Impact of Residual Impurities and Contaminants on Protein Stability

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ABSTRACT: Production of recombinant proteins generates a variety of process-related impurities. The multistep manufacturing processes may introduce many potential contaminants into the final pharmaceutical products. These residual impurities and contaminants can potentially impact the protein stability significantly. In this short review, the authors intend to discuss major sources and types of residual process-related impurities and potential product contaminants, their impact on protein quality/stability, and possible mitigations during product development and manufacturing processes. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci
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INTRODUCTION

Recombinant technology has made possible mass production of proteins for different therapeutic purposes. A variety of expression systems can now be selected for production of proteins of interest with desired post-translational modifications. The expressed protein can be isolated and purified to a high degree traditionally through a combination of chromatographic steps. The purified protein is then put into a stable buffered matrix by ultrafiltration/diafiltration (UF/DF), and filled and possibly lyophilized in final drug product containers for commercialization. The multistep manufacturing processes for the protein drug substance (DS) and final drug product generate a variety of process-related impurities and provide many opportunities for potential product contamination.

Product contamination can be classified generally into two categories—microbiological and nonmicrobiological.[1,2] Although microbiological contamination can be lethal, such events should be preventable through quality assurance of manufacturing processes in appropriately controlled facilities. In addition, all injectable drug products have to meet sterility and endotoxin requirements using standardized test methods before product release. In comparison, there is not a standard set of tests to determine the presence of nonmicrobiological contaminants during the manufacturing process. This is mainly because each process may have a unique source of nonmicrobial contamination and the excipients used may differ in type and origin. Therefore, it is almost impossible to establish standardized assays with enough sensitivity to cover all possible contaminants in a biological product. Occasionally, suspicion of a potential product contamination starts with observation of an aberrant release/stability result, an unexpected clinical event, and/or a change in the pattern of efficacy and/or safety of a drug product (DP). For example, around 2008, an acute, rapid onset of serious side effects resembling an allergic-type reaction (hypotension, nausea, and shortness of breath) were observed clinically after administration of certain lots of heparin.[3] Later, it was confirmed that the vials of heparin were adulterated with oversulfated chondroitin sulfate, which was associated with the adverse reactions.[4,5]

The above example highlights the importance of controlling the level of impurities and contaminants in a drug product. In many cases, however, impurities or contaminants are not linked to aberrant release/stability results, and/or changes in clinical observations. The first step in probing any apparent product contamination is to examine the quality of the DS and DP at the time of release. A significant change in product quality at the time of release and/or a change in stability behavior during subsequent storage could be an indication of potential product contamination, which would lead to an investigation and potentially, a corrective action to prevent or minimize future product contamination.

In this short review, the authors intend to discuss major sources and types of residual process-related impurities and potential product contaminants, their impact on quality/stability, and possible mitigations during product development and manufacturing processes. International Conference on Harmonisation (ICH) Q6B defines contaminants as any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the DS or DP. Therefore, any nonproduct-related and nonprocess-related impurities or substances can be considered as contaminants, which are either detectable or nondetectable and volatile or nonvolatile, deriving from any manufacturing step, environment, product excipient, or the container/closure system. The goal is to promote awareness of potential product contamination, the origin of these contaminants, and their potential stability impact. The increased awareness would help in successful commercialization of effective and safer drug products. Description and discussion of the residual process-related impurities or different contaminants follow sequential stages of a protein product manufacturing process—(1) DS manufacturing, (2) evaluation and selection of drug product excipients, (3) evaluation and selection of a product container/closure system, and (4) finally, DP manufacturing. The main residual process-related impurities or contaminants are summarized in Table 1.

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Table 1. Summary of Main Residual Process-Related Impurities and Potential Product Contaminants, Possible Sources, Stability Effects, and Possible Mitigations

Impurities/Contaminants (Alphabetical)	Possible Sources	Potential Stability Effects	Possible Mitigations	Representative References
Carbon dioxide	Product contact with dry ice, supercritical fluid	Acidification- and interface-induced protein degradation	Choice of proper packaging systems; avoidance of contact with dry ice and supercritical fluid	6
Cleaning agents (hypochlorous and peracetic acids, etc.)	Use of cleaning agents and/or inadequate rinsing	Cleaning agent-induced protein degradation, for example, oxidation	Establishment of proper cleaning procedure	7,8
Formaldehyde/formic acid	Raw materials, excipients, processing aids	Chemical degradants, adducts, aggregates	Control of the quality of raw materials, excipients, storage conditions	9,10
Fatty acids/lipids	Host cells; expression, harvesting, raw materials, excipients	Interaction-induced instability, for example, aggregation	Optimization of processes, control the quality of raw materials, excipients	11–13
Free radicals	Raw materials, excipients, irradiation	Oxidation, clipping	Control of the quality of raw materials, excipients, lighting conditions, formulation selection	14,15
Metals	Process equipment, raw materials/excipients	Aggregation, oxidation, hydrolysis	Use of disposable containers; control of material quality; use of metal chelators	16–18
Moisture	Inadequate packaging systems	Moisture-induced degradation	Choice of proper containers, closures, processing conditions, formulation selection	19
Non-metallic surface materials or leachables	Resins, filters, tubings, containers, closures, product contact materials	Indirect effect, interaction-induced instability, for example, aggregation	Control of material quality, optimization of process conditions, formulation conditions	20–22
Nucleic acids	Host cells; expression and harvesting	Interaction-induced instability	Optimization of purification processes	23,24
Organic solvents	Processing aids	Aggregation	Minimize use or removal of organic solvents	20,25
Oxygen	Air entrapment, inadequate packaging systems	Oxidation, degradation	Optimization of process conditions, packaging systems	26
Particulates—glass-derived	Glass containers and apparatuses	Protein adsorption, aggregation	Control of container quality, formulation conditions	27,28
Particulates—nonglass-derived	Non-glass containers, product contact surfaces, air	Protein adsorption, aggregation	Control of equipment quality, processing conditions	29–31
Peroxides	Raw materials, excipients	Oxidation, aggregation	Control of the quality of raw materials, storage condition, formulation selection	13,32
Protease/host cell proteins (HCPs)	Host cells; expression and harvesting	Proteolysis, interaction-induced instability	Use of protease inhibitors, optimization of manufacturing processes, formulation selection	33–35
Reducing sugars/polyols	Raw materials, excipients, formulation	Glycation, aggregation	Control the quality of raw materials and excipients, formulation selection	36–38
Silicone oil	Containers, closures, syringes, and so on	Aggregation	Minimize use of silicone oil, use of a surfactant and/or a suitable packaging system	39,40
Vaporized decontamination agents	Clean rooms, isolators	Oxidation, aggregation	Control of the air composition in processing areas	8
Tungsten	Syringes	Aggregation	Optimization of the syringe manufacturing processes, type of syringes	41,42

