

CELL CULTURE TECHNOLOGY FOR PHARMACEUTICAL AND CELL-BASED THERAPIES

edited by
Sadettin S. Ozturk
Centocor, Inc.
Malvern, Pennsylvania, U.S.A.

Wei-Shou Hu
University of Minnesota
Minneapolis, Minnesota, U.S.A.



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Downstream Processing

Ron Bates

Bristol-Myers Squibb, Syracuse, New York, U.S.A.

INTRODUCTION

The production and recovery of recombinant proteins, although common, is by no means simple, and requires skills from many disciplines including molecular biology, biochemistry, genetics, and engineering. Since Watson and Crick's discovery of DNA (1), a lot of research has focused on the upstream side of recombinant protein production, and numerous well-established vectors are available along with documented procedures to create the desired expression system. Protein production involves manipulating the cells to generate the desired protein, but just getting the cells to produce the amino acid sequence of interest does not guarantee a usable protein. The protein may not fold correctly, it may not have its activity, or it may be degraded either within the cell, in the culture medium, or during the recovery operations. The culture medium where the product is produced, and hence from where the product must be removed, can be a very complex mixture of polysaccharides, salts, proteins, amino acids, lipids, proteases, nucleic acids, steroids, vitamins, growth factors, whole cells, prions (if animal-derived supplements are used), and adventitious agents. Proteins are recovered from complex mixtures, and often the recovery and purification of that protein may not be economically feasible. It has been estimated that the expense attributed to the purification of a recombinant product can approach 60% (2) to 80% (3) of the total production cost. The successful production of a biological product must involve teamwork between the upstream and downstream engineers, since every component added to boost protein production must eventually be removed.

The terminology in this chapter includes numerous downstream processing terms, and several of the key terms are defined here. The terms adsorbent, particles, stationary phase, and resin all refer to the packing material in a chromatography column. Solute refers to a component to be recovered, separated, or purified. The terms solute, protein, biomolecule, and component are used throughout this chapter interchangeably. Throughput is defined as the amount of product processed per unit time; thus a higher throughput refers to a larger amount of material processed in the same unit of time. Productivity is throughput per unit of measure, like milligrams of product per gram of stationary phase per day, or mg/g/day. Recovery is the amount of

material obtained from the feed, and a high recovery means that very little product is lost during processing.

Downstream processing in cell culture technology is defined as a series of unit operations resulting in a purified therapeutic protein, and this chapter focuses on the purification of therapeutic proteins produced in genetically engineered cell cultures. It is assumed throughout this chapter that the desired protein is secreted into the cell culture medium, and only separation techniques directly used to purify and recover proteins are discussed. Identification techniques such as Western blotting or SDS-PAGE are not included in this chapter.

PURITY REQUIREMENTS FOR BIOLOGICAL PURIFICATION PROCESSES

Most therapeutic proteins are parenteral (i.e., injected or infused directly into the body) and thus require extremely high purity, and they must have a statistically low chance to carry harmful substances such as viruses, nucleic acids (or fragments of DNA/RNA), pyrogens (or endotoxins), or immunogenic proteins. Isoforms of proteins, i.e., different glycosylation patterns, and dimers, may also need to be removed during purification, but these will have to be addressed on a case-by-case basis.

The high purity must be achieved with a high product recovery in a reasonable amount of time to make the process economically acceptable. Thus, the number and complexity of the unit operations should be kept to a minimum. Unfortunately, there are no general purification schemes since all therapeutic proteins and their production medium are intrinsically different. There are, however, common steps and unit operations that should provide the reader with a good starting point. To remove harmful agents, such as virus particles, endotoxins, and other impurities it is good practice to employ several orthogonal purification techniques. For example, a purification process could involve three unit operations—ion exchange, affinity, and size exclusion—each having an orthogonal basis for purification.

POTENTIAL PRODUCT CONTAMINANTS DERIVED FROM ANIMAL CELL CULTURE PROCESSES

Viral Contamination

The presence of viral contamination in biotechnology and especially in cell culture processing is a major concern. As is addressed elsewhere in this book, viruses can be introduced into the process by numerous routes, including but not limited to the following (4–6):

- source of cell lines from infected animals
- viral establishment of the cell line
- use of contaminated reagents or equipment
- improper handling of the cell line.

There are three common, complimentary methods applied to reduce the risk of viral contamination in a protein production process (4–6):

- selection and use of virus-free cell lines and raw materials

- ensuring an adequate clearance achieved by downstream processing
- meticulous testing after appropriate steps to show absence of contaminating virus.

The selection of a tested (for the absence of viruses) cell line, and the use of raw materials shown to be free of viruses are essential in the production of therapeutic products by cell culture technology. Even with a "virus-free" cell line, the absence of viral contamination is not guaranteed, and the downstream processing must be shown to have a sufficient viral clearance. Several techniques are known to have good viral clearances, such as chromatography, but verification of viral clearance is necessary and should include viral spikes and challenge studies utilizing appropriate viral agents, e.g., generic as well as specific types of virus particles. Furthermore, meticulous testing for the absence of infectious viruses must be conducted at appropriate stages during the recovery process.

Endotoxins

Endotoxins (also called pyrogens) are lipopolysaccharides consisting of three completely different sections. There is a nonpolar lipid region, often called Lipid A, an oligosaccharide core and a heteropolysaccharide region, often called O-Antigen (7). Endotoxins are found in the cell membrane of gram-negative bacteria, and the release of endotoxins occurs during cell division, cell growth, and cell death. Unfortunately, endotoxins can be found just about everywhere, so removing them from a process, even a nonbacterial cell culture, can be quite problematic. The European Pharmacopoeia reports the threshold level of endotoxins for intravenous use at 5 endotoxin units (EU) per kilogram of body weight per hour (8), where an EU represents the biological activity of the endotoxin. This requirement does not mean that ultra-low endotoxin levels are needed in every case. For drugs only requiring low doses, the endotoxin level can be much higher than for an antibody, for example, with a dose of a gram or more.

Ideally, in cell culture supernatants the endotoxin level should be zero, but this is usually not the case. Endotoxins are found everywhere, including the process water used in the cell culture. Endotoxin-free water can be used, but it is quite expensive and not always available. Petsch and Anspach (7) provided a table showing the endotoxin concentrations in common sources. Tap water has an endotoxin concentration of 1–20 EU/mL, and murine IgG1 cell culture filtrates have concentrations <100 EU/mL. This can be compared to a high-density *Escherichia coli* culture with a concentration after homogenization of $>2 \times 10^6$ EU/mL.

Due to the high likelihood of endotoxins in the process fluid, endotoxin removal should be built into the downstream processing. The standard protein purification protocol often includes an ion-exchange step, a hydrophobic interaction step, and some sort of size-based separation. These three steps frequently provide an adequate endotoxin clearance, but the product should be tested for the presence of endotoxins. Bischoff et al. lowered a culture's endotoxin level from 1.2×10^7 to <0.22 EU/mL using a three-step process: ultrafiltration, anion-exchange, and immobilized metal affinity chromatography (IMAC) (9).

The selective removal of endotoxins from protein solutions is extremely variable. Adsorptive chromatographic methods are frequently used, and, for most basic proteins, anion-exchange chromatography is effective. The endotoxins, with their highly negatively charged oligosaccharide core, bind strongly to the positively

charged stationary phase, and the proteins are either not bound or eluted with weak mobile phases (10–13). Reduction in endotoxin levels by three to four orders of magnitude are commonly seen. Process streams containing acidic proteins should not be processed through an anion-exchange step with the hopes of reducing endotoxin levels. In this situation, hydrophobic adsorption could be utilized, exploiting the lipid region of the endotoxins. Affinity resins, such as Protein A, also provide a good endotoxin clearance for both basic and acidic proteins.

In addition to nonspecific adsorbers, numerous endotoxin-specific adsorbents are commercially available. These so-called detoxy gels include but are not limited to Polymyxin B, histamine and histidine, and polycationic ligands (such as poly ethyleneimine). Petsch and Anspach provide an excellent review article on endotoxin removal, complete with structures of the endotoxin-specific ligands (7).

Nonadsorptive methods are also used to remove endotoxins, but with less success. Ultrafiltration is used to remove endotoxins from water streams, but it is not very effective for protein solutions, due to the large relative sizes of both the endotoxins and the desired protein. Li and Luo (14) used a salt to aggregate endotoxins for easy removal by ultrafiltration, and two-phase aqueous extraction with detergents is occasionally used for pyrogen removal (15–17).

Cell-Related Impurities

While making the desired protein, cells need to produce a variety of components to survive and replicate, including nucleic acids, other host cell proteins, proteases, lipids, etc. All of these need to be removed from the desired protein. Ideally, whole cells are removed from the product-containing medium without cell rupture, thus limiting the quantities of cell-related impurities, but whole-cell removal does not guarantee the absence of cell debris. Additionally, even when the intact cells are removed, it is still necessary to select the unit operations to ensure that any trace quantities of cell-related impurities are removed.

GENERAL PRINCIPLES FOR THE SELECTION AND SEQUENCE OF DOWNSTREAM PROCESSING STEPS

The process goal is to recover a protein with an acceptable impurity profile in sufficient quantities, and each step in the process must be chosen for a specific reason. For example, a size-based technique could be used as a final step to remove a low-level dimer impurity. This size-based operation would not, however, be very useful at removing a deamidated version of the desired protein. The process should therefore be designed for the earlier steps to remove the deamidated form. Ultimately, the entire purification scheme should be optimized, not necessarily each step.

Downstream processing of biomolecules from recombinant sources can be loosely broken down into three generic zones: clarification and initial purification, intermediate processing, and polishing, with one or two unit operations per stage. Figure 1 shows a typical flow sheet for a protein recovery process highlighting the three stages and commonly used techniques for each stage. It is possible to combine or overlap the zones, but all three stages are often needed to provide adequate purification and clearance of viral particles and endotoxins. Some proteins have been recovered in one stage, but this is rare, and it would be difficult to convince the safety and analytical departments of the robustness of the process for adventitious agent removal.

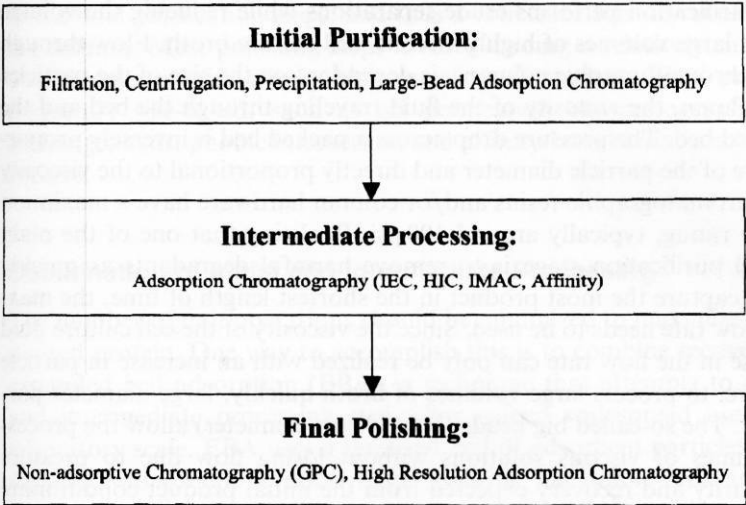


Figure 1 Overview of typical protein purification process.

The single most used purification technique in the downstream processing of therapeutic proteins is chromatography. It is employed throughout the downstream processes, but, to meet the specific requirements of each zone, different sized chromatographic resins with different efficiencies, selectivities and flow restrictions are used at each stage of the purification (18), as is described below.

Occasionally, the purification process can be set up such that minimal processing is needed between chromatography columns. For example, ion-exchange steps often leave the product-containing effluent in a high-salt solution, and hydrophobic interaction columns use high-salt solutions as feed. Therefore, the effluent from an ion-exchange column can occasionally be added to a hydrophobic interaction chromatography (HIC) column with minimal preconditioning (often just the addition of more salt). The reverse is possible as well, i.e., the low-salt effluent from a hydrophobic column can often be directly loaded onto an ion-exchange column.

