supernatant containing the soluble integral membrane proteins to a fresh tube and assay for protein (*see* **Notes 13** and **14**).

3.4 Dialysis

1. Prepare the dialysis tubing by boiling a section in wash buffer for 10 min (*see* **Note 15**). Then boil the dialysis tubing in distilled water for 10 min, followed by washing thoroughly in distilled water.
2. Transfer the solubilized membrane protein fraction into the dialysis tubing (*see* **Note 16**) which is securely closed at one end by either tying a double-knot in the tubing or securing it with a plastic clamp (*see* **Note 17**).
3. Remove air bubbles and seal the dialysis tubing using a double-knot or plastic clamp, allowing for a volume increase during dialysis. Check the integrity of the seal to ensure no leakage occurs.
4. Place the tubing in a beaker containing a large external volume (approximately 5 L) of the appropriate buffer (example listed in Subheading 2.4) or water. Dialyze with gentle stirring at 4 °C. Change the external buffer regularly.
5. When the dialysis is finished, remove the dialysis tubing and wash the outside. Hold the tubing and carefully remove the upper knot or clamp. Using a Pasteur pipette, transfer the protein fraction to a new tube (*see* **Note 18**).

3.5 Removal of Nonionic Detergents by Chromatography

1. Before starting, ensure that the protein fraction containing the nonionic detergent (e.g., Triton X-100) has a concentration of >1 mg/mL (*see* **Note 19**) and that the molecular weight of the protein to be recovered is large enough to avoid entrapment in the pores of the affinity matrix.
2. Apply distilled H_2O to the column matrix, followed by blocking buffer. Next, apply washing buffer to the column and repeat wash step.
3. Transfer the protein fraction to the column matrix (*see* **Note 20**).
4. Collect 0.5–1 mL fractions and assay for protein (*see* **Note 10**).

3.6 Membrane Protein Concentration and/or Buffer Exchange by Ultrafiltration Using Spin Filter Devices

The protocol described below outlines the method for concentrating a ~70 kDa protein using a 2 mL 30 kDa spin filter device and has been adapted from the Amicon Ultra Centrifugal Devices User Guide [24].

1. Insert the spin filter into the filtrate collection tube.
2. Pipette up to 2 mL of the protein sample into the spin filter column. Cover with the concentrate collection tube by firmly pushing the tube onto the column (*see* **Note 21**).
3. Transfer the spin filter device into the centrifuge with the membrane panel facing the center of the rotor for maximum efficiency. Ensure the spin filter device is fully seated within the rotor and that all spin filter devices are counterbalanced.

4. Centrifuge at 7500 × *g* for between 10 and 60 min in a fixed angled rotor centrifuge (*see* **Note 22**).
5. Following centrifugation, remove the spin filter device and separate the filter column from the filtrate collection tube.
6. To collect the concentrated protein at this point, invert the spin filter column and concentrate collection tube and centrifuge at 1000 × *g* for 2 min, ensuring that all spin filter devices are counterbalanced. For best results, carry out this step immediately following **step 5**.
7. For buffer exchange, reconstitute the concentrate from **step 6** in the required volume of the new desired buffer. It is best to assess the protein concentration from **step 6** (*see* **Note 10**) for reconstitution at the required concentration.

4 Notes

1. Triton X-100 is one of the oldest classical nonionic detergents in use. It has a CMC value of approximately 0.2 mM with a temperature-dependent micelle size of 60–90 kDa [25].
2. Make a stock solution of 20% Triton X-100 by adding 2 mL Triton X-100 to 8 mL PBS and stirring gently until fully dissolved. Store the stock solution at 4 °C. Triton X-100 is corrosive and toxic. Avoid contact with the eyes and wear appropriate personal protective equipment when handling.
3. Bio-Beads are macroporous polystyrene beads and have a high surface area that adsorbs organics with a molecular weight of <2000 from aqueous solution. They may be used to remove Triton X-100 from protein fractions. Due to the presence of linear alkyl hydrophobic groups, Triton X-100 has a high micelle molecular weight value and does not pass through dialysis membranes. Detergents with a low micelle molecular weight and high CMC (e.g., bile acids and their derivatives) can be removed by dialysis (*see* Subheading 3.4).
4. Bovine serum albumin is used as a bulk carrier protein to saturate nonspecific protein binding sites and minimize protein loss during this procedure.
5. Spin filters are disposable membrane-containing ultrafiltration devices that are used for protein purification, buffer exchange, detergent removal, and protein concentration. Spin filter devices commercially available from Merck (e.g., Amicon Ultra Centrifugal Filter Devices), ThermoFisher Scientific (e.g., Pierce Protein Concentrators), and other suppliers are available in a range of molecular weight cut-offs (e.g., 3, 10, 30, 50, and 100 kDa) and volume sizes. An appropriate volume filtrate collection tube and concentrate collection tube are

provided as part of each spin filter device. The membrane filter molecular weight cut-off should be selected based on the size of the desired protein. For example, if the target protein is 78 kDa, purification through a 30 kDa filter would appropriately remove detergents of reasonable micelle size while allowing retention of the target protein.

