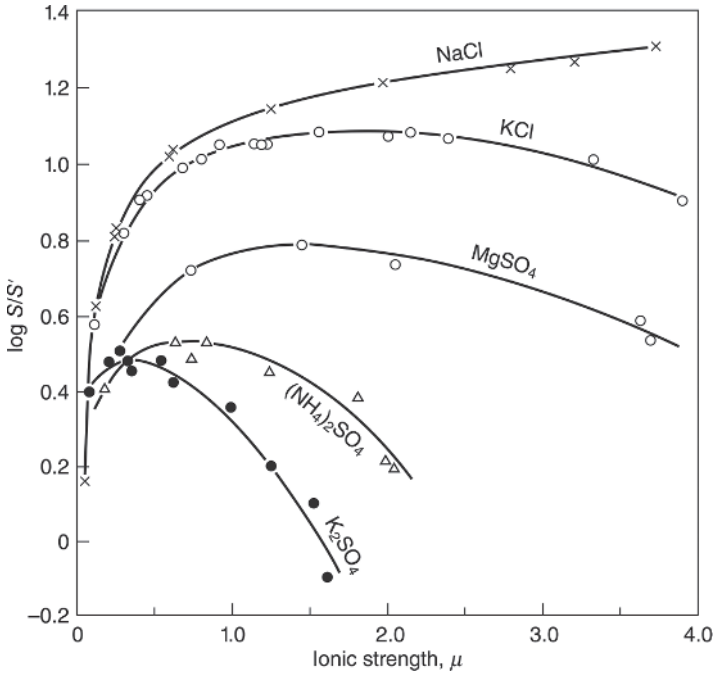

Figure 1.23 Solubility of carboxyhemoglobin in aqueous solution with different electrolytes at 25 °C. *S* and *S′* are used in lieu of *w* and w_0. Source: Data from Green 1932 [28].

of the salt added. This behavior can be seen, for example, in Figure 1.23 for carboxyhemoglobin. Adding NaCl increases the solubility, which is referred to as "salting in," but adding potassium or ammonium sulfate at concentrations above about 0.4 M depresses the solubility, which is referred to as "salting out." As can be seen in Figure 1.23, for this protein, magnesium sulfate results in salting in at low ionic strengths and in salting out at high ionic strength. In any case, it should be recognized that these effects are quantitatively and sometimes even qualitatively different for different proteins.

Protein solubility trends can be described by the extended form of the Debye–Hückel theory. Accordingly, we have:

$$\log \frac{w}{w_0} = \frac{0.5 \cdot z_1 \cdot z_2 \sqrt{I}}{1 + A\sqrt{I}} - \kappa_s I \tag{1.17}$$

where w is the protein solubility in the actual solution, w_0 is the solubility of the protein in water, z_1 and z_2 are the salt charges, and κ_s and A are salt- and protein-specific empirical parameters. At high ionic strengths, Eq. (1.17) reduces to the following log–linear relationship:

$$\log \frac{w}{w_0} = \beta - \kappa_s I \tag{1.18}$$

which, as seen in Figure 1.24, is observed for many different proteins.

The effect of the salt type on protein solubility has been formally described for the first time by Hofmeister [30] who ranked the anions and cations according

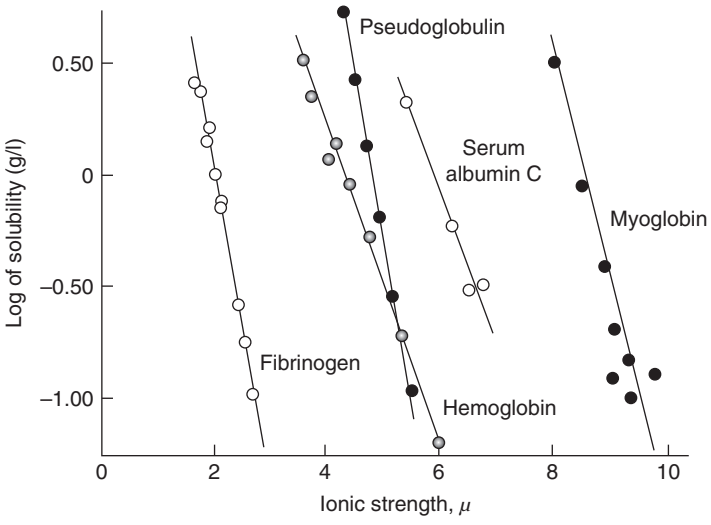

Figure 1.24 Solubility of various proteins in ammonium sulfate solutions. Source: Cohn and Edsall 1943 [29]. Reproduced with permission of American Chemical Society.

to their ability to precipitate proteins. This ranking is described by the following *Hofmeister series* or *lyotropic series*:

$$\text{Anions}: \quad SO_4^{2-} > Cl^- > Br^- > NO^{3-} > ClO^{4-} > I^- > SCN^-$$

$$\text{Cations}: \quad Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+$$

A simple interpretation of this series is that certain ions bind water molecules tightly, thereby decreasing the ability of the protein to stay in solution. Although both the cation and the anion in a given salt are important to this property, the contribution of the anion is usually dominant. Interestingly, the ions in the Hofmeister series also correlate with the so-called Jones–Dole B-coefficient and the entropy of hydration so that both appear to be related to the effects of salts on the structure of water. Finally, it should be noted that, in practice, the selection of salts for use in downstream processing depends not only on the Hofmeister series but also on factors such as price, availability, biocompatibility, and disposal costs.

1.2.2.6 Chemical Stability

Two different types of chemical stabilities should be considered for proteins: the *conformational stability* (or *thermodynamic stability*) and the *kinetic stability* (or *colloidal stability*). The conformational stability of a protein is described by the free energy ΔG of the equilibrium between native and the unfolded states. The transition of the native folded form, N, into the unfolded form, U, is described by the following quasi-chemical reaction:

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U \tag{1.19}$$

Table 1.8 Thermodynamic stabilities of proteins.

Protein	Conditions	Free energy of the unfolding reaction ΔG (kcal/mol)	Melting temperature (°C)
Horse cytochrome c at pH 6 at 25 °C	0 M urea	31.3	n.a.
	2 M urea	22.3	n.a.
	4 M urea	14.2	n.a.
	6 M urea	3.2	n.a.
Hen egg white lysozyme at pH 3.0	24 °C	41.0	n.a.
	40 °C	30.4	n.a.
	55 °C	14.7	n.a.
	75 °C	−5.9	n.a.
Bovine chymotrypsinogen at melting temperature and pH 2.0	0% glycerol	0.015	42.9
	20% v/v glycerol	0.146	44.9
	40% v/v glycerol	0.235	46.2

Source: Data for chymotrypsinogen are from Gekko and Timasheff 1981 [31].

where k_1 and k_{-1} are rate constants. The corresponding equilibrium constant $K_{eq} = [U]/[N]$ is usually very low in aqueous solution as protein folding is generally thermodynamically favored as a result of the concentration of the hydrophobic residues in the protein core. The corresponding ΔG is given by the following equation:

$$\Delta G = -RT \ln K_{eq} \tag{1.20}$$

Representative values of ΔG are given in Table 1.8 along with the corresponding "*melting temperature*," which is defined as the temperature at which half of the protein is in the unfolded state. *Kosmotropic* (or cosmotropic) *salts* and polyols such as sorbitol or sucrose stabilize proteins while *chaotropic salts* or urea at higher concentrations have a destabilizing effect on protein conformation.

Kinetic stability, on the other hand, can be described by the following equation:

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_2} A \tag{1.21}$$

which shows a further kinetically driven step from the unfolded state to an irreversibly aggregated state A. Proteins with a high k_2 exhibit low kinetic stability. The overall stability thus depends on both thermodynamic and kinetic effects. It is possible, for example, for an added salt to decrease kinetic stability while enhancing overall stability as a result of thermodynamic effects. This is often difficult to predict, however, so that, in practice, overall stability and shelf life are measured empirically [32].

1.2.2.7 Mechanical Stability

It is a frequent misconception that proteins are not mechanically stable because of the contradictory reports in the literature. Proteins are highly mechanically

Table 1.9 Typical shear rates encountered in bioprocessing operations.

Operation	Shear rate, $\dot{\gamma}$ (s^{-1})
Expanded beds	<10
Packed beds	$<10^3$
Stirred tanks	10^2–10^3
High pressure homogenizers	10^6

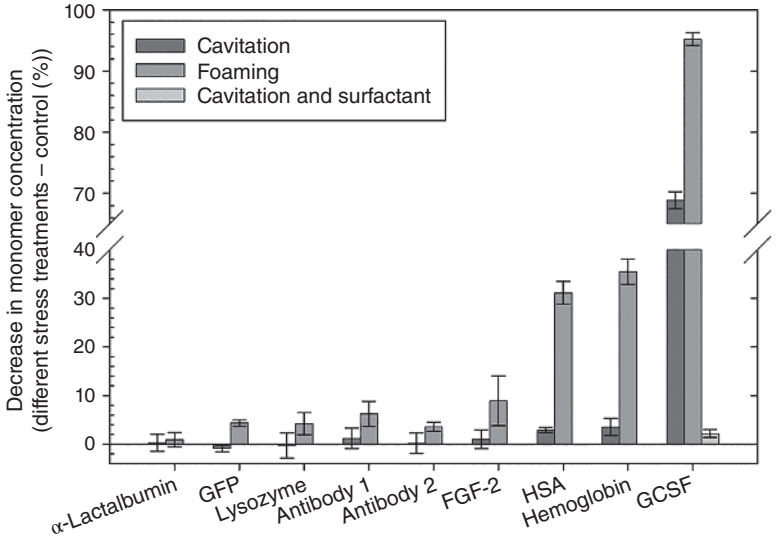

Figure 1.25 Comparison of protein aggregation from cavitation and foaming for nine different proteins. Data points represent the means of three experiments minus control ± standard deviation. Addition of surfactant decreases the effect of cavitation dramatically. Source: Adapted from Duerkop et al. 2018 [8].

stable. They resist shear rates up to $10^8\ s^{-1}$. This is 10 times higher than the shear rates obtained in a high-pressure homogenizer (see Table 1.9). Also, high protein concentrations suppress cavitation. Thus, it is almost impossible to unfold an average protein by a mechanical force under ordinary process conditions. Proteins may be sensitive, however, to unfolding at air–liquid interfaces. Therefore, it is highly recommended to avoid foaming when working with protein solutions. In addition, at very high velocity, cavitation may occur, where gas bubbles are generated. This in turn may lead to protein unfolding or aggregation (see Figure 1.25).

