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Unit processes
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Chapter 6

Downstream process

The target protein, which is produced by the cells either through secretion in the medium (mammalian cells) or intracellularly as inclusion bodies, must be isolated from the host cells and purified to remove or reduce product- and process-related impurities to produce a high-purity final product. Centrifugation, filtering, chromatography purification, precipitation, and concentration (ultrafiltration/diafiltration (UF/DF)) are some of the unit activities used in downstream processing. The nature of the contaminants varies. The overall downstream process design should be strong and consistent in order to ensure that these contaminants are eliminated or kept within defined safety limits. The existence of adventitious viruses should be addressed in the process design for mammalian processes or processes that utilize raw materials of animal or human origin to ensure there is no possibility of viral contamination in the final product. Downstream processes may include more than one orthogonal step for impurity removal. Platform processes allow for a starting point for the process design of molecules that belong to the same class, but further optimization specific to the target protein is necessary.

6.1 Overview

The crude protein is generated in the upstream process and, while the conditions in the upstream process influence the type of protein, the downstream process determines the final product. The desired therapeutic product may be secreted in the medium (as with mammalian cell culture). In microbial cells, it could be the biomass itself or an intracellular or extracellular component. Upon completion of fermentation, separation, recovery, and purification of the product from the host system constitutes downstream processing. Typically, the hand-off between upstream and downstream occurs at the recovery step, i.e. harvest (clarified supernatant or cell pellet). Recovery of the product implies isolation from the cell mass, irrespective of whether the expression system is mammalian or microbial cells. Cell removal is usually achieved by centrifugation or filtration. Harvest of culture broth,

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the recovery step (clarified supernatant or cell pellet), is discussed in chapter 5. This chapter provides essential elements of recovery and purification of biological products with information about future trends.

This chapter will consider the two most used production systems, *Escherichia coli* and CHO, and present the purification and product concentration strategies for these systems in preparation for the final formulation and fill-finish of the drug substance/drug product (DS/DP) (figure 6.1).

The first step in selecting downstream processing methods is to understand the features of the protein in question, its stability profile, and the circumstances that may affect its structure. The primary phases in downstream processing schemes (figure 6.2) are capture, intermediate purification, and polishing (or ultimate purification), each serving a specific purpose. The primary goal of the capture step, the first of the orthogonal chromatographic unit operations, is to isolate and stabilize the target protein while also removing key host cell components. The purification stage's second phase removes the majority of the contaminants, such as nucleic acids and host cell proteins. This stage may be optional, and its inclusion is dependent on the design of the succeeding chromatography processes as well as the impurity and purity profile of the target protein. Polishing, the final purification stage, strives to achieve the utmost purity of the product while also removing any product-related impurities such oxidized and deamidated species. The target protein, the product quality qualities, and the product requirements all have a role in the technique selection or combination.

A downstream process's overall workflow and endpoint are always the same: ensuring the product meets its quality attributes and purity profile, creating the product profile, and establishing the critical product quality attributes for the target

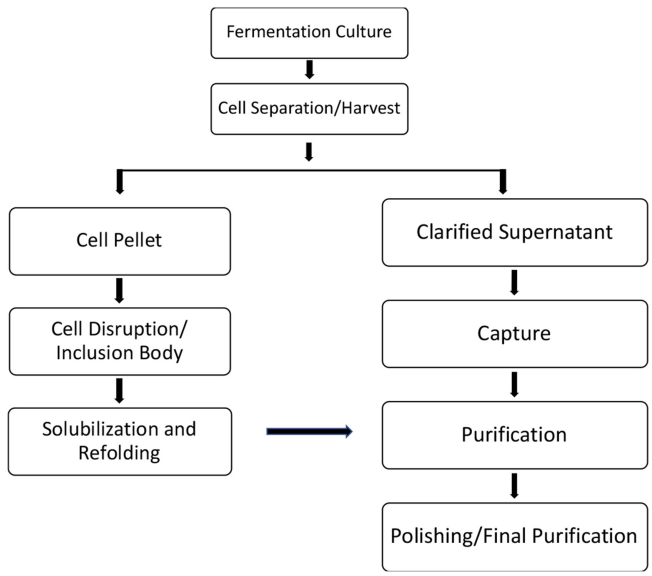

Figure 6.1. Overview of downstream unit operations for purification of recombinant protein.

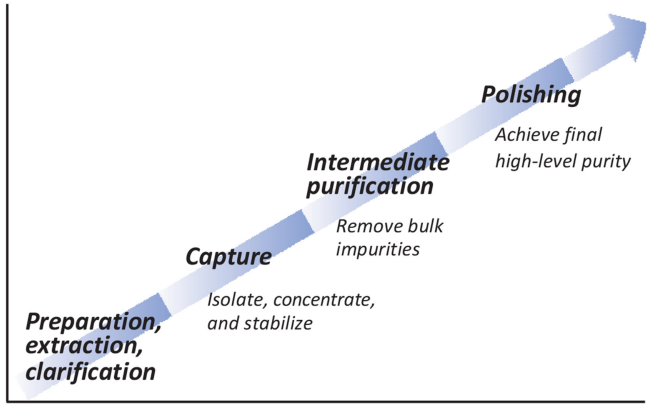

Figure 6.2. A three-stage purification strategy. Credit: Copyright Cytiva Life Sciences.

Table 6.1. Protein properties and applicable chromatography methods.

Target protein property	Applicable chromatography purification method
Specific ligand recognition	Affinity chromatography (AC)
Metal ion binding	Immobilized metal affinity chromatography (IMAC)
Charge	Ion-exchange chromatography (IEX)
Hydrophobicity	Hydrophobic interaction chromatography (HIC)
Size	Gel filtration (size-exclusion chromatography)
Isoelectric point	Chromatofocusing

molecule at the start of process development activities and process design. Purification and effective recovery are critical components in the success of the upstream and overall bioproduction processes. To attain a high degree of purity and assure safety, the chosen purification process must be robust, easy to scale up, and capable of handling and effectively removing or lowering both product-related and process-related contaminants. Quality expectations for recombinant proteins are always a prime concern, and they will remain very stringent. This is due to initial concerns about the safety risk concerning immunogenic effects for products that may not be entirely human-like or other hazards that arise due to potential contamination of the product by the host cell system (e.g. mammalian cells).

The appropriate chromatography separation methods are chosen based on the differences in the target protein's properties and other substances present in the sample. Chromatography, the workhorse for protein purification, is a continually evolving high-resolution technique. Both traditional and highly sophisticated techniques take advantage of both the protein's physical and chemical properties, its interactive nature, and the nature of the product variants. Table 6.1 and figure 6.3 show examples of protein characteristics employed in various chromatography methods.

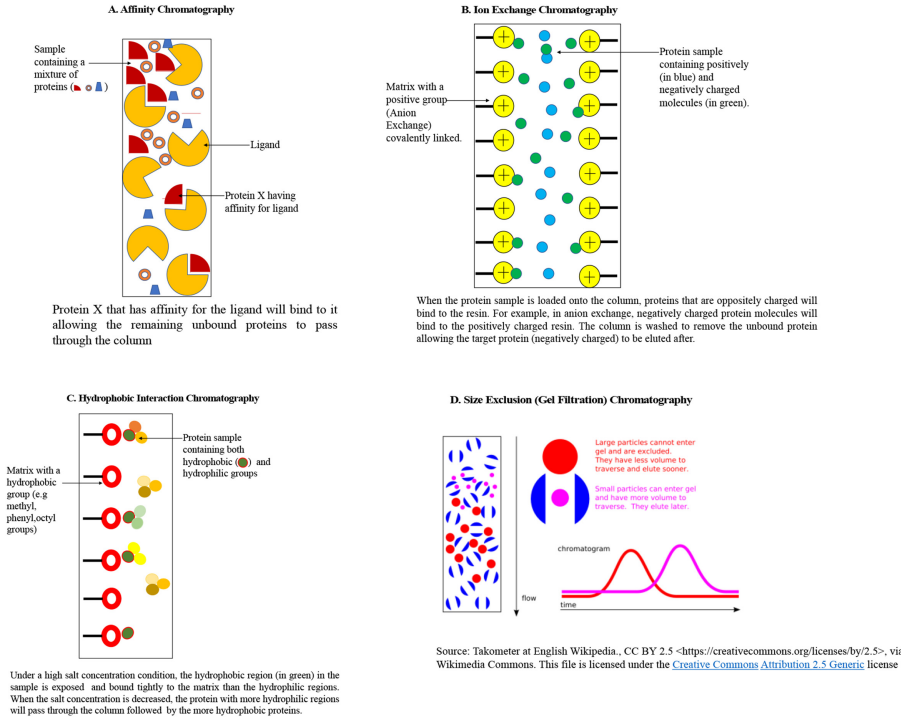

Figure 6.3. Schematic presentation of the various chromatography methods. Source: Wikipedia: Takometer.

Look for qualities such as dynamic binding capacity (DBC), yield, quality, HCP removal, and purity when selecting a resin.

Maximum productivity and cost savings are accomplished by reducing the number of processes to a minimum without affecting the ultimate yield. The downstream processing steps, on the other hand, are crucial to the product's protection, thus they must be closely monitored. The goal of purification is to remove or reduce impurities and pollutants to levels that are below the acceptable level for patient safety. The types of impurities that dictate the type of purification strategy necessary for the downstream process are referred to as process-related and product-related impurities in biological manufacturing.

Product-related impurities in the drug substance are molecular variations formed during manufacture and storage that differ from the intended products in terms of operation, protection, and efficacy. The structural differences in proteins could be caused by the host organism's cellular mechanism in protein creation or some processing activity. From the standpoint of patient safety, these deviations may or may not be acceptable. Substances are product variants that have attributes (such as activity, safety, and efficacy) that are comparable to the target product. To distinguish between contaminants and product-related chemicals, a detailed characterization of the product and its variants is required.

Process-related impurities are contaminants that are linked to the manufacturing process. Among them are proteins from the host cell, DNA, and raw materials (e.g. cell culture media, antibiotics, chromatographic media used for purification, and specific buffer components). Impurities in the upstream process can come from either the cell substrate or the cell culture. The contaminants produced from the cell substrate are affected by the expression system. Cell culture-derived contaminants, on the other hand, are affected by upstream process parameters, which affect whether the product is secreted into the medium or comes as an intracellular product. Impurities are also influenced by downstream process conditions and source materials. Chromatographic media, buffer components, and leachable components are all examples of these.

The major purpose of the downstream process is to reduce contaminants through a succession of purification procedures so that the final product fulfills purity criteria.

Monitoring is required for downstream unit operations and screening the product at every stage for its purity profile, stability, and biological activity. The emphasis on characterization, stability, and overall product profile increases as the target molecule progress through the stages from development to toxicology to clinical, safety, and efficacy testing. It is only when the upstream and downstream processes are locked that the rest of the testing begins, making these processes pivotal in the development timeline of biopharmaceuticals.

6.2 *E. coli* system: recovery and purification

The recombinant protein production in *E. coli* happens either in the intracellular, periplasmic, or extracellular spaces, however, most recombinant protein production occurs intracellularly as insoluble aggregates called inclusion bodies (IBs).

Inclusion bodies are dense protein clumps that can be found in the cytoplasm and periplasm. The density of the IBs is helpful for the separation process compared to the cellular detritus. The protein is generally stable in the IB form and is a suitable stage for pausing operations during manufacturing campaigns. The inclusion bodies often comprise greater than 90%–95% of the protein, reducing the downstream purification burden. The protein is inactive and insoluble in form. The protein must therefore be converted into its active, native conformation. The functional protein is then purified and concentrated to yield the product ready for formulation.

Typical steps before purification to convert the insoluble protein form to soluble, active form include the following:

- The cells are broken to release the inclusion body.
- The inclusion body is separated from the cell detritus.
- Co-precipitated pollutants are removed by body washing with inclusion.
- The inclusion body is solubilized to denature it in preparation for refolding.
- The process of restoring a protein to its active state is known as refolding.

The additional steps to recover the inclusion body and renature the protein to its native state are often considered processing challenges.

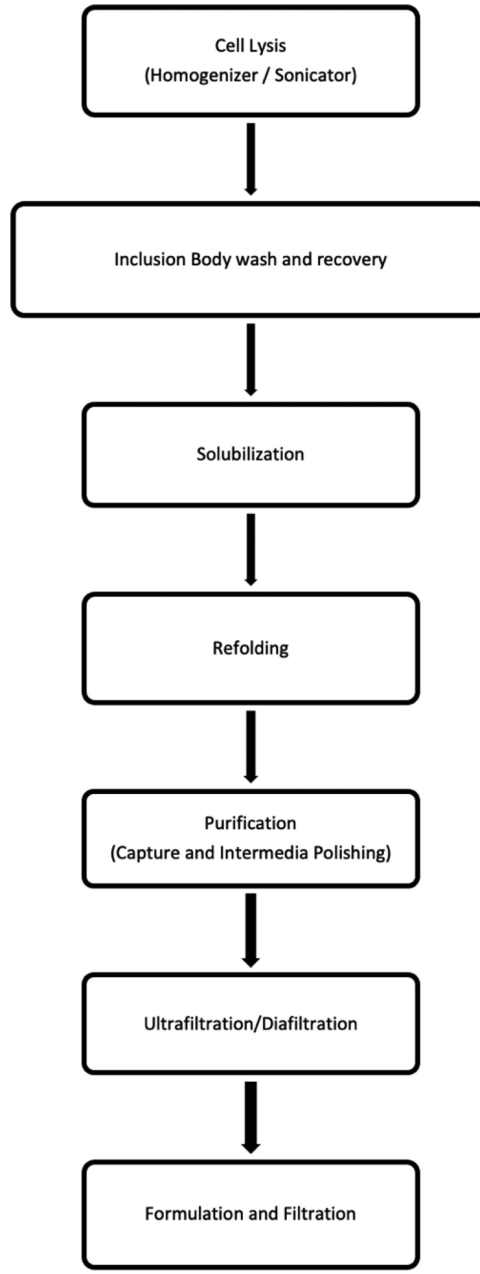

Figure 6.4. Flow chart for downstream processing of recombinant protein expressed in *E. coli.*

Figure 6.4 depicts a typical downstream process for a recombinant protein generated as inclusion bodies in *E. coli*.

