Current Perspectives on Stability of Protein Drug Products during Formulation, Fill and Finish Operations

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Commercialization of protein-based therapeutics is a challenging task in part due to the difficulties in maintaining protein solutions safe and efficacious throughout the drug product development process, storage, transportation and patient administration. Bulk drug substance goes through a series of formulation, fill and finish operations to provide the final dosage form in the desired formulation and container or delivery device. Different process parameters during each of these operations can affect the purity, activity and efficacy of the final product. Common protein degradation pathways and the various physical and chemical factors that can induce such reactions have been extensively studied for years. This review presents an overview of the various formulation-fill-finish operations with a focus on processing steps and conditions that can impact product quality. Various manufacturing operations including bulk freeze−thaw, formulation, filtration, filling, lyophilization, inspection, labeling, packaging, storage, transport and delivery have been reviewed. The article highlights our present day understanding of protein instability issues during biopharmaceutical manufacturing and provides guidance on process considerations that can help alleviate these concerns.

Introduction

The term "formulation, fill and finish" refers to the series of processing steps that are needed to turn a purified drug substance into the final dosage form, the finished product, for the market (*1*). The formulation step involves taking the purified protein at the desired concentration and dispensing it with the correct excipients that can ensure product quality and integrity during the subsequent fill/finish steps including filtration, filling, lyophilization, packaging, storage, transport and delivery. A robust formulation would need to keep the biopharmaceuticals stable not only during shelf storage but also during these manufacturing steps. At the same time, key operating and process parameters should be optimized to obtain a robust manufacturing process. The problems for protein therapeutics could be very different from the traditional small-molecule pharmaceutical processing and may require special handling and storage conditions to ensure product quality (*2*, *3*). For instance, protein thermal instability is one of the main reasons why protein drugs need to be maintained under cold temperatures during storage and transport to achieve longer shelf life. Similarly, other stresses such as photo exposure and mechanical agitation could also impact the stability of protein products.

Proteins are large macromolecules made up of a sequence of amino acids and characterized by a unique three-dimensional structure corresponding to their biologically active state. The native structure of a protein molecule is the result of a fine balance among various interactions including covalent linkages, hydrophobic interactions, electrostatic interactions, hydrogen bonding and van der Waals forces. Intraprotein and protein−solvent interactions both play an important role in maintaining the protein structure and its stability. The free energy of unfolding has been generally reported to be quite small, in the range of 21−63 kJ/mol (*2*). Since the folded state of protein is only marginally more stable than the unfolded state, any change in the protein environment may trigger protein degradation or inactivation.

The degradation pathways for protein therapeutics are many and often complex. Simplistically speaking, these pathways can be divided into two categories: physical degradations, which do not involve covalent bond modifications, and covalent modifications (for reviews, see refs 1−5). Physical degradations are most commonly manifested by protein aggregation. This type of degradation involves assembly of monomeric units of proteins, and dimerization is a common occurrence within these set of events (*6*). Higher order protein oligomers are often referred to as "high molecular weight species" or protein aggregates (*4*−*8*). These protein aggregates can be either soluble or insoluble. Recent evidence has suggested that protein aggregation occurs by a specific association of partially denatured polypeptide chains, as opposed to nonspecific co-aggregation (*7*, *8*). Protein aggregation can be assessed by a variety of techniques, such as size exclusion chromatography, field flow fractionation and analytical ultracentrifugation for soluble aggregates (*9*) and light obscuration/scattering techniques for insoluble aggregates (*10*). A second type of protein degradation is a change in the secondary, tertiary or quaternary structure of the protein, which does not involve protein−protein interactions. Biophysical techniques, such as circular dichroism, FT-IR and fluorescence are usually employed to assess such structural changes. These two degradation pathways can be intimately linked: a change in protein structure often precedes protein aggregation phenomena (*4*). Native aggregates are those in which there is only an assembly of protein monomers without a change in structure, whereas there is a change in protein