Clarification and Initial Purification

In the initial product conditioning, the main goal is to reduce the working volume of the process stream and to remove cell debris and some harmful contaminants. The resolving strength of unit operations in the first stage is not as important as speed and recovery. Typical unit operations include filtration, chromatography, precipitation, and centrifugation. For example, a centrifugation and/or filtration step can be used to separate whole cells from the product-containing supernatant (called clarification). Subsequently, the product-containing material can be applied directly onto a chromatographic column for a crude purification, thus removing proteases and other harmful components as well as reducing the volume of the feedstock. For monoclonal antibodies, this first column is often a Protein A column (see section on Recovery of Monoclonal Antibodies later in this chapter). Ion-exchange and hydrophobic interaction columns also work well during the initial purification stage.

The initial purification performs crude separations while reducing the volume of feedstock, often large volumes of highly viscous, cell culture broth. Flow through packed beds, like chromatographic columns, is dependent on the size of the particles packed into the column, the viscosity of the fluid traveling through the bed and the length of the packed bed. The pressure drop across a packed bed is inversely proportional to the square of the particle diameter and directly proportional to the viscosity of the solution. Chromatographic resins and/or column hardware have a maximum allowable pressure rating, typically around 100 psi. Realizing that one of the main goals of the initial purification stage is to remove harmful degradants as quickly as possible and to capture the most product in the shortest length of time, the maximum attainable flow rate needs to be used. Since the viscosity of the cell culture fluid is fixed, an increase in the flow rate can only be realized with an increase in particle diameter. Therefore, to process large volumes of broth quickly, large diameter particles must be used. The so-called big beads (100–300 μm diameter) allow the processing of large volumes of viscous solutions without losing flow due to pressure limitations. The purity and recovery expected from the initial product conditioning are on the order of 70% and 85%, respectively; after clarification, typically only one purification step, utilizing unit operations that have high loading capabilities with high to moderate recoveries, is employed before the intermediate processing stage.

Intermediate Processing

The intermediate processing stage removes most of the remaining contaminants, such as virus particles, nucleic acids, endotoxins, and other host cell proteins, that were not removed in the initial purification stage. Typically, two or more orthogonal processes are used to ensure adequate impurity removal and adventitious agent clearance. Unit operations with a large capacity, high recovery and good resolving power, such as adsorption chromatography, endotoxin gels, and membrane processing are commonly employed in the intermediate processing stage. Chromatographic processes in the intermediate purification stage often utilize 30–100 μm particles to achieve high recovery and purity over one or two complimentary steps. The chromatographic processes involve the use of many types of adsorptive resins including ion exchange, hydrophobic interaction, and affinity-based. The effluent from one column often requires additional processing, such as buffer exchange, and/or a concentration step, before the next purification step. More information on chromatographic theory and practice is given below.

Polishing

The final polishing stage removes impurities closely related to the desired protein, such as deamidated isoforms and aggregates, and often involves only one unit operation having both very high resolution and extremely high recovery (typically >95%). Chromatographic resins used for polishing are frequently high-performance particles (10–30 μm diameter) that offer good resolution but at reduced flow rates and loading capacity. In general, the feedstocks for a final polishing step are very clean, often with only one or two minor impurities, and small diameter particles, with very high efficiencies, are required to remove these impurities. Unfortunately, these highly efficient particles also limit the processing capability (i.e., productivity) of the chromatographic step. Only small volume feedstocks are used

in the final purification steps, and thus a concentration step is often required before the column. Most polishing steps involve loading a relatively small amount of feed onto a column with a very high recovery of product. Final processing resins are frequently size-based, such as gel permeation chromatography (GPC), but ion exchange, hydrophobic interaction, and affinity-based resins are also used with good success.

Combination of Initial and Intermediate Processing

The number of unit operations should be minimized to maximize the recovery of the desired protein. One way to accomplish this is to combine recovery steps. Recently, expanded bed adsorption (EBA), a technique that attempts to combine the initial and intermediate processing steps, has gained widespread use, especially on the laboratory scale. EBA uses a fluidized bed of adsorbent particles with a large intra-particle void volume to allow large particles, like whole cells, to pass unadsorbed, while secreted molecules, like proteins, are adsorbed onto the resin. Once the feedstock is passed over the column, the direction of flow is switched, resulting in a typical packed bed column. Thus, unclarified cell culture broth can be used as feedstock resulting in fairly pure protein in only one step. EBA has been successfully applied to unclarified cell culture broths without any detectable cell damage (19) and is explained in more detail below.

Global Process Requirements

The goal of downstream processing is the recovery of active protein in sufficient quantity at adequate purity. To meet this goal, several unit operations need to be developed, optimized, and implemented, but it is not necessary to achieve the highest possible resolution from each step, especially if it is at the cost of recovery of product or productivity. The requirements of the process should be achieved globally. The goal of each step should focus on the strength of that step, and it should also consider the strengths of the other steps in the process. For example, if a troublesome impurity eluted closely to the desired product in an ion-exchange step, the ion-exchange step could be developed to remove that impurity. Unfortunately, adjusting the conditions to remove that impurity may negatively affect the throughput of the step, and it could also affect the overall yield by requiring the step to discard the portion of the column effluent that contains the impurity. Another approach would be to determine if a different unit operation would remove that impurity more efficiently: in the previous example, if the impurity is a dimer it may be easily removed by a GPC column. During the development phase of the process, the strengths of each processing step should be maximized to produce the most productive process while still maintaining recovery and purity.

INITIAL PRODUCT CONDITIONING

The following section delineates the strengths and weaknesses of methods used for clarifying cell culture broths, for removing proteases and other harmful contaminants, and for the reduction in the working volume of the process stream. Common initial steps are membrane processes, like filtration, and centrifugation to remove

whole cells and cell debris. After cell removal, crude chromatography, extraction, and precipitation are popular secondary steps to eliminate contaminants from the desired protein.

Membrane Processing

Membranes are an integral part of most protein purification schemes. A standard first step in the recovery of secreted proteins is to simply pass the cell-containing culture fluid over a membrane, where the permeate flows through the membrane and the cells are retained on the membrane surface (20). Additionally, the concentration or buffer exchange of process streams is frequently accomplished using a membrane unit. Membranes can be chemically inert, i.e., nonadsorptive, or contain functional groups to adsorb solutes. Nonadsorptive membranes separate solutes on the basis of size; a liquid (with or without particulates) is forced through a porous membrane. The small molecules (molecules smaller than the diameter of the pores) pass through the membrane, while the larger molecules are retained (21). Typically, only crude separations, large molecules from small molecules, are achieved using nonadsorptive membranes, but this crude separation can offer a very good recovery of the desired product.

There are two main classes of nonadsorptive membrane processes in the pharmaceutical industry, microfiltration (MF) and ultrafiltration (UF), although nanofiltration and reverse osmosis (RO) are also used to a lesser extent. MF is defined as the clarification or removal of microparticulates from a liquid stream. These microparticulates can be cells, cellular debris, aggregates, precipitates, bacteria or any other nondissolved substance in a cell culture medium. MF membranes are classified by the diameter of the smallest particle that is retained by the membrane, typically on the order of 0.1–10 μm . UF is the separation of dissolved components based on molecular size, such as the recovery of peptides from a cell culture supernatant, removal of protein dimers or elimination of virus particles from a process stream. Membranes used for UF are classified by molecular weight cut-off (MWCO), which is the molecular weight in Daltons (Da) of the smallest molecule that is retained by the membrane. Typical UF membranes range from 1000 to 500,000 MWCO. Nanofiltration is similar to UF but with MWCO ranging from 100 to 1000 Da. Reverse osmosis membranes are capable of separating salts from aqueous solutions and are classified by the percent rejection of a salt (typically sodium chloride).

Microfiltration is usually operated in a dead-end configuration where the solution is forced through a membrane situated perpendicular to the direction of flow. Dead-end filtration units are commonly used in applications where large differences between solutes exist, such as the removal of whole cells from a culture medium. Unfortunately, the buildup of retained material on the membrane surface results in a reduction of the fluid flow passing through the membrane, and this buildup requires frequent cleaning or replacement of the membrane.

Ultrafiltration processes are operated in either dead-end or cross flow (also known as tangential flow) configuration. Cross-flow filtration (CFF) utilizes a membrane situated parallel to the direction of flow, and thus only a portion of the feed flows through the membrane. The feed is kept under pressure and forced to flow along the surface of the membrane, preventing a large cake from forming on the surface of the membrane and allowing much longer operation lifetimes than dead-end filtration. The permeate side of the membrane in CFF is either kept at ambient pressure or under vacuum to promote flow through the membrane. The stream that does

not pass through the membrane is called the retentate and exits the unit at a higher solute concentration. The concentration of the retentate is determined by the ratio of permeate to retentate flow. This ratio is controlled by altering the operating conditions, e.g., MWCO, feed pressure, feed flow rate, permeate pressure. The most common applications of CFF in the downstream processing of proteins are the concentration, buffer exchange and desalting of protein streams, such as after a chromatography column.

The difference between the feed pressure and the permeate pressure of membranes is called the transmembrane pressure (TMP). The TMP is used to monitor the filtration step (both in dead-end and CFF). As the TMP rises to a predetermined level, the operation needs to be stopped, and the membrane needs to be either cleaned or replaced.

There are various types and sizes of membrane units available commercially, such as tubular, hollow fiber, and flat sheet (cassette type, spiral wound units, etc.) (21), and they can be manufactured with functional groups, much the same as chromatography resins but with the opportunity for better productivity. Derivatized membranes utilize adsorption to selectively bind solutes from a process stream. Adsorptive membranes offer better separation efficiencies, but they are more difficult to develop and often get replaced by a chromatographic column. Membrane suppliers produce large lines of adsorptive membranes including ion exchange, hydrophobic, affinity, endotoxin, and viral-specific functional groups. There are numerous reports of researchers using derivatized membranes, such as Lee et al.'s work with an affinity membrane to purify enzymes (22), and most derivatized membranes are used similarly to chromatography resins, explained in more detail below.

Another benefit of membrane processing is the ease of scale-up. The process is scaled directly from the laboratory based on the cross-sectional area, assuming the same geometry at both scales. The accompanying flow rates are also scaled based upon the ratio of the cross-sectional areas, i.e., a five-fold increase in cross-sectional area results in a five-fold increase in flow rate, throughput, and membrane area. The pressure drop and process time should be the same in both the laboratory and manufacturing facility.

Centrifugation

Centrifugation is used to separate or concentrate materials suspended in a liquid medium, based on sedimentation rates in an increased gravitational field. Two particles of different masses will settle in a tube at different rates in response to gravity. In the downstream processing of biomolecules, the particles are usually cells, subcellular organelles, viruses, and large molecules such as protein and nucleic acid aggregates. Typically, a centrifugation step in cell culture processing results in a solid pellet (which is discarded) and a clear supernatant (containing the product).

The increased gravitational field is generated in the centrifuge by the revolution of a rotor around a fixed point. The centrifugal force (measured and reported as multiples of the force of gravity) generated by this rotation is used to increase the settling rate of biomolecules, and it is proportional to the rotation rate of the rotor (in rpm) and the distance between the rotor center and the centrifuge tube. Therefore, a given centrifuge may use multiple rotor sizes to produce different separation conditions. Each centrifuge has either a specific graph (a nomograph) or a table relating rotation rate (rpm) to centrifugal force for each size of rotor it accepts. The centrifugal force is also dependent upon the design of the rotor itself, e.g., fixed angle or swinging bucket.

Centrifugation is frequently used as a first step to separate whole cells from the supernatant. It is also used to separate protein aggregates from the supernatant following a precipitation step. Centrifugation is a well-studied and well-used unit operation (23) with a host of references on its use in bioprocessing (24–35, for example).