1.2.2.8 Viscosity

Many of the solutions and suspensions encountered in bioprocessing are highly viscous. This is especially true for fermentation broths that contain DNA and for highly concentrated protein solutions. In general, viscosity, η, is related to the

Table 1.10 Apparent viscosities of various fluids at 20 °C.

Liquid	Apparent viscosity (mPa s)
Water	1
Glycerol	1070
Ethanol	1.20
Acetonitrile	0.34
Clarified cell culture supernatant	~5
Blood	10
E. coli homogenate	~40
E. coli broth	~20
Penicillium chrysogenum fermentation broth	40 000
Heinz Ketchup	50 000–70 000

shear stress, τ, and the shear rate, $\dot{\gamma}$, by the following equation:

$$\tau = \eta \times \dot{\gamma} \tag{1.22}$$

For Newtonian fluids, η is a constant and the relationship between shear stress and shear rate is linear. For non-Newtonian fluids, however, η varies with shear rate and the relationship becomes nonlinear. For example, the behavior of pseudoplastic fluids is described by the following equation:

$$\tau = K \times (\dot{\gamma})^n \tag{1.23}$$

where K and n are called the *flow consistency index* and *flow behavior index*, respectively. For highly concentrated protein solutions and for many culture supernatants, n is smaller than unity, indicating that the apparent viscosity, $\eta = \tau/\dot{\gamma}$, decreases with increasing shear rate. The ranges of various shear rates encountered in bioprocessing are shown in Table 1.9.

The viscosities of some typical fluids as well those of some of the solutions encountered in bioprocessing are shown in Table 1.10. In general, cell culture supernatants have viscosities lower than 10 mPa s, while cell homogenates are much more viscous with values of η up to 40 mPa s. DNA is, usually, the greatest contributor to the viscosity of raw fermentation broths. Fortunately, however, both genomic and plasmid DNA are very sensitive to shear and are often mechanically degraded early on in the downstream process. DNAse enzymes, naturally occurring or added intentionally, also help degrade these molecules, thereby reducing viscosity. This is especially important for intracellular products that require disruption of the cells with concomitant release of the intracellular components into the product-containing solution.

The *intrinsic viscosity* $[\eta]$ is a measure of a solute's contribution to the solution viscosity and is defined as follows:

$$[\eta] = \lim_{c \to 0} \frac{\eta - \eta_0}{\eta_0 c} \tag{1.24}$$

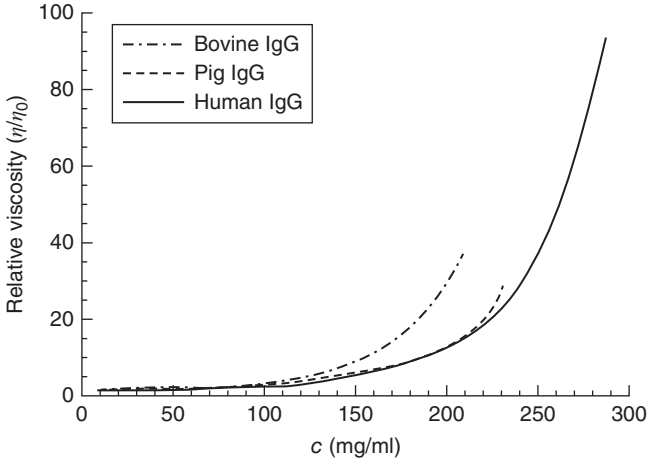

Figure 1.26 Relative viscosity of human, bovine, and pig IgG solutions as a function of IgG concentration at room temperature. Source: Adapted from Monkos and Turczynski 1999 [33].

where η_0 is the solvent viscosity in the absence of the solute and c is the solute concentration. In a semidilute limit, η can be described as a function of c by the following series expansion:

$$\frac{\eta - \eta_0}{\eta_0} = [\eta]c + k_1[\eta]^2c^2 + k_2[\eta]^3c^3 + \cdots \tag{1.25}$$

At very high protein concentrations, however, semiempirical models are needed [33]. As an example, Figure 1.26 shows the relative viscosity (defined as η/η_0) of IgG solutions as a function of IgG concentration. For concentrations lower than about 100 mg/ml, the relative viscosity increases approximately linearly with solute concentration conforming to the first term on the right-hand side of Eq. (1.25). However, at higher concentrations, the viscosity increases almost exponentially.

Table 1.11 provides the intrinsic viscosities of representative biomolecules. As can be surmised from these data, the intrinsic viscosity depends on the shape of the molecule. For instance, rod-shaped proteins have a higher intrinsic viscosity than globular ones. An empirical relationship between $[\eta]$ and molecular mass M_r is given by the Mark–Houwink equation:

$$[\eta] = K(M_r)^a \tag{1.26}$$

where a is a parameter related to the "stiffness" of the polymer chains. Theoretically, $a = 2$ for rigid rods, 1 for coils, and 0 for hard spheres. Empirically, however, values of $a = 0.6$, 0.7, and 0.5 have been found for BSA, ovalbumin, and lysozyme, respectively. Literature data (e.g. see Ref. [35]) suggest a general relationship between intrinsic viscosity and the number of amino acid residues, n_{aa}, which can be expressed as follows:

$$[\eta] = 0.732(n_{aa})^{0.656} \tag{1.27}$$

with $[\eta]$ in ml/g. Accordingly, larger proteins and protein aggregates have higher intrinsic viscosity than smaller proteins and monomeric forms.

Table 1.11 Intrinsic viscosities of various biologically important macromolecules in dilute solutions.

Shape	Solute	Molecular mass	[η] (ml/g)
Globular	Ribonuclease	13 680	3.4
	Serum albumin	67 500	3.7
	Ribosomes (*E. coli*)	900 000	8.1
	Bushy stunt virus	10 700 000	3.4
Random coils (unfolded proteins)	Insulin (A-chain)	2 970	6.1
	Ribonuclease	13 680	16
	Serum albumin	68 000	52
	Myosin subunit	197 000	93
Rods	Fibrinogen	330 000	27
	Myosin	440 000	217
	Calf thymus DNA	15 000 000	>10 000

Source: Sibileva et al. 2001 [34]. Reproduced with permission of Springer.

1.2.2.9 Diffusivity

The *molecular diffusion coefficient* or *diffusivity* in solution, D_0, is a function of the solute size, the viscosity of the solution, and temperature. As previously noted, the Stokes–Einstein equation describes this relationship as follows:

$$\frac{D_0\eta}{T} = \frac{k_b}{6\pi r_h} \tag{1.28}$$

where k_b is the Boltzmann constant and r_h is the solute hydrodynamic radius. The diffusivities encountered in bioprocessing range widely from 1×10^{-5} cm^2/s for salts and other small molecules to 1×10^{-9} cm^2/s for large biomolecules such as DNA. Protein diffusivities in dilute aqueous solution are generally in the range 10^{-6}–10^{-7} cm^2/s. Table 1.12 provides a summary of typical diffusivities in dilute solutions at room temperature.

In general, protein diffusivities are 10–100 times lower than those of small molecules. Plasmids have even smaller diffusivity values – as much as 1000 times lower than for small molecules. Figure 1.27 illustrates the effect of molecular mass on the diffusivity of globular proteins in dilute aqueous solution at room temperature. Tyn and Gusek [36] provide the following correlation for such proteins:

$$\frac{D_0\eta}{T} = \frac{9.2 \times 10^{-8}}{(M_r)^{1/3}} \tag{1.29}$$

where D_0 is in cm^2/s, η in cp, T in K, and M_r in Da, which has accuracy better than ±10%. Both Eqs. (1.28) and (1.29) indicate that the group $D_0\eta/T$ is a constant for a given protein in aqueous solution. This fact can be utilized conveniently to estimate the effects of solution viscosity and temperature on the protein diffusivity. In general, D_0 increases with temperature in part because of the T-term in the denominator of this ratio but, more importantly, because the solution viscosity usually decreases as the temperature is increased. For example,

Table 1.12 Diffusivities in dilute solution at room temperature.