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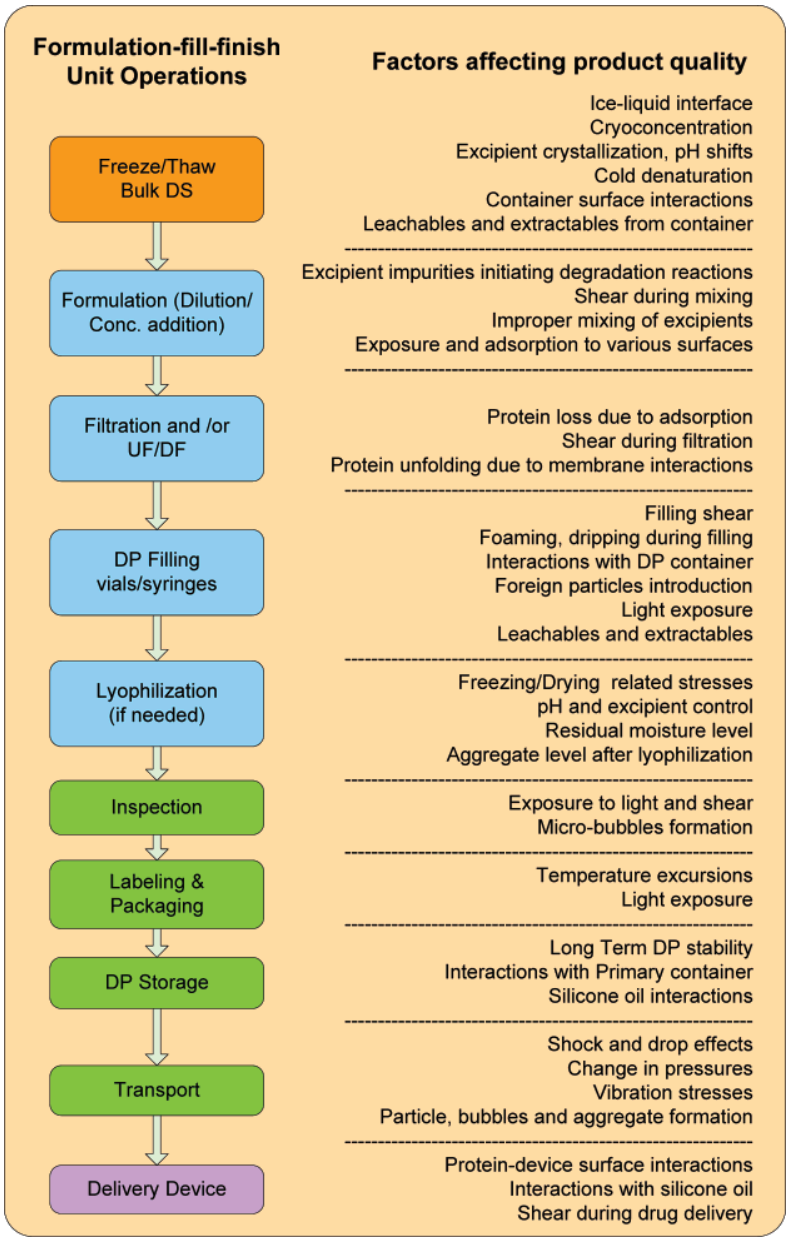

Figure 1. Overview of the several formulation, fill and finish processes and the various factors that can affect product quality during these processing steps

structure (secondary and/or tertiary) in the formation of non-native protein aggregates.

There are several possible covalent modifications in proteins. One common modification is protein fragmentation, which involves the cleavage of a peptide bond. Residue specific modifications include but are not limited to aspartate isomerization, protein oxidation, deamidation, pyroglutamic acid formation and disulfide bond shuffling (*5*). Many times, proteins undergo post-translational modifications, such as glycosylation. Changes in glycosylation patterns under storage and processing conditions are also known to occur (*11*). Further, formulation excipients sometimes have the potential to interact with protein side chains, such as the glycation reaction between reducing sugars and side-chain or N-terminal amino groups (*12*). Covalent degradations that lead to changes in net charge of the protein can be captured by ion exchange chromatography (*13, 14*) and capillary isoelectric focusing (cIEF) (*15*). Weak cation exchange such as peptide mapping, are more comprehensive and have the potential to detect most, if not all, covalent modifications (*13, 16*), but these are more time-consuming and resource intensive.

This review article presents an overview of various formulation, fill and finish operations. The key aspects of processing steps that can affect stability and integrity of a product are discussed. Figure 1 presents the series of operations and various factors that can impact product quality. In the sections that follow, each unit operation is described in detail. The impact of some of the key operating input parameters on protein stability is included, and guidance is provided on scale-down studies needed to evaluate such destabilizing factors. For many fill and finish operations, such as freeze−thaw, mixing, and filtration, the main concern would be physical stability of the protein. However, exposure to light and various manufacturing equipment surfaces could trigger covalent modifications as well.

