EGAS MONIZ SCHOOL of HEALTH & SCIENCE | INSTITUTO UNIVERSITÁRIO EGAS MONIZ

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MESTRADO INTEGRADO EM CIÊNCIAS FARMACÊUTICAS

BIOLOGICALS: PROCESSING AND STABILITY ISSUES

Trabalho submetido por
Ana Carolina Clarindo Silva
para a obtenção do grau de Mestre em Ciências Farmacêuticas

Fevereiro de 2024

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Trabalho orientado por
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Fevereiro de 2024

To my brothers and mother, for always being there, for believing, for all the love…

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Resumo

A agregação proteica representa um desafio importante para a estabilidade dos produtos biofarmacêuticos, tanto em operações unitárias de rotina, como ao longo do prazo de validade do produto.

As operações unitárias como a congelação/descongelação, a mistura, a filtração e o enchimento tendem a induzir a agregação de proteínas, principalmente devido ao stress interfacial, entre outros factores. Em alguns casos, o ajuste da formulação pode reduzir a agregação, enquanto noutros é necessário optar por equipamento mais adequado à composição da solução com a qual se está a trabalhar.

Do ponto de vista comercial, o tipo de agregação mais problemático, uma vez que pode ocorrer depois de o produto já ter sido distribuído no mercado, é a que ocorre ao longo do prazo de validade dos produtos biofarmacêuticos. Trata-se de um processo insidioso, muitas vezes desencadeado pela degradação enzimática de constituintes do produto acabado, que ocorre lentamente ao longo do tempo e pode resultar na recolha de lotes, na possível escassez de medicamentos essenciais, em danos à reputação do fabricante e em prejuízo financeiro. Do ponto de vista clínico, a formação de partículas em biofármacos pode resultar na perda de atividade terapêutica e/ou levar a reações imunogénicas indesejadas, se introduzidos no organismo.

É essencial contornar este problema, uma vez que ele pode afetar os parâmetros de qualidade do produto final. Se for identificado durante o processo de fabrico, são implementadas medidas de controlo em processo (IPC) e de controlo de qualidade (QC), em conformidade com as atuais boas práticas de fabrico (cGMP), para mitigar a probabilidade da sua ocorrência.

O objetivo deste trabalho é realçar operações de fabrico específicas, juntamente com degradação química e/ou enzimática, que possam levar à agregação, assim como explorar medidas preventivas para mitigar este problema.

Palavras chave: Biofármaco, agregação, proteinas, congelamento, descongelamento, filtração, enchimento, degradação química e enzimática

Abstract

Protein aggregation poses a significant challenge to the stability of biopharmaceutical products both in routine unit operations and throughout the product's shelf-life.

Unit operations such as freezing/thawing, mixing, filtration, and filling tend to induce protein aggregation, mainly due to interfacial stress, among other factors. In some cases, adjusting the formulation can reduce aggregation, while in others, opting for more suitable equipment for the intended purpose is necessary.

From a business perspective, the most problematic type of aggregation, as it may happen after the product has already been distributed in the marketplace, is that occurring throughout the shelf-life of biopharmaceuticals. This is an insidious process, often triggered by enzymatic degradation of constituents in the final product, which occurs slowly over time and can result in batch recalls, possible shortages of life-saving medicines, damage to the manufacturer's reputation, and financial loss. From a clinical viewpoint, the formation of particles in biopharmaceuticals may result in therapeutic activity loss and/or lead to unwanted immunogenic reactions if introduced into the body.

It is mandatory to circumvent this problem as it can affect the quality parameters of the end product. If identified during the manufacturing process, in process control (IPC) and quality control (QC) measures are implemented in compliance with current good manufacturing practices (cGMP) to mitigate the likelihood of its occurrence.

The purpose of this literature revision is to highlight specific manufacturing operations, alongside chemical and enzymatic occurrences that may lead to aggregation, and the preventative measures achieve mitigation.

Key words: Biopharmaceutical, aggregation, proteins, freeze, thawing, filtration, filling, chemical and enzymatic degradation

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Abbreviations list

ADC – Antibody-drug conjugate
ADR – Adverse drug reaction
API – Active pharmaceutical ingredient
ASTM – American Society for Testing and Materials
BCT – Bacterial challenging test
BMM – Bottom mounted mixer
CA - Cellulose acetate
CIP – Clean in place
CQA – Critical quality attribute
DLS – Dynamic light scattering
DP – Drug product
DTPA - Diethylenetriamine pentaacetate
DS – Drug substance
EDTA - Ethylenediaminetetraacetic acid
EMA – European Medicine Agency
FFA – Free fatty acids
FINP – Finished product
GMP – Good manufacturing practices
HA – Health authority
HC – Heavy chain
HCP – Host cell proteins
ICH – International Council for Harmonization
IM – Intramuscular
IPC – In process control
IV – Intravenous
LC – Light chain
LPLA2 – Phospholipase 2
mAb – monoclonal antibody
MCO – Metal catalysed oxidation
NIH – National Institute of Health
PDE – Permissible daily exposure
PES – Polyether sulfone
PFS – Prefilled syringes
ppb – Parts per billion
ppm – Parts per million
PS20 – Polysorbate 20
PS80 – Polysorbate 80
PVDF- Polyvinylidene difluoride
QP – Qualified person
ROS – Reactive oxygen species
SC – Subcutaneous
SIP – Steam in place
SUT – Single-use technologies
SvP – Subvisible particles
TMM – Top mounted mixer
VHP – Vapour hydrogen peroxide
VPHP – Vapour phase hydrogen peroxide

1. Introduction

For the past century, there has been significant emphasis within the pharmaceutical industry on the development of biopharmaceuticals because of their vast potential (1). Treatments for diseases that were once believed to be untreatable and deadly are now proving effective. Patients can now look forward to a longer life and an increased standard of living.

Insulin, which is now commonly prescribed to individuals with diabetes, was the first protein drug and represented a significant therapeutic achievement of the past century. It took almost a century to replace animal-derived insulin with that produced by recombinant DNA technology, which permitted the production of a safer insulin product in greater quantities (1).

Biopharmaceuticals demonstrate potential in various medical fields, including hormonal imbalances, autoimmune disorders, and cancer therapies (2). In addition to the therapeutic relevance, the economic importance of these products is enormous. The global spending on biologics exceeded $250 billion in 2019, almost 50% in the United States. By 2026 the biologics market is expected to exceed $700 billion (3).

This industry is subject to strict regulatory oversight, with high standards of manufacturing processes and product quality that must be maintained throughout the shelf life of biopharmaceuticals (2).

Quality issues may arise from manufacturing unit operations, such as freeze and thawing (4), filtration (5), mixing (6) and filling (7), or other factors related to chemical and enzymatic degradation (8). It is thus essential to optimize the processes in order to maintain quality throughout the manufacturing process and prevent potential issues that can arise during the shelf life.

Protein aggregation can occur at all stages of biopharmaceutical production, becoming one of the most common manufacturing-related quality issues (4). Particles can also form at a slow rate throughout the biopharmaceutical shelf life.

Particles may be detected during production or through optical inspection at the end, and in the worst-case scenario, during the shelf life of biopharmaceuticals, which may lead to a patient safety risk and batch recall. A batch recall carries the risk of both financial loss and damage to the manufacturer's reputation. In addition, it can result in a shortage of life-saving medication if no therapeutic alternatives are found in the market. The extent of damage both for the patient and the company can be immense, hence the imposition of strict regulations on the production of such molecules.

The aim of this work was to review the challenges surrounding the production of biological drugs, with a particular emphasis on protein-based biological medications such as antibodies and polypeptides.

2. What are biopharmaceuticals

2.1 Definition of biopharmaceuticals

A biological medicine, or biopharmaceutical, is defined by European Medicines Agency (EMA), as "a medicine whose active substance is made by a living organism" (9). The National Institutes of Health (NIH's), Dictionary of Cancer Terms, offers a more precise definition, stating that it is "a substance made from a living organism or its products and is used to prevent, diagnose, or treat cancer and other diseases. Biological drugs include antibodies, interleukins, and vaccines" (10).