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DRUG SUBSTANCE MANUFACTURING

The drug substance manufacturing process by recombinant DNA technology generally consists of the following steps—establishment of proper cell banks, culture/fermentation production of the protein of interest, isolation of the expressed protein, protein purification, virus inactivation, and finally, UF/DF of the protein into a stable formulation buffer at a desirable concentration. The main residual process-related impurities come from the culture/fermentation process, where a variety of carefully defined medium components are added for cell growth and cell-derived substances are generated. In addition to the process-related impurities, contaminants can be brought into the final DS preparations through any of the above processes. The difference in the type and level of impurities or contaminants in different protein preparations could explain the different extent and mechanism of degradation for recombinant human thrombopoietin (rhTPO),[34] and different aggregation behaviors of three commercially holo-α-lactalbumin products—Sigmaα-La, IEXα-La, and Cα-La, at different pHs.[43,44]

Host Cell Proteins

Host cell proteins (HCPs), as main process-related impurities, are generated during expression of the protein of interest in host cells. The type and level of HCPs can be significantly different depending on the expression system, and the protein purification process. Identification of all existing HCPs can be challenging because of their diversity and relatively low levels. Using LC/MS, more than 20 *E. coli* proteins (ECPs) were identified in a purified Fc fusion protein with the most abundant ones as 60-kDa chaperonin (14.5% of ECP mass; 88 ppm), chaperone protein ClpB (10.6%), and isocitrate dehydrogenase nicotinamide adenine dinucleotide phosphate (7.2%).[33] HCPs can have an impact on processibility of the protein of interest such as protein crystallization[45] and the product immunogenicity potential because of their foreignness.[46,47]

Presence of HCPs may or may not impact the product stability. For example, the aggregation of a model protein was accelerated in the presence of another contaminating protein through protein–protein interactions or disulfide linkages.[48,49] In comparison, as many as seven plasma protein contaminants are present in the anti-D immunoglobulin preparations from seven European manufacturers, for example, α2-macroglobulin, α1-antitrypsin, albumin, and α2-HS glycoprotein, α-lysozyme, and ceruloplasmin, but neither purity nor impurity correlated with the storage stability in terms of protein activity (both at 37°C and 4°C).[50]

In contrast, presence of residual proteases in the DS can significantly influence the long-term storage stability depending on their level in the preparations.[51] Rapid protease-catalyzed hydrolysis was observed for affinity-purified rhTPO at pH 6–8,[34] and a Chinese hamster ovary-derived high-purity human IgG1 mAb.[35] The carboxypeptidase-catalyzed C-terminal lysine clipping in mAb's leads to charge heterogeneity, which may potentially impact the protein stability, as the number of charges in mAb's plays a role in protein aggregation.[52] Proteolysis of whey proteins led to enhanced aggregation because of the higher propensity of hydrolyzed fragments to aggregate.[53] Enhanced aggregation was also observed for fragments generated by autocatalyzing proteins such as botulinum neurotoxin type A upon long-term storage or freeze–thawing.[54] Simultaneous presence of proteolysis and chemical hydrolysis may complicate detection of a proteolytic event.[35] To minimize the impact of residual proteases, the level of HCPs needs to be minimized in the purified DS to an acceptable level. Proteases can be removed by altering the purification scheme,[34] or inhibited by use of a protease inhibitor.[35]

Nucleic Acids

Nucleic acids are polyanions and can bind theoretically to any positively charged proteins at an acidic pH through electrostatic interactions. Interaction of nucleic acids with proteins can influence their stability and function both for RNAs[55,56] and DNAs.[57–59] For example, the well-known DNA-binding proteins, or transcription factors bind to DNA complexes at regulatory loci to regulate expression of a target gene.[60] The association between single-stranded DNA and proteins was shown to be fast (possibly diffusion-controlled) and the binding affinity depends on the length of the DNA.[61,62] Such binding may alter the secondary structure and induces conformational changes in proteins.[23,24,63,64] Indeed, recent studies have demonstrated the possibility of using RNA aptamers for stabilization of proteins against aggregation both in solid and liquid states.[65,66]

In reality, the residual amount of nucleic acid level in a protein product is generally low. The recommended WHO upper limit for a large-dose protein product, such as antibodies, is 10 ng/dose. Assuming a molecular weight of 150 kD for a mAb at a dose of 100 mg and a 100 bp size for a dsDNA, the molar ratio at this limit would be 4E+6. At such a ratio, any stability impact on proteins is unlikely detectable by a traditional analytical method.

Fatty Acids/Lipids

Fatty acids and lipids can be present in the final DS.[67] They are introduced as cell components during expression, as processing aids during purification.[68] or during viral inactivation.[69] Fatty acids and lipids could potentially influence the protein stability, as they can bind tightly to proteins, presumably through hydrophobic interactions. Examples include caprylate interaction with lysozyme,[70] or albumin,[71,72] and lipid interaction with α-synuclein[73] and a variety of enzymes.[74,75] Interactions with lipids clearly enhanced the aggregation of lysozyme[11] and insulin.[12]

In general, because of the limited solubility of fatty acids and lipids, trace amount of such contaminants has not been found to play a significant role in protein stability. Design of a process to remove the residual fatty acids/lipids can be based on the purification of membrane proteins and/or pH adjustment method.[76,77]

Metals and Other Nonmetallic Leachables

Most bioprocessing equipment is made of stainless steel in a variety of qualities, which may corrode to a different degree in solutions of different compositions, especially in chlorinated solutions.[78] Three metal ions—iron, chromium, and nickel—were identified to be the major leachables under various formulation buffer and pH conditions.[17] Both protein and ethylenediaminetetraacetic acid (EDTA) in a product formation have been shown to increase the dissolution of stainless steel,[17,79] and the metal leaching process.[16] NaCl was also able to facilitate corrosion of certain stainless steel components in a low pH solution.[80] In addition, raw materials used in expression and

purification processes may contain certain level of residual metals. For example, immediately after preparation, the Fe content was found to be 734 ppb in 50 mM phosphate buffer, whereas 57 ppb in 50 mM Tris buffer.[81] Therefore, residual amounts of metals are expected to be present in a purified DS.