For the expression of soluble proteins in the *E. coli* cytoplasm there is no formation of inclusion bodies and, as such, this removes additional processing to

recover the desired protein. Some of these cytoplasmic proteins are also able to fold spontaneously under optimum conditions.

The redirection of the intracellular expression of proteins as inclusion bodies into soluble cytoplasmic proteins occurs at the cellular level via modification of the expression mechanism and appropriate vector design. Soluble protein expression is an attractive alternative, however, it may not suit all protein types. Therefore, another critical factor determining this strategy's success is only if the protein is expressed in a high abundance, at least greater than 5% of the total protein, allowing the recoveries to be maximized without additional steps (IB recovery, solubilization, and refolding).

6.2.1 Cell disruption

Cell disruption to extract inclusion bodies (recombinant protein) is performed before the protein is purified following the cell harvest in *E. coli* for intracellular protein synthesis. *E. coli*'s double cell wall is made up of a rich outer lipopolysaccharide (LPS) membrane, an aqueous periplasmic gap, and an internal thin peptidoglycan cell wall. The breaking of these cellular membranes for the substance present in this intracellular area is referred to as cell lysis (the outer LPS-rich membrane and inner cytoplasmic membrane). Cell disruption is either chemical, enzymatic, or mechanical, or sometimes a combination thereof.

The appropriate method to use is determined based on the protein of interest, the technique's impact on product quality, further downstream processing, and overall yield. In addition, the product's characteristics (solubility, location within the cell, and other physical properties), the batch size and operational scale, processing time, process efficiency, and the protein's stability must be considered while evaluating and determining the optimal method.

6.2.1.1 Physical

Mechanical disruption methods such as grinding, high-pressure homogenization, and ultrasound, as well as non-mechanical disruption methods such as freeze/thaw, heat treatment, and osmotic shock, are all used to physically lyse cells.

6.2.1.1.1 Mechanical methods

Mechanical disruption methods are the industry's preferred choice as it renders a high degree of disruption efficiency and ease of scalability. Mechanical disruption uses hand-held grinders or motorized devices with rotating blades that can break down the cells. The most common devices are motorized appliances such as Waring blenders, Polytrons, hand-held grinders such as Dounce, Potter-Elvehjem homogenizers, and high-pressure homogenizers such as the French press.

The Waring blender resembles a typical standard household blender and is best suited for grinding. The Polytron draws the tissue into a long shaft with rotating blades. These two devices are best suited for grinding down soft, solid tissues. The Dounce homogenizer works by pushing the sample between the sides of the tube and the pestle, thus creating shearing forces. The Dounce homogenizer is an effective

tool for lysing tissue culture cells and fine tissue pieces or applications requiring only mild lysis. The Potter-Elvehjem homogenizers grind tissues as well as lysing cells. The sample is placed in the sample tube and the pestle is rotated at a certain speed. This repeated movement of the pestle on the tube causes a shearing force that breaks the cells.

However, the most common industrial device is a high-pressure homogenizer. The samples are forced through a narrow space in this device while applying high pressure through a piston action (e.g. 20 000 psi) onto the sample. As the cells move from high pressure to a low-pressure zone, they expand and rupture. Generally, the samples are passed multiple times through this homogenizer for high lysis efficiency. To minimize protein denaturation, it is recommended to cool the sample (2 °C–8 °C) during homogenization. High-pressure homogenizers are easily scalable and can accommodate various sample volumes (figure 6.5).

A bead mill homogenizer is another commonly used mechanical method for *E. coli* disruption. This method uses beads that are vigorously agitated at high speeds to break and lyse the cells. The cell suspension is passed through a grinding chamber (consisting of a rotating shaft) filled with beads (approximately 80%). When subjected to high speeds, the sheer force and impact from the beads cause the cells to break. The device does not use any external probes and is relatively self-contained, minimizing any contamination risks.

Sonication is another type of mechanical disruption method used for cell lysis. This method uses an acoustic transducer to generate pulsed high-frequency sound waves, which causes microscopic bubbles that radiate through, creating agitation and cell lysis eventually. This method is used for the homogenization of small sample volumes and is effective for bacteria and spores. However, excessive heat is usually generated from ultrasonic treatment and such samples immersed in an ice bath are generally subjected to short bursts.

Although mechanical disruption is very commonly used, there are certain challenges with this method, including heat generation and possibly even causing denaturation of proteins, limited selectivity and shear stress that may be damaging

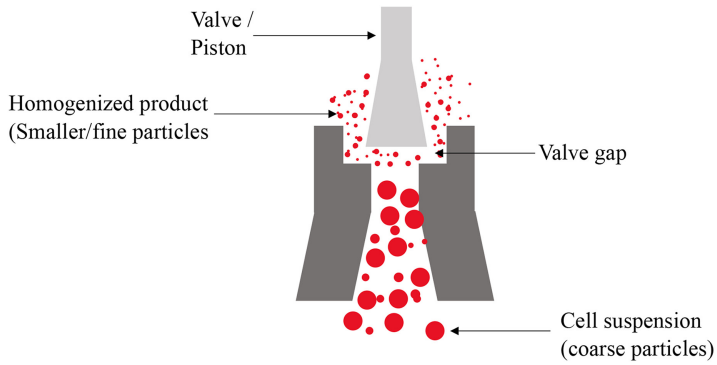

Figure 6.5. Schematic of the working principle of a high-pressure homogenizer.

to sensitive components (e.g. specific proteins), and lastly, the disintegration of the cell debris, which makes the recovery process more contaminating.

6.2.1.1.2 Non-mechanical physical methods

Freeze/thaw: The freeze/thaw method includes freezing a cell suspension (using dry ice, ethanol, or freezer) and then thawing the cells at ambient temperature or at 37 °C. The creation of ice crystals during the freezing process, and their eventual breakdown during the thawing phase, causes cells to expand and then lysis. As a result, many freeze/thaw cycles are required for effective lysis.

Heat shock: Thermolysis imparts heat to the cells that denatures the membrane proteins and lyses the cells. The culture is heated to approximately 50 °C to disrupt the outer membrane or 90 °C for cytoplasmic components.

Osmotic shock: To trigger cell lysis this approach involves a drastic change in the osmolality of the cells. The cells are first placed in a solution with a high osmotic pressure (for example, 1 M sucrose or a high salt solution). This is rapidly shifted to a low concentration solution leading to an osmotic shock onto the cells. Next, the water molecules move from a low salt concentration towards a high salt concentration (so water moves into the cells), causing increased pressure inside the cells resulting in an explosion. Likewise, when cells are exposed to a low concentration solution first followed by a high concentration solution, the water will flow out from the cells leading to rupture.

6.2.1.2 Chemical

In addition to the above mechanical and non-mechanical methods, chemical disruption methods are also in use. These methods use enzymes or chemical additives such as detergents to lyse cells. These agents work by either enzymatically destroying the cell wall or membrane, increasing the osmotic pressure, or precipitating the cell wall proteins.

Detergents work by forming micelles by binding to the lipid membrane protein. Micelle development causes the cell membrane to rupture, allowing internal proteins and inclusion bodies to escape. Based on physical qualities and the type of the action, detergents include:

- *Anionic*: sodium dodecyl sulfate (SDS) is an anionic salt that reorganizes the cell membrane by disrupting protein–protein interactions.
- *Cationic*: ethyl trimethyl ammonium bromide may disrupt LPS and phospholipids in cell membranes.
- Triton X100 is a non-ionic detergent that solubilizes membrane proteins.

Solvent addition, chaotropes, and metal chelators are other chemical additives that are also used for cell disruption. Solvent addition acts by extracting the cell wall's lipid components, increasing the cell membrane permeability to release the intracellular proteins. Examples of commonly used solvents are toluene, dimethyl sulfoxide, and certain alcohols. Chaotropes such as urea may also be used; these agents solubilize membrane proteins. EDTA, a chelator, can bind the metal ions

Mg^{2+} and Ca^{2+} from the cells' outer membrane, resulting in increased permeability, causing the release of intracellular components.

The use of digestive enzymes that can disintegrate the cell wall is another chemical technique for lysing cells. Cell walls and membranes vary between cell types and strains, necessitating the use of enzymes. Lysozyme, for example, is commonly employed to break down the cell walls of gram-positive bacteria. Since the cell walls of gram-negative bacteria differ from those of gram-positive bacteria, lysozyme is required.

The chemical method is easy to implement and scalable, unlike mechanical methods that require intensive capital expenditure. However, some of the limitations of these methods could be the long reaction times for efficient lysis, the burden on downstream operations to demonstrate effective removal of these chemicals, and the risk these chemicals may pose to operators.

Generally, a combination of the various disruption methods is employed in the biomanufacturing processes to overcome lower yield and efficiency limitations. For example, using chemical detergents (Triton X100) as a pretreatment followed by mechanical disruption (high-pressure homogenization) improves the lysis efficiency and reduces the number of passes through the homogenizer. Similarly, chelating agents and detergents are incorporated in buffers to suspend the cell pellet before liquid homogenization. As a result, these combined methods are always more efficient than using a single method.

In the preparation of soluble proteins expressed in *E. coli*, the cells are lysed, and the supernatant is cleared to remove cell debris, lipids, ribosomes, and other particulate materials. The cleared protein is then introduced to the correct chromatographic column for capture.

6.2.2 Inclusion body recovery and separation

Recovery and separation are carried out after the cells have been ruptured and their contents, including inclusion bodies and other cellular components, have been released. To separate the insoluble inclusion bodies from the soluble components and insoluble cell debris, these processes are required.

To increase yield and purity, various separation procedures based on inclusion bodies' density and solubility variations have been applied. The clarifying, purifying, and concentrating of the inclusion bodies before the solubilization and renaturation steps require inclusion body recovery and separation. A combination of methods such as centrifugation and filtration or other alternatives (precipitation, two-phase aqueous extraction) may achieve this resulting in relatively pure inclusion bodies.

Centrifugation is a commonly used technique for the recovery of inclusion bodies. Depending on the scale of operation and the liquid volumes, both benchtop/floor and continuous centrifuge may be used. Choosing a suitable speed and gravitational force (typically between 5000 and 20 000 g) and the high density of inclusion bodies over the cell debris, the inclusion bodies are collected as pellets. The soluble and less dense cell debris is removed in the supernatant, reducing the processing volume.

Another alternative approach to centrifugal separation is microfiltration or ultrafiltration for the recovery of insoluble inclusion bodies. Inclusion bodies are estimated to be between 50 and 1500 nm in size, and choosing an appropriate large pore size filter (microfiltration: 0.05–5 μm; ultrafiltration: 0.001–0.1 μm) achieves a high yield of the inclusion bodies. In addition, filtration benefits enhanced purification by removing soluble impurities, DNA, and toxins released during cell disruption through the large pore size.

Microfiltration eliminates cells and cell detritus from the cell lysate with a size range of 0.2–10 m. Large molecules such as proteins, polysaccharides, and IBs can be removed using ultrafiltration membranes with pore sizes ranging from 0.001–0.1 m. For impurities that may co-precipitate with the inclusion bodies, a diafiltration step using an inclusion body wash buffer to eliminate the other impurities can help. But this may not be particularly suitable for large-scale operations due to the high consumption of wash solution based on the number of diavolumes needed for an efficient process. In addition, it is recommended to maintain low transmembrane pressure (TMP) while processing cell lysate. Still, this evaluation should be made based on the processing time, impact on product quality, membrane fouling, and performance. Typical parameters that should be evaluated for filtration operations include membrane pore size, type of membrane (hollow fiber, flat sheet), permeate flux/fouling, TMP, and cleaning.

Expanded-bed adsorption, aqueous two-phase liquid extraction, solvent extraction, precipitation, and size-exclusion chromatography to isolate and purify inclusion bodies from the cell lysate are other approaches to the standard centrifugation or membrane filtration operations that integrate cell disruption and solubilization.

The selection of an appropriate method, i.e. centrifugation separation, membrane filtration, or a combination of cell disruption and solubilization approaches, is dependent on several factors: the protein of interest, operation scale, facility design and fit, consumption of buffers, overall production process, and the desired level of purity. In addition, the challenges and limitations of each of these methods must also be assessed to make the IB recovery and separation reliable, robust, and economically feasible.

Depending on the approach, sometimes, given the highly insoluble nature of inclusion bodies, integrating wash solutions may be necessary to remove cellular impurities, specifically those that co-precipitate with the inclusion bodies. Despite the high purity of most inclusion bodies, optimizing IB wash solutions will substantially increase overall purity and reduce stream operations. Maximizing purity before solubilization and refolding of the inclusion bodies can improve overall process yield. In addition, the concentration/purity of the inclusion bodies can reduce processing volumes for subsequent steps.

The recovered IB may be washed with solutions having components that can selectively solubilize and remove cell debris, such as low levels of chaotropes, detergents, a combination of salts, and pH, to remove cell debris effectively and other co-precipitated impurities. A careful balance between purity and recovery is required while choosing chaotropes or other agents that can potentially solubilize the IBs, impacting overall yield (for example, using a high concentration of urea

guanidine-hydrochloride can lead to solubilization of the IBs). Centrifugation is commonly used to recover the inclusion bodies after these washing steps. Purified water is used as a last-step wash to remove these chemical agents and other debris before yielding a highly pure IB pellet.

At this stage the concentrated and pure inclusion bodies (IBs) provide an opportunity for long-term freezing (typically −20 °C) of the IB pellet. This allows for flexibility in production schedules, pooling IB pellets from multiple upstream batches for further processing and maximizing downstream operations.