The manufacturing process for biopharmaceuticals is significantly more complex than that used for chemically synthesised drugs. In addition, the regulatory framework for biological medicines is considerably more intricate. The production of a biologic drug substance (DS) comprises therapeutic protein expression, subsequent filtration, purification, buffer exchange, concentration, and formulation steps that deliver the developed bulk drug substance. The DS often undergoes freezing until the initiation of the drug product manufacture (11, 12).

Manufacturing biopharmaceuticals is conventionally divided into two stages: upstream and downstream processes. In the upstream stage, the organism is engineered to produce the desired protein. A replication process follows this step, in which a favourable environment is set to facilitate rapid growth of the organism and protein production. Once the protein of interest is synthesised the downstream process starts with the harvesting and purification steps. This phase refines the protein for its intended use. This is a highly challenging phase because the manufacturer must comply with strict purification regulations. The final product must not contain any debris, remnants of the producing organisms, or unwanted metabolites (12). Therefore, this phase is both demanding and costly in the manufacturing process.

2.2 Characterization of protein based biological medicines

The development of peptide medicines began with the need for a strategy to treat diseases caused by hormone deficiency, such as diabetes. Despite the well-established understanding of the disease's pathophysiology and insulin's role, the task of producing a viable peptide at scale with an acceptable level of purity remained a challenge (13).

Peptides are a distinctive group of pharmaceutical compounds situated at the molecular level between small molecular weight molecules and proteins. Although they differ both molecularly and therapeutically from each other, they provide intrinsic signalling messages for several physiological processes, offering potential for mimicking natural pathways in therapeutic intervention (14). On a larger scale, polypeptides are mainly represented by monoclonal antibodies.

Biopharmaceuticals can be built or coupled into unusual forms, such as:

- Fusion proteins (genetic engineering): Coupling of a biopharmaceutical with another protein (*i.e.*, albumin, FC part of antibodies, transferrin) in order to improve its circulation time and pharmacokinetic profile. Creating a fusion protein can also increase stability in bacterial expression systems (15, 16);
- Protein–polymer conjugates: The conjugation of a biopharmaceutical to a molecule capable of improving its circulation time, among other functions – *e.g.*, PEGylation (17), XTENylation (18), or even increase the specificity of a drug by coupling a small, generally toxic drug (payload) to a highly specific antibody (targeting moiety) capable of selectively delivering the drug to specific cellular targets (mainly used for cancerous treatments) (19);
- Assembly of proteins such as virus-like particles (20) or nanoparticles to create drug delivery systems (21).

3. How the properties of proteins affect the manufacturing process of biopharmaceuticals

Maintaining the stability of biopharmaceutical formulations from manufacturing to administration is a constant challenge for research and development scientists. It is important to consider the factors that affect stability to ensure the effectiveness and safety of these medications. Cold and thermal denaturation, freeze-drying, pH conditions, concentration, ionic strength, agitation, interfacial interactions, and primary packaging design are some of the factors that can affect the stability of biopharmaceuticals (15) that have to be closely monitored.

Some of the challenges of manufacturing these molecules include structural instability caused by unfolding, misfolding, or covalent/non-covalent modifications, as well as aggregation (15).

In fact, proteins are sensitive molecules, and it is a challenge to preserve their intrinsic characteristics throughout the manufacturing process. Firstly, the protein itself may have intrinsic properties that favour aggregation. Additionally, the many stresses that proteins undergo during the manufacturing process can be catalysers for changes in configuration (unfolding, misfolding) promoting aggregation, bioactivity/therapeutic activity loss, possible reduction in concentration, and/or the triggering of immune reactions upon administration. The challenge with these medicines does not stop after fill/finish because there are several additional factors (*i.e.*, agitation during transport, incidence of light, temperature of storage, storage period, adsorption to the container, etc.) that can compromise the quality of the biopharmaceuticals.

4. Mechanisms of protein aggregation

Aggregation is a common challenge in the manufacture and storage of proteins. Protein aggregation can occur naturally, or due to stress, and can affect the bioactivity of biologically active proteins such as antibodies and/or stimulate an immune response.

Aggregates can form because of the delicate nature of these molecules when exposed to various stressful conditions encountered during production, formulation, storage, distribution, and administration. (22).

Proteins aggregate through three main mechanisms, broadly defined by the seeding entity: native monomers, denatured proteins[1], and pre-existing aggregates (23).

4.1 Mechanism #1: Native monomers

The first mechanism relates to the self-aggregation of native monomers into small oligomers (Figuere 1). Because of their intrinsic surface properties, they have a tendency to self-associate, producing small oligomers, considered to be reversible. However, the tendency to form large, potentially irreversible oligomers increases with protein concentration.

Insulin is an example of a protein that naturally self-associates to form reversible and irreversible oligomers, with potential consequences in terms of bioactivity loss (24, 25).

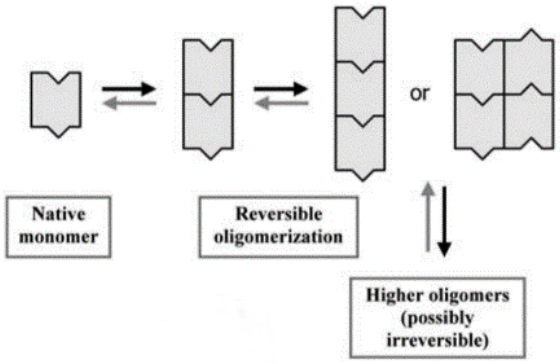


Figure 1. Protein aggregation mechanism #1: Native monomers (Adapted from (24))

[1] The original article refers to conformational change or partial unfolding

4.2 Mechanism #2: Conformationally changed proteins

In the second mechanism, there is an increased tendency for the aggregates not to dissociate because of a conformational change on the monomer that creates the possibility for stronger association (Figure 2) (24).

For protein aggregation to occur, a conformational change must occur first. The ratio of protein prone to aggregation relative to native protein is lower in this mechanism than in the first , where aggregation occurs due to an intrinsic protein characteristic that is independent of conformational changes (25).

This type of aggregation is primarily induced by stress and is therefore the main mechanism associated with events occurring during the manufacturing process. Although irreversible, it can nevertheless be prevented via the correct use of excipients or conditions that can stabilise the protein's native conformation, thus avoiding physical instability with conformational changes that lead to the formation of irreversible aggregates (24).

Such aggregation appears to be a common occurrence for interferon-ɣ (25), for example. A variant of this process can occur when the alteration in protein conformation that leads to aggregation is due to a change in the covalent structure (chemical instability); this event can be triggered by chemical degradation such as oxidation, deamidation, or proteolysis. In this instance, it is crucial to implement measures to enhance chemical stability and avoid further degradation when this mechanism is triggered because the chemically modified monomer can attract both native and other chemically modified monomers. Chemically altered protein aggregates can be particularly immunogenic (24, 25).

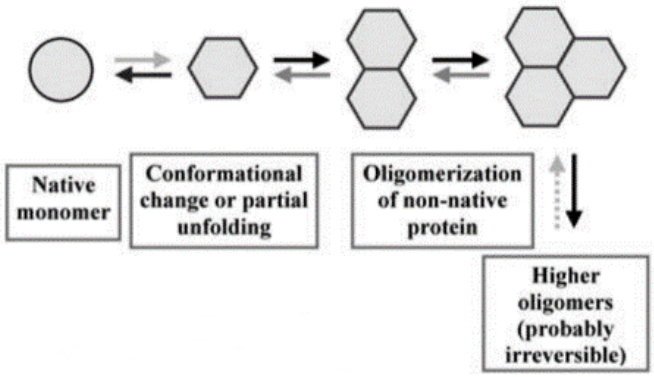


Figure 2. Protein aggregation mechanism #2: Conformational changed proteins (Adapted from *(25))*

4.3 Mechanism #3: Nucleation (pre – existing aggregates)

The third mechanism involves a nucleation event that promotes the formation of visible particles or precipitates (Figure 3). However, when an aggregate reaches a sufficient size, its growth is stimulated by the addition of monomers, resulting in the rapid formation of larger species. The described process generally undergoes a lag phase during which no particles are detected for an extended period. However, larger species suddenly appear and accumulate. When the nuclei are made up of the product of agglomeration itself, they are called homogeneous, and when the nuclei are made up of an impurity or contaminant, they are called heterogeneous. (24, 25).