6. The reconstitution buffer can be the same detergent or salt buffer or can be exchanged to suit downstream applications, for example, removal of higher concentration detergent for a lower concentration to maintain minimum CMC for detergent.
7. The starting material depends on the source from which the membrane proteins are being purified. Membrane proteins can be successfully isolated from plant and animal tissues or cell cultures, bacteria, yeast, and fungi. Animal tissues can be broken with a mixer or blender. Due to the presence of robust cell walls, unicellular organisms like yeast or bacteria and plant cells are more difficult to disrupt. Different techniques for breaking down cell walls include glass bead milling, grinding mills, homogenization, ultrasonication, osmotic shock, repeat freeze-thawing, and enzymatic lysis [15]. If possible, the protein should be prepared from sources where it is in high abundance, as a certain amount of protein may be lost during the purification process. The starting material can be enriched if the target protein is known to be associated with the plasma membrane, mitochondria, or nucleus. During initial steps of membrane protein isolation, cytosolic proteins can be removed to obtain an enriched preparation of membranes containing the protein of interest. Soluble cytoplasmic proteins are extracted by cell disruption in a neutral pH, isotonic, and detergent-free buffer [12], followed by differential centrifugation or purification using sucrose gradient centrifugation.
8. The pH of the working buffer should be tested following addition of any protease inhibitors, as addition of such components may alter the final pH of the buffer.
9. It is worthwhile determining the effect of the high pH buffer on any enzymatic activity the protein of interest may have and considering potential interactions the buffer may have with any column matrix that will be used at later stages in the purification process.
10. Options include measuring ultraviolet absorbance at 280 nm or using one of several commercially available dye-binding assays, such as the Bradford assay or the bicinchoninic acid (BCA) assay.
11. It is worthwhile to keep the butanol phase for protein assays as it may contain extremely hydrophobic proteins that are difficult to solubilize.

12. The effect of the Triton X-100 solubilization procedure on the structural and functional properties of the protein of interest should be evaluated during preliminary screening experiments. In order to maintain catalytic activity, the membrane protein should be dissolved under optimal conditions for stability at a detergent/protein ratio that is not much above the minimal detergent/protein ratio required for solubilization [15]. Additionally, proteins are more susceptible to protease attack following solubilization with detergents, so protease inhibitors are necessary to prevent protein degradation. Premixed cocktails of commonly used protease inhibitors are now available commercially from a variety of companies including Roche, Sigma-Aldrich, and Pierce. It is recommended to carry out purification procedures at 4 °C in order to minimize proteolysis. Additionally, the effects of Triton X-100 on subsequent purification techniques should be evaluated. Replenish protein-stabilizing additives or protease inhibitors if they are removed or inactivated at any stage in the experiment, for example, EDTA is removed by hydroxyapatite chromatography [15]. If possible, minimize any purification steps that add new detergents or alter the original detergent/lipid ratio.
13. It is worthwhile to keep the pellet for protein assays as it may contain extremely hydrophobic proteins that are difficult to solubilize.
14. Due to the presence of aromatic groups, Triton X-100 has substantial UV absorbance at 280 nm [25]; thus, an alternative protein concentration assay should be carried out. For the same reason, Triton X-100 is not suitable for subsequent purification steps involving column chromatography with UV monitoring of the fractions. As an alternative, bile salts and their derivatives including CHAPS and CHAPSO can be used for solubilization.
15. As dialysis tubing is susceptible to cellulolytic microorganisms, gloves should be worn when handling the tubing.
16. A small funnel may be used to aid transfer of the protein fraction into the dialysis tubing.
17. Prior to transferring the protein fraction into the dialysis tubing, the integrity of the membrane and clamp/knot can be tested by applying distilled water or buffer and checking the tubing for leaks.
18. Avoid losing dialyzed samples by carefully opening the tubing over a larger glass beaker to collect any accidental spillage.
19. A high concentration is necessary to allow for any loss of protein during the procedure.
20. Use washing buffer to dissolve the protein fraction for optimum detergent binding.

21. To prevent damage of the device during centrifugation, check that the spin filter column is fully seated in the filtrate collection tube and that the concentrate collection tube is sitting firmly on the filter column.
22. The centrifugation speed and time depend on the molecular weight limit of the filter membrane, the concentrate volume, and the centrifuge rotor (fixed angle, 7500 × *g*, swinging bucket, 4000 × *g*). Refer to the manufacturer's user guide of the spin filter devices of choice for the appropriate centrifugation time.

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