6.2.2.1 Inclusion body solubilization

A critical step after recovery of the IB pellet is to re-solubilize the pellet in preparation for the subsequent renaturation process. The goal of the solubilization procedure is to denature the protein and lower the amount of aggregated protein in the inclusion body. A combination of chemical agents and operating conditions to accomplish this denaturation of the insoluble protein aggregate to a soluble linear polypeptide.

For solubilization of the inclusion body, substantial quantities of a denaturant have traditionally been utilized. Some of the most utilized substances are 6 M guanidine-hydrochloride (Gu-HCl), 8 M urea, detergents, alkaline pH (>9), organic solvents, or N-lauryl sarcosine. Gu-HCl is a powerful chaotrope that is significantly more effective than urea at denaturing excessively aggregated inclusion bodies. Urea solution contains isocyanate, which leads to carbamylation of free amino groups of polypeptides, specifically when incubated for a more extended period under alkaline pH, further affecting the product quality. One concern with using high concentrations of chaotropes is precipitation, affecting overall process yield and subsequent purification steps.

Until incorporating the chosen denaturant into the solubilization technique, it is important to test it with the target protein.

The presence and concentration of a reducing agent, as well as time, temperature, ionic strength, and the denaturant-to-protein ratio, all affect the solubilization success of each denaturant. Table 6.2 lists the experimental starting locations for the solubilization of inclusion bodies. They can frequently be purified at this point using a separation technique that is compatible with the solubilized proteins.

Table 6.2. Example for solubilization design of experiments.

Process parameters	Start condition	Optimization
Buffer	50 mM Tris-HCl, pH 8.0	pH (7.0, 7.5), buffer composition (e.g. EDTA)
Denaturant	8 M urea or 6 M Gu-HCl	Concentration of denaturant
Inclusion of body conc. (mg ml^{-1}, wet weight)	20	Testing a higher and lower concentration (e.g. 10, 40 mg ml^{-1})
Temp (°C)	Ambient	4–30
Time	60 min	15 min to 2+ hours

Many different solubilization techniques (e.g. REFOLD database: http://pford.info/refolddb/) have been published. SDS (10%), N-lauryl sarcosine, or other detergents, as well as pH extremes, are alternatives to the most frequent solubilization agents. For proteins with disulfide linkages a reducing agent such as dithiothreitol (DTT), beta-mercaptoethanol, or Tris 2-carboxyethyl phosphine (TCEP) is commonly used. These reducing chemicals can decrease disulfide bonds and stabilize free cysteines.

For some proteins, mild solubilization solutions with lower chaotrope concentrations have been utilized. The use of mild solubilization conditions has been reported to retain native-like secondary structures, denature the protein to an intermediate state, and possibly improve protein renaturation. Denaturing the protein to an intermediate state rather than an entirely linear polypeptide can improve the yield during refolding. Using this mild solubilization is highly dependent on the protein and IB produced.

Solubilization may have a significant effect on the refolding conditions and, as such, the solubilization buffer and the operating conditions must be designed, considering the renaturation process. For cysteine-containing proteins, isolated IBs usually contain non-native intramolecular and intermolecular disulfide bonds. It may be beneficial to incorporate these reducing reagents combined with the chaotropes to reduce the disulfide bonds. The non-native disulfide bonds can likely reduce the solubility of the IBs in the absence of dithiothreitol, cysteine, or beta-mercaptoethanol. The inclusion of thiol agents is unnecessary for proteins that may already be in a reduced state. Arginine is also commonly employed in the solubilization and/or refolding buffers to prevent protein aggregation in subsequent processes. To avoid oxidation of the free cysteine groups exposed during denaturation and reduction, EDTA may be used.

In addition to the type of denaturant and other additive components, the concentration of these reagents, the ratio of solubilization buffer to the amount of IB pellet, the solubilization reaction time, pH, and temperature are other parameters that must be assessed for optimal conditions.

6.2.2.2 Inclusion body renaturation

Protein refolding is initiated upon the solubilization of IBs to obtain correctly folded proteins. Excess denaturants and reducing agents are gradually eliminated, and reduced proteins must be transported to an oxidizing environment to stimulate the formation of native disulfide bonds. Dilution, dialysis, or on-column processing are all used for renaturation of the solubilized IBs. Refolding is normally done at very low protein concentrations, such as 10–100 g ml^{-1}. This helps prevent aggregation while slowly converting the denatured protein into its native, correctly folded conformation.

6.2.2.2.1 Dilution

Protein refolding by dilution is the most common method, given its simplicity of operation. The solubilized IB is diluted into an appropriate refolding buffer. This dilution reduces the protein concentration and the denaturant concentration and

provides a suitable oxidative environment to enable disulfide bond formation. It is essential to control and maintain a low protein concentration throughout the refolding process to minimize aggregation. A controlled dilution, preferably a pulse or drip dilution, for converting the solubilized IBs into the refolding buffer over a defined time is a preferred method (slow dilution). Flash dilution into excessively large volumes may result in a rapid shift in the environment, causing rapid refolding that can further increase aggregation or misfolding. Continuous mixing of the refolding solution while the denatured protein solution should be performed to avoid aggregation. The optimal dilution best suited for the processing volume without deteriorating the protein quality should be chosen.

6.2.2.2.2 Dialysis

Dialysis is another standard method used to remove or reduce solubilizing agent concentration via the diffusion of components through buffer exchange. During dialysis, the concentration of the solubilizing agent gradually decreases, allowing the protein to refold properly. During dialysis, the sample volume to dialysis buffer ratio should be such that the protein is completely refolded at an equilibrium solubilizing agent concentration. However, dialysis is a slow and cumbersome process, particularly on a large scale.

6.2.2.2.3 On-column

Refolding using packed chromatography columns is a desirable option to the traditional refolding process. The common approaches with this method include:

- Denatured protein immobilization on a matrix and subsequent denaturant dilution to facilitate refolding. This is accomplished by non-specific or affinity interactions, in which the protein is spatially separated. Affinity chromatography with fusion tags is an example of on-column buffer exchange and renaturation. Refolding conditions require careful optimization, as these are dependent on protein–matrix interactions.
- SEC-based denaturant dilution takes advantage of the difference in partition coefficients between the protein and the denaturant components. By limiting the diffusion of different protein forms into the refolding mixture, this approach can prevent aggregation. The material eluted from the column is divided into size fractions. The denatured protein is adsorbed, then washed to remove the denaturant and eluted in the renaturing solution using the bind and elute method.
- A refolding reactor is created by immobilizing folding catalysts on the stationary phase of a chromatographic column.

Some of the advantages of on-column refolding include suppressing unspecific intermolecular interactions, refolding a maximum protein concentration, and, most importantly, integrating purification and renaturation. However, some challenges with on-column renaturation are a possibility for precipitation, column fouling, and difficulty with cleaning. In addition to this, striking a balance between column chromatography operations and renaturation is critical to the refolding process's success.

The removal of the denaturants promotes the native protein conformation. The various components included in the refolding buffer aid correct folding and formation of a native disulfide bond. Depending on the renaturation process type (i.e. dilution, dialysis, or on-column), the time taken for complete refolding is anywhere from a few hours to days. As a result, determining the best refolding conditions can have a big impact on the total yield and efficiency of the process. Two important processes occur during renaturation: first-order refolding and higher-order aggregation. This results in competing for first- and higher-order reactions.

There is no universal refolding process and a method suitable for one protein may not apply to another protein. This is inclusive of the refolding buffer, using additives, and the rate of removal of the denaturant. This ideal protein concentration minimizes aggregation, refolding conditions such as pH, temperature, the volume of refolding buffer to the denatured protein solution, and an adequate processing time. The use of additives in the refolding process ensures native disulfide bond formation, promotes proper folding to the native state, inhibits aggregation, and maintains the protein's stability. Several additives are required to achieve these outcomes. The reaction's pH and temperature can affect protein stability, the rate of disulfide bond formation, and, most importantly, the folding of the protein. A stepwise pH reduction or even stepwise temperature change is evaluated to promote correct folding and precipitating misfolded protein and other unnecessary proteins. Disulfide bond formation is a rate-limiting step, and the rate of renaturation can significantly affect process yield. Therefore it is critical to control the reactions as any rapid changes in the environment can reduce yield as stable intermediates cannot be formed before aggregation. Thus identifying the optimum operating conditions that can influence the efficiency of the process while being suitable for commercial-scale manufacturing is essential.

To determine the progress of the refolding reaction, the percentage of unfolded and correctly refolded protein as a progression of time, representative samples at specific time intervals are taken and analyzed by RP-HPLC. This information is essential, specifically at the early stage of process development, while optimizing the conditions to maximize recovery.

Table 6.3 lists a comparative description of various methods of refolding.

6.2.2.3 Depth filtration for clarification of refolded protein

The refolded protein is generally clarified before proceeding with chromatography purification processes. Clarification by depth filtration using charged depth filters or normal-flow filtration, and sometimes even centrifugation, is used to remove protein aggregates. Given the nature of the refolded proteins and the presence of a broad range of particle sizes, sometimes multiple separation steps are required. Multiple separations are often overcome by using a multigrade depth filter and membrane filtration into a single operation. Although still in use, centrifugation may not be feasible, mainly when working with large processing volumes. The key challenge with the filtration process is minimizing product loss and obtaining a desirable quality of the filtrate to maximize the efficiency of the subsequent purification steps while also protecting the column.

Table 6.3. Advantages and disadvantages of the common protein folding methods.

Refolding technique	Advantages/disadvantages
Dialysis	Time-consuming (can take several days). Requires large buffer volumes.
Dilution	Most preferred. Slow dilution allows for control over protein aggregation; the dilution ratio is broad (ranging from tenfold to a hundred-fold). May require large buffer volumes and handling capacity depending on the dilution ratio.
On-column refolding	Quick, effective, and straightforward. No volume limitations. Unlike dilution, working with high protein concentration is possible, eliminating cumbersome equipment handling and even volume reduction steps. The method is highly dependent on the type of proteins.
SEC	Volume limitations, columns are designed to handle very small volumes. Aggregates formed on the column may be difficult to remove. Similar to on-column refolding, high protein concentrations can be used.

Cellulose depth filters are commonly used in the biopharmaceutical industry for this application. The cellulose filter matrix's thickness and depth trap the suspended particles and separate them from the carrying liquid. The inherently positively charged depth filters generate an electrostatic charge to the negatively charged particles in the feed stream, allowing even small particles to be captured in the matrix that may otherwise escape. Coupling multiple pore size filters in series creates a graded structure that can maximize the surface area and limit fouling due to particle deposition. The final filtrate is then sterile filtered before loading preparation for the subsequent chromatography step. It may be optional to add a microporous membrane downstream of the cellulose filters before the sterile filter. This can help provide an additional layer to filter out other small particles that can likely block the final sterile filter and decrease the throughput of this sterile filter. The pressure and the filtrate turbidity must be monitored during the depth filtration process.

6.2.3 Purification

After clarifying the refolded protein solution, the next set of unit operations cater to purifying the protein to generate a highly pure product that meets its purity specifications. Some proteins are challenging to purify and require physicochemical-based chromatography methods to maximize their yield. Based on the variations in the target protein's characteristics and other chemicals present in the sample, suitable chromatographic separation methods are chosen.

6.2.3.1 Capture

The best chromatographic separation methods are chosen depending on the properties of the target protein and other compounds in the sample. The capturing step aims to bind the product to the matrix (either due to charge, specific interactions, or even affinity) such that impurities do not bind to this, and removal becomes more accessible by elution. The product can be eluted using a step gradient or a linear elution method, resulting in a high product concentration but a modest purification degree. For example, to avoid interactions with proteases, high throughput and short processing times are essential. Specific product variants may impede product recovery and cause further degradation that is difficult to remove afterwards.

The chromatographic process and capture step strategy may differ depending on the target molecule. For example, non-antibodies, oligonucleotides, polysaccharides are more often captured using ion-exchange chromatography (IEX) or hydrophobic interaction chromatography (HIC). Affinity chromatography, on the other hand, is used to capture antibodies (e.g. protein-A, protein-G).

To purify refolded proteins, ion-exchange chromatography (IEX) and, to a lesser extent, hydrophobic interaction chromatography (HIC) have been used successfully as the initial chromatographic stage. These methods have been shown to increase refolded protein recovery by providing stability, reducing aggregation, and efficiently integrating purification for both process- and product-related contaminants. Linear gradient elution for separation is the name given to these methods for purifying refolded proteins. Soluble proteins, expressed in the *E. coli* expression system, are directly loaded onto the chromatography column to separate the target protein from other cellular contaminants, including host cell proteins, after being recovered by cell disruption and clarification nucleic acids and lipopolysaccharides.

Affinity chromatography, used predominantly for the mAb purification process, is also experimented with for large-scale purification of other recombinant proteins (e.g. recombinant insulin, plasminogen, GCSF, IFN-alpha, follicle stimulating hormone)

A high degree of recovery and product stability, and the ability to process high capacity, are the key demands for this capture step.

6.2.3.2 Intermediate purification

Following the capture step, high-resolution methods such as hydrophobic interaction ion-exchange chromatography (anion, cation), size-exclusion chromatography, and reversed-phase chromatography are used to eliminate most impurities such as host cell proteins, nucleic acids, and endotoxins. It is not commonplace to use an AEX as a capture stage followed by a CEX chromatography method as an intermediate purification step, or vice versa. These choices are highly dependent on the target proteins and the impurities profile.

Lower flow rates, gradient elution, and matrices containing smaller particles are employed to improve resolution. Following these stages the commodity's purity is usually 99%.

For the chromatography process, at each purification step some amount of the product is undoubtedly lost. Therefore, there is always a need to reduce the number of purification steps in any biomanufacturing process. Another advantage of reducing the steps is the faster processing time, which helps improve the yield of unstable proteins. Upon conclusion of the capture step, the product quality, the purity and impurity profile of the desired protein, and the intermediate and final purification step's objective are assessed to determine the need for an intermediate purification step. Some processes may eliminate an intermediate purification step favoring a polishing step and reduce overall processing time for economically viable biomanufacturing operations.