The direct effect of metal ions on protein stability has been recently reviewed.[82] The degree of effect may depend on the type and concentration of the metal and especially, the protein itself, as demonstrated for factor VIII SQ,[83] β-lactoglobulin,[84] murine adenosine deaminase[85] tau protein,[86] and rhDNase.[87,88] In a recent report, it was demonstrated that even a small nickel-plated brass connector (for connecting silicone tubings) can leach significant amounts of nickel, copper, and zinc and cause significant protein precipitation within 10 min of product contact at room temperature.[16] Only a few ppm of metal contaminants was enough to induce significant precipitation of a protein, likely due to possible chelation of a transition metal to cross-link the protein monomers.[16]

Often, metals can have an indirect effect on protein stability. A frequently reported metal-related degradation is the catalysis of protein oxidation via the Fenton or Fenton-like pathway.[89,90] This pathway generates hydroxyl radicals for oxidizing proteins. Coexistence of other metals such as aluminum could potentially enhance the Fenton reaction by promoting formation of ferrous ion.[91] The oxidation-sensitive residues in proteins include Met, Cys, His, Trp, Tyr, Pro, Arg, Lys, or Thr.[80,92–94] The catalytic rate depends on the type and concentration of the metal ions. For example, addition of 0.15 ppm (w/w) chloride salts of Fe^{3+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, or Zn^{2+} does not affect the oxidation rate of Met in hIGF-I at room temperature, but when the salt concentration increases to 1 ppm, Fe^{3+} causes a significant increase in oxidation.[95] Significant variation was observed in the determination of methionine oxidation by LC–UV/MS peptide mapping when a trace amount of metals, especially iron, was present in the digestion buffer and chromatographic column.[96] Presence of antioxidizing or reducing agents such as ascorbate or sulfhydryl-containing compounds can further facilitate metal-catalyzed oxidation of peptide or proteins, usually in a concentration-dependent manner.[92,94,97,98]

Transition metals are efficient catalysts of redox reactions and catalyze the "autoxidation" of many biomolecules, likely as a result of binding of transition metals to biomolecules.[99] Autoxidation may lead to formation of not only oxidation products but also cleaved products. Copper (not other transition metals) was shown to induce specific clippings in monoclonal antibodies (IgG1) at the hinge K222–T223 bond.[18] This metal-induced clipping mechanism is different from those for high pH or temperature-induced clipping. Similarly, a trace amount of redox active metal ions (such as Cu^{2+} or Fe^{3+}) catalyzed generation of hydrogen peroxide by radical chain reduction of molecular oxygen into water, which led to fragmentation at the hinge region of a human IgG1.[100]

Transition metals were also shown to accelerate the Maillard reaction, a common degradation pathway between reducing sugars and basic amino acids. Fe^{2+} and Cu^{2+} were shown to accelerate this reaction between several sugars (maltose, fructose, glucose, arabinose, and xylose) and amino acids (aspartic acid, glutamic acid, alanine, leucine, isoleucine, valine, proline, serine, cysteine, phenylalanine, arginine, and lysine).[101] It is possible that the enhanced degradation is through complex formation between sugar molecules and metal ions.[102,103]

Use of a metal chelating agent, such as EDTA, can be effective in inhibiting metal-catalyzed protein degradation such as copper-induced clipping of IgG1,[18,100] or Fe-catalyzed protein oxidation in different buffer systems.[81] Use of diethylenetriaminepentaacetic acid (DTPA) can also be effective in inhibiting Fe-catalyzed protein degradation.[104] On the other hand, use of a chelating agent may lead to safety concerns and is not always beneficial in controlling metal-catalyzed protein degradation. Addition of EDTA actually enhanced Fe^{2+} or Fe^{3+}-induced clipping in monoclonal antibodies (IgG1).[18] The enhanced catalytic activity is likely because of both acceleration of metal leaching from the container in the presence of a chelating agent and an active state of the metal under chelated state. In a recent report, it was shown that ferrous EDTA complex can effectively catalyze oxidation of methionine and histidine residues and also cleavage between Met and His in human parathyroid hormone.[105] Use of DTPA could effectively inhibit the hydrolytic event. It is therefore recommended that the quality of stainless steel in direct contact with proteins should be carefully evaluated and controlled, and a stainless steel/protein compatibility study could be conducted during the development process.

In addition to metals, other nonmetallic leachables can be introduced into the DS preparations as process-related impurities. A common class is leachables from chromatography resins, such as protein A[106] and Strep-Tactin[107] from their respective affinity columns. β-glucans can leach out from cellulose filters and also come from raw materials used in the manufacturing process.[108] The potential nonmetallic leachables from product-contacting material surfaces during protein DS manufacturing was recently evaluated. A total of 13 potential leachable species were identified, including volatile organic compounds, semi-volatile organic compounds, anions, cations, and so on.[20] None of the 13 contaminants was found to interact with the protein or to affect the protein stability/safety. Nonetheless, the level of nonmetallic leachables should be minimized to ameliorate potential safety concerns.

Organic Solvents

Although organic solvents are rarely used in the production of biological products in mammalian cell systems, they have been used in microbial expression systems for production of proteins.[109] Organic solvents have also been used as processing aid in certain cases.[110,111] Solvent detergents are often used for virus inactivation and reduction.[112] Trifluoroacetic acid is often used as a solvent in reversed-phase HPLC for purification of proteins or peptides.[113] Therefore, residual organic solvents can be found in the protein DS.[20]

