# REVIEWS

# Effects of Surfaces and Leachables on the Stability of Biopharmaceuticals

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**ABSTRACT:** Therapeutic proteins are exposed to various potential contact surfaces, particles, and leachables during manufacturing, shipping, storage, and delivery. In this review, we present published examples of interfacial- or leachable-induced aggregation or particle formation, and discuss the mitigation strategies that were successfully utilized. Adsorption to interfaces or interactions with leachables and/or particles in some cases has been reported to cause protein aggregation or particle formation. Identification of the cause(s) of particle formation involving minute amounts of protein over extended periods of time can be challenging. Various formulation strategies such as addition of a nonionic surfactant (e.g., polysorbate) have been demonstrated to effectively mitigate adsorption-induced protein aggregation. However, not all stability problems associated with interfaces or leachables are best resolved by formulation optimization. Detectable leachables do not necessarily have any adverse impact on the protein but control of the leachable source is preferred when there is a concern. In other cases, preventing protein aggregation and particle formation may require manufacturing process and/or equipment changes, use of compatible materials at contact interfaces, and so on. This review summarizes approaches that have been used to minimize protein aggregation and particle formation during manufacturing and fill–finish operations, product storage and transportation, and delivery of protein therapeutics. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:4158–4170, 2011

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## INTRODUCTION

Therapeutic proteins are used to treat a wide range of serious medical conditions, providing substantial benefits to patients. Proteins are complex molecules, subject to both intrinsic variation (e.g., glycosylation pattern and charge isoforms) and a variety of chemical (e.g., deamidation and oxidation) and physical (formation of soluble aggregates, particle formation, and reversible association) degradation pathways. Most common intrinsic degradation pathways for protein therapeutics include aggregation and often particle formation, with the resulting degradation products normally making up a very small mass fraction of the therapeutic protein product. Not all molecular variants or degradation products necessarily result in a loss of efficacy or a decrease in safety. Some types of protein aggregates may elicit immune responses in patients.[1,2] However, the mechanisms for immunogenicity of therapeutic proteins in patients are still not well understood and a link between immunogenicity and aggregates or particles in products remains unclear in many cases.[3,4]

Using state-of-the-art technology, biotechnology companies use formulation and process control strategies to obtain high purity and stability in order to meet a typical goal of a 2-year shelf life.[5] For the general case of bulk protein aggregation as described by Chi et al.,[6] either partial unfolding or aggregate assembly can be the rate-determining step for

**Abbreviations used:** mAb, monoclonal antibody.

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aggregation of proteins. The conformational and colloidal stability of the protein can be optimized through the appropriate use of formulation buffer type, pH, and excipients.[5–10] Similarly, formulation conditions can also be used to maximize chemical stability of proteins.[5–10] High-concentration monoclonal antibody (mAb) products present their own unique challenges such as self-association, viscosity, opalescence, and protein particle formation.[11,12]

Protein stability in bulk solution is only one of the key issues. During manufacturing, final fill–finish, storage, and delivery, proteins may adsorb to surfaces or react/bind with leachables. In some cases, this has resulted in aggregation, particle formation, or adsorption losses.[5,10] Figure 1 depicts some of the processes of how solid and liquid contact surfaces and leachables have caused instabilities in protein products. Adsorption of proteins to surfaces is a complex process that is important in many fields.[13,14] Protein surface adsorption can be driven by a combination of electrostatic forces, hydrophobic binding interactions, and entropy changes due to contributions from both water and protein.[14,15] These surface adsorption processes may be reversible or irreversible and may lead to either unfolding or partial unfolding of the adsorbed protein or only minimal perturbations to the protein structure. Depending on these factors, the adsorption of protein may be minimal and not cause any additional aggregation or particle formation. Simple adsorption can result in a reduction in the bulk protein concentration that can be more of a concern for low concentration formulations. In other cases, protein adsorption could nucleate further aggregation and particle formation. If adsorption is reversible, it is possible that the desorbed proteins may be released in a structurally perturbed form that could lead to further aggregation or particle formation in the bulk.[16] However, the detailed mechanism(s) has