6.2.3.2.1 Polishing

The polishing phase in the purification process removes traces of aggregates (low and high molecular weight proteins), degradation products, or other product variants; prior purification processes have greatly decreased product-related contaminants as a significant process. The purified product is also prepared for formulation or storage as an intermediate product in this final phase. Size-exclusion chromatography and reversed-phase chromatography are two polishing procedures for achieving ultimate product purity.

Often desalting and or buffer exchange are required to change the purified protein composition, which is easily accomplished by size-exclusion chromatography. In addition to enabling the removal of low and high molecular weight contaminants, the protein is also transferred into the desired buffer. This allows for making final adjustments to the conditions of the purified protein for either final storage.

Purification steps may or may not be in tandem, and sometimes various intermediate processing steps may be included in between these chromatography steps. This may be product concentration by TFF, salt precipitation, or even desalting. When the buffer components are modified, desalting is commonly done. This could be done before IEX to remove salt and let the protein sample bind to the column, or after purification to eliminate low molecular weight contaminants, or even after purification for the final pure protein.

A general scheme for the downstream process for soluble protein is shown in figure 6.6.

Three-step and two-step purification schemes for a protein expressed as IBs are shown in figure 6.7.

Upon completing the purification process, the purified protein is mostly concentrated by tangential flow filtration (UF/DF) to prepare the drug substance (lyophilized or sterile solution) before storage until further processing into a drug product.

6.3 Mammalian system purification

Protein production in mammalian cells is typically by secretion of the product into the culture medium. Therefore product recovery starts with separating the cells from the cell culture medium through depth filtration, centrifugation, or alternative

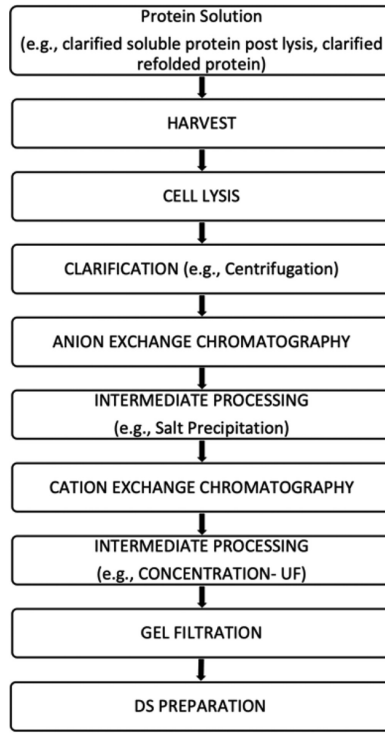

Figure 6.6. Scheme for purification of a soluble protein expressed in *E. coli* (three-step purification strategy).

separation techniques (chapter 5). Since the protein is secreted into the medium, unlike *E. coli*, the separation process is not extensive.

The clarified harvest is subjected to purification and product concentration steps to yield the desired product, meeting the target purity and safety. Understanding the properties of the protein and its contaminants is key to the development of the purification strategy. In addition, the proteins produced in a mammalian system may carry a potential risk of viral contamination. Therefore, steps that target viral inactivation and removal must also be incorporated into the overall scheme of downstream operations.

A general outline for a mAb purification process is detailed in figure 6.8. The mAbs are a unique protein modality in that they all share a common feature via the Fc region. This non-antigen binding site has enabled the development of a platform process. mAbs are bifunctional with a common symmetrical structure—a pair of identical heavy and light chains linked via disulfide bridges.

A three-phase purification strategy may be employed to purify the clarified harvest (CHO process) as the purification of the refolded protein.

- *Capture*: Isolate and concentrate the product.
- *Intermediate purification*: Remove most of the bulk impurities (e.g. host cell proteins, nucleic acids, DNA, endotoxin, and viruses).
- *Polishing*: To achieve high purity by removing any trace impurities and other product-related substances/impurities.

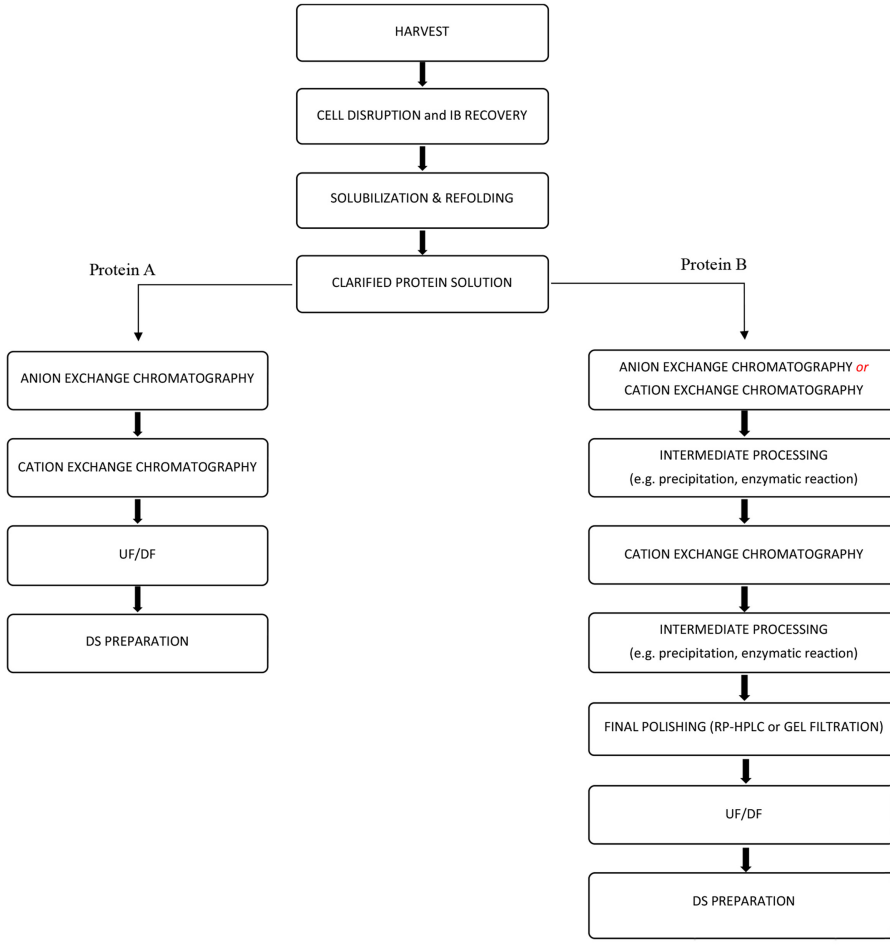

Figure 6.7. Multi-step purification schemes for expressed proteins.

Various chromatography methods that meet the overall purpose and satisfy the criteria to fit into the overall purification strategy are selected and optimized to achieve the desired outcome. However, the number of steps used is not universal. It depends on the proteins' properties, the target purity criteria required, the yield, the identity of the impurities, and the protein's intended use.

Starting with protein-A chromatography and a series of ion-exchange chromatography steps, most of these operations are carried out in a bind and elute mode, except anion exchange chromatography, which uses a flow-through mode for the desired protein.

6.3.1 Capture—protein-A chromatography

A capture step (e.g. IEX, affinity chromatography) targeted to isolate the concentrate should be capable of removal of critical contaminants as well. Generally, this

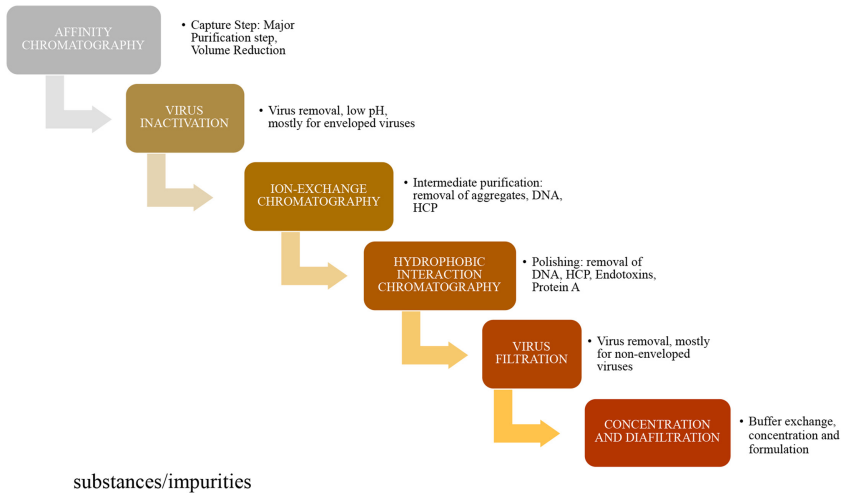

Figure 6.8. Overview of a mAb purification process.

step results in a significant increase in product concentration and purity, further improvised if a highly selective affinity medium is used. Other methods of capture that can also be used include crystallography and precipitation. However, these techniques require additional processing steps (e.g. solid–liquid separation) and are still primitive in success at the industrial scale.

The primary capture for mAb from the clarified harvest is initiated with affinity chromatography. For mAbs, protein-A chromatography has been employed extensively. The consistency of the Fc-portion across mAbs, the targeted binding region for protein-A, has led to protein-A chromatography becoming the ubiquitous method of choice in the downstream purification strategy for capture.

Protein-A chromatography is a type of affinity chromatography in which antibodies bind to an immobilized protein in a specific and reversible way, to form a ligand. Protein-A is a 56 kDa surface protein found in *Staphylococcus aureus*. It has five binding domains that can bind to immunoglobin G's Fc region (IgG). Both native and recombinant forms of protein-A are used as ligands in protein-A resins. The protein-A ligand is coupled to a matrix/medium, typically cross-linked agarose (e.g. Sepharose®), other polyacrylamides, and polymers. Protein-A's high capacity makes it suitable even for large-scale applications—a single coupled protein-A molecule can bind at least two antibody molecules. The recombinant protein-A is further engineered to have a higher binding capacity than its native counterpart. These modifications to the ligand (e.g. single domain multimers/single point coupling, multi-point attachment) for additional benefits, including chemical and thermal stability, have rendered protein an extraordinarily robust and high capacity. Protein-A is also resistant to specific denaturing and chaotropic agents (urea, Gu-HCl) and can withstand a wide pH range of 2–11, making it tolerant to too harsh conditions. Figure 6.9 shows the structure of protein-A and how it binds to proteins.

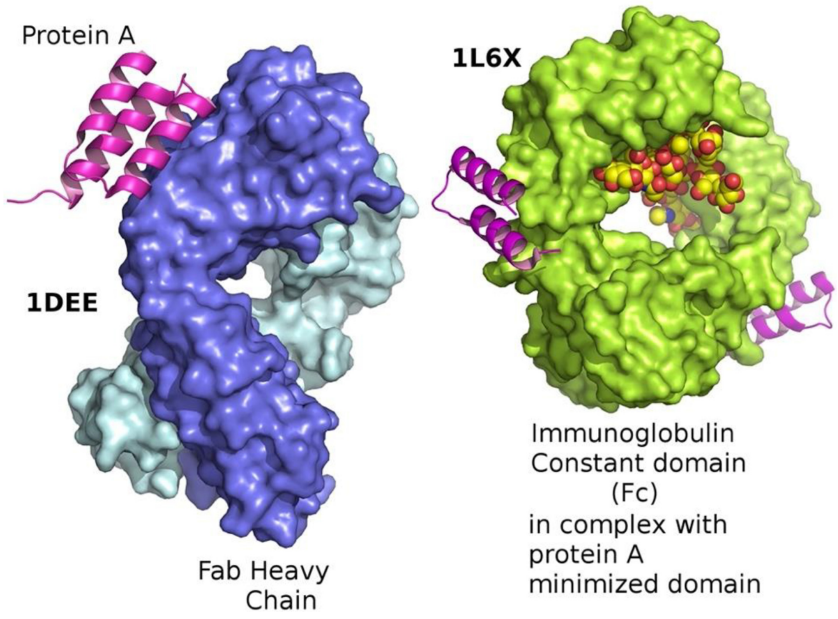

Figure 6.9. Protein-A and it is binding to an immunoglobulin. (Source: E A S, Creative Commons License.)

There are three major varieties of protein-A resins available commercially, based on matrix composition:

- *Agarose based*: e.g. Protein-A-Sepharose® Fast Flow, MabSelect (Cytiva).
- *Glass or silica based*: e.g. Prosep vA and Prosep vA Ultra (Millipore).
- *Organic polymer based*: e.g. polystyrene-divinylbenzene Poros A and MabCapture (Applied Biosystems).

These resins are available for both laboratory scale and commercial-scale processes (< 1 cm to 2 m column diameter) and are resistant to high concentrations of Gu-HCl, urea, reducing agents, and low pH.

Protein-A can also exhibit affinity for specific variants of the Fab region, beneficial for the purification of Fab fragments. Although protein-A is the most widely used purification system for human and humanized mAbs, specific antibodies may not bind or weakly bind to the protein-A ligand and this selectivity can also be used advantageously.

Another alternative to protein-A is protein-G—an immunoglobulin binding protein in group G of the streptococcal bacteria. It varies from protein-A in its binding specificities. Based on the IgG that is being purified, either of these is used to their advantage to aid in the capture step. Some of the key features of these two ligands are as follows:

- Protein-A has a broad species reactivity and binds well to IgG from various species, including humans, rabbits, mice, and cows.

- Protein-G has a stronger binding to IgG from mice or rats. The native protein-G binds to albumin. This binding site has been removed in the recombinant form.
- Protein-A may be a better alternative for separating certain IgG subtypes and other classes of antibodies, as well as eliminating other antibodies, such as cross-species IgG contamination from horse or fetal calf serum. When combined with agarose beads, some of these binding affinities change, for example, rat IgG does not bind to protein-A but does bind to Protein-A-Sepharose (table 6.4).