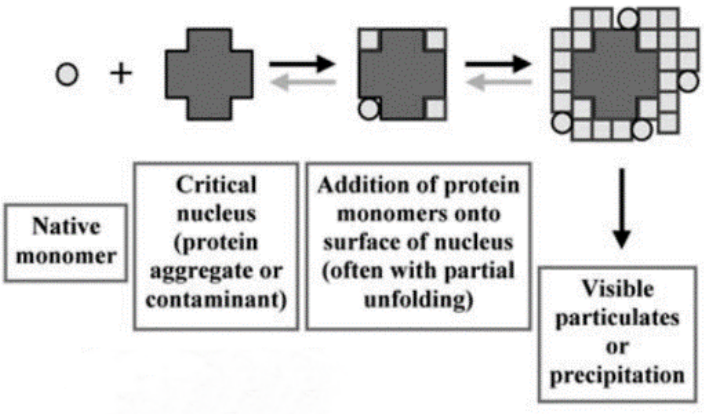


Figure 3. Protein aggregation mechanism #3: Nucleation (pre-existing aggregates) (Adapted from *(25)*)

4.4 Extrinsic / intrinsic aggregation

Beyond the classification based on aggregation mechanisms, solubility, and size, protein aggregation can be classified as intrinsic or extrinsic. Intrinsic protein aggregation, represented by mechanism #1, occurs during protein formulation during the steps of synthesis and purification. Extrinsic aggregation, mainly represented by mechanism #3 arises from protein contact with external sources that occur during processing, such as fibres contaminants, particles from the glass surfaces within containers, particles from stainless steel present in bioprocessing equipment, or silicone oil droplets present inside pre-filled syringes. (23).

Many are the stimuli for the formation of aggregates during manufacturing. The first mechanism (Mechanism #1: Native monomers) is the one that would occur in a more natural way; therefore, it is not triggered by the manufacturing process itself and has the advantage of being, mostly, reversible. The second mechanism (Mechanism #2: Conformationally changed proteins), however irreversible, can be avoided to some extent if the necessary technical mitigation actions are implemented. The use of appropriate formulation/excipients and tight control, performing appropriate in process control (IPC) and critical quality attributes (CQAs) of manufacturing unit operations that are prone to trigger aggregation, are examples of possible strategies. In practice, the most common root cause of extrinsic particles is the introduction of fibres and particles. As a mitigation action, cleanroom garments are worn, and there are elaborately designed cleanrooms for Good Manufacturing Practice (GMP) Class A, B, C, and D. These GMP areas are mostly controlled by continuous particle measurements. The third mechanism (Mechanism #3: Nucleation) is the most problematic for the manufacturing of biopharmaceutical products, especially when considering that the aggregation is preceded by a lag phase, which indicates that this might not be perceived during manufacturing. However, the most problematic challenge for the pharmaceutical industry are issues that arise between the 2 and 3 quarter of the product shelf life (*e.g.* 6 and 15 months after filling, considering a 24-month shelf life). Since those issues are observed only during drug stability testing, in the worst scenario it could possibly lead to a batch recall.

5. Drug substance / drug product / finished product

A drug substance (DS) or active pharmaceutical ingredient (API) is the actual component responsible for the pharmacological activity of a medicine. A biopharmaceutical is the product of the activity of a living organism genetically engineered to produce the desired protein (12), as discussed before.

Once the chosen organism produces the protein, a cascade of operations is initiated to harvest and purify the target protein so that it can be preserved until pharmaceutical processing (12, 26).

Drug product (DP) is used to define the fully formulated product that contains as its API the protein previously produced. Typical biopharmaceutical products are presented as vials (liquid or lyophilized) and prefilled syringes (26). Nowadays, prefilled syringes are often manufactured as a combination device to make the application more user-friendly.

In production, a final product, or finished product (FINP) is a product that is ready for sale. In the pharmaceutical industry, this refers to the primary and secondary packaged medicine, which can be sold to customers after a qualified person (QP) approval, as required in EU countries.

6. Processing drug substance / drug product and FINP

As already mentioned in section 2.1, the manufacturing process of a biopharmaceutical can be roughly divided into two main steps, upstream and downstream processing, in which the first step is related to the production of the DS and the second one is related to its harvesting, purification, and formulation. The result of the operations carried out during upstream and downstream processes is the DP that can now be filled into its primary packaging. Afterwards it goes through visual inspection and, in case a device is used (i.e. pre-filled syringes), it has to be assembled and, finally, the DP can be directed to its final packaging (12, 26). After approval by a quality function (in the EU by a QP), distribution to the countries to be supplied takes place, followed by warehousing, *i.e.*, intermediate storage at the respective country affiliates and shipment to wholesalers. The wholesaler then delivers the medicines to the pharmacies and/or hospital pharmacies.

Upstream processing is the initial stage of the API using living organisms such as bacteria, yeast, or mammalian cell cultures. The process starts with a cell culture that undergoes fermentation, resulting in the production of the designated protein. The main objective of upstream processing is to obtain the desired protein by using well-defined and optimised variables, such as cell line, culture medium, and growth conditions (12). The downstream process, on the other hand, comprises the steps necessary from the harvesting of the target protein to the compound originating the DP.

A number of unit operations are then required to remove significant impurities from the desired protein at a minimum cost, in the shorter period possible, aiming for the highest yield with the highest purity. To stabilise the API solution, it is stored in a buffer containing *e.g.* polysorbate 20 (PS20), polysorbate 80 (PS80), or poloxamer 188 to protect the proteins.

These operations are conducted to purify and concentrate the DS. At the end of the process, the DS can either proceed directly to DP compounding or, more commonly due to materials management, be frozen for later compounding at the manufacturer's convenience (26).

The frozen DS is then thawed and mixed with the necessary excipients to achieve a stable formulation. Subsequently, a final filtration is performed during fill and finish, resulting in the DP, which can be filled into its primary packaging.

Finally, the DP (*e.g.* monoclonal antibody (mAb)) manufacturing process consists of a series of unit operations (Figure 4), which starts with the thawing of the API, pooling/compounding (including dilution)/mixing into the final DP solution, filtration, filling into primary packaging components (vials, syringes, cartridges, etc.), lyophilisation (if necessary), and inspection (6).