The effect of organic solvent on protein stability has been widely studied.[114,115] The effect can vary dramatically, depending on the concentration of the organic solvent and protein. A gradual effect is generally seen with increasing proportions of an organic solvent in proteins such as alpha-chymotrypsin with several organic solvents[116] and human growth hormone with ethanolic solutions.[117] Simulation of the effect of different organic solvents (formamide, acetone, and isopropanol) at nondenaturing concentration on the structure of haloalkane dehalogenases reveals that the behavior of solvent in the vicinity of hydrophobic patches on the protein surface is similar to the air/water interface.[25] Therefore, it is conceivable that such organic solvents would facilitate formation of protein

aggregates via partial unfolding of a protein at the organic solvent/water interface. Indeed, 2,2,2-trifluoroethanol (TFE) was found to enhance α-helical conformations with a simultaneous increase in aggregation of bovine serum albumin (BSA) because of the promotion of intermolecular cluster formation.[118] TFE was also shown to increase the aggregation of several other proteins, including α-chymotrypsin,[119] protein L,[120] and human muscle acylphosphatase,[121] and lentil seedling amine oxidase.[122] In many cases, however, proteins can tolerate a significant amount of organic solvents such as human and bovine IgGs at an acetonitrile concentration up to 50%–60%[123] and lysophospholipase in a 0%–50% (v/v) of water mixtures with organic solvents such as methanol, ethanol and acetonitrile.[124] In some cases, organic solvent, such as DMSO can help to reduce protein aggregation, as observed for arginine kinase aggregation during refolding[125] or convert fibrils to monomers such as 50% AcOH/water for fibrillated human calcitonin.[126] In fact, glycerol is an often-used organic solvent for suppressing protein aggregation in different processes, such as refolding,[125,127] purification,[128] incubation,[127,129] and historically, freeze/thaw.[130,131] The presence of such a residual organic solvent can therefore be acceptable in a purified DS. Details on solvent classification and limits of possible daily intake can be found in ICH Guideline for Residual Solvents (Q3C).

EVALUATION AND SELECTION OF DRUG PRODUCT EXCIPIENTS

In general, drug product excipients are evaluated and selected to make proteins more stable and/or suitable for administration. For these reasons, a drug product may contain part or all of these excipients—a buffering agent, a bulking agent, a tonicity adjustment agent, and a surfactant. Agents of different pharmaceutical grades are made by various manufacturing processes and sourced by several vendors. The purity and/or contaminant levels in these excipients can vary significantly and affect the product quality/stability to different degrees. Even for pure excipients, contaminants can be generated during storage through various degradation pathways. A variety of excipient-derived contaminants and their potential reactions with drug candidates have been reviewed.[132] These excipient-derived contaminants are listed in the following categories—trace metals, peroxides, aldehydes, reducing sugars/polyols, and organic acids.

Trace Metals

Trace amounts of metal ions are ubiquitous in almost all excipients. The contaminating metals in sucrose, one of the major excipients for biologics, can be traced back to the production of sugar canes, which contain varying amounts of metals depending on the soil quality.[133] The level of residual metals can be high so that a simple buffer solution may contain enough residual metal ions to accelerate protein oxidation during the assay process.[81] The variety of effects of metal ions on protein stability has been discussed in the above section. Product excipients have to be carefully screened for residual level of metals for metal-sensitive proteins. As discussed, the effect of trace metals may be mitigated through use of a chelating agent such as EDTA for the inhibition of metal-catalyzed histidine oxidation.[134]

Peroxides

Peroxides are often present in many excipients because of their autoxidation process during storage. Polysorbates are often-used excipients to inhibit protein aggregation, but are good source of peroxides.[135] A major degradation pathway in these materials is autoxidation, especially at high temperatures.[13] Both neat polysorbate 80 and its diluted solutions easily generate peroxides, catalyzed by light, and in the presence of metal.[32,99,136] Because of these influencing factors, the peroxide levels in different lots from the same vendor may vary significantly.[137] Accordingly, peroxide-induced degradation in protein solutions containing PS 80 of different grades from different vendors can vary significantly.[136] Different types of polysorbates are expected to have varying degrees of deleterious effect because of the structural variations.[138]

Peroxides were also found in other common excipients used in protein products such as poloxamer, sucrose, lactose, mannitol, and polyethylene glycols (PEGs).[137] The accelerated oxidation of recombinant human epidermal growth factor in the presence of fructose or PEG 6000 during the freeze/thaw process is likely because of the presence of peroxides in these excipients.[139] Metal can catalyze the formation of peroxides in antioxidants such as ascorbate, and thiol-containing agents[99] or through direct interaction with molecular oxygen via redox reactions.[140] This may explain why the addition of ascorbate, an antioxidant, actually accelerated oxidation of recombinant human ciliary neutotrophic factor (rhCNTF) in the presence of peroxides[141] and oxidative degradation of other drug candidates.[142]

The main effect of peroxides is to catalyze oxidation reactions in proteins. Examples include oxidation of sulfhydryl groups in membrane proteins[143] and oxidation of recombinant human granulocyte colony-stimulating factor during storage.[144] Other oxidation-derived degradation products can also be observed such as high-molecular weight and low-molecular weight species.[136,141] Even in a solid state, a protein can be oxidized by a low level of peroxides in polysorbate 80 during storage.[32]

Peroxides can also oxidize other excipients in a protein product and affect the quality/stability indirectly. Peroxides have been shown to oxidize histidine, an often-used buffering agent, in a pH and temperature-dependent manner, into several products—including 4(5)-imidazolecarboxaldehyde, imidazole-4-acetic acid, 4(5) imidazolecarboxylic acid, and so on.[134]

To minimize the potential effect of residual peroxides, the quality of excipients should be carefully evaluated for peroxide level. An upper limit could be implemented for incoming raw materials. Storage conditions need to be defined to minimize storage-induced peroxide formation. It has been clearly demonstrated that the rate of peroxide formation in polysorbates can be greatly inhibited if stored under nitrogen and/or at a lower temperature.[32] It is therefore recommended to purchase peroxide-containing excipients in single-use containers, and store them under nitrogen at low temperatures.