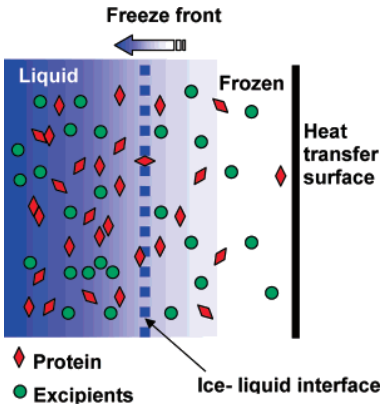

Figure 2. Slow freezing can result in cryoconcentration of proteins and excipients, which can further cause protein aggregation or precipitation. If the freeze front moves slowly, solutes are excluded from the solid−liquid interface, resulting in higher concentration in the regions that freeze later.

determine the overall stability of the molecule to various bioprocessing stresses. In addition to formulation and other unit operations, protein instability issues may arise from interactions of drug product with packaging and components of delivery device. Current understanding of these challenges is included. Photodegradation of light-sensitive products is also discussed in a later section.

Formulation and Fill-Finish Operations

Bulk Freeze−Thaw: Advantages. Bulk freeze−thaw is commonly employed during biopharmaceutical manufacturing to gain operational flexibility while maintaining product quality. A frozen drug substance provides several advantages over liquid storage, including increased product stability, reduced possibility of microbial growth and alleviation of foaming issues during transportation, thereby eliminating the need to perform transport validation. Lowering the temperature to achieve a frozen bulk reduces the rates of degradation reactions and also immobilizes the protein molecule in a frozen matrix, thereby minimizing diffusive collisions that lead to aggregation. Lack of availability of free water also prevents several degradation reactions that are assisted by water, such as peptide bond hydrolysis and aspartic acid isomerization phenomena, further increasing the stability of frozen bulk in comparison to aqueous formulations. This greater assurance in product quality provides flexibility to schedule formulation-fill-finish operations based on the needs of the manufacturing facility. In this scenario, bulk drug substance is stored frozen and when needed is transported to the fill site where it is thawed, initiating a series of formulation and fill/finish steps. The application of freeze−thaw is not limited to storage of drug substance but is also used for the storage of pharmaceutical intermediates and formulated drug products.

Protein Freezing: Stability Challenges. While bulk freeze−thaw offers numerous operational and product quality benefits, it may also prove detrimental to protein stability. Cryoconcentration is one of the common mechanisms through which protein destabilization could occur during freezing (*17*−*19*). As the freeze-front moves during the freezing process (Figure 2), the excipients as well as the proteins get excluded from the ice−liquid interface. As a result the concentration of the liquid bulk (yet to be frozen) close to the ice crystals increases progressively with freezing. Such concentration build up of excipients may

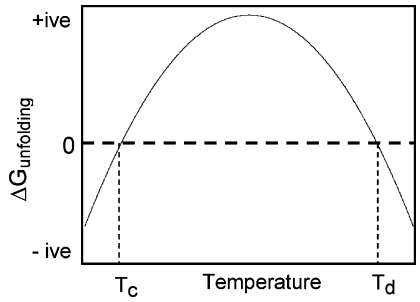

Figure 3. Thermodynamic justification of cold denaturation of protein. The parabolic shape of Gibbs free energy implies that protein unfolding becomes favorable not only at elevated temperatures ($T > T_d$) but also at very cold temperatures ($T < T_c$)

buffer components, which can also result in protein destabilization (*20*). At the same time, increase in protein concentration also increases the possibility of molecular collisions and may result in protein aggregation or precipitation. The extent of cryoconcentration is maximized if the rate of freezing is slow. As a result, uncontrolled freeze−thaw processes, where the freeze front velocities are lower, are impacted to a greater extent by the destabilizing effects of cryoconcentration. One way of minimizing such freeze concentration effects is to reduce the freezing times by increasing the heat transfer from the container. Dendritic ice growth is also preferred in order to minimize cosolute exclusion during freezing. This can be achieved by establishing directional heat flow and avoiding mixing during freezing. Mixing could be detrimental as it would suppress dendritic ice growth, making the ice−liquid interface more flat, and therefore result in increased cryoconcentration.