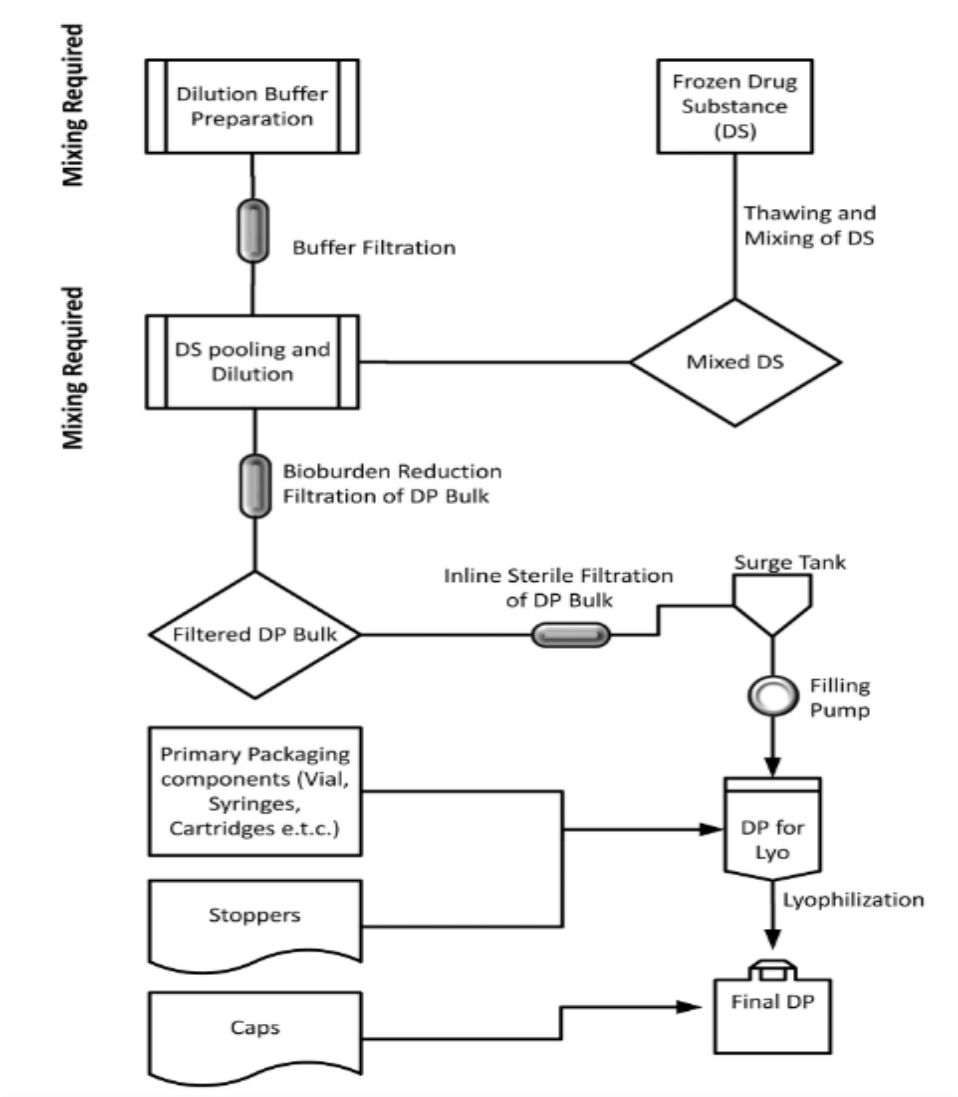


Figure 4. Process diagram of a generic mAb drug product manufacturing (6)

However, most DS formulation is concentrated due to material management, including worldwide shipping and storage management to reduce transport and warehousing costs. In such cases, it is necessary to prepare a buffer solution during the compounding process to dilute the DS to the final concentration. Usually, two filtration steps occur, bioburden reduction filtration and final sterile filtration.

To ensure the convenience of patients and doctors, pre-filled syringes (PFS) have become a focus of development. These devices are growing in popularity because of their convenient administration. Although manufacturing them is more complex and expensive, doctors can treat more patients in the same amount of time, making PFS a competitive advantage.

After aseptic filling into a primary packaging (e.g. a PFS), assembly may still be required (e.g. screwing in a plunger rod, installing a needle safety device). This is followed by secondary packaging, in which the drug product is packaged in a blister, for example, and placed in a folding box together with a leaflet on which the variable batch data (*e.g.* batch number, expiry date) is printed in-line. Some packaging material is printed with the appropriate country design/language (artwork). These FINP are packed into larger transport boxes containing several dozens of drug product packs and stored on pallets. They are then shipped, usually refrigerated, in pallet boxes to affiliates for distribution.

All operations involved in the production, warehousing and distribution of a DP have the potential to cause stress on the delicate structure of proteins. It is thus crucial to ensure that these processes do not compromise the effectiveness and safety of the FINP.

7. Quality impact of the manufacturing unit operations

7.1 Freeze/Thawing

Freezing is a key step in the storage of bulk DS to preserve them and allow greater flexibility in the manufacturing process. The frozen DS remains in storage until required for the manufacture of the DP. When required, the substance undergoes a thawing process to facilitate formulation. The physical alterations brought about by freezing and thawing may result in conformational changes in proteins that could lead to protein aggregation. (4).

Multiple stresses are linked to the freeze-thaw process, including freeze concentration, changes in pH, mechanical stresses, and low temperature, which may result in cold denaturation (27), exposure of protein molecules to the ice–liquid interface, and/or adsorption to the container surface (28).

7.1.1 Trigger factors for protein aggregation during freeze and thawing

- Cryoconcentration: Upon freezing, ice crystals prompt the exclusion of solutes and proteins from the solid phase (27, 29).
- Air Bubbles: Freezing processes can increase the solute concentration, which in turn leads to gas accumulation. This, in turn, increases bubble formation and can cause protein instability due to solution/air interfaces. (27).
- In maximally freeze-concentrated solutions, interfaces may form when the solution is cooled below the glass transition temperature (27); however, storage of the frozen formulation at temperatures above the glass transition temperature results in greater molecular mobility and can result in enhanced degradation, especially protein aggregate formation (29).
- Quasi-liquid layer: This refers to the thin layer of liquid water that forms on the surface of ice crystals. Proteins trapped in this layer may be exposed to destabilising stress caused by an increase in acidity resulting from the negative charge on the surface of ice crystals. (27).

7.1.2 Impact of interfacial stress during freezing and thawing

Interfacial stress is one of the topics that have been pointed out in several studies as particularly important due to the conformational changes that proteins undergo during the freezing and thawing stages.

Freezing and thawing stresses such as formation of ice– water interfaces, buffer-induced pH changes, re-distribution / concentration of solutes, and phase separation can lead to complex changes in the buffer environment and result in protein aggregation. Although the mechanisms responsible for protein stabilisation or destabilisation by buffers are intricate and not thoroughly understood, it has been demonstrated that the characteristics of a protein solution environment, including salt type, buffers, and their concentrations, have a significant impact on protein aggregation (4).

7.1.3 Freezing/thawing time range and formulation

In a study by Jain et al. (2021), it was suggested that a combination of slow freezing and fast thawing would be the least likely to cause aggregates. The opposite combination of fast freezing and slow thawing would cause the highest amount of protein aggregation due to the increase in protein-protein interactions between unfolded proteins and aggregates, which would produce either more aggregates or high molecular weight aggregates. In this scenario, the most significant factor leading to the development of aggregates would be gradual thawing. Other variables, such as sodium chloride and protein concentration, also demonstrate relevance to aggregate formation. Formulations with reduced concentrations of sodium chloride and elevated protein concentrations are less vulnerable to interfacial stress caused by the freeze-thaw cycle (4).

The rationale behind the protection in high protein concentrations relies on the fact that the fraction of protein molecules exposed to the ice-liquid interface would be proportionally reduced, and thus have a positive impact on minimising aggregation.

On the other hand, freezing induced increase in salt concentration can reduce intermolecular repulsion (*i.e.*, colloidal stability) between protein molecules *via* charge shielding, resulting in more favourable intermolecular interactions that lead to aggregation (28).

The stability data available to date demonstrate that the high protein concentration and reduced sodium chloride formulation continues to meet the target criteria for the quality attributes tested under long-term storage conditions (4).

A plan to manage the impact of freeze and thawing in the final product must be devised early in the development phase. However, it is worth to pointed out that freeze-thaw cycling experiments, which are usually performed with small size samples, are not necessarily predictive of long-term frozen stability of proteins in larger amounts (27).

Therefore, one has to consider that most of the studies regarding freeze/thaw stresses were conducted on a small scale and scale-up to the commercial manufacturing stage of drug production, may add additional stress to the proteins (*e.g.* usage of so-called cryo vessels or single unit technologies (SUT) for storage of DS).