Formaldehyde/Formic Acid

Formaldehyde and formic acid are trace impurities that are often present in some pharmaceutical excipients. Lactose from one manufacturer contains 1.0 ppm formic acid and <0.2 ppm formaldehyde, and PEG 4000 from one vendor contains 14 ppm formic acid and 3.6 ppm formaldehyde.[145] Both formaldehyde

and formic acid can be formed from oxidative degradation of polysorbates[9] and PEG.[146] Different polysorbates may have different rates of formation, as demonstrated for polysorbate 80 (faster) and polysorbate 20 (slower) upon incubation at 25°C.[138] Other degradation mechanisms can also lead to formation of formaldehyde such as degradation of Tris buffer.[147]

These trace contaminants can potentially react with amino and/or hydroxyl groups in drugs to form significant amounts of degradants (formates and amides respectively). Hydroxy methyl derivative (formaldehyde adduct) can be formed between the product and formaldehyde during storage because of the degradation of PS 80 or PEG 300.[9] The aldehyde derivative could form a Schiff base with primary amines for further degradation.[10] Even in a solid state, drugs containing amines can react with both formaldehyde and formic acid derived from degradation of PEG during storage.[146] Formaldehyde is an effective cross-linking agent for proteins, producing carbinolamines (methylols) of arginine and lysine, as well as a product ascribed to a methylene cross-link between arginine and lysine.[148]

Similarly, the quality of excipients should be examined for formaldehyde/formic acid contaminants. A limits test could be applied to control the contaminant level and to minimize the impact of these contaminants.[9] Use of an antioxidant in a drug product could inhibit the excipient oxidation, and potentially minimize the impact on product stability.[146]

Reducing Sugars/Polyols

It is well known that proteins may react with reducing sugars and readily form glycation products via the Maillard reaction. Reducing sugar impurities may cause significant protein glycation, depending on the storage temperature. Incubation of a highly glycation-sensitive monoclonal antibody (mAb1), with 5% glucose at 37°C for 5 days, increased the level of glycation product to 80% of the total protein and the majority of glycation was localized to lysine 98 of an unique sequence in the heavy chain complementarity determining region 3.[36] The glycation products can retain or lose activity with greater or less stability, depending on the protein and site of glycation.[149–151] Because of the electrostatic and/or steric effects, the glycated protein can have a higher or lower tendency for aggregation.[37,38]

Sucrose is a widely used stabilizing and/or bulking agent for proteins. Acid-catalyzed sucrose hydrolysis could generate glucose, a reducing sugar, during storage or through high-temperature treatment.[152,153] A monoclonal antibody, MAB001, was shown to aggregate faster in a sucrose formulation relative to excipient-free formulations at pH 4.8 during storage at 29°C because of the sucrose hydrolysis and a higher aggregation tendency of the glycated antibody.[154]

Reducing sugars can induce other type of reactions, even in a solid state. For example, a degradation product, a benzaldehyde derivative, was identified through oxidative deamination of an aminomethyl phenylalanine moiety after storage of a lyophilized cyclic heptapeptide in a mannitol-based formulation at 30°C for a year.[155] A proposed mechanism is the formation of Schiff base adduct intermediate with reducing sugar impurities in mannitol (acting as an oxidizing agent) and subsequent tautomerization and hydrolysis. The reducing sugar impurities (containing sugar aldehyde groups) are found to be 0.1% in mannitol.

It is obvious that reducing sugars should be avoided to prevent the above degradations, and the level of reducing sugar contaminants may also need to be examined. Formulation conditions should be carefully chosen to minimize disaccharide hydrolysis, which may lead to the formation of reducing sugars.

Fatty Acids

Polysorbates contain fatty acid chains and storage of PS 20 and PS 80 can generate fatty acids and their ester derivatives.[138,156] The solubility of these degradants may be temperature-dependent. Their potential effects and removal have been discussed in the above section.

EVALUATION AND SELECTION OF A PRODUCT CONTAINER/CLOSURE SYSTEM

The main purpose of a container/closure system for a drug product is to ensure that the contents will not be lost and foreign materials/microbes will not enter into the system.[157] Common product containers are vials, syringes, and cartridges, made of either glass or plastic materials. Evaluation and selection of a proper container/closure system is one of the key steps in drug product development, as its quality can significantly impact the product stability, safety, and marketability. Changing the container/closure system during product development may require comparability assessment, as the product quality attributes may change because of the differences in construction materials and/or associated leachable/extractable contaminants.[158]

Silicone Oil

Silicone oil is commonly used to coat rubber stoppers to facilitate manufacturing operations or syringe barrels/plungers to facilitate both manufacturing and administration processes. It was estimated decades ago that about 0.15–0.25 mg silicone oil could be expelled from each disposable syringes used by diabetic patient and was recommended for removal for its "potential toxic effect."[159] Around the same time period, insulin product clouding and loss of product activity were observed because of the product contact with residual silicone oil coming from repeated uses of disposable syringes (until consumption of about one-third of the product in vials).[160]

A clear trend today is to market biological products in prefilled syringes for better safety and ease of administration.[161] An obvious concern is the potential long-term effect of silicone oil on protein stability during product storage. Many recent studies have demonstrated a deleterious effect of silicone oil on protein stability. Proteins can be easily adsorbed onto the silicone oil droplet surface, leading to a loss of soluble proteins such as BSA, lysozyme, abatacept, and trastuzumab.[162] The amount of protein adsorbable on the surface of silicone oil may need further investigation, although at least a monolayer of protein molecules was demonstrated in some studies.[163,164] The adsorption of protein at the silicone oil/water interface can be irreversible[39] and protein concentration dependent.[165] Silicone-induced surface adsorption of proteins can facilitate protein aggregation and particle formation.[40,161,163,164] This phenomena is likely similar to air/water interface-induced protein aggregation as such adsorption can potentially induce partial protein unfolding for enhanced aggregation. Silicone-induced protein aggregation was shown to be pH-dependent, as pH regulates the surface charge and hydrophobicity of a protein.[166]

On the other hand, not all proteins are affected by silicone oil in terms of stability. It was shown that an IgG1 was adsorbed on the surface of silicone oil, but adsorption did not stimulate aggregation during isothermal incubation if samples were not agitated.[164] Albinterferon α-2b in a formulation containing 0.1 mg/mL polysorbate 80, 36 mg/mL mannitol, 23 mg/mL trehalose, and 10 mM phosphate at pH 7.2, does not interact with excessive amount silicone oil (six times the normal amount in siliconized glass containers), resulting in no change in product purity, biological activity, and long-term stability.[167] It is possible that the polysorbate in the formulation has certain protective effect.