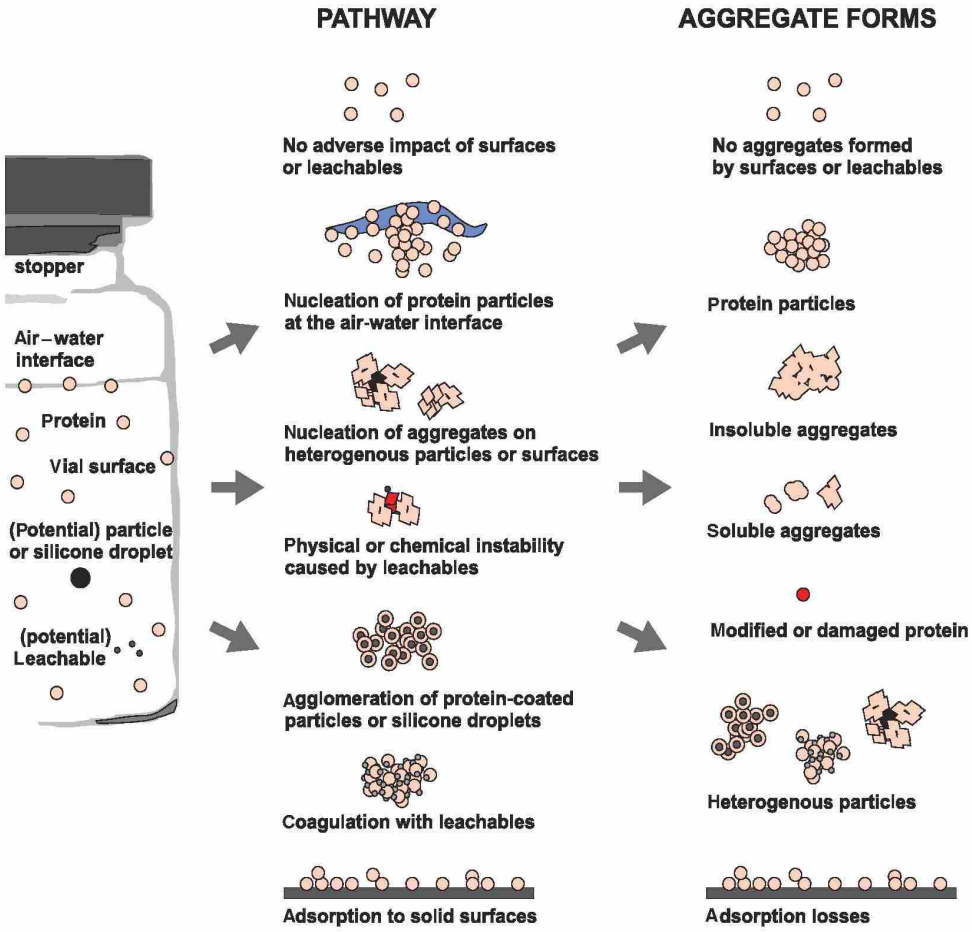


**Figure 1.** Possible physical degradation pathways of proteins caused by interfaces, foreign particulates, and leachables described in this review. The processes in the figure correspond to specific examples that have been published and are discussed in the text. Although the figure shows a vial as one example, these processes may also occur in other upstream operations and in other containers or delivery devices. These examples are also described and reviewed in this work.

not been fully determined for many published reports of adverse protein interactions with surfaces. Figure 1 illustrates alternative mechanisms of particle formation. These include agglomeration of protein-coated particles or silicone oil droplets and coagulation of proteins with leachables. This might occur when a few subvisible particles (SVPs) that were initially colloidally stable (due to a high negative surface charge in the case of glass and silicone oil at common formulation pH conditions) become less stable when the surface charge is reduced by adsorption of protein. These protein-coated particles may then simply agglomerate together to form larger, more easily detectable particles. It is also possible that foreign particles could agglomerate even if there is little or no protein adsorption to the particles. A similar process of binding of leachables to proteins can lead to particle formation through colloidal destabilization of the protein, followed by precipitation of particles. Other leachables may also cause protein damage by directly reacting with the protein, potentially creating an aggregation-competent protein species. The last process for aggregation and particle formation we discuss is exposure to the air–water interface. Air–water interface exposure is one of the more common causes of particle formation and aggregation described in the literature. As with other interfaces, the details of the mechanism(s) of air–water interface-induced aggregation are not well described for many proteins. In this review, we present examples of the published evidence for these aggregation and particle formation processes and discuss rational mitigation strategies. Many of these examples of interface- and leachable-induced aggregation and particle formation processes are specific to certain products or conditions. We also note that detectable leachables may have no adverse impact of product safety, efficacy, quality, or protein stability. In this review, we have included many different examples (even if they are less common) so that the lessons learned may be used to help in the practical resolution of other similar issues in the future.