7.1.4 Step-by-step of freeze and thaw operation

Table 1 gives an example of the manufacturing scale up of a freeze-thawing procedure that utilizes a cryo-vessel, the ongoing practice at Roche Diagnostics GmbH.

All the steps outlined could result in mechanical stress. Pumps may cause conformational changes in proteins, resulting in their aggregation. Chapter 7.4 elaborates on such stresses in detail. Furthermore, adsorption effects may occur and there may be leaching of substances from the tubing and gaskets into the product solution. To ensure quality in a GMP process, it is essential that the entire process undergo qualification and validation.

Table 1. Process flow of the freeze and thaw operation

Freezing – process step performed by a DS site (e.g. Penzberg)	
The API is transferred to a cryogenic vessel using a dip tube that runs from the top to the bottom of the vessel, enabling the inner compartment to be filled.	
Thawing	
Step #1	Silicone oil is pumped through the outer compartment of the vessel allowing the warm up of the API and the beginning the thawing process.
Step #2	A peristaltic pump is used to move the solution from bottom to top to homogenise the API solution and facilitate heat distribution during thawing.
Step #3	The final stage is the transfer of the thawed API solution to a receiving tank, and the purge of the API solution is performed through overpressure (*e.g.*, using nitrogen). The nitrogen is pressured into the cryo-vessel that prompts the API solution to flow through a tube placed at the bottom of the vessel into the receiving tank, from where it can go straight to compounding or be placed on hold for later compounding

7.2 Mixing

Mixing is an essential part of biopharmaceutical manufacturing, the main purpose of which is to homogenise DS and DP. To some extent, degradation can be triggered by mixing through the formation of subvisible particles (SvP) that can affect product quality. Common problems associated with mixing are mechanical shear and air-liquid interfacial stress; however, formulation can play an important role in overcoming these challenges (6).

The air-water interface is one of the stress factors present during mixing, and it can be easily overcome by optimising the formulation. Variables such as pH, ionic strength, and polyvalent ions can influence protein instability during mixing, affecting protein-protein interactions at the air-water interface. Therefore, the development of stable formulations can be a mitigation action to avoid the formation of aggregates while mixing (30).

7.2.1 Filter clogging

Considering that sterile filtration occurs after mixing, one would think that it does not represent a significant problem. However, the formation of particles during mixing presents two main challenges:

- First, particles over 0,22 µm are retained by the filter; if a solution holds a large amount of particles, it can cause clogging and/or fouling of the filter leading to process delays or failures and, more frequently, the need for human intervention with the associated higher risk of breakage of the aseptic barrier and therefore contamination (6, 30).
- Second, particles below 0.22 µm are not held by the filter; those particles will remain in the solution and can act as nucleation seeds, triggering aggregation that can happen months after filling, and in the worst case scenario, could lead to a batch recall (6, 30).

However, mitigation actions can be implemented to reduce the number of particles without taking the risk of breaking the aseptic barrier. Introduction of an additional (= 3rd) filtration (pre filtration) step during the passage of DS to the compounding vessel can diminish the number of particles. As bioburden filtration (0.45 µm filter) occurs after compounding, it is ensured that this is a safe possibility to prevent filter clogging during sterile filtration.

7.2.2 Impact of different mixers on aggregation

Gikanga et al. (2015, 2017) tested different types of bottom-mounted mixers (BMMs) to figure out which process would be more effective. The results, presented in a sequence of two papers, are summarized below.

7.2.2.1 Assessment of Mavag Mavadrive™ Mixer, Magnetic Mixer, LevMixer®, and Mobius® Mixer

In the first paper 4 BMMs (Mavag Mavadrive™ Mixer, Magnetic Mixer, LevMixer®, and Mobius® Mixer) were assessed. The results showed that the Mavadrive™ Mixer and Magnetic Mixer had developed a larger number of subvisible particles (SvP) when compared to the LevMixer® and Mobius® Mixer, the reason being the design of the impeller unit assembly (30).

The Mavadrive™ Mixer presents a gap (tight clearance) of 0.035–0.040 mm between the impeller and the drive unit (Figure 5). The magnetic mixer has the drive unit in close contact with some ceramic beads. In both cases, the narrow gap created seems to be the cause of SvP formation, as it creates a high shear zone (30).

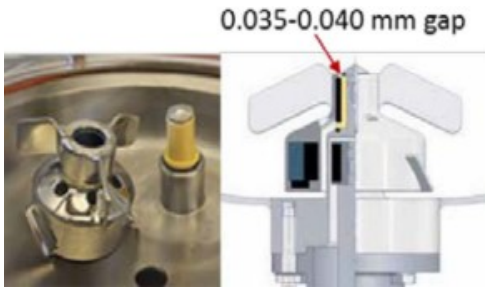


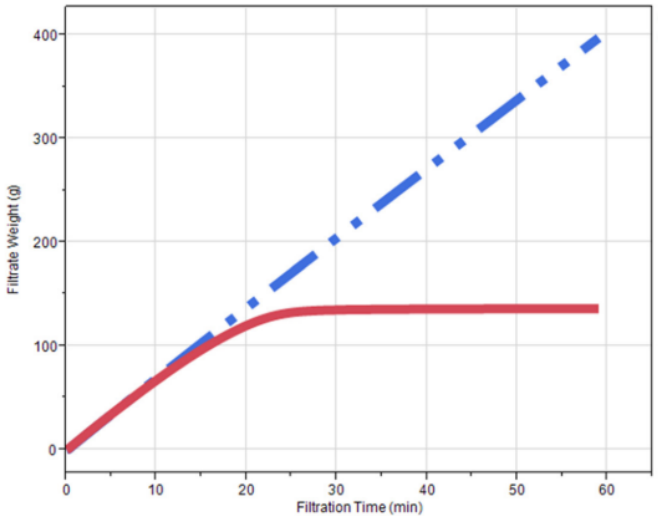
Figure 5. Mavag Mavadrive™ - Gap between the impeller and the drive unit (30)

On the other hand, the LevMixer® and Mobius® Mixer presented a wider gap between the impeller and the drive unit, which did not come in contact during rotation (30).

The tight gap between the impeller and the drive unit seems to exert influence on SvP formation. Therefore, the same products mixed by the Mavadrive™ Mixer and the magnetic mixer are under greater risk of particles being present due to shear stress, as compared with the LevMixer® and Mobius® Mixer.

7.2.2.2 Assessment of the Lignin® mixer: Top-mounted vs. bottom-mounted

The following experiment was designed to ensure the accuracy of the previous findings. A Lignin® mixer equipped with a top- and bottom-mounted impeller was used. Each impeller (top and bottom) was tested separately under the same conditions. The experiment corroborates previous findings. The samples from the bottom-mounted mixer contained >1.00 x 10^5 particles/mL, resulting in significant filter fouling, whereas the sample from the top-mounted mixer contained 3.00 x 10^2 particles/mL. The result was only slightly higher than that of the unmixed control (7.00 x 10^1 count /mL; Figure 6) (30).



Mixing Time (hours)	Particle Count by Coulter Counter (particles/ml)	
	Top-Mounted Impeller	Bottom-Mounted Impeller
0.0	7.00 x 10^1	7.00 x 10^1
0.5	NT	4.53 x 10^4
1.0	2.92 x 10^2	1.05 x 10^5

NT. Not Tested

Figure 6. Particle count after the mixing experiment (30)

7.2.3 Single-use equipment

The commonly used industrial mixers have been, in some instances, replaced by ready-to-use disposable sterile equipment. Biotechnology companies have been showing increasing interest in the use of this disposable equipment; however, there are still challenges to overcome (*i.e.*, extractables/leachables, adsorption and possible environmental impact).