The types of rotors used in centrifuges are fixed angles, swinging buckets, continuous flow, or zonal, depending upon whether the sample is held at a given angle to the rotation plane, is allowed to swing out on a pivot and into the plane of rotation, designed with inlet and outlet ports for separation of large volumes, or a combination of these. The details of even basic centrifuges are too extensive to cover in this chapter. Fixed angle rotors are the work-horse elements of a cell culture laboratory, and the most common fixed angle is a rotor holding eight centrifuge tubes at an angle of 34° from the vertical. In a fixed angle rotor, the materials are forced against the side of the centrifuge tube and then slide down the wall of the tube.

Precipitation

Historically, precipitation was the most common initial purification step, and it was commonly used for fermentations where the product was not secreted. Recently, especially with animal cell cultures, the trend has been to replace traditional precipitation steps with filtration or chromatographic steps, especially in large-scale processes. A brief description of the theory and practice of precipitation, as well as several references, are given below.

The interactions between water and the polar side-chains of amino acids allow proteins to become hydrated and thus soluble in aqueous solutions. When these interactions are prevented, proteins interact with each other and form aggregates that eventually fall out of solution. The purposeful disruption of the water–protein interaction is called precipitation. There are two main classes of precipitation used in the recovery of biologics: solvent and salt (or polymer), and either class is used in two main facets in the biotechnology industry. The first is to remove components that are drastically different from the desired protein (such as proteases or nucleic acids) and prepare crude cell extracts for the next step, i.e., chromatography. The second is to precipitate all the material out of solution to change buffer or provide a storage medium.

Occasionally, once the water–protein interaction has been sufficiently disrupted, the protein aggregates will grow with a density far greater than water and fall directly out of solution, but it is more common, especially in a laboratory setting, to follow the precipitation with centrifugation.

It is also worth noting that precipitation is an equilibrium-driven interaction—the solubility of the protein is reduced but not eliminated, and thus not all of the protein is removed from solution. As an example, consider two solutions of the same protein at 10 and 5 mg/mL under identical conditions. Further assume that the solubility of the protein is reduced to 0.1 mg/mL upon addition of salt. It is obvious that the more concentrated solution will have a greater recovery of protein, due to a larger percentage of protein remaining in solution in the less concentrated sample. Thus, the concentration of a dilute feedstock is often employed directly before a precipitation step to increase the yield.

The solubility of proteins depends on, among other things, the salt concentration and the pH of the solution. At low salt concentrations, the presence of salt stabilizes the various charged groups on the protein, thus attracting the protein into

the solution and enhancing its solubility. As the salt concentration is increased, a point is reached where addition of more salt results in less water available to solubilize the protein. The protein eventually starts to precipitate out of solution when sufficient water molecules are no longer available. These two phenomena are referred to as salting-in and salting-out, respectively. Salting-out of proteins is the most common form of protein precipitation.

Another major factor in the precipitation of proteins is the solution pH. A general rule of thumb for pH effects is proteins are much less soluble at their isoelectric point (pI) than at other pHs. Using organic modifiers or polymers such as polyethylene glycol (PEG) often further reduces the solubility. Protein stability is much more sensitive to changes in pH, however, than to either a change in the organic content of the solution or changes in the ionic strength of the solution. Very slight changes in pH can have drastic effects on protein stability.

Using salts to precipitate proteins out of solution is accomplished in one of the following two methods. The first is to simply add the salt, in solid form, to the feedstock. It is important to note that adding salt to a solution increases the total volume of solution, which must be taken into account if a precise salt concentration is required. The second method involves making up a concentrated salt solution, at the pH and temperature of the feedstock, and adding the saturated solution directly to the feedstock. In the laboratory setting either method is trivial to perform, but on a large scale it is often not possible to use a concentrated solution, since even a 50% saturated solution requires a two-fold increase in the volume of the working stream. In addition, the solubility of proteins in high-salt solutions is typically greater at lower temperatures. Thus a clear solution in a cold box at 2 M ammonium sulfate could become turbid or even show precipitates upon warming to room temperature.

The ability of anions to cause precipitation typically follows the Hofmeister series, i.e., sulfate stronger than acetate which is stronger than chloride, etc.; additionally, monovalent cations are better than divalent cations. Ammonium sulfate is the most commonly used salt due to its high solubility and low cost. The solubility of ammonium sulfate in water is 0.53 kg L⁻¹ or 4.05 M at 20°C, and it is common to see precipitation steps using a 50% ammonium sulfate solution. The recent literature contains numerous examples using ammonium sulfate and other salts to precipitate proteins. Nagashima et al. used a 30–50% saturated ammonium sulfate solution to precipitate glucuronate:baicalein 7-o-glucuronosyltransferase with a 94% recovery (36). Menhard and Zenk used ammonium sulfate (a 0–70% saturated solution gradient) as an initial conditioning step in the purification of acetal coenzyme A (37). Ammonium sulfate precipitation offers several additional benefits over traditional precipitation methods, such as increased stability of most proteins and reduction in proteolysis and bacterial growth (21). In fact, ammonium sulfate precipitates are often the preferred long-term storage method for proteins. Numerous other researchers have used ammonium sulfate in one capacity or another in the recovery of biologics (38–41).

Solvent precipitation involves increasing the organic component of a protein solution until the desired protein falls out of solution. The organic solvent must be miscible with the protein solution, nonreactive with the desired protein, and effective at causing aggregation of the desired protein. The most common solvents are acetone and ethanol, although other alcohols, ethers, and ketones can be used. Aggregation of protein molecules during solvent precipitation is likely caused by electrostatic interactions between the charged groups of individual protein molecules. As the solution becomes more nonpolar, the polar groups on neighboring

protein molecules interact, causing aggregation and eventually precipitation. Unfortunately, too much organic solvent often causes denaturation, so the optimal percent solvent should be chosen very carefully.

Contrary to salt or pH precipitation, solvent precipitation allows for operation at temperatures below the freezing point of water, since all miscible organic solvents lower the freezing point of the solution to well below 0°C. Typical solvent precipitation conditions (using either acetone or ethanol) are 20% solvent v/v on an additive basis (e.g., 20 mL of solvent to 80 mL of aqueous solution) with an aqueous solution having an ionic strength between 0.05 and 0.2 M, operated at 0°C. Generally, precipitation with solvents is greater at lower temperatures, and since proteins are more stable at lower temperatures, this benefit of solvent precipitation can be exploited.

The use of high molecular weight, water soluble, organic polymers is also commonly used to precipitate proteins from solution (21). Solutions of polyethylene glycol (PEG), up to 20% w/v, are frequently used, often resulting in the precipitation of all the protein mass in solution (42). Polyelectrolytes, which are organic polymers with multiple charged sites, are also used to precipitate proteins (43,44). The use of organic polymers in precipitation is very similar to the use of ammonium sulfate. Affinity ligands, either covalently bound to soluble polymers or in free solution, are used to selectively precipitate proteins from solution (45-49). In affinity precipitation, the ligands exploit the protein's specific functionality to form conjugates and precipitate out of solution. The interaction between the protein and the affinity ligand is very similar to the interaction used in affinity chromatography, and, in fact, many of the same ligands are used. Unfortunately, organic polymers are more difficult to remove than either salt or organic solvent from the precipitated proteins.

Aqueous Two-Phase Extraction

Another common initial purification step is liquid-liquid extraction, where solutes partition between two liquid phases. To form two distinct phases, the two liquids must be immiscible, such as hexane and water. Traditional extraction processes use an organic solvent and an aqueous solution to form two distinct phases. Unfortunately, proteins often denature in organic solvents, and thus extraction with an organic solvent is not a viable solution. An alternative is to use two immiscible aqueous phases.

Aqueous two-phase systems form when two solutions containing either two hydrophilic polymers or a polymer and a salt, in the appropriate amounts, are mixed. Typical polymers used in aqueous two-phase systems are PEG and dextran. Ammonium sulfate and sodium sulfate are common salts for the generation of aqueous two-phase systems. Fractionation of solutes between the two phases can be adjusted by slight alterations in the concentration, composition, or temperature of either phase. Aqueous two-phase extraction (ATPE) exploits the subtle differences of the two phases to selectively partition proteins between the two phases, or it can partition proteins away from contaminants like DNA.

The behavior of proteins in the aqueous phases is not well understood, but it is known that altering the composition and/or the concentration of aqueous solutions effects either the hydrophobicity or the ionic nature of that phase. Walter and co-workers offer two good references providing detailed descriptions of different polymer/polymer and polymer/salt combinations, temperature and solvent effects on partitioning, etc. (50,51). In addition to these references, numerous current uses of ATPE can be found for many different proteins (22,52-63) and amino acids (64). Diamond and Hsu report that ATPE is an efficient, economically viable initial

purification step (65). ATPE can offer an alternative to other initial processing steps while still meeting the purity and recovery requirements, and ATPE can be operated in a continuous fashion, thus improving productivity over traditional batch operations.

In addition to the standard polymers, researchers also add specific ligands to polymer chains, e.g., affinity ligands, to enhance the partitioning in ATPE (21). Dyes are also chemically attached to PEG to alter the protein partitioning (66,67). Thermoseparating polymers are used as well. Thermoseparating polymers are polymers that when combined with water and heated above a critical temperature, called the cloud point, form two phases. The polymers are easily recycled by cooling the liquid below the cloud point (61–63,68) thus forming one homogenous phase.

Batch Adsorption

Researchers also use activated porous particles, such as chromatography resins, to capture the desired component by adsorbing the solute onto the resin in batch mode. A typical batch adsorption step involves the addition of the resin to the feedstock solution in a buffer that promotes adsorption of the desired solute. The resin, with the desired component, is then filtered to remove the initial buffer. The filtered resin is combined with a much smaller volume of a buffer that is unfavorable to adsorption. The same type of filtration is again used with the filtrate containing the desired component. The choice of buffer conditions to be used should be optimized to initially only bind the desired solute and close analogs, and the desorption buffer should be strong enough to allow for a large reduction in the overall process volume with a good recovery of product.

Any adsorptive particle can be used in batch mode, such as ion-exchange, hydrophobic, or affinity resins. Gagnon reports the use of anion-exchange chromatographic resins as batch adsorbers to remove host cell membranes, lipids, DNA, and pH indicator dyes from the desired protein (69). The adsorbent in this case is added directly to the harvest fluid, stirred gently for a sufficient length of time, and filtered through a 0.2 μm membrane. In a laboratory setting, this filtration is done in a dead-end configuration (often a stirred-cell) using vacuum to pull the supernatant through the membrane. EBA combines batch adsorption with traditional chromatography to both capture and purify the desired protein, as explained in more detail below.

Product Concentration

The concentration of product streams is very important in protein purification, especially before nonadsorptive purification steps like size-exclusion chromatography (SEC). The most common concentration step in the downstream processing of proteins is ultrafiltration operated in CFF mode; a membrane is chosen such that the desired solutes and large biomolecules are retained, but the liquids and smaller molecules pass through the membrane, thus permitting reduction until the desired process volume is obtained. Other processes such as batch adsorption, dialysis, adsorption chromatography and precipitation also offer significant concentration opportunities at the capture stage, but UF is far and away the easiest and most commonly used.

Although used less than UF, ammonium sulfate precipitation is also used as a concentration step. A saturated ammonium sulfate solution can be added to a post-column effluent, for example, precipitating out all the protein in the solution. After centrifugation, the resulting pellets are redissolved in the appropriate volume of

solution required for the next step. Although dialysis and ultrafiltration are more common, ammonium sulfate concentration does have benefits, especially when stability of the desired protein is a major issue.

Buffer Exchange

The removal of salts and the exchange of buffers is typically accomplished using either UF, dialysis, or GPC. In UF, the desired buffer is added to the protein solution at a rate matching the volume removal rate of the permeate stream.