Affinity gravity flow or a low/medium pressure system are commonly used for chromatography. The binding capacity is determined by the binding strength as well as the flow rate. The ligand leakage is one factor to consider. Protein-A point attachment to Sepharose has been shown to have very little ligand leakage under a variety of elution settings. These ligand contaminants are removed during the polishing using SEC. As an initial capture step, affinity chromatography offers several performance attributes that can streamline the process development for new molecules, such as robustness to solution conditions for loading and washing.

In large-scale mAb manufacturing, protein-A-Sepharose (Cytiva) is the most often employed capture step. The binding ability of this medium is significantly higher than that of protein-G Sepharose. Furthermore, Cytiva's high-performance

Table 6.4. Antibody subtypes and recommended purification media. (Source: adapted from abcam.)

Species	Antibody subtype	Protein-A binding	Protein-G binding
Human	IgG	Strong	Strong
	IgG_1	Strong	Strong
	IgG_2	Strong	Strong
	IgG_3	Weak	Strong
	IgG_4	Strong	Strong
	IgA_1	Weak	None
	IgA_2 Fab	Weak	None
	scFv	Weak	Weak
		Weak	None
Mouse	IgG	Strong	Strong
	IgG_1	Weak	Medium
	IgG_{2a}	Strong	Strong
	IgG_{2b}	Strong	Strong
	IgG_3	Strong	Strong
Rat	IgG	Weak	Medium
	IgG_1	Weak	Medium
	IgG_{2a}	None	Strong
	IgG_{2b}	None	Weak
	IgG_{2c}	Strong	Strong

Table 6.5. Purification options for mAbs and Fc fusion proteins using commercial protein-A chromatography media.

Protein-A chromatography media	Features
MabSelect PrismA	Optimal productivity, cleaning, sanitization, bioburden control, alkaline stability, continuous and batch processes, long resin life, easy cleaning, suitable for small-scale purification and screening, prepacked large-scale columns suitable for commercial manufacturing.
MabSelect SuRe LXMabSelect SuRe	Higher ligand density, good performance, long resin life, easy cleaning, prepacked large-scale columns suitable for commercial manufacturing.
MabSelect SuRe pcc	Continuous processes, long resin life, easy cleaning, prepacked large-scale columns suitable for commercial manufacturing, suitable for small-scale purification and screening.
MabSelect	Low to medium titers, a limited number of cycles, suitable for small-scale purification and screening, prepacked large-scale columns suitable for commercial manufacturing.
MabSelect Xtra	High titers, higher ligand density, a limited number of cycles, suitable for small-scale purification and screening, prepacked large-scale columns suitable for commercial manufacturing.
TOYOPEARL AF-rProtein-A HC-650M	IgG binding capacity of >65 g IgG l^{-1} resin.
Prosep Ultra Plus	Large-scale, cost-effective, suitable for high titer, increased capacity and productivity, process flexibility, prepacked, ready-to-use and disposable columns, high throughput, not suitable for cleaning with sodium hydroxide.
Poros MabCaptureA	Highest dynamic binding capacity, process flexibility (shorter bed height, fast flow rate).

medium gives a high resolution as well as crisp and concentrated peak elution. Table 6.5 shows an example of commercially available protein-A chromatography medium. Each resin should be evaluated for protein-A leachability.

Furthermore, the degree to which protein aggregates form should be regarded as a selection factor. Protein-A resins have a dynamic binding capacity of 15–100 g mAb $^{l-1}$ resin, depending on the kind of mAb, adsorbent, and flow velocity (table 6.5).

The typical binding and elution conditions suggested for this medium are summarized in table 6.6. For the enhanced performance of protein-A chromatography, it is critical to optimize some of the process variables such as binding conditions, including the buffer composition and pH to achieve efficient binding, washing conditions, including the optimal composition of the column wash solution

Table 6.6. Binding and elution conditions commonly used with Protein-A Sepharose® chromatography purify IgG from different species. (Source: adapted from *Affinity Chromatography Vol 1: Antibodies*, Cytiva LifeSciences (formerly GE Healthcare).)

Species	Subclass	Protein-A binding pH	Protein-A elution pH
Human	IgG_1	6.0–7.0	3.5–4.5
	IgG_2	6.0–7.0	3.5–4.5
	IgG_3	8.0–9.0	⩽7.0
	IgG_4	7.0–8.0	3.0–6.0
Mouse	IgG_1	8.0–9.0	4.5–6.0
	IgG_{2a}	7.0–8.0	3.5–5.5
	IgG_{2b}	~7.0	3.0–4.0
	IgG_3	~7.0	3.5–5.5
Rat	IgG_1	⩾9.0	7.0–8.0
	IgG_{2a}	⩾9.0	⩽8.0
	IgG_{2b}	⩾9.0	⩽8.0
	IgG_3	8.0–9.0	3.0–4.0 (using 3 M potassium isothiocyanate)

or an excessive number of washing steps that can likely weaken the ligand binding and decrease yield, and most importantly the elution conditions. Any variations in these parameters can cause differences in protein-A's affinity, purity, and impurity levels (table 6.6).

The protein-A chromatography stage is a popular alternative for capturing recombinant mAbs due to its resilience. Depending on the target protein's intended function, such as for diagnostic purposes, purification simply using protein-A chromatography may be sufficient. During this stage process-related contaminants such as host DNA, HCP, and virus are significantly removed.

Despite the universal use of protein-A chromatography, there are certain limitations to this method. The high cost of the resin and cleaning procedures for repeated use that can potentially be a cause for cross-contamination, and even affect the resin's performance due to caustic cleaning (0.1 N NaOH) solutions and leaching. The non-specific binding of impurities such as host cell proteins, DNA, and other cell culture-derived impurities is referred to as chromatography. Ion exchange and other alternatives are being investigated to overcome these limitations with protein-A chromatography. Expanded-bed chromatography is also an advantageous method for binding protein from crude cell culture media.

6.3.2 Intermediate purification and final polishing

The intermediate purification step separates the target protein from contaminants, such as other proteins, viruses, endotoxins, and DNA. The resolution and the specifications of this step are highly dependent on the protein-A eluate sample, its properties, and the final product specifications that need to be met. If the protein

purity attained from the capture step is high, the intermediate step may not be required, and the process moved to one or more polishing steps to increase the purity of the final product. The resolution for separation of the sample's protein components, expected from the final polishing, is high. The binding capacity, resolution, and selectivity for the intermediate purification step are important since a significant number of impurities may still be present if the capture step product had lower purity. A continuous gradient or a multi-step elution may be used to elute the target protein.

6.3.3 Polishing

The binding of impurities such as host cell proteins, DNA, and other cell culture-derived impurities in chromatography is non-specific. For example, if the target protein has a neutral to basic pI, then flow-through mode will be suitable to remove the contaminants. Unlike the target protein, the conditions chosen would allow for acidic impurities to bind to the column, not bind. The bind and elute modes are preferred for a target protein with pI in acidic to below the neutral range. The target protein is eluted first under a high salt concentration condition, leaving most impurities bound to the column.

In CEX or AEX, the resin, loading conditions, the impurity profile, the amount of product loaded, the charge variant profile, the chromatography conditions would need to be optimized to maximize efficiency and recovery. The most common variables for this step are the column loading, wash, and elution buffer compositions.

Based on the impurity profile and the target protein, hydrophobic interaction, mixed-mode chromatography, and occasionally ceramic hydroxyapatite are used in addition to ion-exchange chromatography.

Hydrophobic interaction chromatography can be employed as a polishing step after IEX or as an intermediary step after protein-A. In flow-through mode, HIC can efficiently remove a large number of aggregates, resulting in a high yield. It can give high resolution and separation of process-related and product-related contaminants in the bind and elute mode.

In multimodal chromatography, the resin used combines different interaction types to separate the target protein and impurities. These interactions could be ion exchange, hydrophobic, and even hydrogen bonding. The selectivity that these resins offer may be different from the standard single-mode chromatography resins, allowing them to be suitable for various conditions, for example, high and low conductivity or pH. Examples of commercially available multimode resins are Capto MMC and Capto Adhere (Cytiva). The multimode chromatography method can effectively remove aggregates, host cell protein, and leach protein-A.

With its unique separation capabilities, unprecedented selectivity, and resolution, ceramic hydroxyapatite (CHT) is utilized to efficiently remove nucleic acids, viruses, macromolecules, and other proteins. CHT has been effectively employed as a polishing step in large-scale mAb purification to remove dimers, aggregates, and leached protein-A using a gradient elution method.

Polishing steps ensure the removal of adventitious and endogenous viruses. Overall, the purification strategy should streamline the process development effort, ensuring the product quality attributes are achieved, reducing the cost to make the process economically viable, and minimizing any downstream processing complexities.

6.3.4 Viral removal, inactivation, and filtration

The final product must be free from any contamination, including bacteria, mycoplasma, and virus. Mammalian cells are known to express endogenous virus-like particles, posing a significant safety risk. mAbs, derived from mammalian cell systems, must meet the viral safety specifications due to potential contamination from enveloped and non-enveloped viruses. Characterizing the host cell line, analyzing all raw material sources for adventitious agents, particularly animal-sourced agents, and eventually testing goods at various stages of the production process as well as testing the finished product are all part of this process. A viral clearance must show that the manufacturing process can handle and effectively remove viruses.

As per the current regulations, from a safety perspective therapeutic products derived from mammalian cells can contain less than one virus particle per million doses; this is equivalent to approximately 12–18$\log_{10}$ clearance for endogenous retroviruses and 6$\log_{10}$ clearance for adventitious viruses. Thus virus removal strategies are designed into the overall production process at the purification steps. In addition, orthogonal virus clearance steps are used in mAb processes so that the viruses may be cleared by at least one mechanism if they are not cleared by the other.

The viral removal or inactivation of the virus is achieved by subjecting the solution containing the target protein to conditions that denature the virus proteins but do not degrade the active ingredient. The most used methods are low pH inactivation, detergents, viral filters, heat treatment, irradiation, and chromatography.

6.3.4.1 pH treatment

The treatment of protein-A eluate under low pH conditions is effective against enveloped viruses. Typically, incubating the protein solution at a low pH (around 3.0–3.5) for at least an hour has been commonly used in mAb processes. Even high pH treatments, particularly sodium hydroxide for cleaning chromatography columns, have been effective against enveloped and non-enveloped viruses. Extreme pH exposures can significantly reduce the virus (approximately > 4.0$\log_{10}$).

6.3.4.2 Virus filtration

Virus filtration employs a size-based approach for viral clearance. This method is used effectively as a complementary method to pH treatment in mAb processes. Virus filters are currently available in the form of ultrafilters or microfilters, which have very small pores. Polyethersulfone (PES), polyvinylidene (PVDF), and cellulose make up most virus filters. These filters are categorized based on their size distribution as retrovirus and parvovirus filters. Some of the commercially available viral filters are summarized in table 6.7. The most prevalent and common

Table 6.7. Commercially available virus filter.

Company	Product	Material	Virus	Log reduction value (LRV) claimed by the manufacturer	Virus size (nm)
Asahi-Kasei	Planova 15N	Cuprammonium regenerated cellulose	Hepatitis A virus (HAV)	>6.7	27–32
			Parvovirus B19	>6.1	18–26
			Plum pox virus (PPV)	>4.6	18–24
	Planova 20N	Cuprammonium regenerated cellulose	Parvovirus B19	>4.9	18–26
			PPV	>4.0	18–24
			Xenotropic murine leukemia virus (XMuLV)	>3.1	80–110
	Planova 35N	Cuprammonium regenerated cellulose	HIV	>7.3	80–120
			PPV	< 1.0	18–24
			SV40	>7.8	40–50
	Planova BioEX	Hydrophilized PVDF	Mouse minute virus (MVM)	>4.8	18–24
			Amphotropic murine leukemia virus (A-MuLV)	>5.2	80–130
			PPV	>5.3	18–24
Millipore	Viresolve NFR with retropore membrane	PES	Retrovirus	>6	80–130
	Viresolve Pro (Viresolve Pro device, Viresolve Pro Shield prefilters)	PES	MVM	⩾4.0 log	18–24
			Parvovirus	⩾4.0	18–26
			XMuLV	⩾5.0	80–110
Pall	Pegasus Prime	PES	Parvovirus	>4	18–26
Sartorius	Virosart® CPV	PES	PPV, MVM	>4	18–24
			MuLV	>6 log	80–130
	Virosart® HF	PES	Small non-enveloped virus (e.g. MVM, vesivirus, parvoviruses)	>4	18–30
			Large enveloped viruses such as MuLV	>6	80–130

viruses are summarized in this table. It is not all-inclusive, and other viruses may also be present as potential contaminants.

Filter fouling is a regular occurrence, and it is caused by aggregates, debris, and DNA. Fouling can have a major impact on the virus filter's performance. Prefilters can help you avoid this (for example, negatively charged Pall Mustang S used as prefilter before a Viresolve Pro virus filter removes high molecular weight proteins). The viral filter's membrane permeability is controlled within a specific range, allowing for the overall filtration process. However, the throughput of the viral filters can vary from lot to lot. Additionally, the variability in the protein solution's viral filter burden can also add to the throughput variations. Therefore, when evaluating viral filters for large-scale manufacturing, it is imperative to test the filters' performance using a worst-case protein feed sample.

The integrity of the virus filters is checked both before and after use. These filter integrity tests are conducted to ensure that the filter is free of defects and damages, to confirm the filter's performance, to ensure that it meets the manufacturer's specifications, to ensure that the filter is correctly installed for use in the process, and, most importantly, to conduct end-user virus retention studies. The bubble point, forward flow, water intrusion, and binary gas tests are only a few of the most used non-destructive tests. The filtration process is repeated if the post-use filter integrity test fails. The suppliers of filters typically work with the drug manufacturers to facilitate these requirements.