The advantages in the use of these technologies include reduction of cross contamination, no need for cleaning or sterilisation validation, simplifying the processes and saving time. Furthermore, the size of the equipment can, to a certain extent, be adapted to batch size,

allowing flexibility of manufacturing, avoiding leachables from the stainless steel vessels (31).

A small-scale study carried out by Nakach et al. (2022) revealed that, even under much higher mixing stress conditions than those found in commercial scale, monomer and high molecular weight species remain constant over the time of the study (4-hour stress test). A random variability was observed in particle formation; however, it was attributed to contamination of the sample. Dynamic light scattering (DLS) was carried out to monitor the particle size variation between samples before and after the stress test; no variation was found, and there were no visible changes in turbidity (31).

Industrial mixers used in the manufacturing of biologics today still present the characteristics of the equipment used in small molecules manufacturing. To better accommodate the production of biologics, the structural and processing characteristics of the different manufacturing processes have to be considered. This would improve the quality and efficacy of the biologic drugs, as well as the manufacturing efficiency, by implementing systems and technologies which have shown less quality issues (related to aggregation) and optimised manufacturing processes. The use of ready-to-use disposable equipment (Figure 7 & 8) seems to be a good replacement for commonly used mixers.

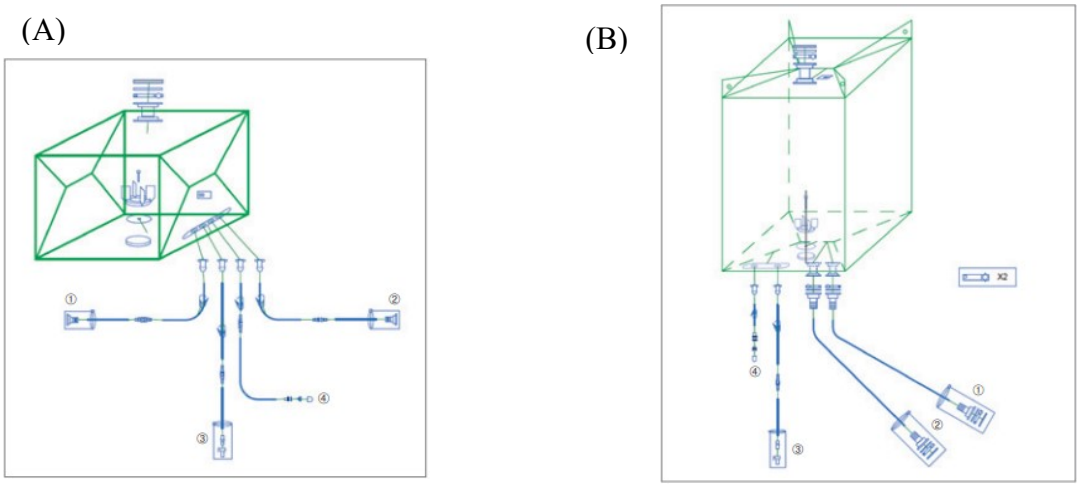


Figure 7. Flexel® Bags for magnetic mixer; (A) 50 L to 1.000 L; (B) 1.500 L to 2.000 L (32)

7.3 Filtration

Due to the thermal liability of protein-based pharmaceutical products, the state-of-the-art method to ensure aseptic processing is through filtration (33-35).

Bioburden reduction filtration can retain particles and microorganisms larger than the filter pore size (0.45 μm). Depending on the filter material and/or the solution properties, components from the formulation (*e.g.,* surfactants) and/or the protein itself can be adsorbed to the filter which can cause conformational changes that can lead to aggregation, resulting in filter fouling, clogging, or even passage of aggregates through the filter membrane into the filtered solution. Materials from the filter can also be released into the filtered solution (33).

After compounding, the pooled bulk solution goes through a first bioburden and particle reduction filtration (0.45 μm). It can then be either stored at 2–8°C for period of time pre-determined by the manufacturer, in which the quality of the DP is not affected, or taken straight to filling, where a second filtration, aseptic filtration (0.22 μm), is performed), completing the aseptic procedure. The bulk solution is then filled into sterilised and depyrogenised vials, cartridges or PFS (33, 34).

7.3.1 Criteria for selecting the filter

Filters must be selected taking into account several criteria (34, 36, 37), which may be summarized as: (1) capability to retain microorganisms, *i.e.*, remove the bioburden and provide a sterile filtrate; (2) compatibility with the product (*e.g.,* solvent) and process (*e.g.*, scale-up for in-line filtration); additionally, the filter should be (3) non-toxic and it should not (4) adsorb components (*e.g.,* surfactant) or add extractables to the formula; (6) allow in-place integrity test prior to and after filtration; and (7) permit sterilisation procedures, including in-place sterilisation, without compromising its integrity.

7.3.2 Integrity test

The pore size of the membrane follows the American Society for Testing and Materials (ASTM) specification; to meet the relevant aseptic standards required for parenteral drugs, the nominal pore size must be 0.22 µm or less. Filters must be validated using a bacterial challenge test (BCT). The integrity test (IT) requires a retention of at least 10^7 organisms/cm^2 of filter surface area, and the filtration product must be a sterile effluent. The standard microorganism used for the test is *Brevundimonas diminuta* (ATCC 19146) because of its small size (0.3 µm mean diameter). The integrity test must be performed before and after the filtration of the product of interest.

7.3.3 Adsorption to the filter membrane

Surfactants, such as PS20, PS80, and Poloxamer 188, are commonly used throughout the manufacturing of pharmaceutical products to prevent interfacial interactions (38-40). Surfactants mainly protect proteins from adsorption through competitive bidding to surfaces, thus preventing proteins from reaching liquid–solid (solution – filter membrane) interfaces and binding to them.

Mahler et al., 2010, analysed how PS80, in a monoclonal antibody solution, binds to filter membranes during sterilising filtration. Different filter materials, Polyvinylidene difluoride (PVDF), Polyethersulfone (PES), Cellulose Acetate (CA), and nylon, from different suppliers, were used. The first study was carried out at a laboratory scale, where the extent of protein bidding to each of the filters was assessed. As such, two solutions with and without polysorbate were filtered. The results confirm that the binding of proteins to filter membranes is significantly higher for solutions without PS, especially for nylon and CA filters.

The factors affecting adsorption onto the filter membrane are outlined in Table 2.

Although small amounts of protein loss usually does not impact the final dose, diluted protein solutions can be affected by it. Adorption of proteins to filter membranes can trigger conformation changes; these proteins can be released into the filtrate in their denatured form, possibly leading to aggregation (33, 38, 39).

Table 2. Formulation and filter characteristics that affect filtration – Adapted from (38)

Filter membrane	Formulation and process parameters
Composition of filter membrane	Composition and chemical nature of the raw materials
Chemical nature (cationic / anionic / non-ionic)	pH of the formulation
Eletrostatic forces (hydrophilic / hydrophobic)	Ionic strength
Surface area and mass of filter membrane	Temperature of the process

Filter selection must meet stringent requirements to ensure sterile and stable products. If denatured proteins or filter particles break through the filter membrane and enter the filtrate, the consequences can be significant because of the significant time required for particle aggregation. Therefore, these consequences may not become clear until the final product reaches the market, potentially resulting in product recall, as discussed before. Excess particles within the solution can block the filter, increasing the risk of contamination through human intervention. In such cases, a third filtration may be advisable to reduce particle counting and blockage of the filter. Blocked filter can require human intervention, such as the opening the sterile circuit.

While performing filtration of low-concentrated solutions, care must be taken to avoid possible protein loss due to filter adsorption, which can ultimately alter the final product's desired concentration.

7.4 Filling

Filling is a critical step in biopharmaceutical production, performed at the end of the process. It involves precise filling (IPC of the conformity of filling weight) of the solution into the primary container using volumetric pumps. However, this step can also result in the formation of aggregates due to interfacial stress, such as that caused by air or solid-liquid interfaces.