Several approaches can be taken for mitigation of the impact of silicone oil. Use of alternative coating technologies (BD-42) for glass syringes may be a solution for silicone-sensitive proteins.[161] Baked-on silicone may restrict its interactions with proteins better than sprayed-on silicone[168] Addition of a surfactant in a protein formulation can effectively decrease protein surface adsorption to silicone oil.[39,162,169,170] The reduction in surface adsorption can inhibit or even prevent silicone-induced protein aggregation or particle formation. For example, addition of PS 20 greatly inhibited silicone oil-induced monomer loss[164] or the formation of IgG1 aggregates in IgG1 molecules.[40] The surface adsorption and aggregation of Abatacept at the silicone oil/water interface can be prevented in the presence of polysorbate 80.[165]

Extractable and Leachables

Assessment of product leachables/extractables from a container/closure system is one of the key tasks during the drug development process and through product approval.[171] Leachables from product container/closure systems can cause serious safety concerns.[172,173] For example, leachables from the stoppers of PS 80-formulated Eprex product were, albeit debatable, considered to have contributed to the increased incidence of pure red cell aplasia.[156,174]

Metal ions can leach from different glass or plastic packaging materials. The type and level of metal leachables are dependent on the type of containers, incubation temperature, and formulation composition.[21] Their effects on protein stability have been discussed extensively in the above section. Tungsten, a key metal contaminant identified in recent years, was shown to play a key role in protein aggregation.[41] This metal is used during syringe manufacturing and can leach out in significant amounts in the funnel region of a prefilled syringe and facilitate formation of protein particles.[175] Tungsten at ppm levels was adequate to facilitate formation of subvisible particulates or precipitates in monoclonal antibody formulations.[176,177] Formation of protein precipitates can occur in seconds. Tungsten-induced protein aggregation/particle formation is not too surprising as this metal can easily bind to many proteins.[178] Binding alters the conformational structure of a protein and can accelerate formation of protein dimers and aggregates.[42] Tungsten-induced protein aggregation is dependent on solution pH, the tungsten species, and the tungsten concentration.[41]

Measures to minimize the possible effect of tungsten include redesigning of the manufacturing process, wash procedures, and use of formulation approaches.[175] Since formation of tungsten polyanions is very limited at higher pHs, formulation of proteins above pH 6.0 would be preferable to minimize tungsten-induced protein aggregation/precipitation.[177] Use of a cationic surfactant cetyl trimethyl ammonium bromide was shown to effectively inhibit tungsten-induced structural changes and formation of subvisible particles.[176]

Other metal leachables/extractables from borosilicate vials could also interact directly with a protein product or form other complexes with formulation excipients to indirectly influence the stability of proteins. It was recently demonstrated that storage of a phosphate buffer in borosilicate vials at 40°C led to the formation of aluminum phosphate particulates within months because of the interaction of phosphate with leached aluminum, and the extremely low solubility of the aluminum salt.[179] Products containing sulfate may have the potential to interact with leached barium to form barium sulfate particles.[180] These particles could act as "seeds" to promote protein aggregation as demonstrated for glass particles (see below).

Leachables/extractables can cause changes in solution pH to facilitate protein degradation. Both the container and stopper can leach basic or acidic components. Glass surfaces can leach alkali components causing pH changes over time, especially under basic conditions. High temperature treatment may accelerate the process. For example, the pH of WFI in different type I glass vials generally increases in a fill volume-dependent manner after autoclaving.[181] In comparison, the pH of a glutaric acid solution in glass vials dropped significantly because of the dissolution of silicon and release of silicates.[27] Stoppers of different composition immersed in 0.9% benzyl alcohol solutions can cause pH change of the solution upon storage. Maximum pH change from pH 6.5–9.1 was observed within 10 days of storage at 40°C for one type of bromobutyl stoppers, whereas no significant change in pH for chlorobutyl or ultralow extractables bromobutyl stoppers.[182]

Other stopper leachables/extractables can potentially influence the stability of proteins. Thiuram disulfides, frequently used as accelerators in rubber stoppers, was found to leach or be extracted from stoppers to interact with captopril (a thiol-containing drug) through thiol-disulfide exchange.[183] Many proteins contain free thiols and therefore, are subject to such degradations by these leachables/extractables.

Many protein products need to be diluted in saline or other diluents for intravenous infusion. The leachables/extractables coming from infusion bags can potentially influence protein stability. In fact, commercial 0.9% saline solutions in bags often contain significant amounts of leachables. A total of 24 different organic contaminants were found in 0.9% saline solutions packaged in polyvinyl chloride (PVC) bags from Australasia, Europe, and North America.[184] Diethylhexyl phthalate (DEHP) and 2-ethyl hexanol, a DEHP breakdown product, were found in all samples. Processing conditions for bags may influence the leaching process. Sterilization by ethylene oxide increased leaching of a plasticizer—di-(2-ethylhexyl) phthalate (DEHP), in PVC bags.[185] A zinc salt of 2-mercaptobenzothiazole (vulcanizing agent) leached out from the stopper of 100 mL saline polyolefin bags and caused a loss of dulanermin after freezing.[186] Replacement with nonlatex stoppers corrected the problem.

As discussed, container/closure leachables/extractables may play a significant role in protein product stability. Early evaluation of container/closure suitability should be conducted before initiation of any clinical trials. Improvement of the manufacturing processes and surface treatment can be effective in minimizing the level of leachables/extractables. Fluorotech coating provides an effective means to minimize stopper leachables.[174]

Formulation approaches can also be applied to reduce the impact of system leachables/extractables, such as avoidance of a basic pH, phosphate buffer, or sulfate in the design of a protein formulation.

Glass Particulates

Particulates in a drug product can come from a container/closure system. Dissolution of a glass container during storage leads to formation of subvisible glass particulates before glass flakes (delamination) become visible.[27] Delaminations have led to more than 20 product recalls in the last few years, including the recall of Procrit and Epogen injections by Amgen.[187] Many factors can influence the glass dissolution and delamination process. Primary glass-related factors include the glass manufacturing process, surface imperfection/morphology, alkalinity, or treatment.[188–190] Product-related factors include formulation pH, composition, and storage temperature and duration.[191] Dissolution and delamination can occur at a neutral or basic pH, especially in citrate, tartrate, or phosphate containing solutions.[192]

Presence of glass particulates can have a negative impact on product quality/stability, which may lead to product recalls. It has been shown that nanometer-sized hydrophilic silica particles are effective seeds to facilitate extensive aggregation of recombinant human platelet-activating factor acetylhydrolase and particle formation.[193] This occurred following protein adsorption on the particle surfaces. Several antibodies were shown to be easily adsorbed irreversibly on the surface of glass microparticulates.[28]

Glass delamination does not appear to be easily noticed as an issue in the early stage of the product development process, as assessment of product compatibility with a container/closure system takes a long period of time. Use of stressed conditions such as high temperature or temperature cycling may facilitate early identification of any dissolution or delamination issue. Monitoring other parameter changes such as pH and appearance of subvisible glass particles could also be an option. In general, the quality of glass containers and their processing conditions need to be carefully controlled to minimize potential structural change. Coating the glass surface can mitigate glass dissolution or delamination.[27] Another option to bypass this issue is to use polymeric containers, which may have other issues, such as gas permeation.