Dialysis involves using a piece of tubing or a bag deliberately manufactured to allow small molecular weight solutes to pass unrestricted while prohibiting larger molecular weight (>15 kDa for example) solutes from passing through the material. A typical dialysis step involves loading the concentrated sample into a dialysis bag and placing that bag into a vessel filled with the desired buffer under mild agitation. Sufficient time is allowed for equilibrium to be reached and the process is repeated. The equilibrium nature of dialysis necessitates at least one repetition of the process. For example, if the buffer volume is 50-fold greater than the sample volume, the maximum dilution of the original sample is 51-fold.

Dialysis is a time-intensive unit operation. Typically, a well-mixed sample can reach 90% of equilibrium in 2–3 hr (21); however, the remaining 10% can take appreciably longer. The simple operation and equipment needed to perform a dialysis step makes it a very popular laboratory technique, but it is less useful in large-scale operations.

Gel permeation chromatography also exchanges buffers by size-based operations. In GPC, the proteins are sterically prohibited from entering the pores of the column packing, and thus they elute much faster than smaller ions. A buffer exchange, GPC step involves the following: equilibrating a column packed with the adsorptive stationary phase with the desired buffer, load the sample onto the column, and immediately after the sample has been loaded, flow the desired buffer through the column. The desired solute should elute (in the desired buffer) much faster than the original buffer/salt. A rule of thumb for GPC is that the volume of feed loaded onto the column should be <5% of the total column volume to ensure adequate distance between the back edge of the solute band and the front edge of the original buffer band.

Gel permeation chromatography is typically much faster than dialysis and is used more frequently on large scale. More information on GPC is given below.

CONVENTIONAL CHROMATOGRAPHIC METHODS

General Principles of Chromatography

Chromatography is one of the oldest and most widely used purification methods, with Tswett (70) generally being credited with its invention. Since Tswett's early work separating plant dyes, much has changed in the "state of the art." In the 1950s column chromatography emerged, drastically increasing the speed and efficiency of chromatography, but the column packings used in those first columns were structurally soft, thus prohibiting the use of high flow rates. By the early 1960s, rigid column packings and better solvent delivery systems were available, and high-performance liquid chromatography (HPLC) was born. HPLC is used today in every recovery and purification laboratory in the biotechnology industry.

Chromatography encompasses a wide range of separation modes that can be categorized loosely as either: nonadsorptive (e.g., size-exclusion) or adsorptive (e.g., ion-exchange). The adsorptive modes of chromatography include (but are not limited to) ion-exchange, hydrophobic interaction, reversed-phase, affinity, and immunoaffinity. In this chapter, chromatography refers to the batch-wise loading of feed onto a column packed with particles to separate moieties into distinct zones (i.e., peaks) that are kept apart by zones containing only mobile phase (i.e., "empty zones").

Chromatographic theory revolves upon the amount of time solutes spend interacting with the stationary phase. The most common way to represent this interaction is called the capacity factor, k' , defined as:

$$k' = \frac{\text{time spent in adsorbent}}{\text{time spent in liquid phase}} = \frac{t_r - t_0}{t_0} \quad (13.1)$$

where t_r is the retention time of the solute, and t_0 is the retention time of an unadsorbed solute. Figure 2 shows an ideal chromatogram with two components, and this figure, hopefully, provides a graphical explanation of the terms used above and below.

For components to be separated on a chromatography column, the time the individual solutes spend in the adsorbent must be sufficiently different. Typically, larger differences in k' values lead to better separations. The separation between two peaks is called the separation factor (α) and is defined as the ratio of the capacity factors of each component:

$$\alpha = \frac{k'_2}{k'_1} \quad (13.2)$$

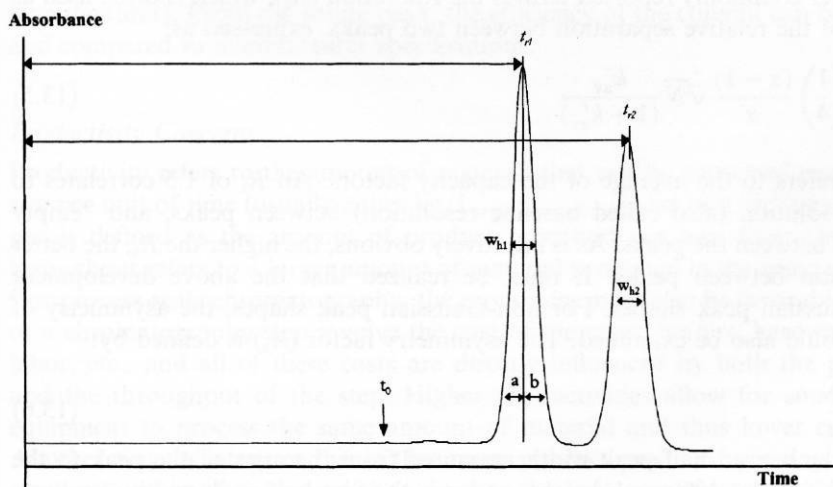


Figure 2 Generic elution chromatogram. A standard two-peak separation separated by a small empty zone. The y-axis is the response from the detector and the x-axis is the time. The variables are further explained in the text with t_r referring to retention time of each component and w_h referring to the width of each peak.

It is worthwhile to note that a large α does not guarantee a good separation. The retention times used to calculate k' are the time when the peak apex exits the column, and there is no factor in α to account for the shape of a peak. Thus, a situation can arise where a large difference in retention times exist, but where the widths of the peaks are sufficiently large and overlap, preventing pure product from being recovered.

Extremely large peak widths can result from an inefficient column or from poorly chosen elution conditions resulting in nonspecific binding or gradual desorption. Large peak widths can be wide Gaussian-shaped peaks, or they can be the result of tailing or fronting. Generally, the larger diameter resins have wider peaks and lower column efficiencies.

The efficiency of a column is typically reported as either the number of theoretical plates, N (analogous to the number of plates for a distillation column) or the height equivalent to a theoretical plate (HETP). Both N and HETP are related to the width of the protein band exiting the column. This can be clearly seen from the following relations:

$$N = 5.54 \left(\frac{t_r}{w_h} \right)^2 \quad (13.3)$$

$$\text{HETP} = \frac{L}{N} = \frac{L}{5.54} \left(\frac{w_h}{t_r} \right)^2 \quad (13.4)$$

where w_h is the peak width at half peak height and L is the column length. Smaller plate heights (HETP values) are analogous to higher efficiencies. The efficiency of a column is often reported as the number of plates per meter of bed length, and efficiencies for a 90 μm packed bed are on the order of 5000 plates/m. Smaller (and hence more efficient) particles should have considerably more plates per meter.

Another commonly reported term is the resolution (R_s), which is often used as a measure of the relative separation between two peaks, expressed as:

$$R_s = \left(\frac{1}{4} \right) \frac{(\alpha - 1)}{\alpha} \sqrt{N} \frac{k'_{av}}{(1 + k'_{av})} \quad (13.5)$$

where, k'_{av} refers to the average of the capacity factors. An R_s of 1.5 correlates to complete resolution (also called baseline resolution) between peaks, and "empty zones" exist between the peaks. As is intuitively obvious, the higher the R_s , the better the separation between peaks. It must be realized that the above development assumes Gaussian peak shapes. For non-Gaussian peak shapes, the asymmetry of the peak should also be examined. The asymmetry factor (A_s) is defined by:

$$A_s = \frac{b}{a} \quad (13.6)$$

where b is the second half peak width measured from the apex of the peak to the point on the peak at 10% peak height, and a is the first half peak width also measured at 10% peak height to the apex of the peak, as shown in Fig. 2. Acceptable asymmetry factors range from 0.8 to 1.2. Peaks that have long fronts or tails are undesirable, since even if extremely asymmetric peaks are well resolved, the purification is achieved at the cost of throughput and productivity.

The brief theoretical development given above can be used to determine how well a packed chromatography column will separate the desired protein from the other components. It can also be used to compare separations using different conditions. The efficiency of the column, coupled with the resolution between the desired protein and the closest troublesome impurity, can be used to screen columns for applicability. Once columns providing the necessary resolving ability are determined, the amount of protein that the column can separate per batch needs to be examined. Obviously, to maximize productivity, the most protein that can be loaded onto the column while still meeting the required purity needs to be determined.

The ability of a chromatographic resin to adsorb a protein is called the protein uptake capacity or the binding capacity. Resin manufacturers report protein binding capacities at specific conditions, and it is important to note that the binding capacity can be very dependent on the specific conditions. In addition, the model proteins used by the manufacturers do not have the same binding capacity as the desired protein, but, in a pinch, they can be used for comparison. There are two types of binding capacities reported by manufacturers and in the literature: batch and dynamic. Batch (or static) capacities are determined using batch adsorption experiments. Dynamic binding capacities involve measuring the breakthrough of proteins through a packed column under given conditions at specific flow rates. The capacity, whether it is dynamic or batch, depends on the properties of the adsorbent, the properties of the protein and the experimental conditions. The column capacity is especially important during scale-up, where the size of the column is an important consideration.

The column hardware and packing technique are as important to the success of the chromatographic step as the choice of the adsorbent and the experimental conditions. Each resin manufacturer supplies a suggested packing method and packing solvent. After packing the column, the bed should be visually inspected for channeling, and an efficiency test should be run. The efficiency test is run with a nonadsorbing substance (like acetone for an ion-exchange resin) or a compound of known characteristics (like the protein of interest or a test compound suggested by the manufacturer). From the solute peak, the efficiency of the column can be calculated and compared to manufacturer specifications.

Productivity Concerns

Productivity refers to the amount of material that can be processed per equipment size per unit of time (usually either $\text{kg}/L_{\text{stationary phase}}/\text{year}$ or $\text{g}/\text{m}^2/\text{day}$). Throughput is defined as the amount of product processed per unit time; thus a higher throughput refers to a larger amount of material processed in the same unit of time. For process-scale chromatography, the economics must also be favorable. The costs of a chromatography step involve the cost of the resin, buffers, hardware, utilities, labor, etc., and all of these costs are directly influenced by both the productivity and the throughput of the step. Higher productivities allow for smaller sizes of equipment to process the same amount of material and thus lower capital costs. Another way to interpret this is that more product produced per day relates to a smaller number of days needed to operate the unit operation, but if bigger units are used to shorten the campaign length, the increased capital costs could outweigh the reduction in campaign length. The throughput and productivity of a chromatographic step are dependent on many factors including the size and type of resin used, the buffering species, the flow rate, the separation efficiency, the length of the

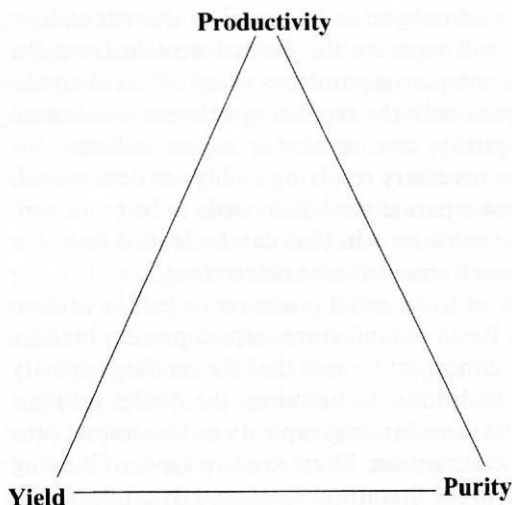


Figure 3 Productivity triangle. The maximization of the productivity of a unit operation is dependent on two main constraints, yield and purity.

column, the capacity of the resin, etc. Unfortunately, it is not possible to maximize all of these factors. The productivity triangle shown in Fig. 3 displays the tradeoff that must be made in preparative chromatography. To maximize productivity, either the yield or the purity can be optimized, but not both. For example, consider optimizing (hence maximizing productivity) an initial purification step where yield is much more important than purity. High capacity, large diameter resins should be used to increase flow rate through the packed bed, but large diameter resins are typically not very efficient, and thus, the purity of the product will be low. The separation resulting from this step will be crude at best, but the feedstock will be processed quickly; this is especially important in the initial purification, where proteases could be present in the feedstock.