7.4.1 Aggregation during filling

Adsorption, friction, and cavitation are potential triggers for protein aggregation during filling, but they do not appear to be the primary contributors. In contrast, shear stress is involved in protein denaturation and the resulting aggregation. However, protein unfolding would necessitate a shear rate exceeding 10^7 s^{-1}, while during filling, the commonly applied rates are 10^5 s^{-1}. Therefore, at this rate, other factors must collaborate to promote denaturation (41).

Other factors can have an impact on product quality, such as physical grinding (*i.e.*, solution trapped between moving parts), rising of temperature generated during pumping, foaming, contact time with pump material, material compatibility, and interaction with processing aids, (*e.g.*, silicone oil) (42).

7.4.2 Shear stress and adsorption

The adsorption of proteins onto solid surfaces may trigger conformational changes, potentially resulting in aggregation, depending on the structural stability of the protein and the hydrophobicity of the surface. The theory that shear stress alone is not the cause of aggregation suggests that other contributors, such as the nature of the material, play a role in the initiation of aggregation mechanisms during pumping. Therefore, variables such as the nature of the materials in contact with the protein solution and the type of pump for the intended purpose need to be carefully assessed (7).

Although the adsorption to surfaces is protein and surface specific, a study conducted by Roffi et al. (2021) ranked the adsorption of a given protein to three different pumps (ceramic rotary piston pump, stainless steel rotary pump and peristaltic pump) at manufacturing scale. The study concluded that the ceramic rotary piston pump produces more particles, followed by the stainless-steel rotary piston pump and the peristaltic pump. Mechanical disruption of the adsorbed protein film may cause the formation of nucleation particles in the final protein solution (7). An earlier similar study conducted by Nayak et al (2011), a decade ago, achieved comparable findings when examining the fill-finish process of a specific protein using five

pump systems (ceramic rotary piston pump, stainless steel rotary pump, rolling diaphragm pump, peristaltic pump and time-pressure filler). The study concluded that the rolling diaphragm pump, peristaltic pump and time-pressure filler produced significantly fewer particles than the rotary piston pump. (42)

Protein unfolding in ceramic rotary piston pumps and peristaltic pumps cannot be attributed to cavitation, given the mild pressure drop. However, this cannot be completely ruled out in cases of time-pressure, where a larger pressure drop occurs (7).

7.4.3 Characteristics of dosing systems

7.4.3.1 Piston pump

The device is characterised by a stainless steel or ceramic piston (Figure 9), which, when activated, creates a pressure gradient that sucks the solution into the cylinder. The piston then rotates 180° on its own axis to dispense the solution. A downward movement ejects the solution into the needle. The pump's advantage lies in its accuracy and precision when handling small filling volumes (<0.3 mL). However, its disadvantage is that different pumps are needed for different ranges of volumes, and there is a hypothesis suggesting that piston pumps cause more aggregation than other pumps due to a high count of SvP particles in solutions filled by a piston pump (43, 44).

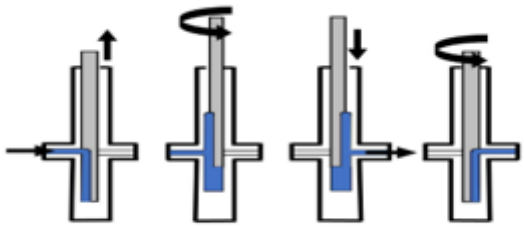


Figure 8. Piston pump
Adapted from (43)

7.4.3.2 Radial peristaltic pump

The radial peristaltic pumps (Figure 10) comprise a rotor that drives a counterpressure plate, which is equipped with three rollers responsible for exerting pressure on the fluid within the tubing. This pressure gradient advances the fluid continuously, enabling the bulk solutions to be consistently drawn into the pumping system. The tubing diameter, elasticity, number of rollers, counterpressure, rotation angle and size of the pump head play a crucial role in determining the dose (volume of solution). The benefits arise because of the solutions contacting solely with the tubing, rather than the pump mechanism, which minimises extractables, leachables and particles. However, a precise assessment of the tubing material is necessary to avoid shedding of the tubing into the solution (43, 44).

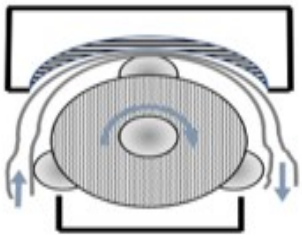


Figure 9. Radial peristaltic pump
Adapted from (43)

7.4.3.3 Linear peristaltic pump

The physical principle used in the linear peristaltic pumps (Figure 11) is the same as that in the radial peristaltic pumps. However, this pump has multiple horizontal piezo actuators that move differently in each part of the cycle. The solution flows from top to bottom, and once the tubing is filled with the solution, a central piezo actuator moves forward, compressing the tubing and stopping the flow of the solution. The piezo actuators located below move at a 60° angle in relation to the central actuator, successively propelling the solution downward. The piezo actuator located upward moves in the same way but backward. This motion prevents unwanted dripping. Volume is measured by the number of cycles, quantity of displaced actuators, actuator size, and tubing elasticity (43, 44).

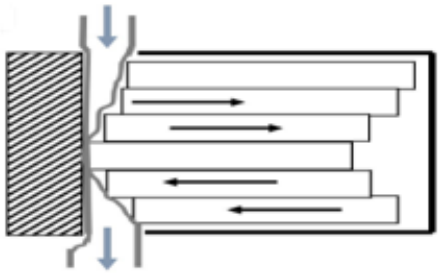


Figure 10. Linear peristaltic pump
Adapted from (43)

7.4.3.4 Time-pressure filling system

In the time-pressure system (Figure 12), the solution is stored in a tank and overlaid with an inert gas, typically nitrogen, at a constant pressure. The solution is then distributed through the tubing to the filling needles. The valve controls the filling volume by opening for a set period of time to adjust the pressure and diameter of the tubing. Because of changes in density and viscosity, the time pressure system is affected by temperature. It is essential to use computer systems and technical experts to ensure the reliability of the process. Nonetheless, among the filling systems described, it is considered as the least precise (44).

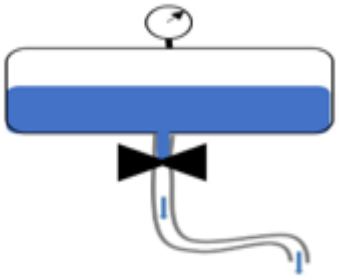


Figure 11. Time-pressure system
Adapted from (43)

7.4.4 Filling of low volumes

Low volumes pose a challenge in the filling process to ensure dosing accuracy. Intravitreal medications require low filling volumes, typically less than 200 µL. The piston pump is the most precise means of measuring low volumes, despite generating more shear stress than other pump types. However, there has been a trend towards using radial peristaltic pumps, which generate less shear stress and have limited surface contact (only with tubing).

Nevertheless, these pumps are not as precise as piston pumps for measuring volumes below 200 µL (44).

Special requirements for particles in ophthalmic medications are defined by the United States Pharmacopeia (USP), which differs from the standards set for parenterals.

A study by Dreckmann et al. (2020) found that piston pumps generate the most SvP > 2 µm compared to radial and linear peristaltic pumps, and produce the highest amount of protein degradation when filling a protein solution. The primary source of shear stress on piston pumps is friction caused by the piston's contact with the cylinder in a grinding motion. Both peristaltic pumps exhibited high shear rates in the region where the tubing material is compressed either by the rollers (radial peristaltic) or the piezo actuators (linear peristaltic). However, the shear stress affects the solution near the tubing walls more than the solution in the centre of the tubing, which is relatively protected. When comparing the particle count of the piston pump with that of the peristaltic pumps, it was found that the piston pump had a particle count that was 20 times higher than that of the peristaltic pumps. In a subsequent pump evaluation using a liposome model sensitive to shear stress, it was discovered that the radial peristaltic pump generates an increased shear rate compared with piston pumps. This is contrary to what is reported in the literature, which suggests that piston pumps are the most susceptible to shear stress (43). In conclusion, although the piston pumps produced a greater quantity of particles, they did not present the highest shear rate, suggesting that other factors may have contributed to the high particle count.