## MANUFACTURING AND FILL–FINISH OPERATIONS

Manufacturing of therapeutic proteins is a complex process, which begins with production of the protein in cells cultured in a bioreactor wherein the protein is exposed to a multitude of solution species in the growth medium. The protein is then separated from the cell culture media by filtration or centrifugation. Recovery from inclusion bodies and refolding are performed if necessary. In downstream protein purification, viral inactivation and removal steps are often performed (e.g., low pH incubation, nanofiltration, and solvent–detergent addition). Multiple chromatography (e.g., affinity, ion exchange, and hydrophobic interaction) and filtration steps are used to purify the protein further. The protein may be concentrated and formulated using diafiltration. The formulated bulk may then be frozen or held before the final sterile filtration and fill–finish operations. Following the final sterile filtration step, the product is filled into vials, syringes, or cartridges. Each of these steps may expose the protein to interfaces (i.e., solid–liquid and air–liquid) under a variety of solution conditions. In this review, we focus on downstream examples of interfacial protein instabilities, although many of the aggregation and particle formation processes to which proteins are exposed during downstream unit operations could also be relevant to the cell culture environment.

### Diafiltration

Air bubble entrainment and/or microcavitation have been cited as a cause of aggregation during diafiltration operations.[17–19] Adsorption to solid surfaces, contamination by particulates, and increased rate of aggregate assembly due to mixing could also be causes of aggregation.[19] Simple adsorption losses and fouling of the protein onto the membrane can also occur. For instance, deactivation of aminoacylase was directly caused by adsorption losses to an ultrafiltration membrane surface.[20] The type and brand of filtration membrane have been shown to result in different levels of protein adsorption.[21]

Process controls may be used to minimize aggregation during diafiltration by optimization of the operation parameters such as the transmembrane pressure and cross-flow rate.[22] It has been suggested that reducing turnover of the air–water interface and bubble entrainment would also reduce the formation of particles in biotherapeutics during diafiltration operations.[18,19] It is possible that some formulation excipients can provide additional protection during diafiltration. This of course depends upon whether excipients are added during the diafiltration operation or afterward by addition of a concentrated stock of the excipients. Although addition of a surfactant can suppress the formation of aggregates at the air–water interface when the protein is also exposed to shear,[23] this strategy may not be practical for diafiltration operations. Surfactants are normally added after the diafiltration operation because of the difficulties in controlling and predicting the final surfactant level in the retentate.[24]

### Freezing and Thawing

Freezing is a common unit operation during the production of therapeutic protein products. Bulk intermediates are often frozen to increase their stability during production hold steps and freezing is the first step in lyophilization. Freezing and thawing can trigger aggregation and particle formation in proteins by

various different mechanisms.[25] Storage temperature and freezing rate are important parameters for frozen stability. One other factor that is the focus of this review contributing to the overall destabilization of the protein is the choice of container material. For example, polytetrafluoroethylene and other commercial freezing containers fostered more aggregation than polypropylene during freeze–thawing of an $IgG_2$.[26] Cryoprotectants, such as sucrose or trehalose, are often added to protein formulations to protect against freezing and thawing damage.[25]

The ice–solution interface itself can be destabilizing to proteins: increased intermolecular β-sheet content was measured by infrared spectroscopy for two different proteins adsorbed to the ice surface.[27] Polysorbate addition has been shown to reduce formation of nonnative intermolecular β-sheet levels in proteins adsorbed to ice interfaces.[27] In this case, the ability of polysorbate to reduce such structural changes in ice-adsorbed protein molecules was protein specific.[27] Polysorbate 20 protected Factor XIII during freeze–thawing by competing with the partially unfolded protein for interfaces.[28] Additionally, polysorbate 80 protected hemoglobin from damage at interfaces during freezing.[29] The 40% or greater loss of interleukin-11 (IL-11) activity caused by adsorption to glass lyophilization vials was prevented by polysorbate 20, although for complete protection during lyophilization, trehalose and human albumin were also necessary.[30]