6.3.4.3 Other methods

Chromatography. Some of the chromatography methods used to purify mAbs are also efficient in separating enveloped and non-enveloped viruses from the target protein, for example affinity chromatography. This option only acts as a complementary method to other effective treatments discussed above, and the effectiveness is also dependent on the purification conditions and the virus properties. Evaluations are made to assess the capability of the chromatography operation in providing a consistent clearance.

Detergent. This method is used predominantly for the manufacturing of blood products. Sodium cholate and Tri (n-butyl) phosphate (TNBP) are effectively used to treat plasma-derived products to inactivate enveloped viruses. The detergent treatment inactivates the virus by solubilizing its lipid membrane and preventing it from binding to or infecting cells. However, detergents cannot be used against non-enveloped viruses.

Heat treatment. This method has been used for virus removal in human plasma-derived and animal-derived products. This method uses wet and dry heat treatment in which a viral protein structure is altered, inactivating the virus. Heat treatment is effective against the enveloped and non-enveloped virus. However, the non-enveloped virus needs an extremely high temperature for effective inactivation. This may be a cause of concern on the target protein, possibly denaturing or impacting its functionality.

Irradiation and UV. Gamma irradiation is used for animal-derived raw materials. Viruses such as reovirus and CVV have been rendered non-functional

with this method. Tight control of operational parameters is required to ensure consistency, effectiveness, and reproducibility. A low dose of UV-C radiation (254 nm) can destroy the viral nucleic acid for ultraviolet-based treatment. This wavelength has no impact on the target protein. Some resistant strains of parvovirus have shown to be more prone to inactivation using UV-C.

6.3.4.4 Virus removal validation

Irrespective of the virus removal method chosen, the focus must be on the robustness, reliability, and effectiveness of consistently removing the virus. This must be a risk-based approach and may necessitate more than one inactivation or removal method in the therapeutic protein's manufacturing process to effectively remove both enveloped and non-enveloped viruses. Virus validation studies that use model viruses representing actual process conditions are routinely performed and required to meet regulatory expectations.

The FDA Guidance 'Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin' (1997) on effective virus removal states the following:

> Confidence that the infectious virus is absent from the final product will result in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The virus protection evaluation procedure includes selecting virus-free cell lines, inspecting unprocessed bulk, evaluating the downstream process' capacity to eradicate or clear viruses, and ultimately testing the product to confirm the absence of contaminating viruses.

The validation of virus removal throughout downstream processing is aided by an examination of the cell substrates, raw materials employed, virus inactivation/removal, and the final product test. Retrovirus or adventitious viral contamination raises concerns about product protection. Even if there is no virus infectivity or reverse transcriptase activity in the master cell bank (MCB) or working cell bank (WCB), electron microscopy can detect virus-like particles (VLPs). Microscopic examination does not determine the biological relevance of suspicious particles, particularly their infectivity, such as the presence of vast numbers of A-type particles in hybridoma cells, where infectivity is extremely low or non-existent. Despite the discrepancy between the number of virus-like particles and their infectivity, the total reduction factor is commonly used to compute the particle number.

It is hard to rule out the possibility of an unknown virus with unknown and potentially hazardous physiological implications. The occurrence of the viral contamination complicates the development of a specific assay. Furthermore, without an accurate and responsive assay, monitoring the presence of the virus and its removal or inactivation in the protein drug's downstream phase is difficult. Preventive measures include extensive testing of the producer cells for specific viruses and testing for the adventitious virus at various stages during fermentation.

Thus there is a far greater emphasis on validating the viral clearance steps, and this is facilitated by a virus challenge or spiking study in which the viruses are added to the product at known titers and then monitored through the entirety of the process at each step (using an infectivity assay).

The viral clearance/titer decrease (given as $\log_{10}$) is calculated at most, if not all, of the stages. The use of identical viruses from the same genus or family as the virus(es) or non-specific viruses that are closely related to the known or suspected virus is required for the study of viral clearance or inactivation. The procedure determines the overall level of viral reduction achieved during the operation (of viruses known to be present). Assume that the deliberate insertion of the virus to the unit operation application sample exhibited sufficient clearance in the selected steps. Any unit operation of the downstream process should not be tested in this circumstance. Due to the difficulty of conducting viral clearance research, the focus is on a few but efficient unit procedures for virus elimination. The fact that one-log reduction factors have no effect on the overall clearance factor (typically 2–3 in a downstream process). Although determining excess clearance (clearance minus risk) can be difficult, it should be a critical component of any endeavor to limit the risk of viral infection.

Experiments with virus spiking should not be performed in a cGMP facility. This is an exception in large-scale production. To avoid any possible virus burden at the polishing stage, virus validation experiments should be undertaken in small-scale trials (with scalable equipment), particularly around the capture and intermediate phases. Steps that are likely to clear the virus should be considered individually when there is enough virus to make an informed conclusion. Cleaning in place and output of frequently used chromatographic columns and filter systems should be confirmed. Fractional factorial designs are the ideal for investigations since downscale factors of 100–1000 can be obtained.

Virus clearance tests are usually carried out twice during the process and product lifecycle. The first is linked to the production of clinical phase I material that demonstrates the eradication of at least two separate viruses. The second image depicts the eradication of four different viruses during the manufacturing of phase III material. The xenotropic murine leukemia virus (XMuLV), minute virus of mice (MVM), simian virus 40 (SV40), and pseudorabies virus (PRV) are the most often used four virus models because they span a wide spectrum of traits that are reflective of other potential adventitious agents.

Validation model viruses must be comparable or identical to viruses suspected in the cell line or closely linked to viruses that could infect the cell, such as retroviruses for recombinant or hybridoma cells. To attain the ideal reduction factor for viruses, the model virus should be produced to high titers and detectable in a responsive but easy test. When concentrating a virus solution to improve the volumetric titer, use caution—viral particle aggregation can result in increased but negligible mechanical removal via filtration or a decrease in inactivation due to viral particle defense at the aggregate's center. Model viruses used as an unknown source of infection include SV40, human poliovirus 1, animal parvovirus, a parainfluenza virus or influenza virus, Sindbis virus, RNA viruses, and murine retroviruses.

When using mammalian cell culture or biologic materials, virus clearance is achieved through downstream processing; the efficacy of these processes is validated in the same way that sterility testing for bacterial contamination is done, so a statistical sample of the lot can be checked for compliance with sterility testing protocols. To examine the effect of viral clearance, spiking experiments for the appropriate unit processes are used. To ensure that virus distribution is balanced for the individual intermediates of such an operation, the virus titer of the load is calculated and compared to the (residual) virus titer of the product containing fraction after processing, such as the flow-through or eluate of a chromatographic process or the permeate of a filtration process.

In most cases virus inactivation is a two-step procedure (fast phase 1 and slow phase 2). Samples taken at several time intervals, with at least one time point less than the minimum exposure time, are used to create an inactivation curve. Quantitative infectivity assays should be sensitive and reproducible, and they should be done with enough repetitions to ensure statistical validity. Assays for detecting viral contamination produce extremely variable findings due to the biological nature of the test techniques, needing extensive validation of assay accuracy, reproducibility, repeatability, linearity, limit of quantitation, and detection limit. The necessity of objective statistical assessment has been emphasized by the FDA's 'Points to Consider', the EMEA's 'Notes for Guidance', the ICH Guidelines, and other sources. In quantitative virus clearance research, plaque formation and cytopathic assays are the two most common *in vitro* assay methodologies. Both assays have been validated and are used routinely to determine viral titers. Q5A, the fourth phase of the ICH Harmonised Tripartite Guideline, delves deeper into tests.

Until the process samples are titrated, the influence of the buffer solutions must be investigated for interactions with the detector cells or a decrease in the model virus's infectivity. Detector cells for virus titration include SC-1 cells (retrovirus), CV-1 cells (SV40), L 929 cells (reovirus), and Vero cells (PI3). The XC plaque assay can identify retroviruses over a longer length of time. After inoculating SC-1 cells with the appropriate sample, the cell layer is UV-irradiated before being overlaid with XC cells after a certain cultivation period. After the plaques have formed on the cell layer, they are counted. Based on the physical structure of a retrovirus-infected cell monolayer, the titer is quantified in plaque or concentrates forming units (pfu/FFU). On the detector cells, the cytopathic effect (CPE) of viruses that do not cause plaques or foci is detected. The titer is expressed as the tissue culture infectious dose for 50% of the total cell number (TCID50). The balance of virus distribution across the entire process cycle, including corresponding fractions such as washing and regeneration phases of chromatography or the retentate of filtration, is unlikely for most processes due to denaturation of the virus by caustic solutions commonly used for regeneration or capture of virus particles within the membrane matrix of a filter. For the approved viruses, the maximum achievable titer is in the range of 10^7–10^9 ml^{-1}, which is further decreased by 1 log by the needed spike of 1:10–20 for a single validation experiment. Another disadvantage is the technical difficulty of titrating all the process fluids because the detection cell determines the volume of a sample. A sample volume of 0.1–1.0 ml is commonly used for titration.

The unit activity reduction factor is calculated using the volume of the process fluids and the viral titers determined for the load and the product containing fraction after processing. The reduction factor is commonly stated in $\log_{10}$ units, with the 'individual reduction factor' R_i for each unit operation. During the purifying process, specific reduction factors combine to generate the overall reduction factor for a virus. On the other hand, the accumulation of virus clearance can only be claimed for process steps that represent diverse physicochemical measures. Based on cell assay variability measured on a logarithmic scale, a logarithmic reduction factor in the order of 1, i.e. a 90% reduction in titer, is regarded insignificant for virus clearance. To provide a numerical value for the virus burden of a cell culture fluid now of harvest, electron microscopy is employed to quantify viral particles in a specified volume. This approach, on the other hand, raises new problems, such as how representative a few millimeters of a sample may be at cell culture fermentation sizes ranging from a few hundred to several thousand liters.

Furthermore, the cells in culture are grown to densities of 10^6–10^7 cells per milliliter, reducing the number of cells analyzed by EM to around 10^3 ml^{-1} by several logs. It takes a lot of practice to identify virus particles and distinguish them from particulate matter created during the preparation process, and establishing the viral identity is difficult. Objects emerging from sample preparation, such as high-speed centrifugation of cell culture supernatant, are one example; centrifuged pellets often contain complex aggregates that obfuscate information needed to distinguish virus shapes.

A risk assessment technique for validating a purification process is evaluated on a case-by-case basis, taking into account the cell substrate, the nature of any viruses identified, culture methods, the target protein, the purification process design, the product's intended use, patient population, and dosage and frequency of administration, among other factors.

Outsourcing viral clearance validation investigations to specialist laboratories has become commonplace. However, a certain viral set up in a medication manufacturing facility may not be possible in terms of time, cost, or other resources.

6.4 Product concentration

Tangential flow filtration (TFF) unit operations are common to products expressed in *E. coli* and CHO systems and are used to clarify, concentrate, and purify proteins. The fluid is injected tangentially along the membrane's surface in a TFF system. Some of the feed components are forced across the membrane and onto the filtrate side by the pressure supplied to the system. Particles and other molecules that are too large to pass through the membrane pores are kept and swept up by the crossflow that sweeps across the membrane continually, keeping the membrane from becoming clogged and enhancing throughput (figure 6.10).

TFF is further subdivided based on the size of the molecules isolated in the feed. The most common form of TFF is ultrafiltration (UF). Proteins are separated from the buffer components in this process (e.g. buffer exchange, desalting, and concentration). The membrane pore sizes range from 1–20 nm that can easily separate

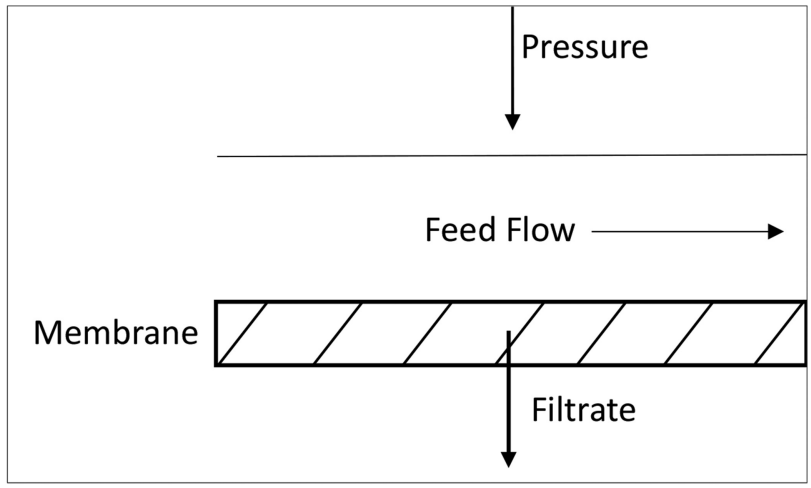

Figure 6.10. Tangential flow filtration.

Table 6.8. Commercial membranes and properties.

Commercially available membranes	Properties
Ultracel®	Regenerated cellulose
Ultracel PL	Regenerated cellulose, NMWL: 1–300 kD
Ultracel PLC	Composite regenerated cellulose, NMWL: 5–1000 kD
Biomax®	Polyethersulfone-based, NMWL: – 5 to 1000 kD

proteins in the range of 1–1000 kD (nominal molecular weight limit (NMWL)). Larger molecules (protein products) are held in ultrafiltration, a pressure-driven process, enabling smaller molecules to pass freely. The separation is primarily caused by changes in the filtration rates of the various components through the membrane as a function of applied pressure.

Polymers such as polysulfone, polyethersulfone, polyvinylidene fluoride, and regenerated cellulose are often used in ultrafiltration membranes. A microporous structure facilitates and controls selectivity, molecule retention on the membrane surface, and filtration flux (e.g. polyethylene). The membranes are reused effectively without much deterioration in their performance or with the risk of contamination. This is mostly due to their resistance to strong cleaning agents, acids, and high temperature tolerance. Due to their superiority regarding permeability and retention, cellulose membranes are most widely used for protein applications. High retention is critical to the process's success when choosing the right membrane, its rating in terms of the nominal molecular weight limit (NMWL), chemical compatibility, and fouling limit (table 6.8).