Solute	Solvent	Solvent viscosity, η (mPa s)	Diffusivity, D_0 (10^{-5} cm^2/s)
Benzoic acid	Water	1	1.00
Valine	Water	1	0.83
Sucrose	Water	1	0.53
Water	Ethanol	1.1	1.24
Water	Glycol	20	0.18
Water	Glycerol	>120	0.013
Ribonuclease (M_r = 14 kDa)	Water	1	0.120
Albumin (M_r = 65 kDa)	Water	1	0.060
IgG (M_r = 165 kDa)	Water	1	0.037
pDNA (M_r = 3234 kDa)	Water	1	0.004

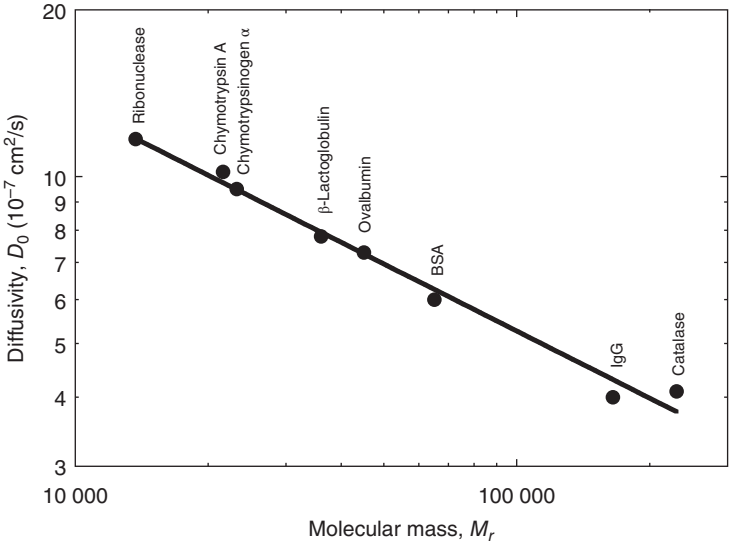

Figure 1.27 Diffusivity of globular proteins in dilute aqueous solution at room temperature. Source: Adapted from Tyn and Gusek 1990 [36].

going from 25 to 4 °C, the viscosity of a dilute aqueous solution increases from about 1 mPa s to about 1.5 mPa s. As result of this relationship, D_0 is expected to become only about 60% of the value at 25 °C. Understanding this effect is important for scale-up as biochromatography processes are often developed in the laboratory at room temperature but then scaled-up for operation at different temperatures, usually lower than room temperature in order to minimize product degradation and inhibit the potential growth of microorganisms.

Predicting the diffusivity of nonglobular proteins is more complex as their shape and not just their radius affect D_0. In general, for the same molecular

mass, the diffusivity of an elongated macromolecule, such as a fibrous protein, is substantially lower than that of the same molecule in globular form. Thus, unfolded proteins have smaller diffusivities than the corresponding folded ones. Plasmids, which are rod shaped in their supercoiled conformation, have much smaller diffusion coefficients than proteins with a stronger dependence on molecular mass (2/3 power) than that suggested by Eq. (1.29) (see Ref. [37]). Linear polymers have even lower D_0-values that are nearly inversely proportional to molecular mass. Polymer–protein conjugates, such as PEGylated proteins, have smaller diffusivities than the corresponding unconjugated protein. In this case, both the protein molecular mass and the polymer molecular mass contribute to the value of D_0, but the former according to the 1/3 power while the latter according to the first power of M_r. The following equation has been developed by Fee and van Alstine [38] to predict the hydrodynamic radius r_h (in nm) of PEGylated proteins:

$$r_h = 0.082(M_{r,\text{protein}})^{1/3} + 0.373 + 0.000\,11M_{r,\text{PEG}} \tag{1.30}$$

where $M_{r,\text{ protein}}$ is the molecular mass of the protein and $M_{r,\text{ PEG}}$ is the molecular mass of total conjugated PEG polymers. The value of D_0 for the conjugated protein is found replacing r_h in Eq. (1.28) with the value of r_h predicted by this equation. Examples of chromatography of PEGylated proteins including high loading conditions can be found in Ref. [39]. Finally, virus and virus-like particles exhibit diffusion coefficients consistent with Eq. (1.28) if they are spherical but substantially smaller values if they are rod shaped.

In general, if the shape of a protein or of a bioparticle can be approximated as an ellipsoid with radii r_a and r_b, using the average radius $(r_a + r_b)/2$ in place of r_h in Eq. (1.28) results in an error of less than 15% in the predicted value of D_0 if $r_a/r_b < 2$. Several approaches are available for the experimental determination of diffusivity and are reviewed, for example, by Cussler [40]. Commonly used approaches for proteins include DLS (described above), diffusion cells, Taylor dispersion-based methods, and microinterferometry. Such measurements may be needed for molecules that have complex or unknown shapes such as in the case of aggregates.

1.3 Bioprocesses

This section discusses commonly used expression systems and the general structure of downstream processes needed to achieve the desired product purity. Special emphasis is placed on the production of recombinant proteins by fermentation and cell culture, which play a major role in industrial biotechnology.

1.3.1 Expression Systems

Many different expression systems have been developed for recombinant proteins, ranging from very simple bacteria to plants and animals. However, the number of host cells actually used in the industrial production of biopharmaceutical proteins is quite limited. The most popular bacterial strain is *E. coli* BL21, which is used for the production of proteins whose biological activity does

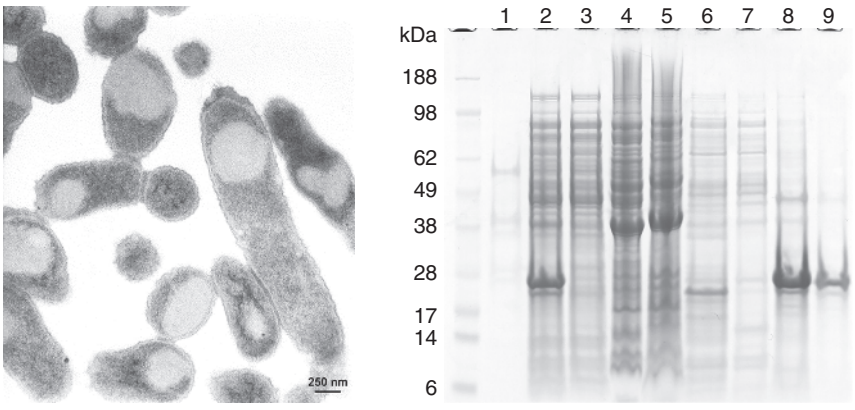

Figure 1.28 (a) Electron micrograph of *E. coli* cells overexpressing a recombinant protein as inclusion bodies. (b) SDS–PAGE under nonreducing conditions. Lane 1: culture supernatant; lane 2: homogenate; lane 3: soluble fraction; lanes 4–7: supernatants of wash steps; lanes 8 and 9: insoluble fraction containing the product.

not require post-translational modifications. Protein expression in *E. coli* can occur in three different ways. The protein can be secreted into the periplasm, which is the space between cell membrane and cell wall; it can be expressed in the cytoplasm as a soluble protein; or it can accumulate in the cell as inclusion bodies. Each system is effective for different proteins. The cytoplasm of *E. coli* is a strongly reducing environment that hinders the formation of disulfide bridges, whereas the periplasm offers a more oxidizing environment, which allows such a formation facilitating folding. Therefore, antibody fragments and antibody-derived molecules (the so-called "new formats") are often expressed into the periplasm. Some proteins that are toxic to the cells or that have a short half-life in the cytoplasm or periplasm life have been successfully produced as inclusion bodies. In this case, the protein forms aggregates that cannot be attacked by proteases. On the other hand, although often not fully denatured, proteins expressed as inclusion bodies are generally not in their native conformation. Thus, a refolding process is typically required to generate a fully active form. Although refolding can be costly, on balance, this approach is frequently economically viable, as expression levels in inclusion body system are extremely high and simple washing procedures can be used to remove most host cell proteins resulting in relatively high initial purities. Figure 1.28 shows an example of *E. coli* cells overexpressing a protein as inclusion bodies and the corresponding SDS–PAGE analyses at various stages in the process.

The yeast cells *Saccharomyces cerevisiae* and *Pichia pastoris* have been successfully used for over expression of various recombinant proteins including insulin and albumin. *S. cerevisiae* is also used for the production of Hepatitis surface antigen. However, mammalian cells are used for the majority of biopharmaceutical proteins. Although mammalian cell culture is generally more complex, these cells can perform complex post-translational modifications, such as glycosylation, which are often critical to proper biological and

pharmacological activity. Chinese hamster ovary cells (CHO) are the most commonly used mammalian expression system, especially for recombinant antibodies. The human cell line PerC6 has been developed more recently and is also used for production of some recombinant proteins. CHO and PerC6 are able to overexpress antibodies in concentrations as high as 15 mg/ml. Such high product titers are achieved mainly because the expression of proteins in these systems is generally independent of growth. As a result, the cells can be maintained in a perfusion bioreactor for long times, e.g. up to 30 days, in a viable productive state and can be cultivated to very high densities, e.g. up to 2×10^8 cells/ml.

Other mammalian cell lines used in recombinant protein production include baby hamster kidney (BHK) cells, Vero cells, and Madin–Darby canine kidney (MDCK) cells. Several coagulation factors that require γ-carboxylation are produced in BHK. Vero cells, derived from monkey kidneys, and MDCK cells are used for the production of vaccines. Insect cells such as cells from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* (Sf9) have also been proposed for overexpression of recombinant proteins and, in particular, for the expression of virus-like particles and virus base gene therapy vectors. These cells can be infected with insect viruses such as the Autographa californica nuclear polyhedrosis virus, also known as baculovirus. Although easy to handle, so far, processes including insect cell systems have not been licensed for the industrial production of biopharmaceutical proteins.