The productivity of each step needs to be examined to maximize overall process throughputs. It is also important to realize that optimization of each step does not always transfer to the optimal overall process, and hence the global goal must also be taken into account during the development of each step, as discussed above.

Adsorptive vs. Nonadsorptive Chromatography

Chromatography is the use of resins to selectively retard the flow of solutes across a packed bed. The resins used in chromatography are grossly classified as either adsorptive or nonadsorptive. Solute physically bind, or adsorb, onto the surface of the stationary phase in adsorptive chromatography. For example, ionic interactions between a charged solute and the oppositely charged stationary phase cause adsorption in ion-exchange chromatography (IEC). A typical adsorptive chromatography cycle involves loading the feedstock onto the column, washing the less strongly bound impurities from the column, eluting the desired component with a stronger eluent, removing the more strongly adsorbed solutes with a harsh mobile phase, and regenerating the stationary phase for the next cycle. However, care must be used when selecting the conditions for adsorption to ensure that the protein of interest does not bind too strongly and require harsh, possibly denaturing, elution

conditions. In any adsorptive chromatographic separation, it is desirable to optimize the conditions to allow for the weakest binding of the solute to the stationary phase. Strongly bound solutes require large volumes of mobile phase or harsher mobile phases to desorb. The binding, washing and eluting conditions are chosen in adsorption chromatography to suit the situation. Adsorptive chromatographic methods provide many options: the solute of interest can be strongly bound to the stationary phase, while the impurities are either not bound or weakly bound; the desired protein can be weakly bound to the stationary phase, while impurities are either not bound or very strongly bound; or the product can be unretained by the column while the impurities are adsorbed by the stationary phase.

The use of nonadsorptive resins in chromatography mostly refers to GPC, which is also known as SEC and gel filtration chromatography. In GPC, the separation is based on size: larger molecules are restricted from the smaller pores of the resin and thus elute faster (and in less volume) than the smaller molecules that have access to a larger volume of the stationary phase. There is no adsorption and thus no concentration of the solute bands. A concentration step is frequently required before this chromatographic method. Furthermore, as the solute band disperses (due to diffusion) while it passes through the stationary phase, the volume of the feed must be less than the elution volume of the desired component, to allow for complete separation. One useful benefit of the inert nature of the stationary phase in GPC is the use of mild operating conditions, often simply the eluent from the previous step, can be used.

Gradient vs. Isocratic Elution

Isocratic elution refers to the case where the solutes are eluted off the column using the same mobile phase as the loading buffer. Isocratic elution is very effective at separating solutes that have similar binding affinities. If the solutes encompass a large range of affinities, then isocratic elution becomes less attractive due to the retention time and volume of mobile phase needed to elute the strongly retained components as compared to the weakly retained components. One instance where isocratic elution is frequently used is where the desired solute is not retained by the column, but the impurities that need to be removed are. The feed can be continuously loaded onto the column until the active binding sites on the column are occupied by the impurities. The column can then be washed to remove the impurities and equilibrated with the starting buffer to begin another purification. Isocratic elution is also used when the solutes are weakly bound to the adsorbent. Another benefit of isocratic elution is that since the loading and elution buffers are the same, no equilibration between cycles is needed, and in some applications, where equilibration steps are long, this is extremely beneficial. It is important to realize that the longer the solutes are on the column in isocratic elution, the wider the peak gets.

Gradient elution can be either continuous or step-wise. In a continuous gradient, the strength of the mobile phase is continuously increased to selectively desorb solutes. The shape of the gradient can be anywhere from convex to linear to concave, although linear is the most common. Continuous gradient elution allows for the separation of solutes having drastically different binding affinities, in a relatively short time and small volume. Unfortunately, before the next cycle can be started, the column needs to be equilibrated with the loading buffer, thus lengthening the effective cycle time. Step gradients involve the instantaneous change in mobile phase

composition (e.g., 10–40 mM NaCl) allowing for the separation of solutes having vastly different binding characteristics in a relatively short amount of time. Step gradients are commonly used in systems purifying more than one component. Another common example of step gradient elution is where the impurities are not bound, but the desired solute is. Here, the feed is loaded onto the column until the stationary phase is saturated with the product. Next, a wash step with the loading buffer can be used to remove any unbound or weakly bound components still in the stationary phase matrix, and finally, the desired solute can be eluted with a step change to a stronger eluent. The adsorbent will then need to be equilibrated before the next cycle. In large-scale chromatographic processes, isocratic or step gradients are preferred due to inherent complexities associated with generating a reproducible, continuous gradient.

Types of Resin Selectivities

Ion-Exchange Chromatography

The charged nature of proteins is exploited in IEC to differentiate between individual proteins, as well as between nucleic acids and proteins. IEC involves the reversible adsorption of charged molecules to an oppositely charged functional group immobilized on a particle. Under certain conditions, some biomolecules bind irreversibly to ion-exchange resins, and it is recommended that another form of chromatography be used for this situation. The desorption of the desired solute from the stationary phase (referred to as the elution of the solute) should occur under nondenaturing conditions. IEC is the most commonly used form of chromatography to separate proteins due to its simplicity, nondenaturing operating conditions, and relatively low cost (71).

All proteins have a backbone of amino acids, which may or may not be charged. In a sense, proteins can be thought of as large polyions, with the charge dependent on the pH of the solution. Of the 20 naturally occurring amino acids, lysine, arginine, and histidine (below pH 7) are positively charged, and aspartate, glutamate, and cysteine (above pH 8) are negatively charged.

Additionally, at neutral pH, the N-terminal amines are positively charged, and the C-terminal carboxylic acids are negatively charged. The remaining amino acids do not contribute to the overall net charge on the protein. They do, however, affect the binding of the protein to various chromatographic resins, due to nonspecific interactions. It is also worthwhile to note that the charge on these amino acids is affected by both the neighboring amino acids and the pH of the local environment. The point at which the number of positive charges and negative charges are equal (i.e., where the protein is electrostatically neutral) is called the pI of the protein. The pI is useful in determining which type of ion-exchange resin to use. This will be explained in more detail below.

An IEC step, like most adsorptive chromatography steps, involves four main stages: equilibration of the stationary phase, adsorption of the sample or solute onto the stationary phase, desorption of solute, and regeneration of the stationary phase in preparation for another run. Occasionally, a wash or cleaning step is used either to flush solutes less strongly bound to the matrix before the elution of the desired protein or after the elution of the desired protein to remove strongly bound components. Sanitization might also be needed, depending on the process.

The first stage of an ion-exchange operation is the equilibration of the charged stationary phase with a buffer at a specific ionic strength and pH, thus associating the charged functional groups with simple counter-ions (typically either sodium or

chloride). The equilibrium conditions should be chosen such that the adsorption of the desired compound is favorable but not too strong or irreversible.

The second stage is the loading of the feed onto the stationary phase in either the equilibration buffer or another buffer favorable to adsorption. During this stage, the solute molecules either displace the adsorbed counter-ions and bind reversibly to the stationary phase or they travel through the column unretained. A more detailed discussion on the pros and cons of using the stationary phase to bind the desired solutes or the impurities is given elsewhere in this chapter.

The third stage involves changing the conditions of the mobile phase to be unfavorable for ionic interactions, by either increasing the ionic strength or adjusting the pH. This can be done via a constantly changing gradient or via one or more step changes. On a large scale, step changes or completely isocratic operation are preferred.

The fourth stage in an IEC step is to regenerate the resin and prepare it for the next cycle. Regeneration typically involves a very high-salt buffer to remove all bound components and fully associate the functional groups with counter-ions. The nature of the regeneration buffer is dictated by the feed and by the process. Regeneration can involve extremely high pH solutions, like 1 N NaOH for complex mixtures.

The type of ion-exchange is classified by the functional group attached to the stationary phase. Anion-exchange chromatography involves a positively charged stationary phase (or column matrix) with negatively charged counter-ions. Negatively charged solutes (also called anions) exchange positions with the counter-ions and adsorb onto the stationary phase until either displaced by a compound with a higher affinity or until the charge is altered by changes in the mobile phase pH. Cation-exchange chromatography works in much the same way, but with a negatively charged matrix and positively charged counter-ions.

Anion- and cation-exchange chromatography are further subdivided into two classes, strong or weak—e.g., strong anion-exchange chromatography—based on whether or not the functional group carries a constant or pH-dependent charge. Resins classified as weak have functional groups with nominal pK_a s, thus allowing for a variable charge over a pH range. Table 1 lists the types of exchanger and common functional groups attached to the stationary phase. Levison et al. present physical data on 70 commercially available ion-exchange resins (72). The authors list experimentally derived data on swelling, ion-exchange capacity, protein binding capacity, flow rate/pressure drop, and overall performance.

The type of ion-exchange resin to be used in a chromatographic step depends on three important factors: scale (or scale-up), specific requirements of the step (including purity, capacity, speed, and resolution), and the pI of the protein of interest. If a chromatographic step is to be scaled-up, the resin needs to be available in sufficient quantities. A great method developed in the laboratory is worthless if the resin is not available in large enough quantities. Additionally, the resin available in bulk should have the same characteristics as the analytical-size column; no *surprises* should occur upon scale-up. The best way to verify this is to pack an analytical column from the same lot of material to be used on large scale and confirm that the performance is the same.

When choosing resins, the specific requirements of the step should also be taken into account. For example, if the step is to be used as a capture step, then throughput and yield are more important than resolution and purity; in turn a large diameter resin should be used. Alternatively, if the chromatographic step is needed as

Table 1 Types of Ion-exchange Chromatography

Type of exchanger	Functional group	Common name
Strong anion-exchanger	Quaternary amine (<i>N,N,N</i> -trimethyl-2 hydroxy-propylamine)	Q
Weak anion-exchanger	Tertiary amine (diethylaminoethyl)	DEAE
Strong cation-exchanger	Sulfonic acid (sulfoxyethyl or sulfoxymethyl)	S
Weak cation-exchanger	Carboxylic acid (carboxymethyl)	CM

a final polishing step, high resolution is the most important criteria, and a high-efficiency small diameter resin should be used. Most ion-exchange resin manufacturers offer a full line of stationary phases from high performance, small beads to the so-called "big beads."

The choice of the functional group attached to the particle is dependent on the pI of the protein, the pH range over which the protein is stable, and the types of impurities present. At a pH below its pI, a protein has a net positive charge (more positively charged amino acids than negatively charged ones) and will adsorb onto a negatively charged stationary phase, i.e., a cation-exchange resin. Conversely, at a pH above its pI, a protein has a net negative charge and will adsorb onto an anion-exchange resin. It should also be noted that although net charge is a good first approximation, it does not always provide the necessary information. It has been postulated by many researchers that protein adsorption occurs via charged patches on the protein, and these charged patches do not necessarily reflect the overall net charge. Although very important, this discussion is beyond the scope of this chapter.

Righetti and Caravaggio (73,74) and Malamud and Drysdale (75) present tables containing the isoelectric points of many common proteins. Unfortunately, most cell culture researchers are working with proteins that are not going to be listed in these tables. In these situations, a protein titration curve can be generated via batch experiments at different pH values or electrophoretically. Modern electrophoresis equipment allows for fast, simple isoelectric point determination using isoelectric focusing (IEF). In addition to pI, the pH range over which the protein is stable is also important. If the protein is stable over a region above its pI, an anion-exchange resin should be used; conversely, if it is stable below its pI, a cation-exchanger should be used. For proteins that are stable above and below their pI, either type of ion-exchange resin can be used. Simple batch adsorption experiments with several resins at various pH values provide enough information to determine a starting point for column development work. Batch adsorption experiments (with several resins) could also be used to determine situations where the protein of interest is bound to the stationary phase and the contaminants are not. Adsorption experiments are an effective way to quickly screen a host of resins under numerous conditions. Two model proteins, lysozyme and bovine serum albumin (BSA), are commonly used to determine the type of ion-exchange resin that is needed. Lysozyme is an extremely basic protein (pI > 10) and is not retained on an anion-exchanger under normal conditions. It is, however, retained quite well on cation-exchangers. BSA

($pI = 4.7$) provides the opposite situation; BSA is retained strongly on an anion-exchanger at neutral pH, but is unretained at pH 4.5 or less (76). It is, however, retained on a cation-exchange resin at pH values < 4.5 .