Virus filtration and high-performance tangential flow filtration are two types of UF systems commonly utilized in the manufacturing process (HPTFF).

The separation is accomplished via HPTFF using variations in size and charges. The disparities between the product and impurities can be further increased by changing the buffer pH and ionic strength to maximize efficiency. In specific mAb purification techniques, HPTFF effectively eliminates host cell proteins (HCP) and host cell DNA contaminants.

Diafiltration (DF) is a TFF technique that converts a protein buffer into a buffer that can be kept, stabilized, and given. It is used in conjunction with ultrafiltration or other separation processes to boost product yield or purity. During diafiltration operations, the buffer is inserted into the recycle tank while the filtrate is removed from the device service. In most procedures, the target protein product is present in the retentate, therefore diafiltration washes components out of the retentate into the filtrate, combining buffer exchange and undesired impurity removal/reduction into one technique. If there is liquid in the filtrate, diafiltration washes it through the membrane and into a storage jar. Diafiltration can be done in two ways: batch and continuous volume. In batch mode, a large amount of buffer is applied to the recycling tank. After the retentate has been concentrated, a new volume of buffer is supplied to the tank for further concentration until the retentate volume has reached a particular level. This process is repeated until the desired number of diavolumes is obtained. There is no level control in batch mode. In the constant volume mode, a continuous operation, the retentate volume is maintained by introducing a buffer to the recycling tank at the same rate as the filtrate is removed. This approach is deemed more effective than batch mode because the product concentration at which this procedure is conducted cannot be regulated in batch mode. The DF step's main purpose is to eliminate any undesirable buffer (or contaminant species) components from the retentate product. As a result, the yield is affected by the quantity of diavolumes.

The UF/DF formulation step is an essential and widely used step that processes the highest product concentration, mostly in its final formulation, the core to downstream processing. UF/DF operations continue to evolve with an emphasis on robustness, high yield, and cost-effectiveness. UF/DF is also standard for other buffer exchange and product concentration processes before or between chromatography steps.

A retentate tank houses the target protein product, a membrane assembly retains the feed, and a feed pump drives the feed through the membrane with tangential flow and pressure. The feed and retentate are recirculated through the filter assembly in the traditional batch mode operation for multiple passes until the desired protein concentration is achieved. In addition to these components, a process monitoring tool, equipment for testing product recovery, cleaning, etc, are also used to ensure consistency in operations and achieve the desired yields. TFF is made with both traditional stainless steel TFF systems and more recently developed single-use methods. Hollow fibers or cassettes are used to construct these. An automated single-use system with disposable flow routes and lower volume hold-up offers flexibility, reduces cross-contamination hazards, and allows for a quicker turnaround due to less cleaning tasks (figure 6.11).

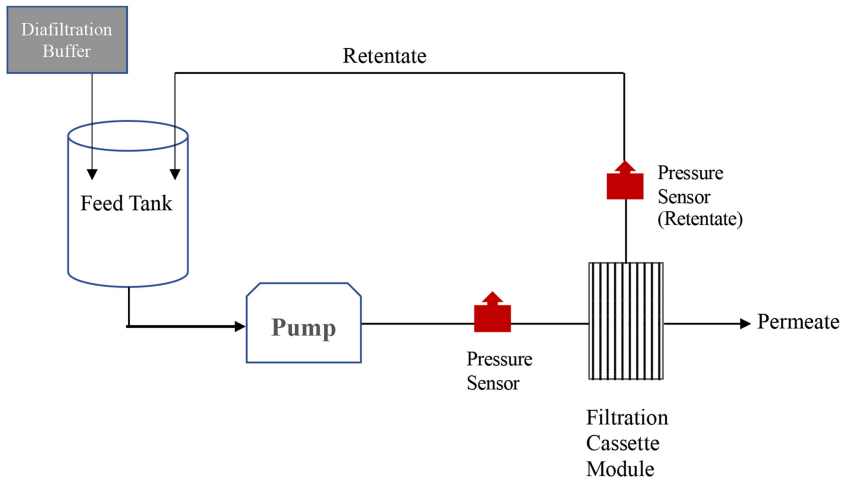

Figure 6.11. Schematic of a UF/DF system.

Single-pass TFF (SPTFF) is gaining more use in recent years. It is an enhanced version of the batch mode operation wherein, after a single pass through the filter assembly, the retentate is sufficiently concentrated, eliminating recirculation. This high throughput and recovery may be attributed to the increased residence time (due to reduced flow rates or longer path lengths) in the feed channel. This operation is particularly useful for volume reduction steps, generally between chromatography steps or other intermediate steps with large processing volumes.

Irrespective of the operation mode, it is important to determine and define the process objectives: final product concentration, impurity removal, volume reduction, and buffer exchange. Understanding this and establishing the criteria to meet will help maximize the process efficiency, accurately plan out future scale-up operations for robustness and consistency at the commercial scale. Optimizing the process parameters for this unit operation is crucial to minimizing product loss. Some of the critical process parameters to consider for the TFF (UF/DF) process are:

- Transmembrane pressure (TMP) is the differential in pressure between the two sides of a membrane. This parameter determines the amount of pressure or force required to propel the feed through the membrane. The filtrate flow increases until it reaches a plateau when TMP is increased to a specific level. As the protein concentration rises or the feed flow rate declines, the TMP decreases until the flux reaches a plateau (figure 6.12). The TMP required for the process is set for optimal activity when the flux is high without imposing undue pressure or striking a high protein concentration at the membrane.
- The crossflow rate is when the solution flows across the membrane as it passes through the feed channel, and the feed channel type determines it. At the same TMP, a higher crossflow rate results in a higher flux, which also means more product passes, leading to product degradation. As a result, it is critical to offset the increase in flux with more pump passes and hold-up volume for the proper crossflow rate.

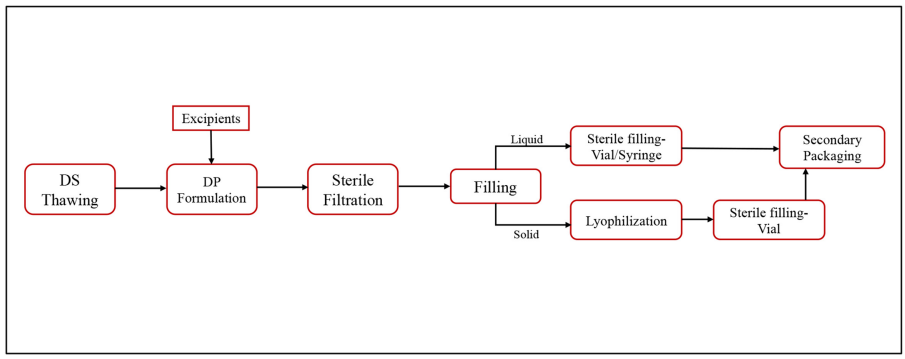

Figure 6.12. Schematic for DP preparation activities.

- The membrane area is determined based on the total processing volume and the process flux. While choosing a longer process time may lead to a smaller membrane area requirement and less hold-up volume, this should not be so high as to affect the product quality (degradation or contamination). Additionally, it is always recommended to incorporate a safety margin when choosing the membrane area requirements to benefit unforeseen circumstances with fouling and accommodate any lot-to-lot variabilities in the feed stream.
- Filtrate control is used in most applications wherein the target product is in the retentate, does not have a set point for filtrate control, and may operate unrestricted.

An additional factor to consider is for diafiltration operations added in with ultrafiltration, the point at which to start the diafiltration process must be determined. A high concentration formulation is generally preferred, mainly when the injection volume is low, usually in the case of most mAbs administered subcutaneously. However, other drug modalities such as antibody–drug conjugates, bispecific antibodies, cell, and gene therapies have necessitated improvisations to the existing TFF systems, e.g. single-use systems, SPTFF, additional monitoring capabilities, and pre-sterilized plug-and-play options.

6.5 Analytical methods

Downstream processing is entirely concerned with decreasing or eliminating process and product-related contaminants, with the goal of eventually generating a pure product. During the manufacturing process, in-process testing is performed to adjust process parameters, which leads to overall process control. These analytical procedures are the foundation of phase analytical technology (PAT). Monitoring product-related impurities such as high molecular weight and low molecular weight species, glycan variations, and charge variants is critical for overall product protection (for mAb products). Today's methodologies have evolved greatly, making in-process testing a critical tool. Improvements include high throughput

technologies that enable rapid sample processing, a faster turnaround that can support downstream operations effectively and efficiently, particularly for operations that are dependent on a specific analytical testing, example, loading and optimal operation of the downstream unit operation requires knowledge of the product concentration (i.e. titer) in the cell culture media, and subsequent product pools, similarly for product concentration steps. Also, while determining the suitability of testing for large-scale operations, the nature of the product at that stage, e.g. susceptibility to degradation, must be considered to evaluate process hold conditions and minimize the testing time in such instances.

For recombinant protein produced by *E. coli*, the scanning electron microscope (SEM) is used to assess the morphology and size of inclusion bodies using cell disruption and recovery analysis (CDRA). In addition, the impacts of cell disruption tactics on inclusion body size and structure, as well as the impact of cell culture conditions on inclusion body development, can be evaluated using SEM. In traditional plating methods the lysate's representative samples are plated before homogenization on selective and non-selective media and incubated for 12–24 h. The number of cells present on the agar plate is counted to determine the homogenization efficiency after each pass. Like the harvest and clarification procedures, turbidity measurements are useful for monitoring any other centrifugation process for IB recovery.

SDS-PAGE is a valuable tool for determining the purity of inclusion bodies during recovery operations, in particular for devising wash solution strategies and evaluating the decrease of host cell-related protein contaminants. This approach can also be used to investigate protein solubilization and refolding. The presence of intermolecular (and sometimes intramolecular) disulfide bonds can be detected analytically by SDS-PAGE under nonreducing circumstances by treating proteins successively with iodoacetamide (to avoid fake disulfide exchange) and then with SDS in the absence of reductant. RP-HPLC, SDS-PAGE, and analytical SEC are some of the more obvious and clear options for quickly assessing the degree of denaturation or refolding. Still, even surface plasmon resonance (SPR) and light scattering may also be used. Both qualitative and quantitative methods are useful in determining the appropriate conditions that can support solubilization and refolding.

Other standard analytical tests are intended for determining the protein titer, in addition to monitoring the process-related and product-related removal through the various unit operations. Methods for protein titer estimation are UV280nm, RP-HPLC, Bradford Assay, Lowry Assay, and even Octet, ProA HPLC. As for aggregates analysis, the most used methods for low molecular weight and high molecular weight species estimation are SDS-PAGE, CE-SDS, and even analytical size-exclusion methods. Finally, charge variant analysis is typically performed using CEX-HPLC analysis and iCE.

For monitoring process-related impurities, there are several commercial HCP kits available with the option also to customize the anti-HPC-antibodies. These are ELISA-based, but several automated immunoassay systems also have their own HCP kits for use.

6.6 Downstream processing equipment and system components

Downstream processing includes various equipment and other supporting components (also commonly referred to as skids) that vary in complexity. Equipment specific to downstream operations typically include:

- Buffer preparation and storage systems.
- Cell disruption and centrifugation (microbial processes).
- Purification systems/chromatography separation.
- Filtration systems (membrane filtration, depth filtration, etc).

The equipment used for the above processes must offer flexibility to support various scales (lab, pilot, and commercial-scale production). The downstream processing equipment should ensure no degradation or damage to the product due to equipment choice. This can also impact the overall yield and recovery of the process. A drug manufacturer must work with equipment manufacturer(s) to ensure the user requirement specifications (URS), designed for the specific process, are met for the process needs. With major advancements in bioprocessing today, manufacturers can cater to these requirements. One such is the ability to design and manufacture fully automated skids to carry out these purification and membrane filtration operations, including the sub-steps such as cleaning, disinfection, inline buffer dilution, and buffer exchange gradients. Disposable chromatography skids are gaining popularity due to the fast turnaround and the omission of cleaning and cleaning validation. Skids allow for an efficient set-up and streamlined processing. Each unit or system component should be designed and included, keeping in mind the process and desired functions from the unit. It is essential to plan and provide each piece of equipment considering the process requirements and its functional operation (figure 6.11).

The main design features include the .following:

- A process control unit/functional system in which the product is processed (e.g. chromatography columns, membrane filters, etc).
- The automation system enables the sensors' connection/communication with the detection units in the process control system to allow operation and monitoring. Various hardware/software platforms with optional customizable configurations are provided.
- Inlet and outlet systems (e.g. a buffer tank, holding tank, etc) are connected to the process control unit and other secondary accessories, including piping, valves, pumps, etc.
- Measurement devices/probes/sensors are used to control and monitor the process (e.g. pH, conductivity, UV, and pressure sensors).

Once successfully designed, the manufacturing skids are usually factory tested before being delivered to the user facility. Typically, for onsite installation, a check for specifications via installation, and installation and operational qualification exercises are performed before using the manufacturing operations equipment.

6.6.1 Sensors, probes, and meters

Online or inline monitoring and controlling of process parameters are common with upstream bioreactor operations. For downstream processes, commonly monitored process parameters include but are not limited to:

- Flow rate, pH, conductivity, and UV for chromatography unit operations.
- Pressure and flow rate/speed for clarification and filtration.
- Flow rate, concentration factor, pressure, conductivity, protein concentration, and temperature.

Monitoring some of these parameters helps serve as a signal for feedback control loops to optimize the process. Additionally, the data from monitoring are typically stored in the automation system, allowing trend analysis and inclusion in the batch documentation (e.g. UV analysis, chromatogram from a purification process, pressure monitoring for a TFF system (TMP)). pH, conductivity, temperature, and pressure are other commonly monitored parameters using either probes or sensors used inline or offline (table 6.9).