In the past, transgenic mammals were considered as excellent production systems, as proteins can be secreted into their milk with titers up to 5 mg/ml. Two decades ago, such titers could not be achieved with mammalian cell culture. Thus, these expression systems were often preferred. Modern advances in cell culture, however, have made it possible to achieve routinely even higher titers in bioreactors while avoiding the enormous complexities of dealing with animals and allowing much more robust operation in a closed, more controllable system. As a result, transgenic animals no longer play a significant role in biopharmaceutical manufacturing.

Plants and plant cells are also potential candidates for expression of proteins. Although different from those that occur in mammalian cells, post-translational modifications are also possible in these organisms. However, expression in leaves, stems, or seeds presents significant recovery and purification challenges because the tissue or seeds must be ground up, pressed, or extracted yielding very complex mixtures. An interesting expression system is rhizo-secretion, where the protein is secreted into a cultivation fluid from hairy roots. Plant cell cultures, on the other hand, present fewer downstream processing difficulties as these cells grow in very simple media. Currently, one licensed therapeutic protein – Elelyso (taliglucerase alfa) – produced in carrot cells is on the market. There is a lot of research in this field with many ongoing trials. Most probably, there will be more therapeutic proteins manufactured by plant cell culture or plant cells in the future. In addition, other manufacturing applications of plant cell culture are progressively tested such as industrial enzymes, polymer degrading enzymes, feed additives, and edible vaccines, which require less purification efforts [41].

1.3.2 Host Cell Composition

The composition of the host cells has important effects on downstream processing, especially when the product is expressed intracellularly, as in this case, cellular components are the major impurities. However, host cell components are also found extensively as impurities in secreted products, as cell lysis always occurs at least to some extent during fermentation and cell culture. In fact, in some cases, cultivation procedures that yield high titers, such as those used for antibody production, result in partial lysis of the cells, which, in turn, causes contamination of the product with host cell components. An overview of the composition and physical characteristics of the major host cells is given in Tables 1.13 and 1.14.

As can be seen in Tables 1.13 and 1.14, mammalian cells contain more protein but less nucleic acids compared to bacteria. Bacteria and yeast also contain numerous cell wall components. These components are frequently insoluble and are efficiently removed during early processing steps, but these early steps are affected significantly by the cell density and the broth viscosity. Extremely high

Table 1.13 Composition of common host cells for expression of recombinant proteins.

	Composition (% dry weight)					
Organism	**Proteins**	**Nucleic acids**	**Lipids**	**Cell count per ml**	**Dry mass (mg/ml)**	**Wet mass (mg/ml)**
E. coli	50	45	1	10^{11}	20	100
Yeast	50	10	6	10^{10}	80	400[a)]
Filamentous fungi	50	3	10	10^{9}	130	400[b)]
Mammalian cells	75	12	Up to 10	10^{7}–10^{8}	0.17–1.7	1–10

a) For high density culture of *P. pastoris* grown on glucose medium.
b) For high density culture of *P. pastoris* grown on methanol.

Table 1.14 Composition of single cells for expression of recombinant proteins.

Component	**Amount per CHO cell[a)]**	**Amount per *E. coli* cell**
Total DNA (pg)	7 ± 1.2	0.017[b)]
Total RNA (pg)	18 ± 3.0	0.10
Total Protein (pg)	146 ± 22 (2.4×10^7 with an approx. M_r of 40 000)	0.2 (3×10^6 molecules with an approx. M_r of 40 000)
Dry weight (pg)	263 ± 31	0.4
Wet weight (pg)	2500	2
Diameter	18 µm	0.5 µm × 3 µm
Volume (cm^3)	$1.7 \times 10^{-9} \pm 0.2$	

a) Data kindly provided by Nicole Borth from BOKU.
b) A fast-growing *E. coli* cell contains in average the genome in fourfold repetition. Each genomic DNA weight about 0.0044 pg.

cell densities can be obtained for yeast, particularly for *P. pastoris*, for which cell densities up to 400 g of cells per liter have been reported. Such suspensions are extremely difficult to clarify; often, the suspension must be diluted in order to be able to centrifuge the broth. Nucleic acids are present in the form of DNA and all kinds of RNAs. These compounds result in high broth viscosity but are often rapidly degraded by mechanical shear or by endogenous nucleases.

A final consideration is the mechanical stability of the host cells. Bacteria and yeast cells are generally mechanically very stable and shear rates above $10^6\ s^{-1}$ are necessary to break these cells. Such high shear rates are attained only using special equipment such as high-pressure homogenizers or French presses. By comparison, mammalian cells are much weaker. The burst force needed to destroy a yeast cell is in the range of 90 μN, whereas the burst force for mammalian cells is in the range of 2–4 μN. The burst force increases with culture length in a batch culture, which is consistent with the observation that older cells are more difficult to disrupt.

1.3.3 Culture Media

Modern biopharmaceuticals are commonly produced with the so-called defined media whose components are chemically defined. In the past, yeast, meat, and soy extracts, produced by proteolytic degradation and extraction, were commonly used for cultivation of bacteria and yeast cells. The standardization of such raw materials was extremely difficult, resulting in substantial batch-to-batch variations. Similarly, until recently, it was common to supplement cultivation media for mammalian cells with fetal calf serum in concentrations up to 10%. Beside added complexity and cost, such supplements can introduce undesirable adventitious agents, such as prions, which can significantly increase the downstream processing challenge. Although testing for such agents may still be required, the use of defined media greatly simplifies downstream processing.

Media for industrial cultivation of bacteria are usually very simple and provide the essential sources of carbon, nitrogen, and phosphate needed by these simple organisms. Examples are given in Table 1.15. Sometimes, a cocktail of trace elements is added, but frequently trace elements already present in the water are sufficient.

When the fermentation pH is controlled by the addition of NaOH, conductivities as high as 40 mS/cm can be reached at the end of the cultivation period. Such high conductivities can interfere with downstream processing operations such as ion exchange requiring dilution or diafiltration steps. This difficulty may be circumvented using ammonia for pH control, which typically results in lower conductivity of the culture supernatant.

In addition to the salts and sugar that are required for cell metabolism, production of recombinant proteins in bacteria typically requires the addition of an inducer, such as isopropyl-β-D-thiogalactopyranoside (IPTG), which is used to activate protein expression when a certain cell density is reached. The use of natural compounds as inducers is advantageous as such species are readily degraded in the culture and are not significant impurities. On the other hand, detergents or oils added to the culture as antifoaming agents, although present in relatively

Table 1.15 Composition of defined culture media for cultivation of *E. coli*.

Compound	Concentration (mg/ml)
Glucose	1.0
Na_2HPO_4	16.4
KH_2PO_4	1.5
$(NH_3)_2PO_4$	2.0
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.01
$FeSO_4 \cdot 7H_20$	0.0005

small amounts, can affect downstream processes as they tend to foul membranes and chromatography matrices.

Culture media for yeast are similar to those used for *E. coli*. Methanol is used frequently used as the inducer for systems based on the alcohol oxidase promoter (AOX) expression system. Mammalian cell culture, on the other hand, requires much more complex media including glucose as carbon source, amino acids, vitamins, inorganic salts, fatty acids, nucleotides, pyruvate, and butyrate. This basal medium is supplemented with proteins for oxygen transport, hormones, and growth factors. Oxygen transport proteins such as transferrin have bound iron. In order to create a totally protein-free medium, these proteins are often replaced by iron chelators such as ferric citrate, ferric iminodiacetic acid, ferric ammonium citrate, and tropolone (2-hydroxy-2,4,6-cycloheptatriene-1-one). These compounds can, however, interfere with downstream processing. For example, under slightly acidic conditions, ferric citrate forms a gel, which is difficult to separate from proteins and other biomacromolecules.

Several other additives that may be present in cell culture media also impact downstream processing. pH indicators, such as phenol red, added to laboratory-scale culture media often bind to ion exchange resins and are best avoided for large-scale cultivation. Hydrophilic polymers, such as poly(propylene glycol) or poly(ethylene glycol), are often needed in concentrations up to 0.02% to protect the cells from shear stress.

pH in mammalian cell culture is typically regulated by addition of CO_2, although high-density cultures may require addition of NaOH. Final conductivity values of less than 17 mS/cm are typical, making direct capture by ion exchange easier compared to capture from yeast and *E. coli* homogenates.

1.3.4 Components of the Culture Broth

In general, before harvest, the culture broth contains the following components: intact cells, debris from lysed cells, intracellular host cell components, unused media components, compounds secreted by the cell, and enzymatically or chemically converted media components. Oxygen is depleted because during primary

recovery, the oxygen supply is shut down and the residual dissolved oxygen is rapidly consumed. The low oxygen content can induce necrosis, and cells may rapidly die and lyse during this phase. Some cell types begin autolysis after just 30 minutes without oxygen. As a result, depending on the cell type, rapid separation of the cells from the broth supernatant is necessary to keep host cell impurities low. The culture broth may contain high concentrations of CO_2, which is produced by the residual cells and fragments and shifts pH to acidic region. CO_2 has a much higher solubility than oxygen in aqueous solutions, so that substantial amounts can be present. Dissolved CO_2 can be rapidly liberated when the pH is lowered to conduct certain downstream processing steps, thereby forming bubbles that may then enter chromatography columns and disrupt the packing.