The choice of buffer conditions, both equilibration and elution, are quite product-specific, but the following can be used as a general guideline. For an anion-exchanger, the starting pH should be as high as possible, taking into account product stability, with an ionic strength that is as high as possible to allow the product protein to bind but as many impurities as possible to flow through the column unretained. Typically, the equilibration buffer should be 0.5–1 pH unit higher than the pI with a salt level of 50 mM. The buffering species used in the equilibration buffer should not interact chemically with the product and should provide an adequate buffering capacity at the desired pH. More information about the choice of buffering species is provided below. The elution buffer is usually at the same pH as the equilibration buffer with a higher concentration of salt, typically 0.5–1.0 M. Gradient elution involves gradually blending the elution buffer into the equilibration buffer until either 100% elution buffer is being used or the desired protein has eluted. A cation-exchanger would use a presaturation buffer that is 0.5–1 pH unit below the pI of the protein and the same 50 mM salt concentration; the elution condition would again be at the same pH and 0.5–1.0 M salt.

For both anion- and cation-exchangers, a third buffer—often called a cleaning buffer—is employed after elution to remove strongly bound components. The cleaning buffer typically has a much higher salt level, often as high as 4 M, and occasionally is at pH extremes, such as pH 13 or pH 1.

In certain instances, the buffer conditions can be manipulated to allow either the product to flow through the column unretained and the impurities to be bound to the adsorber, or the product to be bound and the impurities to be unretained. This is especially useful to remove nucleic acids (which are negatively charged) from a basic protein on an anion-exchange column. Care must be taken when using chromatography resins to bind impurities since not all impurities are likely to bind to the resin. If used early in the purification process, flow-through columns are extremely productive, but it is generally accepted that a binding step (i.e., where the desired solute is bound to a resin) is needed downstream of a flow-through chromatographic step.

The choice of buffering species is very important and should not be taken lightly. The most important criteria include using buffering species that provide buffering capacity at the desired pH, do not interact chemically with the protein and are nontoxic. Interaction of the buffering species with the adsorbent is the subject of some debate. If the buffering species is such that it is oppositely charged than the adsorber, then when the buffering species is charged, it will compete with the protein for sites on the adsorber. Fortunately, the capacity of the adsorber for small ions (like charged buffering species) is usually much greater than that for proteins, which minimizes the competitive adsorption effects due to the ability of small ions to bind to many more adsorption sites. To be safe, the buffering species should form ions of the same charge as the adsorber and thus not compete for binding sites. There are numerous published tables and charts providing pK_a s of different buffering species (77–80), but many of these species are hazardous and/or toxic. Good et al. (81) published a list of biologically favorable buffering species, the so-called “Good’s Buffers,” that are commonly used in the downstream processing of proteins. Lastly, some situations require volatile buffers, although this is less common; examples of these are formic acid, acetic acid, ammonium acetate, and ammonium carbonate.

IEC, like all adsorptive chromatographic methods, can be operated using either isocratic or gradient elutions, although most isocratic elutions actually involve the use of step gradients, i.e., the instantaneous change from one buffer to another. Additionally, it is worth noting that the traditional sense of gradient elution involves the precolumn mixing of two streams to generate a gradually changing mobile phase. This gradient can be of ionic strength or pH. The use of salt (or ionic strength) gradients is explained above.

In the late 1970s and early 1980s Sluyterman and co-workers developed a technique, termed chromatofocusing, utilizing internally generated pH gradients (82–88). The technique utilizes a column with a buffering capacity, like DEAE, and an eluent containing a low ionic strength, synthetic polymer mixture containing thousands of individual buffering species with a broad spectrum of pK_a s and thus a very linear titration curve and an even buffering capacity over a large pH range. Their method involved equilibrating a weak-base ion-exchange column with a buffer containing a simple basic buffering species (same charge as the adsorbent) at a high pH. The protein sample is adsorbed onto the adsorbent, and then the focusing buffer (titrated to a lower pH with acetic acid, for example) is pumped through the packed column. A linear pH gradient is generated internally (without precolumn mixing) by the titration of the adsorbent by the polyampholyte buffer. In use, these polyampholyte buffers have many limitations, such as their high cost and their known propensity to associate with proteins, thereby restricting the widespread acceptance of chromatofocusing and its use on a preparative scale.

Chromatofocusing typically results in very high resolution and is frequently used as a final polishing step. Nagashima et al. used chromatofocusing as a final polishing step, even though they used a weak-base ion-exchange step in the intermediate processing step (36). Sluyterman and Wijdenes (84) experimented with using simple buffering species to internally generate continuous pH gradients, but concluded that the polyampholyte buffers produced better pH gradients. Recently, investigators have developed techniques involving retained, internally generated pH gradients using simple buffering species to avoid the complications associated with polyampholyte buffers (89–93). Frey and co-workers developed a chromatofocusing technique using pH step gradients (containing one or multiple pH steps) (89,90). Their procedure involved equilibrating the column at one pH with one counterion (acetate, for example) and eluting at a lower pH with a second counter-ion (chloride, for example). Bates and co-workers expanded the earlier work using simple buffering species to generate linear pH gradients to separate proteins (91–93).

Hydrophobic Chromatography

The separation of biological components by hydrophobic interactions was first suggested by Tiselius in the late 1940s (94), and since then, reversed phase chromatography (RPC) has been widely used for the analysis of a variety of substances (95). Hydrophobic interactions are the binding of hydrophobic moieties in the presence of water (96).

The term reversed phase arises from operating a column in a *reverse mode* to what was traditionally done (called normal phase). In normal phase chromatography, the solute partitions between a polar stationary phase, often underivatized silica, and a nonpolar, organic mobile phase. In RPC, long alkyl chains are bonded to the silica backbone creating a hydrophobic, nonpolar stationary phase. The solutes partition between the hydrophobic stationary phase and the polar mobile

phase (e.g., water). The strength of the mobile phase is increased by adding an organic modifier (like acetonitrile or methanol) to the water, thus decreasing the surface tension of the stationary phase, and desorbing the solute. RPC is typically operated using a continuous, linearly increasing gradient of organic modifier, i.e., from 5% to 95% acetonitrile, generated by precolumn mixing of the two "pure" solutions.

Reversed phase chromatography is usually performed with small particles (e.g., 10 μm diameter) and is a very efficient step offering the potential for high resolution. Highly polar proteins are eluted under mild conditions in RPC, and in these instances RPC is used for low-loading, high-resolution steps, i.e., final polishing steps. Comuzzi et al. used RPC after a heparin affinity column to purify milligram quantities of RANTES (regulated on activation, normal T cell expressed and secreted) (97). The authors concentrated the effluent (by ultrafiltration) from the affinity chromatography step and loaded it onto a C-4 column. The RANTES was eluted using a 60-min, linear acetonitrile gradient with an approximate 80% yield. Other researchers have also used RPC in the purification of proteins, but RPC is not as common as a similar technique, HIC, in the downstream processing of proteins.

Hydrophobic interaction chromatography (HIC) and RPC are similar techniques, separating solutes based upon interactions between surface hydrophobic patches on the protein and the hydrophobic ligands on the surface of the stationary phase. RPC typically uses long alkyl chains having 4, 8, 12 or 18 carbon atoms, while HIC adsorbents contain short alkyl chains, 8 carbon atoms or less, or phenyl groups. Additionally, the capacities of HIC columns are much less than the capacities of RPC columns, due to the degree of substitution of the functional groups. In contrast to the high organic content of RPC mobile phases, HIC utilizes aqueous mobile phases that are less likely to denature proteins; the solutes are adsorbed on a mildly hydrophobic stationary phase with a high-salt aqueous mobile phase and eluted with a low-salt aqueous mobile phase. The high-salt concentration of the loading buffer increases the surface tension of the mobile phase, thus favoring adsorption onto the stationary phase (98). This "hydrophobic effect" is defined as an unfavorable interaction between a nonpolar moiety and water (99). The "salting out" of proteins enhances binding in HIC, and hence the precipitation-promoting salts (PO_4^{3-} , SO_4^{2-} , NH_4^+ , K^+ , etc.) from the Hofmeister lyotropic salt series (100) are preferred, but not required, in HIC. After adsorption, the solutes are desorbed off the stationary phase using a mobile phase containing a lower concentration of salts, at the same pH. Horváth and colleagues have published a series of articles related to binding in HIC revolving around the solvophobic theory (97,101–104). Nagashima et al. used a HIC step consisting of a phenyl functional group as an intermediate purification step to purify an enzyme (36). Numerous other groups have reported the use of HIC in the recovery of proteins (105–112).

In HIC, pH effects are worth considering, but care should be taken to not denature the protein of interest. Several pH values should be tested within the stability range of the protein; this can be accomplished in a batch adsorption mode before column studies are initiated. Frequently, increasing the pH (and thus deprotonating amino acids) will decrease the hydrophobic interactions.

Temperature effects have also been shown to be important in HIC (102,103), but it must be realized that temperature control on a large scale is not as easy as in the laboratory.

The development of a HIC step should involve examining several salt concentrations, pHs, and ligand types. The loading of the feed onto the hydrophobic resin should be performed under the lowest possible salt concentration where the desired

protein binds, but conditions where precipitation occurs should be avoided. During method development, the screening via batch adsorption experiments should focus on initial pH and the choice of salt. A common starting point for method development is 1.0M ammonium sulfate at a pH where the protein is stable. The ligand attached to the stationary phase is also important, and several different ligands should be examined. Once the resin and loading conditions are set, the type of elution needs to be determined, either isocratic or gradient. A gradient elution would involve simply decreasing the salt concentration of the mobile phase by precolumn mixing of the high salt buffer with a low or no salt buffer until the desired protein elutes. Gradients are typically easier to develop, since a gradient with a fairly shallow slope works most of the time. Isocratic elution offers the possibility for better resolution between components, as described above, but they frequently require a larger volume of mobile phase to elute the desired component. Determining the optimal isocratic conditions is also a time-consuming operation, often involving numerous iterations.

Thiophilic Chromatography

Thiophilic chromatography was first described by Porath and colleagues (113–115) in their efforts to purify immunoglobulins from serum and since has been used by other researchers to purify proteins from serum, ascites fluid, hybridomas, and prostate-specific antigen proteins (112–126). Thiophilic chromatography goes by many names such as T-Gel, thiophilic interaction chromatography (TIC), and thiophilic affinity chromatography. Thiophilic interactions result from having a nucleophilic molecule three atoms away from the sulfone group (21), such as mercaptoethanol bound to divinyl sulfone-activated agarose. Other novel ligands are also used (116, for example).

Thiophilic interactions between the sulfone-thioether ligand and active sites on the protein are very specific, promoted by water structure-forming salts (like sodium sulfate) (113,118,119), and adsorption is enhanced by high-salt concentrations. Similar to HIC, loading occurs using high concentrations of sodium, potassium or ammonium sulfate, and elution is accomplished by decreasing the salt concentration either via continuous or step gradients. Berna et al. compare and contrast the adsorption of proteins from serum in TIC and HIC; they concluded that both TIC and HIC are salt-promoted, but the salt promotes different types of weak molecular interactions in each mode (117). Sulfate salts have been shown to increase binding, and chloride salts have been shown to decrease binding (21).