Contaminants from Other Product Contact Materials

Other product contact materials may release substances to cause product degradation. Storage of recombinant human vascular endothelial growth factor with several dressing materials showed enhanced formation of protein oxidation and formation of high-molecular-weight protein adducts due to the release of peroxides and other leachates from Adaptic dressing.[194] Polymers have been widely used for preparation of various drug delivery systems. Processing of these polymeric systems may generate contaminants to destabilize proteins. Extrusion of a poly(lactic acid)-based delivery system containing vapreotide, a somatostatin analogue, led to formation of 6% lactoyl lactylvapreotide conjugate as a result of reaction of the lactide with the primary amine at the N-terminal Phe of the peptide.[195] Gamma-irradiation can cause formation of free radicals in polymers and often increases leaching. Polymers with high melting points and crystallinity showed the highest yields of radicals observed at room temperature.[196] As the life time of free radicals varies from seconds to years,[197] it is expected that these radicals would interact with a protein during long-term storage.

Gaseous Contaminants

Gases can permeate through a container/closure system and facilitate degradation of protein products. It is well known that the moisture content for a lyophilized product is a critical parameter for controlling the rate of protein degradation.[198] It was observed a long time ago that moisture content of a lyophilized BCG vaccine in vials sealed with rubber stoppers increased during storage.[199] It was demonstrated that gray butyl stoppers can take up water rapidly during steam-autoclaving, release water rapidly during subsequent oven-drying, take up water slowly during freeze-drying and allow water to permeate through them when stored in a water-saturated atmosphere. Among 12 types of rubber stoppers tested, the gray butyl stoppers and the silicone stoppers showed the lowest water uptake. Similar results were reported later by Sasaki et al.,[19] who demonstrated that moisture can transfer from different elastomer stoppers to the lyophilized drug product during long-term storage under humid conditions through two processes—moisture transfer from the stoppers (desorption) in the early period of storage, and external moisture permeation through the stoppers during the later period of storage. Moisture permeating through noncoated stoppers was more of a concern, as the initial moisture transfer from these stoppers can be controlled by adjusting the stopper drying time. Similarly, high-density polyethylene (HDPE) cannot prevent moisture migration and a trace amount of moisture can be steadily taken up by $CaCl_2$ in HDPE bottles, depending on the storage temperature and relative humidity.[200]

Molecular oxygen can permeate through many plastic containers. The half time required for a nitrogen-filled plastic Crystal Zenith (CZ)-resin vials to reach the ambient oxygen level was estimated theoretically and experimentally to be only 15 days.[26] Low-density polyethylene (LDPE) bottles are permeable to oxygen and the migrated oxygen is responsible for the considerable loss of Thiomersal through oxygen-induced degradation.[201] Formulation excipients can potentially alter the oxygen permeability in different types of containers such as LDPE, polypropylene (PP), or polycarbonate.[202] Thus, PP disposable syringes are considered inadequate against permeation of ambient oxygen, even though they can be used to hold solvents such as glycol, ethanol, or water for long-term storage at different temperatures.[203] These results indicate that plastic containers may be inappropriate for packaging oxygen-sensitive proteins even with a headspace filled with nitrogen.

Storing products at low temperatures may compromise the container/closure integrity and enhance chance of contamination by gaseous substances. It was observed that storage of a biologic product in glass vials with rubber stoppers at −80°C led to a higher headspace pressure than that stored at −20°C.[204] This is because of an ingress of cold dense gas into the vial headspace with a compromised seal elasticity at a low temperature, as the glass transition temperatures of many rubber stopper formulations are in the range −55°C to −70°C. In a recent report, it was shown that carbon dioxide managed to migrate into a variety of containers—cryogenic vials, conical tubes, glass vials, on dry ice, dropped the pH of a solution,

and caused protein aggregation.[6] It is also the author's experience that carbon dioxide can migrate into a drug product in PETG bottles during shipping with dry ice. Possible mitigations include segregation of dry ice from product containers, additional packaging of a product in multiple sealed bags, and release of trapped carbon dioxide in the container before thawing.

DRUG PRODUCT MANUFACTURING PROCESS

A drug product manufacturing process generally consists of the following steps—thawing of DS, transfer DS into a compounding tank, compounding/mixing, particle-reduction prefiltration into a holding tank, sterile filtration and filling, and if necessary, lyophilization before packaging. In these steps, contaminants can be introduced such as residual sanitizing agents, materials leaching from product contact surfaces, and contaminants from the environment of the production area.

Residual Sanitizing Agents

Most manufacturing tanks/equipment need to be cleaned for repeated usage. Residual sanitizing agents in tiny quantities could cause product instability.[7] A few such agents are strong oxidizers and residual amounts can cause significant protein oxidation. For example, hypochlorous and peracetic acids are commonly used as disinfectants and both have been shown to effectively oxidize several amino acids—cysteine, tryptophan, and methionine.[205] In addition, hypochlorous acid causes tyrosine oxidation, leading to protein aggregation. Trace amounts of sanitizing agents have been shown to enhance the oxidation of IL-2 mutein, including Spor-Klenz (mixture of peracetic acid and hydrogen peroxide) and $NaClO_3$.[8] The presence of as little as 0.001% of Spor-Klenz could accelerate oxidation of IL-2 mutein not only during the lyophilization process but also during storage in the lyophilized state.[8]

Particulates

The level of particulates, either subvisible or visible, in parenteral products are strictly regulated, as particulates are believed to have clinical implications.[206–208] Particulates can be introduced into product containers throughout the product manufacturing process. Even under the strict regulatory guidelines, the classified manufacturing areas generally have a residual amount of airborne particulates, which can diffuse into the product vials.[209] Filtration is an integral step during aseptic processing and the quality of filters can be critical. It has been demonstrated that significant amounts of particles ($\geq$1 micron) can be shed into buffers or protein solutions from different types of syringe filters during filtration.[29] The shed particles increased the rate of particle formation in the keratinocyte growth factor 2 solution, especially under agitation.[210] Preflushing the filters with buffers or protein solutions may or may not reduce the level of particles in the filtrate.