Intracellular host cell components appearing as impurities in a culture supernatant can be estimated from the following equation:

$$\begin{pmatrix}\text{Impurity}\\ \text{concentration}\end{pmatrix} = \begin{pmatrix}1 - \text{Fraction of}\\ \text{viable cells}\end{pmatrix} \times \begin{pmatrix}\text{Cell}\\ \text{count}\end{pmatrix} \times \begin{pmatrix}\text{Amount of}\\ \text{impurity per cell}\end{pmatrix}$$

where the amount of intracellular impurity components per cell can be estimated from Tables 1.13 and 1.14. Thus, from a downstream processing perspective, high cell viability is desirable. This is not always possible, however. For example, in high-titer antibody production by cell culture, the cells are often lysed in the final stage of cultivation. As a result, mammalian cell culture supernatants from cultivation with defined media contain host cell proteins in the range of 1–3 mg/ml (1000–3000 ppm). These levels must be reduced to less than 100 ppm in the final product.

1.3.5 Product Quality Requirements

Biopharmaceutical product quality and process validation are subject to regulations by the individual governments. In the United States, the regulatory framework is published in the Code of Federal Regulations 21 (21 CFR), Subchapter F Biologics. The US FDA is responsible for its implementation. In the European Union, the regulatory framework is still under the sovereignty of the individual member states, although an EU-wide umbrella organization, the European Medical Agency (EMA), has been founded with the goal of harmonizing the EU regulatory structure. The existence of multiple regulatory frameworks adds complexity to the global biopharmaceutical industry. Thus, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (https://www.ich.org/home.html) has been established to develop a common international regulatory framework. ICH brings together the regulatory authorities of Europe, Japan, and the United States, as well as experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration. The purpose is to make recommendations on ways to achieve greater consistency in the interpretation and application of technical guidelines and of the requirements for product registration. Although progress is being made in the harmonization process, the regulatory framework of each country remains in effect.

The guidelines ICH Q5B (Analysis of the expression construct in cell lines used for production of rDNA-derived protein products), ICH Q5D (Derivation and characterization of cell substrates used for production of biotechnological/biological products Share), and ICH Q6B (Specifications: test procedures and acceptance criteria for biotechnological/biological products) regulate the medicinal products derived from recombinant DNA.

The following Categories of Therapeutic Biological Products are reviewed and regulated by the Center for Drug Evaluation and Research (CDER), a suborganization of the US FDA:

- Monoclonal antibodies for in vivo use.
- Proteins intended for therapeutic use, including cytokines (e.g. interferon), enzymes (e.g. thrombolytics), and other novel proteins, except for those that are specifically assigned to Center for Biologics Evaluation and Research (CBER, e.g. vaccines and blood products). This category includes therapeutic proteins derived from plants, animals, or microorganisms and recombinant versions of these products.
- Immunomodulators (nonvaccine and nonallergenic products intended to treat disease by inhibiting or modifying a pre-existing immune response).
- Growth factors, cytokines, and monoclonal antibodies intended to mobilize, stimulate, decrease, or otherwise alter the production of hematopoietic cells in vivo

The following categories of therapeutic biological products are defined and regulated by the Center for Biologics Evaluation and Research (CBER), also a suborganization of the US FDA:

- Cellular products, including products composed of human, bacterial, or animal cells
- Gene therapy products
- Vaccines
- Allergenic extracts used for the diagnosis and treatment of allergic diseases and allergen patch tests.
- Antitoxins, antivenins, and venoms
- Blood, blood components, plasma-derived products (for example, albumin, immunoglobulins, clotting factors, fibrin sealants, and proteinase inhibitors), including recombinant and transgenic versions of plasma derivatives (for example clotting factors), blood substitutes, plasma volume expanders, human or animal polyclonal antibody preparations including radiolabeled or conjugated forms, and certain fibrinolytics such as plasma-derived plasmin, and red cell reagents.

It is possible that certain recombinant protein products will fall under the regulatory oversight of multiple regulatory bodies simultaneously resulting in further complexities.

The manufacture of biological products for pharmaceutical applications must follow general guidelines that are established by the regulatory framework. Three keywords summarize the principal product quality requirements: *purity*, *potency*, and *consistency*. Industrially, these requirements must be met with

processes that are economically viable and that can bring products to the market rapidly. Downstream processes must be designed to obtain sufficient purity while maintaining the potency or pharmacological activity in a consistent manner.

1.3.5.1 Types of Impurities

The purity requirements of therapeutic proteins are defined by ICH guidelines. Purity requirements for biopharmaceuticals vary depending on the particular application, dose, and cell line used in the manufacturing process. Thus, it is not possible to specify absolute values. In the ICH guideline ICH Q6B, a distinction is made between *process-related impurities* and *product-related impurities*. Process-related impurities are derived from the manufacturing process, from the cell substrates, from the cell culture components, and from the downstream processing. Examples are host cell proteins, host cell DNA, inducers, antibiotics, and other media components, as well as ligands or other chemicals leached from chromatography media. Product-related impurities are defined as molecular variants arising during manufacture and/or storage, which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety. Examples are precursors, certain degradation products, aberrant glycoforms, and aggregates. An important distinction can be made, however, among the different types of impurities between *critical impurities* or *noncritical impurities*. This is determined during process development by risk analysis studies. A noncritical impurity is an inert compound without biological relevance. This can be, for instance, residual PEG from an extraction process or a harmless host cell component such as a lipid. On the other hand, endotoxins or growth factors secreted into the culture supernatant are examples of critical impurities, as they can exert adverse biological activity. These impurities have to be traced throughout the process and extensive testing and documentation of their removal is generally required.

Contaminants in a bioproduct include all adventitiously introduced materials not intended to be part of the manufacturing process, such as microbial proteases and/or microbial species. Contaminants should be strictly avoided and/or suitably controlled with appropriate in-process acceptance criteria or *action limits* for drug substance or drug product specifications (see ICH guideline Q6B). For adventitious virus, mycoplasma, or prion contamination, the concept of the action limit is not applicable. For this case, the strategies proposed in ICH guidance documents Q5A (Quality of Biotechnological/Biological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin) and Q5D (Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products) should be considered. The starting material should be preferably free of the agent. Spiking experiments at small scale must be conducted to demonstrate clearance and their presence in final product must be strictly controlled. Finally, bioburden, originating from microbial contamination from air or personnel or from inadequately cleaned equipment, can also have serious effects and must be carefully monitored and controlled.

Table 1.16 summarizes the measures required to demonstrate the removal of virus, mycoplasma, prions, and other impurities. This demonstration is

Table 1.16 Measures typically required to demonstrate the removal of adventitious virus, mycoplasma, prions contaminants, and impurities.

Measure	Virus, mycoplasma, prions	Other impurities
Spiking experiments to demonstrate clearance	Yes	No
Starting material preferably free of agent	Yes	No
Clearance measured at each step	No	Yes
Control of final product	Yes	Yes

Table 1.17 Typical virus clearance values of retroviridae virus for purification of recombinant antibody (LRV).

Step	LRV	Minimum LRV	Maximum LRV
Protein A affinity chromatography	5	2	8
Low pH chemical inactivation	5	2	8
Cation exchange chromatography in bind elute mode	3	1	7
Anion exchange chromatography in flow-through mode	5	2	8
Virus filter	5	2	8

Minimum LRV and Maximum LRV define the range of LRV values reported in regulatory submissions.
Source: Data from Miesegaes et al. 2010 [42].

usually done experimentally using scale-down models because, obviously, it would counterproductive to intentionally contaminate the production plant with an adventitious agent. For these determinations, also known as spiking experiments, a bolus of an adventitious agent, e.g. a virus, is added to the raw feed stream entering a purification process step. The virus titer before purification $a' = \log_{10}(\text{Feed titer})$ and after purification $a'' = \log_{10}(\text{Harvest titer})$ is determined and the log-virus reduction factor (LVR) is calculated as follows:

$$\text{LVR} = \log_{10}(\text{Feed titer}) - \log_{10}(\text{Harvest titer}) = a' - a'' \tag{1.31}$$

In order to account for the effect of volume changes (e.g. a mere 1 : 10 dilution results in a LVR of 1), the following individual reduction factor, R_i, is also calculated:

$$R_i = \text{LVR} - \log_{10}\frac{V'}{V''} \tag{1.32}$$

where V' and V'' are the feed and harvest volumes, respectively. Finally, the LVR of the individual process steps are added together to arrive at a cumulative LVR for the entire process. Table 1.17 illustrates a typical virus clearance levels for various steps of an antibody purification process.

Although almost all mammalian cells are infected with virus making viral clearance validation obligatory, efforts are frequently made to omit these procedures by utilizing platform processes that have demonstrated clearance efficiency.

Table 1.18 Examples of input and output parameters in a chromatographic separation process of proteins.

	Parameter							
Input parameter	pH	Temperature	Ionic strength	Load	Flow rate	Column height (residence time)		
Output parameter (performance)	Purity	Concentration	Stability	Yield	DNA content	Host cell protein content	Endotoxin content	Column back pressure

1.3.5.2 Validation

Validation is a critical aspect of biopharmaceutical process development. According to existing ICH definitions, critical parameters that may influence product quality have to be validated. After validation, a standard operating protocol (SOP) is established, which describes the process and the allowed variations. *Critical operational parameters* are defined as a limited subset of process parameters that significantly affect critical product quality attributes when varied outside a meaningful, narrow (or difficult to control) operational range. Consider, for example, the operation of a chromatographic purification step. As shown in Table 1.18, this operation will require the definition of a number of operating conditions as inputs, which, in turn, will result in certain performance characteristics as outputs.