The rate of cooling and the degree of supercooling affect the number and sizes of ice crystals and the time the protein is exposed to the ice interface. Each of these variables could potentially influence the extent of freeze–thawing-induced protein aggregation. Because there are multiple variables in freeze–thaw stress, experimental studies to test the sensitivity of the specific protein formulation to realistic and worst-case freeze–thaw stresses can be used to determine appropriate mitigation strategies.

### Sterile Filtration and Fill–Finish

Sterile filtration and fill–finish operations may exert adverse effects on stability by exposing the protein to production equipment surfaces (e.g., those presented by membranes, tubing, and pumps). In an engineering approach, choice of equipment to minimize air–water interface exposure and turnover, particle shedding, leachables, and cavitation can be employed to eliminate or minimize suspected causes of aggregation.[19] This type of optimization should be performed while also maintaining product homogeneity (i.e., ensuring mixing is adequate) and sterility, and overall robustness and quality. These same strategies could also be useful to minimize aggregation or particle formation in other upstream unit operations. Formulation approaches can also be very effective at reducing adverse interactions with interfaces. For instance, the aggregation leading to membrane fouling during sterile filtration of human growth hormone was found to be caused by adsorption to hydrophobic interfaces and could be mitigated by addition of surfactant.[31] Differences in the magnitude of protein adsorption has been observed between different types and brands of sterilizing filters (e.g., polyvinylidene fluoride (PVDF), polyethersulfone (PES), cellulose acetate (CA), and Nylon).[21] Various filters were found to adsorb polysorbate 80, requiring appropriate setup of the preflush step to avoid decreasing the levels of surfactant below the intended value for the final protein formulation.[21] Interestingly, it has been found that cellulose could preferentially adsorb soluble aggregates of a mAb from solution, although this did not have any adverse effect on the protein stability in bulk solution.[32]

Stainless steel is ubiquitous in protein production equipment and has been reported to be a cause of protein aggregation or fragmentation in several cases: submicron steel particles shed from a pump in the laboratory environment caused "agglomeration of protein-coated particles" (see Fig. 1) and/or nucleated formation of larger aggregates of a mAb[33]; Fe ions caused hinge-region fragmentation of a mAb[34]; exposure to the stainless steel surface combined with additional shear stress resulted in aggregation of a mAb that exhibited a first-order dependence on protein concentration[35]; Fe ions leached from steel caused oxidation and aggregation[36]; Fe ions directly bound to a protein resulting in conformational destabilization followed by aggregation,[37] and surface-induced soluble aggregation of a mAb had a second-order dependence on steel surface area and a zero-order dependence on bulk protein concentration that could be completely suppressed by polysorbate.[38] Stainless steel surfaces typically are "passivated" or "electropolished" to create a more corrosion-resistant chromium oxide-rich surface layer. Factors that may impact the protein in solution include the following: the grade of steel alloy, the frequency of passivation, and chemical exposures of the steel. The impact of the formulation may play a particularly large role in the potential adverse interactions; for instance, exposure of steel to chloride ions at low pH has been shown to result in corrosion and release of Fe ions that subsequently catalyzed the oxidation of methionine residues.[36] Stainless steel exposure is an example of where there may be multiple distinct causes of aggregation or particle formation: the steel surface itself, steel particles shed from equipment, and the Fe ions leached from steel equipment. These examples would correspond to the scenarios of "physical or chemical instability caused by leachables" (Fe ions), "nucleation of aggregates on heterogenous particles or surfaces" (steel surface), and "agglomeration of protein-coated particles" (steel particles) shown in Figure 1. Here,