Proteins could also be susceptible to spontaneous unfolding at cold temperatures, referred to as “cold denaturation” (*21*). This effect is primarily attributed to the weakening of the hydrophobic effect with decrease in temperature. The thermodynamic justification of the cold denaturation temperature can be explained by the parabolic shape of the Gibbs free energy function as shown in Figure 3. A favorable negative free energy of unfolding favors thermal denaturation at higher temperature (T_d). At lower temperatures, it is possible that $\Delta G_{unfolding}$ may become negative again below a certain critical temperature (T_c), resulting in protein unfolding. Such cold-induced denaturation phenomena, though rare, have been reported for certain proteins (*22*, *23*). Further review of this phenomenon can be found in the literature (*21*).

Very fast freezing rates can also prove to be detrimental to proteins (*24*). During freezing, protein molecules can concentrate and get unfolded (*25*, *26*) on the ice−water interface, implying loss in protein activity. When freezing rates are very fast (e.g., submerging container in liquid nitrogen (*17*)), smaller ice crystals are formed and result in a large ice−liquid interfacial area (*25*, *26*). Increased protein aggregation and decreased activity have been reported for liquid-nitrogen-based freezing systems (*27*, *28*). Fast freezing can also trap air that would be released during thawing and may cause protein denaturation on air−liquid interfaces (*29*, *30*).

Thawing Frozen Protein Solutions: Stability Challenges. Frozen bulk needs to be thawed before it can be formulated and processed. Thawing can cause further stress and damage to the protein. Slow thawing rates can result in ice recrystallization with small ice crystals growing into larger ones. Proteins may get denatured at ice−liquid interfaces and lose their activity (*25*, *26*). Cryoconcentration created during freezing can further

Table 1. Comparison of Freeze−Thaw Parameters for Carboy and Celsius Pak

process parameters	carboy (10 L)	Celsius-Pak (16.6 L)
freezing time[a] (h)	17.1 ± 0.9	1.6 ± 0.2
FFV[b] (mm/h)	7.3 ± 0.4	25.5 ± 2.5
thaw time (h)	150 ± 15[c]	2.5 ± 0.5
thaw type	static	dynamic
solution homogeneity after thaw	non-homogeneous	homogeneous

[a] Freeze time refers to time taken by the solution to go from +3 °C to −5 °C. [b] Freeze front velocity [c] Thaw time reported in the table refers to the time needed to thaw 8.5 kg of protein solution in a 10 L carboy at 2−8 °C.

during freezing may be detrimental to proteins, appropriate mixing during thaw is the key to minimize recrystallization and cryoconcentration related effects. Very slow mixing would contribute to longer thaw times and also would not be able to homogenize the solution (concentration gradients from the freeze step would continue to exist and may further increase). On the other hand, very high mixing rates would result in protein shearing, excessive foaming and plausible protein denaturation on the air−liquid interface. The mixing parameters should therefore be optimized to enhance thawing without affecting product quality.

Freeze−Thaw Technologies And Process Scale-Down. The challenges faced during the freeze−thaw process would be dependent on the technology employed for the large-scale process. Most of the stability issues discussed above occur when very slow freeze−thaw rates are applied, which is usually the case for uncontrolled rate technologies (*17*). For example, polycarbonate carboys (10 L and 20 L) are commonly used to freeze and transport bulk drug substance. Freezing is conducted by placing these carboys in walk-in or upright freezers at −30 or −80 °C. Since the path lengths are large and the heat flux is slow, the process times for the freeze−thaw operations could be very long as shown in Table 1. As a result cryoconcentration becomes an important factor governing product quality in these containers. Controlled rate technologies such as Celsius Paks or Cryovessels, on the other hand, can achieve faster freezing and thawing rates by using a combination of small path length and increased flux for heat transfer (*18*). Table 1 shows a comparison of these process times for the uncontrolled rate (carboys) and controlled rate (Celsius Pak) technologies. It has also been shown in literature that the extent of cryoconcentration is minimal for Celsius Paks (*18*).

The effects of bulk freeze−thaw on the product are protein-specific. It may not affect product quality for some protein solutions but may have negative effects on others. As a result, prior to large-scale processing, each product should be evaluated for the impact of multiple freeze−thaw operations on product quality. For early-stage products where product availability may be limited, scaled-down studies can be performed to mimic large-scale freeze−thaw process. For uncontrolled freeze−thaw processes, usually a smaller bottle with (a) surface area to volume ratio similar to that of the large scale and (b) material of construction identical to the large-scale container can be used. Freeze−thaw profiles from large-scale processes can also be mimicked on small scale using a controlled rate freezer. However, certain phenomena, such as cryoconcentration, could be process-scale-dependent and difficult to mimic in a small container. It is usually feasible to mimic the impact of protein−container interactions during freeze−thaw in smaller-scale experiments.