In order to validate the process, the input parameters must be varied over suitable ranges and the corresponding outputs measured. The critical parameters are then defined based on these experiments, which are usually performed at small scale. Suitably narrow operational ranges are established for these parameters as well as for noncritical parameters. As the latter do not affect critical product quality attributes, their ranges will normally be broader than those for critical parameters (see Figure 1.29).

Quality by design (QbD) is a global regulatory initiative having the goal of enhancing pharmaceutical development through the proactive design of pharmaceutical manufacturing process and controls that consistently deliver the intended product characteristics. The "*design space*," a critically important concept in QbD, is defined as the range of conditions under which the process can be operated while maintaining the desired product quality. Although there is no regulatory requirement to have a design space, it is very commonly used to set operating limits. A design space can be described in terms of ranges of material attributes and process parameters. The design space can also be described through a complex mathematical model. The design space can be determined by the following:

- First-principles approach, which is a combination of experimental data and mechanistic knowledge of chemistry, physics, biology, and engineering to model and predict performance.
- Statistically designed experiment such as design of experiments (DOE).

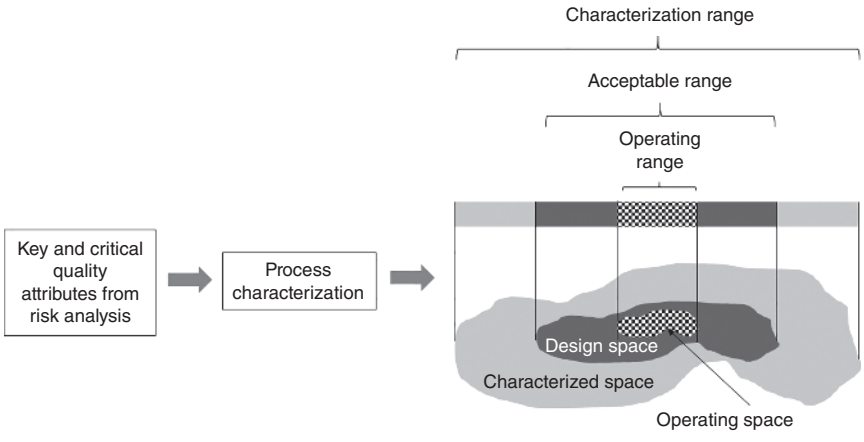

Figure 1.29 Definition of operation ranges for critical process parameters.

- Scale-up correlations, which are semiempirical approaches to translate operating conditions between different scales or pieces of equipment.

A design space allows more operational flexibility and is highly encouraged by health authorities.

Process analytical technologies (PAT) concepts are required to ensure consistent quality of the biopharmaceutical. PAT is a system for designing, analyzing, and controlling processing through timely measurements of critical quality and performance attributes of raw materials, in-process streams, and process parameters with the goals of ensuring final product quality.

1.3.5.3 Purity Requirements

It is difficult to describe absolute purity requirements as they depend on the intended use of the biopharmaceutical, dose, and risk–benefit ratio. Table 1.19 provides only approximate values meant to serve as general guidelines.

Aggregates are an important concern for many biopharmaceutical proteins. It has been shown that aggregates can induce immune reactions or cause other side effects. Moreover, aggregates may constitute seeds for precipitation, thereby reducing the shelf life of a product. As a result, controls on the percentage of dimers, oligomers, and higher aggregate forms have been tightened and the maximum allowed values reduced. The leakage of ligands or other leachable chemicals from chromatographic media and membranes is also an important concern because these materials can be immunogenic or toxic.

Viral contamination is obviously a critical issue as in the past it has been responsible for many iatrogenic diseases, such as those occurring because of contaminated blood products. As absolutely complete removal of these adventitious agents is not possible, limits are often established on the basis of a risk–benefit analysis. For example, the World Health Organization (WHO) accepts for a vaccine one adverse case in 10^9 applications – hence, the probability value of 10^{-9} value suggested by Table 1.19. More recently, limits on the allowed amount of host cell DNA per dose have been relaxed from 10 pg per dose to

Table 1.19 General guidelines for purity, consistency, and potency of protein biopharmaceuticals.

Criteria	Requirement
Purity	
Specific protein content	>99.9%
Dimer/oligomer content	<1.0%
Ligand leakage	Usually <1 ppm
Virus content	Absence with a probability of $<10^{-9}$
DNA content	<10 ng/dose
Endotoxin content	<5 EU/(kg h)
Prion content	Absence with a probability of $<10^{-9}$
Consistency	
Microheterogeneity	Permitted, but consistent
Impurities	Permitted, but consistent
Potency	
Folding	Correctly folded
Mutations	Correctly expressed, no mutations
Processing	Correctly processed

10 ng of DNA per dose. Clinical practice and postmarket studies have shown that, in general, host cell DNA does not pose a high risk, except for some potent compounds such as growth factors or hormonally active compounds.

Many biopharmaceuticals do not consist of individual molecular entities – rather, they consist of a large number of similar isoforms or variants (some recombinant antibody products contain as many as 2000 identifiable variants). Because the biological and pharmacological activity can vary dramatically among different isoforms, it is important to maintain the distribution of these variants within established acceptable ranges. Because of the complexity of bioproduction systems, similar consistency must also be maintained for the impurity profiles, as determined from analytical assays, in order to assure product safety. Finally, test systems must be established to control the potency in vitro and, where necessary, in vivo.

1.4 Biosimilars

A *biosimilar* is a product that is similar in terms of quality, safety, and efficacy to an already licensed reference biopharmaceutical defined as the "originator product." Most protein biopharmaceuticals comprise a high number of molecular entities or variants. These molecular entities are dependent on the primary sequence of the protein, on the nature of the expression system, and on the production process. Even minute variations on the production parameters and

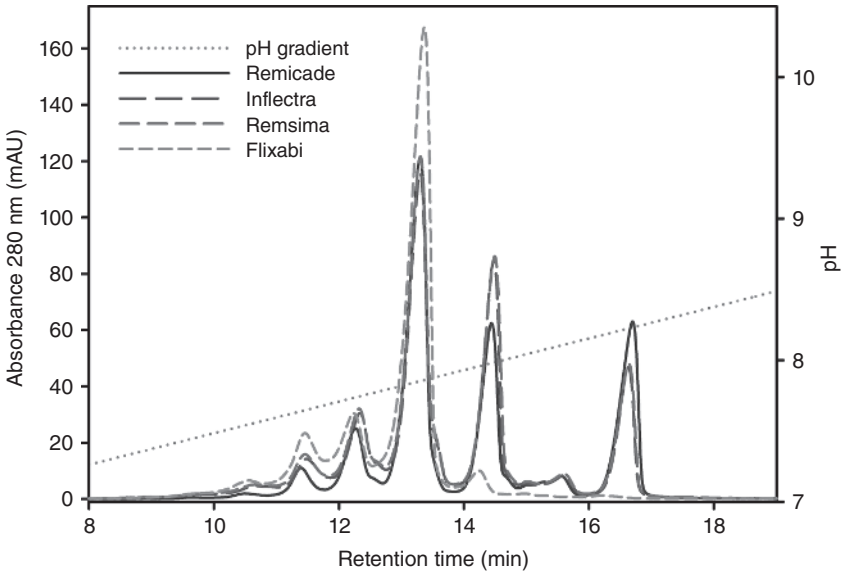

Figure 1.30 Example of differences in variant composition between originator product and various biosimilars. The variants in each product were separated by analytical cation exchange chromatography with a linear pH gradient. The originator antibody Remicade is compared to the three anti-TNF-α biosimilar antibodies Remsima, Inflectra, and Flixabi. Source: Adapted from Beyer et al. 2019 [43].

materials can affect the composition of product variants. Therefore, it is usually not possible to develop a perfect copy of a biopharmaceutical (see Figure 1.30 as an example). The most important goal is thus to achieve similarity with respect to clinical properties.

1.5 Role of Chromatography in Downstream Processing

Chromatography is the principal tool for the purification of biopharmaceuticals. This can be explained by certain advantages of chromatography over other unit operations. Firstly, chromatography provides very high separation efficiencies, which allow the resolution of complex mixtures of components having very similar molecular properties. Properly designed chromatography columns can have the separation efficiency of hundreds or even thousands of theoretical plates. By comparison, extraction and membrane filtration are usually limited to only a few theoretical plates. Secondly, chromatography columns packed with high capacity adsorbents are ideal for capture from the dilute solutions found in bioprocessing. In such systems, a large volume of solution can be contacted efficiently with a small amount of a high-capacity adsorbent packed in a column, resulting either in the rapid concentration of the product. When operated in a flow-through mode, such columns can result in the nearly complete removal of contaminants present in small concentrations without affecting the unbound product. By comparison, liquid–liquid extraction systems typically require similar volumes of the two

phases in order to function properly, so that concentration is not very feasible. A further advantage is that chromatography can be performed in an almost closed system and the stationary phase can be easily regenerated. Finally, chromatography is well established in many practical biopharmaceutical manufacturing processes, and suitable equipment and packing materials are readily available. A perceived disadvantage of chromatography is the difficulty of scale-up within the constraints of the biopharmaceutical industry. However, as will be shown in the remaining chapters of this book, proper application of engineering tools in combination with adequate measurements allows the design of optimum columns for large-scale applications. Indeed, as shown by Kelley [3], chromatographic purification processes can be considered and can be technically and economically viable for protein purification at scales as high as 20 tons/yr. Although no current product is currently made at such a large scale, the popularity of biopharmaceuticals is increasing rapidly so that one could envision such scales in the future.