Timely monitoring and information on critical process parameters online can significantly contribute to the product quality, a better understanding of the process, its impact on the product, and an efficient process. The design and specification of sensors depend on the unit operation and the product/protein type. For example, in chromatography operations, inline sensors should be capable of detecting proteins, nucleic acids, carbohydrates, salts, flow rates, and a safety check for pressure.

Table 6.9. Recommended online monitoring for critical downstream processing steps.

Process stage	Process parameters monitored	Process attributes
Harvest and clarification	Flow rate	Efficiency
	Pressure	Filter membrane
	Turbidity	clogging Clarity of the supernatant
		Protein concentration (product)
Homogenization and IB recovery	Pressure	Homogenization efficiency
	Number of cycles	Protein concentration (product)
	Temperature	Amount of IBs
Purification	pH	Protein concentration (product)
	Flow rate	Purity and impurity profile (e.g. aggregates, residual DNA, HCP, etc)
	A280 (UV)	
	Conductivity	Column performance/resin lifecycle
	Binding conditions	
	Elution conditions	
Concentration	Diafiltration volumes	Protein concentration (product)
	Concentration factor	Purity profile
	Flow rate	Impurity profile
	Turbidity	

Typically, offline analysis instruments are used to determine product quality—which may be due to the processing conditions.

6.6.1.1 UV monitoring

UV spectroscopy is a critical attribute for most of downstream unit operations. UV measures the absorption from aromatic acids (phenylalanine, tyrosine, and tryptophan) in proteins, typically in the wavelength around 280 nm (and sometimes up to 340 nm), to scan different proteins in a mixture as they elute from the column. UV monitoring is a critical feature in chromatographic separation, and some systems include an auto-zero function to normalize the baseline from where the peak UV absorption starts. In certain TFF applications, a UV cell may be installed on the permeate side to monitor the protein breakthrough through the membrane. This is useful for high concentration proteins. Alternatively, the UV sensors may also be installed in the retentate line to determine the retentate recovery to minimize the final product's dilution and maximize the overall yield.

Fourier transform infrared (IR) spectroscopy characterizes proteins based on the amide bond's vibration in the polypeptide (secondary structure). FTIR allows for changes in the protein structure to be assessed.

6.6.1.2 pH monitoring

pH measurements are used as in-process controls, criteria for buffers (equilibration, elution, etc), and a medium that can provide a suitable environment for reactions. pH probes, either glass or plastic, are calibrated before each new operation/run. A pH probe should not be exposed to extreme conductivity values.

6.6.1.3 Conductivity monitoring

Conductivity measurements are used for chromatography and filtration operations monitoring. Conductivity is used to monitor or control gradient operations (e.g. ion-exchange chromatography or hydrophobic interaction chromatography) wherein the salt concentration is increased for elution. In diafiltration operations, measuring conductivity helps monitor the operation's progress indicating when the target process conductivity is met. Conductivity measurement also allows for detailed knowledge of the filters' equilibration preparation for the filtration operations and even as an endpoint for cleaning (CIP). The probes are used in the retentate and or permeate lines to monitor buffer exchange and measure conductivity during the rinsing operation.

6.6.1.4 Pressure sensor

Pressure sensors are commonly used in all operations in bioprocessing, and in most instances, they act as a safety measure—risk to the equipment and the operator. For example, in TFF operations, pressure sensors measure the transmembrane pressure (TMP), the pressure differential across the cassettes, and high pressure on the system. If the pressure goes high, it is critical to shut off the feed pump. Likewise, in chromatography columns and even for depth filtration systems, monitoring the pressure drop over time is a performance indicating parameter. Typically, inline

diaphragm sensors are used for product wetted applications, and other sensors or even gauges may be used for non-product contact areas.

6.6.1.5 Temperature

In downstream processing, temperature-controlled operations or process steps are not required. However, some operations are performed under specific temperatures in rare instances. These operations are carried out either in the manufacturing area set and maintained at that temperature. For refolding operations that may require a controlled temperature, tanks (jacketed vessels) are connected to a recirculation chiller to help keep the desired temperature.

The effects of temperature on pH and conductivity measurements can be important. Calibration and measurement must be done at the same temperature, or temperature compensation must be done manually using the meter's temperature control option. Some pH meters have built-in functions for automatic temperature compensation. Buffers have varying pH values at different temperatures, so it is crucial to utilize the buffer's value at calibration temperature. An NIST calibration is included with most new meters. It is sometimes a good idea to keep track of the temperature, pH, and conductivity of samples.

6.6.1.6 Flow-meters

Flow-meters are used to control the flow rate of a certain operation by monitoring flow rates in real time. Flow-meters are commonly used in chromatography and filtration systems. Inline and non-invasive flow sensors with high accuracy are used frequently as built-in components. These sensors can detect flow rates in pipes based on bidirectional measurements. In TFF systems a mass flow or magnetic inductive flow meter may measure the retentate and permeate sides' flow. Flow rates are an essential parameter, specifically when scaling up operations, so the system's performance is comparable across various scales. Magnetic flow-meters are less expensive than mass flow-meters, but they require a minimum conductivity in the liquid whose flow rate is being measured.

6.6.1.7 Air sensors

Air sensors detect the end of a sample or buffer solution and prevent air from entering the system. These sensors are used in the chromatography system and protect the column from damage by air while also enabling automatic loading. The air sensor is a non-invasive device.

6.6.2 Flow path

A key component in chromatography and filtration systems is the flow path, and these can be either plastic or stainless steel. A disposable flow path is a preferred option in bioprocessing operations. It allows for easy plug-in and plug-out options. The flow paths are pre-assembled and pre-sterilized as a quick plug-and-play option. Additionally, the disposable flow path kits are supplied with extractable and leachable documentation to support regulatory requirements (e.g. Cytiva flow

path kits, QuantaSep 300 SU system with disposable flow path Sepragen, PROCONNOX by Repligen). Thus, the flow path should accommodate even low-volume processes.

6.6.3 Pumps

Pumps play an important role in both upstream and downstream operations, with applications ranging from pumping medium and culture to buffer solutions and extremely delicate protein solutions. Selecting a pump for a particular process, many factors need close consideration. These include the flow rate requirements, physical properties of the liquid, shear sensitivity, and any pressure requirements.

As fluid enters the head of a peristaltic pump, the fluid is trapped between the two rollers. As the fluid moves through, a void may be followed, causing the tubing to be occluded by the rollers. An alternative fluid and void presence in the tubing causes the fluid flow to be pulsated instead of a smooth or continuous operation. All pumps produce pulsations at varying degrees. A pulse-free flow allows for better and accurate control of flow rates and monitored time operations. Pulsation can be reduced by using a pump head with adjustable occlusion; other solutions include a multiple roller design, a pulse dampened, and modifying the discharge tubing layout.

The suction lines are often neglected with pump systems, but piping or tubing transports the source's fluid material. Priming of suction lines is required, and this functionality may be an additional feature of the pump itself—as is if it is self-priming or non-self-priming. Self-priming pumps can draw the feed liquid even from an empty pipe, whereas non-self-priming pumps need to have their lines primed with the liquid before use.

A shorter suction line is likely to improve the operation's efficiency as less energy is used while increasing the pump's longevity. This can also minimize cavitation, which can significantly deteriorate the pump's performance and durability. Cavitation occurs when air pockets are created with the fluid due to pressure changes (the liquid stream's pressure falls below the vapor pressure of the liquid being pumped). These tiny bubbles can then implode, causing damage to the surrounding parts. Different pump types may have specific requirements for the suction line for a smooth operation; for example, for a centrifugal pump, the suction line should be direct and short. The pump inlet is the highest point on the suction line, and the suction line must be tight and free of air leakage for the pump to work. The length of the suction line in a magnetic drive pump should be held to a minimum and mounted with a steady increase to the pump to avoid air pockets. The suction pipe's diameter should be at least as wide as the pump's suction. For diaphragm pumps, the suction line must be non-collapsible and of the reinforced type.

The most used pumps for bioprocesses are the following:

- *Peristaltic pumps*: These pumps are best suited for single-use or disposable applications, mostly due to the tubing type (flexible, silicone, etc) these pumps can handle. Peristaltic pumps are used for low-pressure applications, and one of the main limitations of this pump type is the possible breakage of the tubing due to the constant movement and friction created by the pump head.

This can lead to product loss as well as a risk of contamination of the product. Hence, using it for highly viscous liquid or high flow rates may not be recommended with these pumps.

- *Rotary lobe pumps*: Suitable for liquids with a viscosity greater than water, as well as huge volumes of liquid. Pumping action is generated by rotating lobes. These pumps are susceptible to hard particles in the fluid being pumped, and as a result, the lobe and pump housing may be damaged. A variable frequency drive uses a flow meter to control the pump's speed.
- *Diaphragm pumps*: Most used for downstream operations (chromatography). These pumps come in a variety of pressure ratings and have been successfully employed in high-pressure chromatography applications requiring pressures more than 100 bar.

6.6.4 Valves

Valves organize the flow of products and buffers pumped through the system. When a liquid is pumped via a valve, the pressure-flow characteristics are different. Stainless steel, polypropylene (PP), polyvinylidene difluoride (PVDF), and polyether ether ketone are common materials for valves (PEEK). Different types of valves are used for various operations in the manufacturing of biopharmaceuticals. Pinch valves are often used for soft and flexible tubing in single-use systems, diaphragm valves are used for low-pressure operations, and ball valves are used widely in HPLC systems.

6.6.5 Inline filtration (sterile filtration and particle filtration)

Filtration is a frequently used operation during the various stages in the downstream process, including clarification of harvest, preparation of buffers and solution, and during the chromatography and final filtration steps leading to the finished product. These filtration operations could be microfiltration (clarified harvest), ultrafiltration (concentration, buffer exchanges, etc), and nanofiltration or direct flow filtration through sterilizing grade filters (for the elimination of microbes, viruses, final formulation).

Standard flow filtration includes passing the feed directly through the membrane without crossflow (as seen in TFF). Direct flow or dead-end filtering are terms used to describe this process. NFF is often utilized in chromatography unit operations, as well as any operation requiring particle removal and sterile filtration, such as virus filtration. NFF protects and safeguards the chromatographic column by eliminating particles from the buffer and feeds, as well as reducing bioburden. The filtration area, porosity, and configuration of the cartridges should be assessed to determine optimum filtration parameters. Pressure sensors may be placed upstream and downstream of the filter unit to monitor and control the filtration operation, estimate the pressure differential to determine when the filter needs to be replaced. For particle and microbiological removal, membrane pore diameters range from 0.1 to 10.0 μm. Pre-and post-use filter integrity testing are performed on all filters used in the upstream and downstream operations. Examples include the bubble point test,

forward flow test, water intrusion test, and binary gas test. The ability of a wet membrane to keep gas from flowing freely is measured using the bubble point and forward flow tests. A dry hydrophobic membrane is utilized as a barrier to the free flow of water, and a non-wetting fluid is used in the water intrusion test (HydroCorr test). A mixture of two gases with large permeability variances is used in the binary gas test. The test entails determining the composition of the gas mixture upstream and downstream of a wetted membrane. If the post-use integrity test fails, refiltration is conducted.

Overall, for a bioproduction process, the choice of equipment and its specification should be based on operational purposes. Considering process intensification gaining ground in bioprocessing, small-scale equipment may also be beneficial, in particular for meeting the product amount requirements for toxicology and clinical studies. Smaller skids also have the added benefit of compatibility and ease of implementation of single-use technologies.

Because most downstream processing equipment is made of stainless steel, operations that need extreme conditions may not be suitable owing to corrosion risks. Plastics including polypropylene, EPDM, PTFE, and PEEK are common in tubing, measuring cells, valves, and gaskets. pH and UV sensors are made of glass. The majority of the components, including stainless steel, are deemed extractable. Drug manufacturers need to work cohesively with suppliers for testing and supporting documentation purposes. As the target product progresses with increasing purity towards the final drug substance or drug product preparation, the demand for sterile or aseptic conditions with control for bioburden becomes more rigid. Therefore, the hygienic design of process flow paths, product contact surfaces is essential. Traditional cleaning methods use caustic solutions in varying concentrations, e.g. column cleaning, filter membrane cleaning, equipment, and other consumables. Steam sterilization may also be applied if required. Single-use systems replace several of these components due mainly to the reduced cleaning procedures and faster turnarounds.

6.7 Conclusion

The success of developing a robust downstream process and meeting the product quality requirements is highly dependent on the efficiency and recovery of each unit operation. Product losses can significantly impact the economic viability of the product. Therefore, a systemic approach and identifying the objectives and expectations of each unit operation is essential. A three-stage purification plan, for example, does not necessitate three purifications as chromatography phases in all manufacturing processes. Purity standards and the protein's intended usage should be the driving factors. While a platform process makes it easy to have a typical operation sequence in a multi-product facility involving similar products, variations to this platform approach should be evaluated.

Some limitations are inherent in most downstream operations. For example, chromatography resins are a significant limitation in chromatographic procedures, particularly in resin lifetime, lot-to-lot variability, cleaning, regeneration processes, and viral clearance capacity.

Process optimization activities aimed at reducing processing times, process intensification to minimize process steps must be explored. Strategies such as scaling media volume based on impurity can reduce resin quantity and buffer consumption while enabling the process to be more economically viable. Process efficiency, robustness, and scalability should be a priority. Modern approaches such as integrating a chromatographic step into a multimodal chromatography process, continuous processing, options that can reduce facility footprint, the advent of single-use systems that eliminate costs, complexities with packaging, cleaning, and maintenance have been on the rise in recent years. However, implementation is still limited, but these technologies do present opportunities for productivity and flexibility.

Post drug substance (DS) formulation, unit operations for preparing final drug product (DP) including formulation, filling, and primary and secondary packaging. An outline for DP preparation is illustrated in figure 6.12.