Pumping during the filling process can lead to generation of particles. It was clearly shown that pumping with a positive displacement piston led to shedding of stainless steel particles in the size range of 0.25–0.95 micron , which accelerated formation of particles in an IgG solution.[211] The formation of these particles is facilitated through interaction with the protein, an irreversible adsorption of IgG on the surface of stainless steel microparticles.[30,31]

Leachables/Extractables

Most product compounding and holding tanks are made of stainless steel. As discussed above, metal ions can leach into the drug product solutions and have a significant impact on product quality/stability. Long-term storage stability studies are often needed to determine whether any residual amount of metal ions would have a significant effect on protein stability. Even though a low level of residual metal may not influence protein stability, their level should not exceed the limits recently proposed in United States Pharmacopeia chapter <232> (http://www.usp.org/usp-nf/official-text/revision-bulletins/elemental-impurities-limits-and-elemental-impurities-procedures).

Silicone tubing is commonly used for filling drug products because of its high flexibility and durability. Subjecting the silicone tubing to dynamic flow (simulated use) extractions led to identification of many extractables.[212] The primary organic extractables were a homologous series of silicone oligomers and other extractables including dioctyl phthalate, dioctyl adipate, phthalates, a series of alkyl phenols, and decomposition products of Irganox-type antioxidants, depending on the type of tubing evaluated. Inorganic extractables associated with many of the tubings included Ca, Mg, Zn, and B. In general, the extracted levels of targeted leachables under simulated use (flow) conditions is low and may not present any harmful effects.

Filters may release extractables/leachables, in addition to particle shedding. Extractables/leachables from three different filter membranes—polyvinylidine fluoride (PVDF), polyethersulfone (PES), and mixed cellulose ester (MCE), were compared by soaking the triple-sterilized filters in pH 5.5 histidine buffer.[22] These extractables/leachables generally destabilize the protein, resulting in an increase in aggregation, oxidation, and acidic species formation. Extractables/leachables from PVDF or PES showed a significant protective effect and those from MCE showed a destabilizing effect on both visible and subvisible particle formation of an IgG2.

The above studies suggest that leachables/extractables, while important for product safety considerations, may or may not have a negative impact on product quality/stability. Strategies for minimizing leachables/extractables can follow what has been discussed above.

Environmental Contaminants

Manufacturing environments must be clean and free of harmful contaminants. Various clean areas/rooms for different types of manufacturing operations have been defined based on the particle counts in the air. Although this is important and critical, such a classification system may not be adequate to prevent potential product contamination by gaseous substances, as the air composition of a clean room is not a parameter for routine inspection and, as far as we know, not required by any regulatory agencies.

Any harmful gases in the air of a manufacturing area could diffuse into the product container and alter the product quality or stability. For example, hydrogen peroxide is a commonly used solution for effective decontamination of isolators because it can be easily vaporized. Because of its high oxidizing potential, its vapor can be a good source of product contamination. An excellent example was the investigation on degradation of IL-2 mutein by vaporized hydrogen peroxide and other sanitizing agents.[8] In this report, higher-than-normal protein

oxidation was observed first in stability samples, which led to a systematic investigation. It was demonstrated that a vaporized oxidizing agent can diffuse into a product vial, placed 12 inches apart from the vapor source, in an open environment. For oxidation sensitive products, monitoring the composition of air for presence of any oxidizing agents may be necessary, if such agents are routinely used in the production area. Minimally, reduction of such agents to a safe level should be verified.

Lighting conditions in the manufacturing area could be a source of potential product contamination due to the light-induced generation of free radicals or peroxides, leading to protein degradation and thus product-related impurities.[14,15] These free radicals and peroxides can lead to formation of a variety of protein degradation products, such as fragmentation and aggregation.[14,213,214] Exposure of monoclonal antibody IgG1 to light led to formation of nonreducible covalent aggregates[213,215] and a significant increase in turbidity.[15] Formation of aggregates could be seen after light exposure for 1 min.[215] Aggregation is likely attributed to photo-induced cross-linking reaction(s) such as those between Cys and Trp residues and photolysis of native disulfide bonds for new disulfide bond formation/exchange.[216–218] Light sensitivity of a protein candidate should be evaluated before exposing the protein without protection from light for a prolonged period of time during both DS and DP manufacturing processes.

CONCLUSIONS

Introduction of process-related impurities and potential contaminants into final products can occur throughout the DS and drug product manufacturing processes. These substances vary widely in terms of physical state and chemical properties, and may potentially impact the protein stability (Table 1). Although many process-related impurities are routinely monitored, contaminants are generally not, at least not by release assays. This is because the level of these contaminants in a drug product is often too low to be detected by traditional analytical methods, and does not lead to serious safety concerns.

Since many process-related impurities are generally known, their presence can be controlled to an acceptable level without significant impact on drug product stability. On the other hand, many contaminants are adventitious with unpredictable impact on product stability. A general strategy for minimizing any potential effects of contaminants on drug product stability/quality is to understand the type and source of possible contaminants and to minimize their direct or indirect contact with the product through accurate and consistent control of raw materials, excipients, container/closure systems, and finally, manufacturing equipment and processes. Early and thorough evaluation of product compatibility with materials of possible contact would help to identify potential issues. It is noted that identification of any low-level process-related impurities or contaminants may require use and/or development of more rapid and sensitive analytical technologies such as ICP-MS/NMR for a range of impurities, from small molecule components to higher molecular weight leachables[219] and advanced LC–MS for identification of trace protein impurities.[220,221] A contaminant, not detectable by a sensitive assay, may have to be concentrated for easy detection/identification.[222]

This mini review highlights the variety of process-related impurities and contaminants that can be introduced into a product. These known impurities and product contaminants need to be controlled. Unknown impurities and contaminants may surface any time, when different raw materials and altered processes are introduced. There is a possibility for unexpected product stability/quality issues resulting from unanticipated impurities or contaminants. Therefore, scientists involved in developing and commercializing biopharmaceuticals need to keep these considerations in mind when designing appropriate control strategies for manufacturing DS and DP.

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