Figure 1.31 illustrates the structure of a generic process for the recovery and purification of a biological product produced by microbial fermentation or animal cell culture. The initial steps where cells are separated are often referred to as *primary recovery*. These steps require different strategies depending on whether the product is secreted into the culture medium or expressed in the cell, either as inclusion body, in soluble form in the cytosol or periplasm, or anchored in the membrane. Generally, chromatography plays a minor role in these initial steps, which are focused on the removal of suspended solids such as cells or cell debris. Sedimentation, centrifugation, deep bed filtration, and microfiltration or combinations thereof are normally used for these early steps. However, chromatography, implemented through the use of fluidized or expanded beds, can also be used for the direct capture of secreted proteins from cell culture supernatants.

In these systems, the liquid flows upward through an initially settled bed of dense adsorbent particles. Above a certain flow velocity, the bed expands and the particles become fluidized allowing free passage of cells and other suspended matter while the product is directly captured by the adsorbent. The approach can be effective for dilute suspensions. However, as bed expansion is directly influenced by the feed density and viscosity, the operation tends to be critically affected by variations in the composition of the broth. In practice, the high viscosity and cell density encountered in modern fermentation technology (up to 400 mg/ml wet cell mass for *P. pastoris* or 200 mg/ml for *E. coli*) make it difficult to implement this approach reliably at the industrial scale. An alternative possibility for early capture without clarification is to use adsorption beds packed with large particles, sometimes referred to as "big beads." If the particles are larger than about 400 μm in diameter, the interparticle spaces are sufficiently large to allow passage of small cells and cell debris. Although the efficiency of capture is reduced by the diffusional limitations that accompany the larger particle diameter, the ensuing reduction in the number of processing steps can provide overall economic and operational advantages. Unlike expanded beds, packed bed processes are not very sensitive to feed viscosity so that reliable operation with large diameter beads can be achieved even with viscous feedstocks.

As can be seen in Figure 1.31, following primary recovery, the general downstream processing scheme consists of successive *capture*, *purification*,

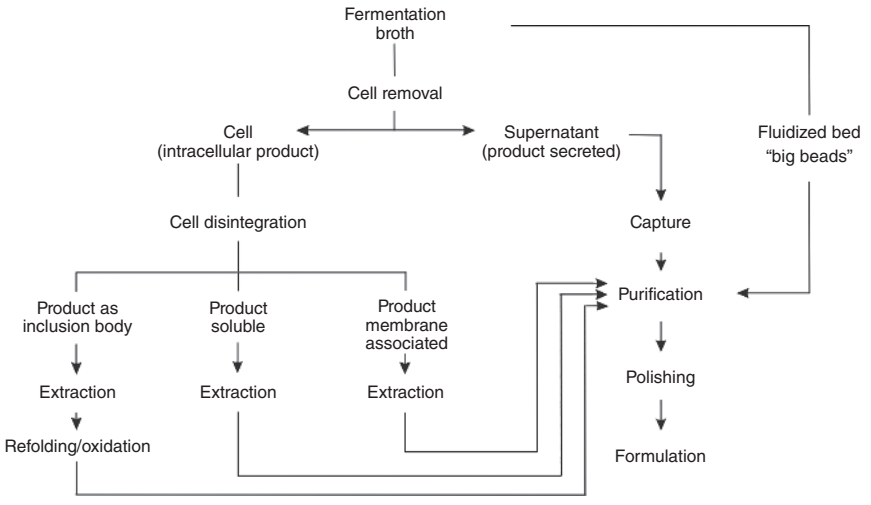

Figure 1.31 Generalized downstream processing flow sheet for purification of proteins starting with the unclarified fermentation broth. Note: "big beads" are large-size (400–500 μm diameter) chromatography media used to directly capture small proteins from viscous solutions that could contain some particulate matter.

and *polishing* steps, each comprising one or more unit operations. With only a handful of exceptions, current industrial processes for biopharmaceuticals almost exclusively employ chromatography for these three critical steps.

For purification of recombinant antibodies, capture is almost always realized using a selective adsorbent comprising Staphylococcal Protein A immobilized in porous beads (see Figure 1.32). This highly selective ligand allows direct loading of the clarified culture broth on the capture column, which binds selectively the antibody. In the subsequent steps, purification and polishing are conducted with ion exchange and hydrophobic interaction columns to remove host cell proteins and aberrant protein variants. Note that intermediate, nonchromatographic steps are also included. Firstly, incubation at low pH for virus inactivation and then "virus filtration" are implemented for viral clearance. Secondly, an ultrafiltration/diafiltration step is included for buffer exchange and final formulation.

Besides purification, chromatography also finds other uses in bioprocessing. An important example is the use of chromatography to facilitate refolding of solubilized protein, which is sometimes a bottleneck in industrial processes. Without simultaneous separation, misfolding and, especially, aggregation compete with the correct folding pathway. Aggregation may originate both from nonspecific (hydrophobic) interactions of predominantly unfolded polypeptide chains as well as from incorrect interactions of partially structured folding intermediates. As can be seen in Figure 1.33, aggregation reactions are second- (or higher) order processes, whereas correct folding is generally determined by first-order reactions [44].

In practice, refolding conditions (e.g. denaturant concentration) are adjusted so that the equilibrium distribution favors the formation of native protein (i.e. $k_2 \gg k_3$). The formation of intermediates is generally very fast so that k_1 can be

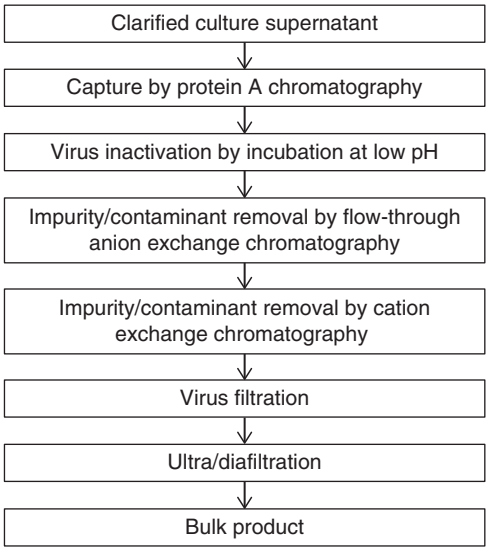

Figure 1.32 Example of a process flow diagram for the purification of recombinant antibodies.

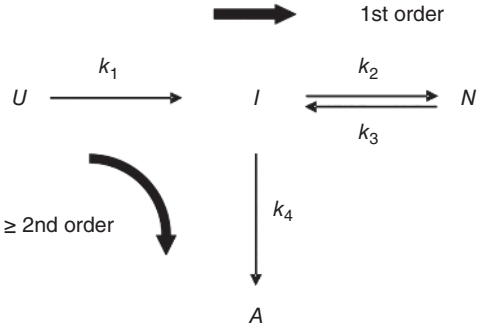

Figure 1.33 Simplified reaction scheme for protein refolding with aggregation of intermediates.

neglected. For the case where $k_3 \rightarrow 0$, we effectively have competing first- and second (or higher)-order reaction. For these conditions, refolding in a batch system is described by the following equations:

$$\frac{d[U]}{dt} = -(k_2[U] + k_4[U]^n) \tag{1.33}$$

$$\frac{d[N]}{dt} = k_2[U] \tag{1.34}$$

where the brackets denote concentrations, k_2 is the net rate constant of folding, k_4 is the net rate constant of aggregation, and n is the reaction order. An analytical solution of these equations is available for $n = 2$ and is given by the following equation [45]:

$$Y(t) = \frac{k_2}{[U]_0 k_4} \ln\left\{1 + \frac{[U]_0 k_4}{k_2}(1 - e^{-k_2 t})\right\} \tag{1.35}$$

where Y is the yield of the refolding reaction and $[U]_0$ is the initial concentration of unfolded protein. As time approaches infinity, the final yield of native protein

Table 1.20 Refolding and aggregation rate constants of a protein in refolding by batch dilution and matrix-assisted refolding using size exclusion chromatography.

Process	Folding k_2 (min^{-1})	Aggregation k_4 (ml/(mg min))
Batch dilution	0.0012	0.3
Matrix-assisted refolding by SEC	0.0012	0.01

Source: Data from Schlegl et al. 2005 [46].

is then given by the following equation:

$$Y(t \to \infty) = \frac{k_2}{[U]_0 k_4} \ln\left(1 + \frac{[U]_0 k_4}{k_2}\right) \tag{1.36}$$

This result suggests that dilution (i.e. low $[U]_0$) is a simple and effective way of ensuring high refolding yields. Although this is effective and widely used in practice, the ensuing large solution volumes complicate further downstream processing and increase cost. Refolding in chromatographic columns, also known as matrix-assisted refolding, can be a valuable refolding alternative to reduce the need for extensive dilution. The underlying mechanism leading to improved folding in chromatographic columns is not completely understood and may depend on the specific nature of the protein and the selected conditions. However, the effects can be dramatic as shown, for example, in Figure 1.33. In this case, refolding was conducted by separating the denaturing agent (urea) from the unfolded protein by SEC, thereby allowing refolding to occur within the column. This resulted in a greater yield of folded protein compared to a simple dilution process. The apparent aggregation rate constant in this case was about 30 times smaller compared to that for the dilution process (Table 1.20). A possible explanation of this result is that aggregation may be inhibited within the matrix pores by steric hindrance allowing a greater portion of the protein can follow the path toward correct folding.

In the example given in Figure 1.34, the unfolded protein was passed over a size exclusion column and the denaturant was slowly removed. Comparison of kinetic constants between conventional refolding by dilution into a refolding buffer and matrix-assisted refolding confirms that aggregation is suppressed in the column (Table 1.20).