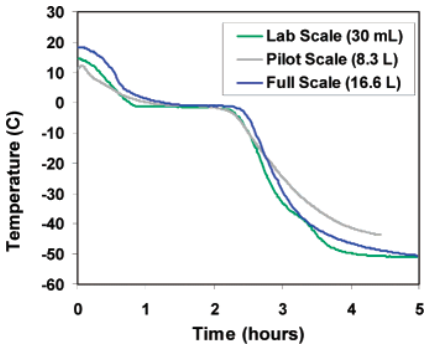

Figure 4. Scalability of controlled rate freeze−thaw as observed for Celsius Pak technology at different scales: 30 mL, 8.3L and 16.6 L.

path length. For example, the path length for 30 mL, 100 mL, 8.3 L and 16.6 L Celsius bags is identical (42 mm) and helps make the process scalable. The heat transfer fluid temperature profile over time can be programmed to achieve the same freeze−thaw profiles at all scales. Figure 4 shows how the thermal control unit of the Celsius technology can be used to obtain similar freeze−thaw profiles for lab-, pilot- and full-scale systems. This provides the flexibility to conduct stability characterization studies at lab scale with very limited material. While such disposable bag technology offer numerous advantages with the freeze−thaw process, the final impact on the product quality is also governed by the impact of the container. Issues such as increase in protein concentration associated with the loss of water vapor from plastic bags have been reported for prolonged storage at room temperature (*31*). The product-packaging interaction, robustness of the bag's mechanical properties, permeability of the bags and the level of leachables and extractables should be characterized in detail to ensure that no impact on product quality over a period of storage time is observed.

Formulation Step. The first step after thawing the bulk is to formulate it with the right buffer and to the target concentration. The formulation step involves adding the desired excipients at target concentration and adjusting pH, conductivity and protein concentration (*32*). The final dosage form for the drug product could be different from that of the bulk drug substance. Sometimes it is operationally more favorable to store a drug substance at a higher concentration than the drug product, and therefore a dilution step would be needed during formulation. In other cases, a buffer exchange may be required between drug substance and drug product. To perform buffer exchange, a UF/DF step may then be required. There can be logistical challenges with the implementation of this step, such as whether to perform it at the bulk manufacturing site or at the drug product fill and finish site. One of the main challenges during the UF/DF process is arriving at the target bulk pH at the end of operation. Recent work by Stoner et al. have provided the groundwork for this phenomena and have provided a mathematical tool to calculate how much pH adjustment to make prior to the step to hit the target pH at the end (*33*). Similarly, a concentration step may also be needed if the drug product is formulated at a concentration higher than that of drug substance. High product concentration and viscosity could pose further challenges to membrane filtration during the concentration step. Impact of filtration on product is further discussed in the next section.

The purity of the excipients could be another key factor affecting product quality at this step (*34*). Certain impurities in the raw materials can trigger degradation reactions. Using

need to be carefully evaluated. Exposure to different surfaces including tubing and tanks could also affect protein stability. Surfactants, such as polysorbate 20, added to the formulation buffer as stabilizers, could get adsorbed on these surfaces and lower the surfactant concentration, resulting in protein destabilization. Leachables and extractables (especially if disposable bags are being used) from the various contact surfaces also have the potential to affect product integrity.

Insufficient mixing during the addition of excipients could alter product quality due to solution inhomogeneity and also result in the final drug product not being able to meet its specifications. Excessive mixing, on the other hand, could create large shear stress that can denature proteins. Physical instability of proteins arising from mechanical stresses such as stirring and shaking in presence of various contact surfaces has been widely reported in literature (*35*−*39*). Air−liquid interfaces created during the mixing and pumping processes are another source of protein denaturation. Pumping can also result in addition of foreign particles in the final solution that can further trigger protein aggregation. The order in which excipients are added is also important in determining product quality. For example, addition of polysorbate is often performed after any UF/DF step in order to minimize loss due to membrane interaction.