correct identification of the cause of the aggregation, fragmentation, or particle formation is crucial for creating an effective mitigation strategy. Addition of a surfactant would be expected to reduce aggregation induced by surface adsorption, yet may not be effective in eliminating oxidation, fragmentation, or conformational destabilization caused by Fe ions. Rather, direct reduction of Fe ion levels by frequent passivation of equipment and avoiding exposures of steel to extreme low pH in the presence of chloride or other corrosive ions might be a better strategy to eliminate negative effects of Fe ions on protein stability.[36] Addition of metal chelators has also been shown to be effective in eliminating the multiple adverse effects of Fe ions on protein stability, although care must be taken in the choice and level of the chelator.[37] Nucleation of larger visible particles from smaller steel particles shed from pumps may not be completely suppressed by surfactant.[26] In this scenario, a change in the process equipment has been shown to be effective. For instance, protein particle formation during filling of an IgG was eliminated by replacement of a radial piston pump with a rolling diaphragm pump.[39] In some cases, there may be synergistic or compounded effects that may make identification of the problem and correct mitigation more difficult. A good example is where buffer-dependent conformational changes in a mAb increased the exposure of a site sensitive to Fe-catalyzed fragmentation.[34]

Stainless steel is not the only important in-process surface to consider. In recent years, use of disposable containers has become a common practice in various steps of the manufacturing of protein therapeutics. Disposable containers pose potential challenges associated with leachables and possible shedding of particles, and are usually subjected to extensive evaluation by biopharmaceutical companies before implementation.[40]

## CONTAINER CLOSURE

Glass vials with rubber stoppers made of various polymers and coatings are commonly used primary containers for protein therapeutics. Most recently, vials or syringes made of cyclic polyolefin (clear plastic) are being evaluated as options for container-closure materials for some biopharmaceuticals.[41] Container closures can be exposed to various solvents to extract and identify compounds that are then monitored as leachables under realistic product contact conditions.[40] This can result in an identification of a large number of extractables that are often not actually detectable in the formulation upon extended product contact. Direct health-based risk assessments can then be performed based on the extractables–leachables data for a given product configuration.[40] Indirect effects of leachables could potentially include aggregation or particle formation.[40] Detectable leachables may not necessarily have any adverse effects on product safety, efficacy, or quality.

Rapid growth in the applications of targeted biotechnology products is driving the development of alternative delivery systems including prefilled syringes (PFSs), autoinjectors (AIs), and infusion devices. Multiple commercial products are currently offered as PFSs and AI devices, and the number is expected to rapidly grow. The development of PFSs, AI, and infusion devices are associated with potential for component compatibility challenges. These potential challenges include sensitivity of proteins to the silicone oil often used to enhance the gliding performance of the syringe/device, sensitivity to trace levels of metals such as tungsten, which may be used in the manufacturing of glass syringes with staked needles, and potential leachables from the glass, silicone, rubber, and adhesive contact surfaces. These possible adverse interactions are addressed during compatibility and stability studies during development. In addition, various types of syringes are being developed currently by multiple vendors including silicone oil- and tungsten-free syringes, enabling a greater selection of container-closure systems to be potentially chosen from and/or evaluated during development.

### Glass

Borosilicate glass is the most commonly used primary container material for biopharmaceuticals.[41] During development, each product formulation is generally assessed and optimized for stability in glass vials (with stopper). Glass vials surface properties can vary between manufacturers and may change due to interactions with the solution or sterilization procedures, which could potentially result in pitting or delamination.[41–44] Glass has been successfully used for many commercial protein products without causing aggregation or particle formation. Although reports of glass delamination are extremely rare for biotechnology products, the recent voluntary recall of a commercial protein therapeutic because some lots "...may contain extremely thin glass flakes (lamellae) that are barely visible in most cases" shows that delamination is still an important quality factor to be considered.[45] We note that the voluntary recall also states that "To date, there have been no complaints or adverse events reported which can be directly attributed to the presence of glass lamellae."[45]

Excipients can also potentially interact with leachables from glass. Depending upon the exact supplier, glass can potentially leach ions such as barium or aluminum forming insoluble visible particles of barium sulfate or aluminum phosphate when exposed to formulation excipients (sulfate and phosphate).[46] Proteins can adsorb to glass surfaces. In one case, the adsorption of protein to glass was minimized by

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