SEC-promoted refolding is also possible in continuous processes, which can also include a recycling system for aggregated protein. Yield and productivity of a continuous refolding system using pressurized continuous annular chromatography (P-CAC) considering initial protein concentration, residence time, and recycling rate were extensively studied for α-lactalbumin as a model protein [47]. Also, countercurrent chromatography systems such as the simulated moving bed (SMB) can be used for continuous matrix-assisted refolding.

Ion exchange, affinity adsorption, and hydrophobic interaction have also been used to facilitate refolding. A method based on the adsorption of the unfolded protein on an ion exchange resin was introduced by Creighton [48].

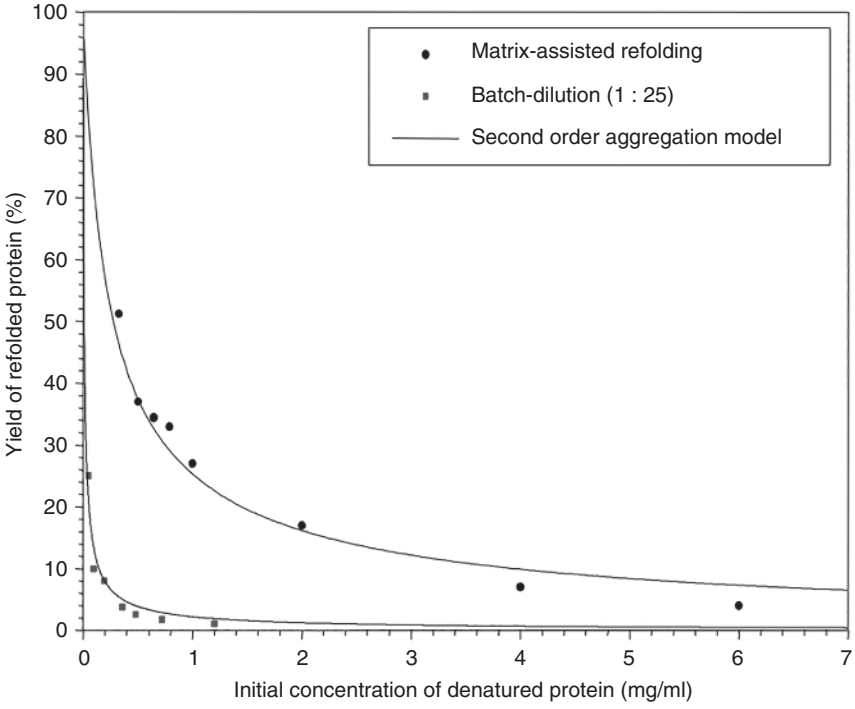

Figure 1.34 Refolding yield of a protein by batch dilution and with matrix-assisted refolding using size exclusion chromatography. Source: Adapted from Schlegl et al. 2005 [46].

Further improvements of this method rely on using more sophisticated buffers during loading and elution. The methods can also be executed in a continuous manner. The surface contact can initiate refolding. In many instances, it has been observed that final refolding takes place after the protein has been eluted from the column. Immobilization of denatured proteins on a solid support often limits its flexibility and therefore its ability to regain the native configuration because of multipoint interaction with the matrix. Introduction of an N- or C-terminal poly-histidine-tag allowed the reversible one-point immobilization of the denatured protein on a solid support based on immobilized metal affinity chromatography (IMAC). Refolding can be achieved by a simple buffer exchange in a stepwise or gradient manner. The use of HIC was described for the refolding of lysozyme, BSA, α-amylase, and recombinant γ-interferon [49].

Immobilized folding catalysts and artificial chaperones have also been suggested as refolding aids. Mimicking in vivo folding systems was a further step of improving in vitro refolding yield. The chaperones or compounds mimicking chaperones are immobilized on a chromatography matrix. The protein solution is passed through such columns. The folded proteins are slightly retarded, and the denaturant is exchanged. The immobilized chaperones prevent aggregation. Thus, a refolding can be achieved at higher concentrations or yield. One has to keep in mind that a chaperone acts in a stoichiometric manner. Thus, large amounts of chaperone protein are necessary to avoid aggregation.

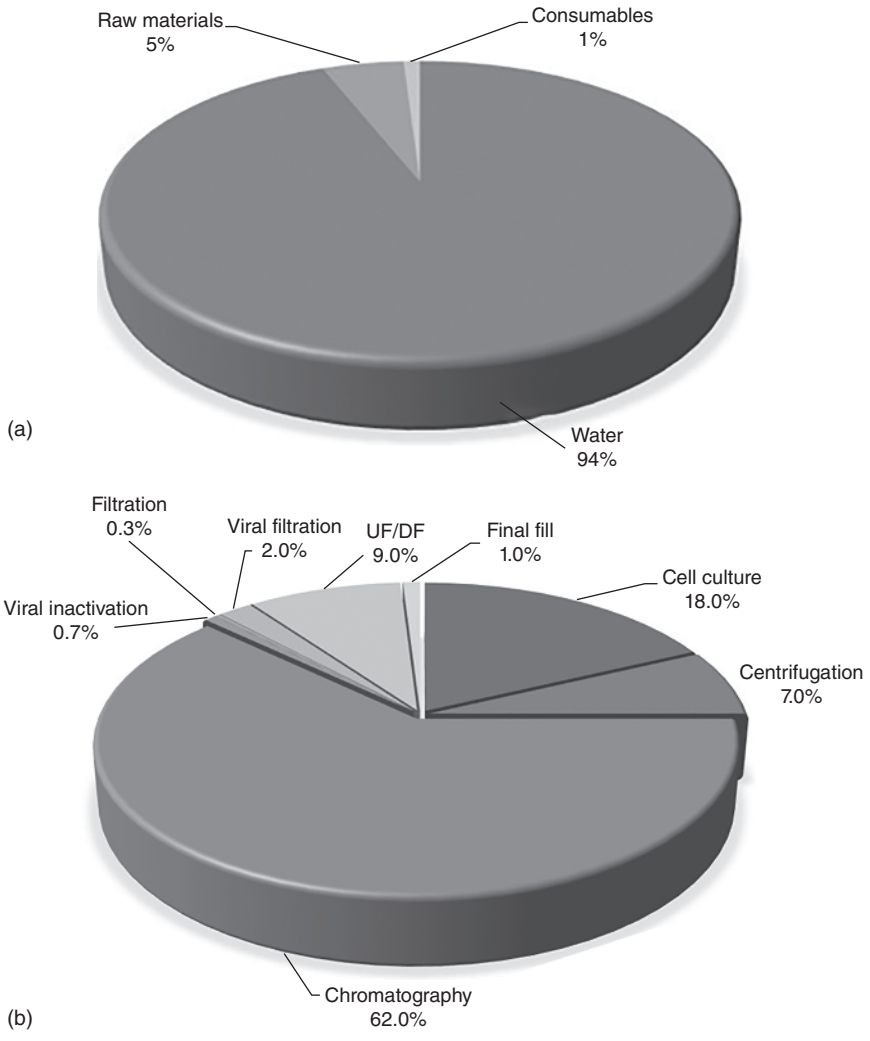

Figure 1.35 (a) Main contributions to the process mass intensity for monoclonal antibody manufacturing. The PMI metric was determined from 14 process datasets from biopharmaceutical firms for both large-scale (≥12 000 l) and small-scale (≤5000 l) antibody manufacturing operations. (b) Percentage of total water use by each unit operation. Source: Adapted from Budzinski et al. 2019 [50].

1.6 Environmental Impact of Biopharmaceutical Manufacturing

The *environmental footprint of biopharmaceutical manufacturing* has been largely neglected in the past. However, water scarcity is increasingly being recognized as a major future threat to business. Especially in arid areas, biopharmaceutical manufacturing may become very expensive because of the large amounts of water used by both upstream and downstream processes.

Additionally, pharmaceuticals have become pervasive in the environment, which poses a risk for people and animals. In response to these pressures, the pharmaceutical industry is focusing increasingly on the environmental impact of pharmaceutical and biopharmaceutical manufacturing aiming to improve the efficiency of its operations. In addition to reducing the environmental impact, processes with a reduced environmental footprint also tend to be more economic. The process mass intensity (PMI), already in use in other industrial sectors, has been proposed as a standardized metric to assess environmental impact in pharmaceutical manufacturing. Such a metric is independent of fluctuations of raw material, water, and energy costs. Total PMI is defined based on the amount of active pharmaceutical ingredient (API) produced as follows:

$$\text{Total PMI} = \frac{\text{Total water, raw materials, and consumables used in the process (kg)}}{\text{Amount of API produced (kg)}}$$

As shown in Figure 1.35 for a typical biopharmaceutical manufacturing process producing a recombinant antibody, chromatography is the greatest contributor to the PMI, especially in terms of the amount of water consumed. There are ample opportunities to optimize chromatography with respect to water consumption including maximizing the utilization of binding capacity in processes, and thus reducing the consumption of elution buffers, implementing process intensification by using smaller, more efficient separation columns that can be washed, eluted, and cleaned with smaller amounts of aqueous buffers, and by implementing process integration, thereby reducing or eliminating intermediate steps, such as diafiltration or dilution, which can consume large amounts of buffers. The engineering fundamentals covered in this book provide, we believe, not only opportunities to maximize productivity and reducing downstream processing costs but also have the potential to help reduce the environmental impact of chromatography processes contributing to our quest for sustainability and to creating environmentally friendly production processes.

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