Small-scale characterization studies should be conducted to evaluate protein stability under formulation conditions. Buffers should be characterized to establish appropriate tolerances around excipient concentrations, pH, conductivity and osmolality. Hold time studies using the worst case scenarios for surface area exposed per unit volume should be designed to study the impact of different contact materials on protein stability. Temperature excursions should also be evaluated through hold time studies at various temperatures over prolonged duration of time. The characterization of the mixing process should include both product homogeneity testing as well as impact of mixing shear on product quality. Based on the tank and impeller geometry and product properties (viscosity and density), bulk and impeller tip shear can be computed for the manufacturing conditions. In the absence of appropriate scaled-down mixing systems, rheometers can be utilized in the lab to expose the product to the maximum applicable shear over the recommended duration of the mixing process. Final samples can be analyzed to assess the impact on product quality attributes. These findings can then be verified with fewer runs on the commercial scale to determine the impact during large-scale processing.

Filtration. After the bulk drug substance has been formulated, it goes through sterile filtration. Sterile filtration is usually performed with a 0.22 μm filter to make sure that the bulk is free from viable micro-organisms. An additional in-line filtration step might be incorporated just before filling. Dual filtration prior to filling may also be employed for risk mitigation in the scenario of a filter failure. The protein solution as a result could see multiple filtration steps before being filled as a final dosage form in the drug product container. It is therefore important to evaluate the impact of these filtrations on product quality. Sterile filtration at high trans-membrane pressure could stress the protein while pushing it through the filter pores.

The protein can also selectively bind to the membrane resulting in either misfolding on the membrane surface or protein loss. It is therefore important to study the compatibility of the product to the membrane material. Figure 5 shows the binding of a protein drug product (at 1 mg/mL) to PVDF membrane. It

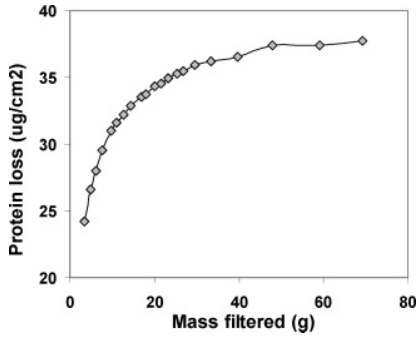

Figure 5. Protein adsorption on PVDF membrane as measured during sterile filtration through 0.22 μm filter

for low concentration products if the batch size is relatively smaller or if the bulk is not being pooled after filtration and before filling.

Similarly other formulation components, e.g., surfactants that are added as stabilizers, can get adsorbed on the membrane surface. This will cause the surfactant concentration in the solution to go below the target, which might result in product destabilization. Recent study by Mahler et al. (*40*) reported minimal loss of polysorbate 20 due to adsorption on filter membrane and also suggested that protein in formulation could influence surfactant concentration during dialysis process. While such losses may not be significant, it is advisable to test polysorbate concentrations under the final scale process conditions. A larger filter area will reduce filtration time, but at the same time it will maximize the protein and excipient losses associated with membrane adsorption. As a result, scale-down studies should be conducted prior to large-scale processing to assess the impact of filtration on product quality and to recommend the optimum filter size and the membrane type for the manufacturing process. Other filtration process parameters such as the trans-membrane pressure across the membrane, the temperature of the bulk and the liquid flow during filtration should also be evaluated for their impact on protein stability.

Drug Product Filling. Once the drug substance has been formulated and sterile filtered, it is filled into the primary drug product containers, which are usually vials, or syringes for prefilled injectables. During this step, the drug product not only comes in contact with the primary container but also the various components such as stoppers, plungers, etc. All of these components are subjected to sterilization processes separately and brought together under aseptic processing conditions (*41*). Since there is no further sterilization step, it is critical to maintain the sterility of the drug product during this step. The environment during the filling process could also contribute to foreign contaminants in the final drug product. The container closure systems and the environment of the fill chamber are qualified to be of the highest standards (class 100 room) needed to ensure product quality. Air flow patterns, HEPA filtration, humidity and operation design are used to minimize sources of foreign contaminants such as airborne dust, depyrogenation particles and fibers from operator garments, mobile machine parts and components. In addition to creating additional solid−liquid interfaces that may deactivate proteins, foreign particles pose a significant risk of causing immunogenicity (*42*, *43*).

Interactions with container surface and components, which come in direct contact with the drug product, can also affect protein stability. Siliconized stoppers can contribute to protein aggregation (*44*) and particulate formation in vials. Leachables

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