Affinity and Pseudo-affinity Chromatography

Affinity adsorbers can be loosely classified into two categories: highly specific adsorbers and group adsorbers (21). Highly specific adsorbers use the actual enzyme or substrate or an analog as the ligand, and those ligands are immobilized onto the stationary phase. Group adsorbers use ligands that bind to a wide range of proteins that have similar structures or functions, such as using immobilized Protein A to purify antibodies. Highly specific immunoaffinity resins are either available commercially or easily produced using activated particles that can be loaded with a ligand of choice from resin manufacturers (127, for example), but the specifics involved with choosing and loading of the ligand are beyond the scope of this chapter. For the remainder of this chapter, affinity chromatography will be used to refer to group

adsorbers. A list of common affinity resins and literature references are given in Table 2.

The two most common affinity resins for antibody purification are Protein A and G. Both resins utilize immobilized bacterial proteins that bind specifically to the Fc region of IgG antibodies (128). Protein A is more specific to mammalian IgGs (like fully humanized IgGs of subclasses 1, 2 and 4), while Protein G shows greater specificity towards mouse or rat IgG (128). On either Protein A or Protein G, the loading of the feedstock is done at or near physiological pH and ionic strength, e.g., 10 mM sodium phosphate, 150 mM NaCl, pH 8.0. After loading the antibody-containing feedstock onto the affinity resin, the resin is then washed with 5–10 column volumes of buffer under loading conditions. The elution of the antibody off the column requires a strong elution buffer (e.g., 1.0 M acetic acid, pH 3) and the antibody-containing effluent should be neutralized, dialyzed or diafiltered as soon as possible, to limit the degradation of the antibody in the strong elution buffer. Examples of the use of several resins are given below.

Pseudo-affinity adsorbers, like immobilized metal affinity resins, bind proteins through interactions common to many groups of proteins. IMAC separates proteins based on their affinity for metal ions. Two amino acids, histidine and cysteine, form complexes with transition metals at neutral pHs. Typically, the affinity of a protein for an IMAC column is proportional to the number of surface histidine residues. This is often exploited by adding a poly-histidine fusion to recombinant proteins to simplify the purification. Fusion and tagged proteins are beyond the scope of this chapter, but the reader is encouraged to review the literature on using fusion proteins to simplify recovery using IMAC columns (129–135), affinity tags (e.g., green fluor-

Table 2 Examples of Affinity Chromatography Resins

Ligand	Specificity	References
2'5' ADP	Enzymes which have NADP ⁺ as cofactor	21
5' AMP	Enzymes which have NAD ⁺ as cofactor, and ATP-dependent kinases	21
Blue (Cibacron Blue)	Broad range of enzymes which have nucleotide cofactors, serum albumin, etc.	21,179,180
Bezhydroxamic acid (BHA)	Peroxidase	181–184
Red (Procion Red)	Broad range of enzymes which have nucleotide cofactors, etc.	21
Protein A	Fc region of IgG and related molecules	174,185,186
Protein G	Similar to Protein A, but different affinities for IgGs from different species	187
HCIC	Similar antibody binding affinities to Protein A, but with a different ligand	137–139
IMAC	Proteins containing surface histidines	121,162

escent protein) (38,136–139), or other fusions (140–144). The most common metal ions used in IMAC are Zn^{2+} , Cu^{2+} , Ni^{2+} and Fe^{2+} .

The development of a purification step should begin with several analytical experiments (either in column or batch adsorption mode) to determine the best metal ion to use. Typically, copper offers stronger binding, and zinc is often weaker. Loading of the protein-containing feed solution onto the IMAC column should be done at relatively high ionic strength (0.5–1.0 M NaCl) with a slightly alkaline pH (typical buffers include tris acetate at 50 mM or sodium phosphate at 25–50 mM). After loading the protein, the column should be washed with 5–10 column volumes of the loading buffer to remove weakly bound contaminants. The elution buffer is at the same conditions used for loading but with an increased amount of a substance with a strong affinity for the immobilized metal ion, e.g., ammonium chloride (150 mM) or imidazole (50 mM). EDTA can also be used to elute proteins from an IMAC column, but it must be realized that EDTA will strip the metal–protein complex from the column, thus offering no further resolution. EDTA is frequently used to clean all strongly bound protein from the column matrix. As with all adsorptive methods, isocratic or gradient elution can be used, and it is up to the reader to decide which is best for the situation at hand. Lastly, the issue of metals leaching off the column is worth considering, especially for large-scale operations.

Recently another affinity resin has gathered much attention, especially in the recovery of antibodies. Hydrophobic charge induction chromatography (HCIC) utilizes a ligand that is antibody-selective and allows for elution under much gentler conditions. HCIC was first described by Burton and Harding (145) using a pH-dependent, dual-mode ligand (4-mercaptoethylpyridine, see Fig. 4) that favor hydrophobic binding under loading conditions (physiological pH) and electrostatic repulsion under elution conditions. A typical HCIC step involves equilibrating the column with a 50 mM tris buffer at pH 8 (phosphate-buffered saline, pH 7, is also used) and loading the sample onto the column. The loaded column is then washed with the loading buffer before an instantaneous step change to the elution buffer (50 mM sodium acetate, pH 4.0) rapidly desorbs the antibodies. The pooled antibody containing fractions are then immediately dialyzed (or buffer exchange) to a more suitable buffer. Schwartz et al. (146) compare HCIC to traditional Protein A purification for several systems. They reported recovery values in the 92–98% range with purities >95%, which were at least as good as those realized from a Protein A recovery system. A four-log reduction in viral load was also seen in the HCIC step, and

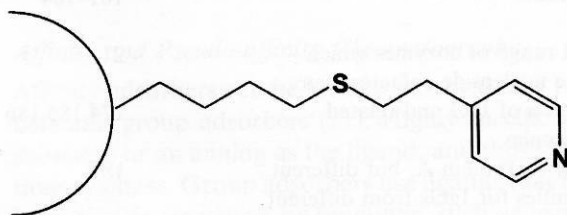


Figure 4 HCIC ligand. At physiological ionic strength and pH values, the aromatic pyridine ring is uncharged and hydrophobic. The 4-MEP is known to bind strongly and selectively to IgG subclasses from most species. When the pH of the solution is dropped below the pK_a of the nitrogen (4.8), the ligand has a positive charge; under such conditions, most antibodies carry a positive charge, and therefore electrostatic repulsion occurs.

significant DNA reduction was realized. Additionally, Guerrier et al. (147) used HCIC to purify milligram quantities of whole hIgG₁ and antibody fragments.

Gel Permeation Chromatography

Gel Permeation chromatography has been used extensively in the purification of proteins, nucleic acids, and other biological macromolecules since its inception (148,149). Gel chromatography (also called GPC, gel filtration chromatography or SEC) is a mild purification technique that separates molecules based on size, using an inert (noninteracting) matrix with a carefully controlled pore structure. The pore structure of the gel matrix is such that smaller molecules are able to diffuse into more of the matrix than larger molecules, and hence the larger molecules elute first from the column followed by the other solutes, in order of decreasing size. It is important to note that the solute "size" reported from a gel chromatography assay is dependent on the shape of the molecule. Two proteins of the same molecular weight can have different elution characteristics depending on their shape, e.g., one is a rod-like molecule and one is spherical in shape. Most commercially available "calibration" standard samples assume a spherical shape.

Gel permeation chromatography is a very mild purification technique that allows operation at conditions suited for the stability of the desired protein, and it can be operated in the presence of cofactors, at high or low pH, at 37°C or 4°C, with or without detergents, etc. It is also a suitable technique for both preparative practices and analytical techniques. Preparative gel permeation chromatography is frequently used as a polishing step where low capacities are acceptable and the feed stock is relatively clean (150). Dimers and degradation products are the most common impurities removed from the product in gel chromatography steps. Additionally, GPC is frequently used as a buffer-exchange step.

It is generally believed that gel filtration separates solutes based on steric hindrances, and that gel filtration can be used to determine the molecular weight of components (151–153). To determine the molecular weight of an unknown sample, a set of standards of known molecular weight is used to determine a calibration curve (also called a selectivity curve). The set of standards would contain similarly shaped molecules with molecular weights greater and less than the expected molecular weight of the desired protein. A typical calibration curve involves plotting the logarithm of the molecular weight vs. the average distribution coefficient ($K_{d,av}$).

$$K_{d,av} = \frac{V_e - V_o}{V_t - V_o} \quad (13.7)$$

where V_e is defined as the volume of mobile phase needed to elute the solute from the column, V_o is defined as the volume needed to elute a solute that does not enter the pores of the resin, and V_t is the total volume of the column. The volume of mobile phase needed to elute the solute from the column can be calculated by multiplying the retention time and the flow rate. The subsequent plotting of the distribution coefficients and the log of the molecular weights of the standards results in a pseudo-linear graph from which the molecular weight of an unknown sample can be determined from its elution volume. Again, it is imperative to realize that the shape of the molecule is very important and can skew the results drastically. Obviously, the relative molecular weights of compounds are less important than purity and recovery when GPC is used preparatively.

NONCONVENTIONAL CHROMATOGRAPHIC METHODS

Displacement Chromatography

Displacement chromatography is a type of elution chromatography where the more strongly bound solutes push (or displace) the less retained solutes from the stationary phase. The different moieties are separated into adjacent zones of highly pure individual components. In contrast to standard elution chromatography, no "empty zones" exist between solute zones. The zones exit the column in order of increasing binding strength in a so-called "displacement train," with the last moiety to exit, having the strongest binding strength, being the displacer.

The displacers can be ampholytes (154,155) indigenous proteins (156), other proteins (157,158), or small molecules (159-161). The presence of a displacer, and hence a displacement train, allows the solutes to be loaded at levels significantly higher than normal elution chromatography, without the detrimental peak tailing that is seen in overloaded elution chromatography. Displacement can occur with any adsorption media, and numerous examples can be found in the literature for displacement in IEC (162), IMAC (163), HIC (164), hydroxyapatite (165), and affinity-based chromatography (166). The choice of displacer is dependent on both the type of resin used and the protein to be purified, and there are numerous alternatives for each type of resin.

The choice of displacer is obviously a crucial factor in the utility of displacement chromatography for the desired solute. Displacers should be chosen such that they are nontoxic, stable, inert with respect to the components in the feed, soluble in the mobile phase, easily removed from the desired solute and have a stronger affinity for the stationary phase than any of the components in the feed. Another factor to consider is the concentration of the displacer. Too little displacer results in broad zones of insufficient purity, and too much displacer results in insufficient volume available to form a displacement train and hence little to no separation between components.

A typical purification cycle consists of presaturating a column with a buffer that is favorable to adsorption. Next, the solute is loaded onto the column followed directly by the loading of a solution containing the displacer. The column is then subjected to a buffer that is unfavorable to adsorption. This elution buffer allows the formation of the "displacement train" and collection of product. The last step involves regenerating the column in preparation for the next cycle.

Expanded Bed Chromatography

As mentioned above, EBA or expanded bed chromatography allows the loading of unclarified feedstocks directly onto a recovery column. Expanded bed chromatography combines the capture and intermediate purification steps by simultaneous utilization of the same resin and equipment for two separate steps. The first part of EBA is the generation of a fluidized bed by an upward flow. The second step is a downward flow resulting in a traditional adsorption chromatographic step. As with any fluidized bed, it is imperative that the flow rate is greater than the minimum fluidization velocity to ensure adequate mixing. A fluidized bed has much greater space between the particles (called intraparticle void volume) than a traditional packed bed, allowing the use of viscous feed solutions at high flow rates with minimal pressure drop. A typical EBA step would involve the loading of a viscous feed solution in such a manner to create a fluidized bed where the cell debris and other nonadsorbed material flow through the bed. This is followed by a wash step to remove debris and

unbound components from the resin. After completion of the loading and washing steps, the direction of flow is reversed and a standard elution chromatography step is run. The type of elution depends on the nature of the separation and the type of functional group attached to the bead. Many researchers have reported the use of EBA to purify proteins from bacteria, yeast and mammalian cultures (167–175). Garke et al. used EBA with cation exchange resin as an initial step in the purification of recombinant human basic fibroblast growth factor (173). Feuser et al. discussed the use of EBA for the purification of monoclonal antibodies (MAbs) and report that hybridoma cells interact with an expanded bed of cation exchange resin but do not interact significantly with Protein A adsorbent (19). Fahrner et al. reported that expanded bed Protein A chromatography had similar purification results as typical fixed bed chromatography (174). The removal of whole cells from product-containing supernatant is a common use of EBA, but if the cells rupture, proteases, nucleic acids and other undesirable moieties will be released. Fortunately, several researchers have shown that EBA is a mild technique, even for shear sensitive hybridoma cells (175), and thus cell rupture is usually not an issue.

DESIGN OF PURIFICATION PROTOCOLS

Although, there are no generic protein purification procedures, several guidelines will assist in the method development process. To ensure adequate purity and adventitious agent clearance, two to three orthogonal purification techniques should be used in the recovery process. It is important to maximize the strengths of each unit operation and not to demand too much from each step. The design of the purification protocol should utilize techniques that are adequate for that stage of purification; for example, a size exclusion chromatographic method should not be used as a primary recovery step; instead an UF step should be used if size-based retention is desired. Lastly, the entire purification protocol must be chosen such that the protein is not denatured or rendered inactive.

Recovery of Non-MAb Proteins

A generic protocol utilizing the techniques and guidelines explained above for the purification of most non-MAb proteins would include the following steps:

- Initial purification—microfiltration to remove the whole, intact cells, followed by either a low resolution chromatography step or an ammonium sulfate precipitation to crash most proteins out of solution.
- Intermediate—HIC and IEC (order interchangeable) to remove impurities with different hydrophobic and ionic characteristics, respectively, as well as to drastically reduce the DNA/RNA and viral load.
- Polishing—HIC or IEC using very high-resolution particles followed by GPC to remove any dimers.

In between unit operations, buffer exchange and concentration steps will occasionally be needed. Of course, this “generic” protocol does not work in all instances, and most certainly it is not the most productive or efficient process. Several simple modifications to the protocol can be quickly made based upon known attributes of the protein. For example, if surface histidines are present, an IMAC column can replace either the HIC or the IEC step in the intermediate stage. Lastly, nucleic

Table 3 Examples of Protein Purification

Protein	Primary recovery	Intermediate purification	Final polishing	References
UDP-glucuronate	Precipitation	AEC HIC	GPC CF	36
MT5-MMP	Centrifugation	AEC	IAC	188
2-hydroxy- isoflavanone dehydratase	HAC Precipitation	AEC HIC AEC	CF GPC	189
4-hydroxy- benzoate glucosyl- transferase	Precipitation GPC	AEC GPC HAC Affinity chromatography	CF AEC	180
UBGAT	Precipitation GPC	HIC GPC	CF	112

AEC, anion-exchange chromatography; CF, chromatofocusing; HAC, hydroxyapatite chromatography; IAC, immunoaffinity chromatography; GPC, gel permeation chromatography.

acid and virus-specific affinity resins can be used in the protocol to ensure adequate clearance. Table 3 presents several purification schemes used by other researchers.

Recovery of Monoclonal Antibodies

The recovery of monoclonal antibodies has been covered in some detail above in various sections, and the references provided in those sections contain many purification protocols. An article by Iyer et al. (176) provides information on many different issues that arise during the development of an antibody purification process. Most MAb purification schemes employ similar chromatographic techniques, e.g., Protein A, IEC, HIC, and affinity, used in various orders depending on the impurities present. Other researchers have developed one-step antibody purification methods using novel affinity or immunoaffinity ligands (127, for example). As provided in the previous section for non-MAb proteins, below is a quick, generic protocol for the purification of MAbs:

- Initial purification—centrifugation, depth filtration or microfiltration to remove whole, intact cells, followed by big-bead Protein A, HIC or batch adsorption or chromatography.
- Intermediate processing—Protein A to bind all the antibodies, followed by IEC to purify based on ionic characterization.
- Polishing—GPC or high-performance cation-exchange chromatography to remove dimers or high-performance IEC (like chromatofocusing) to remove close analogs based on charge.

Again, the buffer exchange and concentration steps have been omitted, but they will most likely be needed between the techniques. If EBA is used, the microfiltration step can be eliminated. In addition, endotoxin-specific columns (so-called detox gels) are frequently utilized in-between purification zones to ensure adequate endotoxin removal.

The current industrial trend is to avoid using Protein A, thus avoiding potential leachable or extractable issues with the column matrix. In these non-Protein A processes, the protocol is similar to that used for non-MAb proteins discussed above. Yazaki et al. (177) discuss the purification of antibody fragments using a nonaffinity-based purification process. The authors utilized batch adsorption of an anion-exchange resin followed by vacuum filtration through a 0.2µm membrane to clarify the harvest supernatants. The clarified broth was then passed through two complimentary chromatographic columns (with dialysis before and after each column) to produce sufficient quantities of minibody and diabody fragments for both preclinical and early clinical supplies. Directly following clarification and dialysis, the broth was applied to a ceramic hydroxyapatite (calcium-hydroxide-phosphate functional groups) column at low salt and near biological conditions. After sufficient washing (until the baseline—measured at 260 nm—returned to zero), a linear gradient of K₂HPO₄ was used to elute the desired components off the hydroxyapatite resin. The fractions containing product were then dialyzed and adsorbed onto a strong anion-exchange column. The column was then washed until the baseline returned to zero and eluted with an increasing linear salt (sodium chloride) gradient. The product-containing fractions were then concentrated and sterile filtered. The authors also examined the use of a hydrophobic interaction column instead of the hydroxyapatite column. The product was eluted off the column using a decreasing salt gradient, but formation of a minibody dimer was noticed. The authors reported recoveries of between 40% and 81% for each chromatographic step. The purified product was lastly filtered through a 0.04 µm filter to ensure viral removal.

Additionally, researchers at the University of Minnesota reported using a novel, modified zirconia resin with a step gradient in salt to replace Protein A in

Table 4 Examples of Monoclonal Antibody Purification

Protein	Primary recovery	Intermediate purification	Final polishing	References
MAb24	Ultrafiltration	Protein A	GPC	186
Polyhistidine-tagged antigen	Centrifugation	IMAC	GPC AEC	121
BsMAbs	Centrifugation Ultrafiltration	Protein A	HAC	190
Polyhistidine-tagged firefly luciferase	Centrifugation EBA (IMAC)			191
Peroxidase-tagged antibodies	Precipitation	Affinity chromatography		181
IL-18	Anti-porcine IL-18 affinity chromatography			127

AEC, anion-exchange chromatography; HAC, hydroxyapatite chromatography; IMAC, immobilized metal affinity chromatography; EBA, expanded bed adsorption; GPC, gel permeation chromatography.

the purification of MAbs (178). Numerous other examples are available in the literature, some delineated in Table 4.

SCALE-UP STRATEGIES FOR CHROMATOGRAPHIC PURIFICATION OF BIOLOGICS

Although gram quantities of protein can frequently be produced using bench-top equipment, the need for larger quantities is occasionally required. In the biotechnology industry, this is akin to the advancement of a potential biological candidate. To produce more material, either several batches can be run in parallel or the laboratory scale procedure can be scaled up. The procedure for scaling up, if done properly, is quite simple, but before the scale-up can be done, several factors must be considered. The same resins (or packing material) used in the laboratory should be used on the preparative scale to provide the same separation results. Ensuring that the packing material used in the laboratory is available in sufficient quantities is very important for obvious reasons. Additionally, the cost, continuity and consistency of the bulk resin must be thoroughly examined. The cost of the resin is not always a major part of the recovery cost, especially if the resin can be reused over numerous cycles, but the continuity of the supply of resin and the batch-to-batch consistency are vital to the success of a large campaign. If the resin is to be part of a FDA-filed process, an audit of the manufacturer is advisable, examining factors such as quality assurance protocols, batch sizes, and quality control data. The ability of a resin to be cleaned-in-place (CIP) is very important if resin reuse is planned. The CIP conditions often involve harsh solutions (such as 1 M NaOH), and the chemical stability of the packing material must be confirmed under these conditions. Laboratory studies should include a column lifetime study, where complete cycles (loading, eluting, CIP, regeneration) are continuously repeated until column degradation is noticed. Occasionally, these studies are conducted at elevated temperatures to extrapolate past the number of cycles tested, but these elevated temperature studies should be interpreted carefully. They frequently can provide misleading results. The most accurate method would involve operating the stability testing under the same conditions of the preparative separation.

The actual scale-up is a relatively straightforward task involving several simple steps, listed below:

- The amount of protein to be recovered is needed
- The maximum protein loading at laboratory scale is required
- The effective resin capacity needs to be determined
- The size of the preparative column needs to be calculated.

Before the preparative method can be determined, the mass of protein that is going to be needed at preparative scale must be identified. The mass of protein needed depends on its intended use (preclinical, phase I, phase II, etc.), the size and potency of the bioreactor, and whether or not multiple lots of material will be pooled prior to chromatographic purification. Once the total amount of protein needed is known, the maximum amount that can be loaded onto the laboratory column must be determined. The easiest way to determine the maximum loading is to incrementally increase the amount of feed applied to the column until the purity and/or yield of the desired protein becomes unacceptable. The increase of protein load on the column can be accomplished in two ways, either volume or

concentration overloading. If the step involves adsorption chromatography, and the feed is loaded in a buffer that promotes adsorption of the solutes onto the stationary phase, then the amount of solute added can be increased by simply increasing the volume of the feed solution at the same concentration loaded onto the column. An alternative is to increase the concentration of the feed in order to add the same feed volume but more mass of protein. Adding the same volume of solution is critical in nonadsorptive chromatographic steps.

Once the maximum loading is known, the effective column capacity can be calculated. The maximum mass of protein loaded onto the column ($m_{p,max}$) divided by the total column volume (V_c) yields the effective column capacity, CAP_{effect} :

$$CAP_{effect} = \frac{m_{p,max}}{V_c} \quad (13.8)$$

Having the capacity of the packing material, combined with knowing the mass of protein that is needed per cycle, the volume of the preparative column can be calculated. The quotient of the mass of protein required (m_{prep}) by the effective column capacity yields the preparative volume of resin needed to purify m_{prep} of protein:

$$V_{prep} = \frac{m_{prep}}{CAP_{effect}} \quad (13.9)$$

From the volume of resin needed it is trivial to calculate the dimensions of the preparative chromatography column. Although scale-up based solely on column volumes are routinely performed (i.e., the dimensions of the column are not important) keeping the length of the preparative and laboratory column the same is the safest situation. The length of column is directly related to the efficiency of the column, and it is also directly related to the pressure drop across the column (i.e., longer columns have higher pressure drops, all other factors being equal). Therefore, whenever possible it is best to scale-up based upon inner diameter, keeping the length of the column fixed. Thus, once the volume of the preparative column is known, the required cross-sectional area (and hence the diameter) of the preparative column can be calculated from the length of the analytical column. For example, if 10 L of resin are required and the length of the laboratory column used was 20 cm, the inner diameter (ID) of the preparative column should be 25.2 cm, as seen below:

$$ID = \sqrt{\frac{4V_{prep}}{\pi L}} = \sqrt{\frac{4 \times 10 \text{ L} \times (1000 \text{ cm}^3/\text{L})}{\pi \times 20 \text{ cm}}} = 25.2 \text{ cm} \quad (13.10)$$

Therefore, in this example, the preparative column would be 20 cm $\times$ 25.2 cm ID.

It is also very important to remember that as the purification progresses to large pilot or production scale, the weight of the bed becomes significant. In these instances, extra care should be taken to keep the length of the column short. Most laboratory columns are < 30 cm long, and if the scale-up is performed based on inner diameter, this does not become an issue.

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