7.4.5 Filling and its possible impact on product quality

The filling process is a crucial step in biopharmaceutical production that has a substantial effect on product quality, particularly in relation to the formation of particles, as no filtration is conducted after filling. The complex phenomenon of protein aggregation during pumping is influenced by various factors, including shear stress, adsorption, and other factors specific to the material. Numerous studies have compared various pump types, revealing differences in their ability to generate particles and induce protein degradation.

Piston pumps, known for their precision in handling small volumes, produce higher levels of subvisible particles and contribute to protein degradation. Nevertheless, it is crucial to acknowledge that shear stress alone does not appear to be the exclusive reason for the generation of aggregation during or because of pumping. Other factors, including the composition of materials in contact with the protein solution and the specific type of pump, also have a significant impact.

Radial and linear peristaltic pumps have the advantage of minimising contact between the solution and the pump mechanism, which reduces the amount of extractables, leachables, and particles. The findings of these studies contradict the widely held notion that piston pumps are the most vulnerable to shear stress, highlighting the need for a comprehensive evaluation of all factors contributing to protein aggregation.

Time-pressure filling produces a minimal shear force, which is sufficient to minimise particle formation; however, it is highly sensitive to temperature and the precision is poor, making it more suitable for high volumes.

In summary, understanding the complexities of protein aggregation during pumping is essential for optimising biopharmaceutical production processes. Careful consideration of the pump types and materials in contact with the protein solution is crucial to ensure product quality and minimise the risk of aggregation.

8. Product quality impact of potential chemical and enzymatic degradation throughout manufacturing

Chemical and enzymatic degradation and aggregation of proteins have been extensively reported in the literature as having the potential to trigger an immune response, either acutely or upon prolonged use.

Because of the sensitivity of proteins, even the most basic stimuli can induce conformational changes, leading to physical and chemical stability issues. These issues may compromise the quality of the final product and even trigger immunogenic reactions causing adverse drug reactions (ADRs). Therefore, it is crucial to ensure the stability and quality of proteins in drug development and manufacturing processes (45, 46).

Meeting the critical CQA is essential to ensure the safety and effectiveness of a product from manufacturing until the end of its shelf life.

During the manufacturing of drug products, between other chemical instability triggers, oxidation can result from light exposure, contact with surfaces, and different manufacturing materials that can introduce redox-active metals and leachables into the formulation (11).

8.1 Light exposure

Light exposure during manufacturing, storage, distribution, and administration processes can cause photooxidation. Oxidation may also be caused by redox-active metal ions (iron, copper or nickel). Methionine and tryptophan residues are highly susceptible to oxidation, followed by histidine, cysteine, phenylalanine, and tyrosine residues, whereas strong oxidants are capable of oxidising the peptide backbone (11). Some consequences of exposure to light include heightened peroxide levels, oxidation of specific amino acid residues, alterations in colour, aggregation, and increased acidic species (8, 47).

Molecules are exposed to visible light (> 400 nm) during the manufacturing process, and potential exposure to UV light cannot be excluded. Specific proteins may be vulnerable to a range of wavelengths in the visible spectrum (400-700 nm), whereas short wavelengths (300-

400 nm) could result in higher levels of tryptophan degradants (11), as well as phenylalanine, tyrosine, and sulfur-containing cystine (47). Conversely, longer wavelengths (600-700 nm) have minimal impact on product quality (11).

The critical processes, from manufacturing to administration, that pose high light exposure risk may be summarized as: (a) chromatographic processes – Chromatography columns are mostly made from transparent glass; (b) fill and finish – visual inspection, packaging and long-term storage, and exposure to UV-C light; (c) administration – biopharmaceuticals delivered through IV systems are often diluted into solutions in clear IV bags that are exposed to light throughout the process.

8.1.2 How photodegradation occurs

Photodegradation in the UV region primarily occurs with aromatic amino acids, cysteine, and peptide backbones. This happens due to the excitation of an electron that is promoted to a higher energy state (singlet state), followed by processes such as fluorescence, creation of oxygen species (ROS), and photoionization, which can result in an uncharged radical (48, 49).

The use of antibody-drug conjugates (ADCs) as a therapeutic approach for cancer treatment has brought the issue of photostability to the forefront. This is because many anticancer medications conjugated with antibodies contain photosensitive aromatic groups (49, 50).

Not only are the proteins, as the active pharmaceutical ingredient, susceptible to photodegradation, but some excipients, such as polysorbate, can also undergo photodegradation. This process results in peroxide production within the formulation and can lead to further oxidation of amino acids. Furthermore, metal-catalysed oxidation (MCO) can trigger this reaction (45, 48). Peroxides are capable of oxidising amino acid residues such as methionine and histidine (11).

Photooxidation can also be influenced by the headspace oxigene in the vials or even the type of buffer (48).

8.1.3 Mitigation actions to minimise light degradation during manufacturing and storage

- Use of light sources with restricted output in blue and UV wavelengths (11, 47).
- Wavelenght selective filters/shields (11, 47).
- Time-based exposure controls (most effective mitigation strategie) (11).
- Sacrificial antioxidants (with methionine being the most effective) have limited efficacy on certain amino acids (11).
- Robust secondary packaging is essential for sensitive molecules. In cases where secondary packaging is removed at the final destination due to storage restrictions in sterile areas, light-protective primary packaging, such as amber or yellow glass, becomes necessary to ensure product quality (11, 45).

8.1.4 How excipients can provide protection against photooxidation

Even when fulfilling all requirements to promote an optimum environment for the manufacturing of biopharmaceutics, it is still impossible to completely avoid exposure. Normal manufacturing, laboratory, testing, distribution, and administration conditions can lead to excessive light exposure for vulnerable protein-based products.

Due to the inevitable exposure of proteins to light during the manufacturing to administration process, regulatory agencies conduct rigorous photostability studies when evaluating protein-based products for marketing approval (48).

Shah et al. (2018) evaluated five excipients (amino acids: methionine, tryptophan and arginine; sugars: sucrose and trehalose) in relation to their protective potential against an IgG1 mAb. The study adhered to the International council for harmonization (ICH) guidelines (Q1B, 1997) and examined two groups of samples; one group was stored in clear vials and the other was protected from light with a tin foil covering. During the SDS-PAGE analysis, bands of approximately 25 and 50 kDa, representative of the light chain (LC) and the heavy chain (HC) respectively, were observed in all samples. However, in the group of samples exposed to light, two additional bands of approximately 75 and 150 kDa were observed, indicating oligomerization, which previous studies suggested could be a consequence of HC-LC or HC-HC cross-linking (50). The only exception to the finding was the sample with methionine as a protective agent, which showed only one of the two high

molecular weight bands, approximately 75 kDa. None of the high-molecular-weight bands were detected in the dark control samples. The findings were further confirmed through the use of DLS, which demonstrated that the sample containing methionine had a hydrodynamic radius similar to that of the dark control sample without methionine. This suggests that methionine played a protective role in shielding the antibody from photooxidation.

8.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is commonly employed in the pharmaceutical sector because of its sterilising qualities. It is highly effective against bacteria, viruses, and spores, whilst also being environmentally acceptable (51, 52). It requires short cycle times and has been proven to be safe and effective in reducing microbial load to an acceptable level, creating a sterile environment within isolators for aseptic filling, as well as in the sterilisation of secondary packaging materials (53, 54).

The concentration range typically employed during a cycle is between 400 and 1300 ppm (v/v). Nonetheless, concentrations as high as 6000 ppm (v/v) have been utilised in diverse applications (54).

Owing to its characteristics, hydrogen peroxide is becoming a preferred decontaminating agent for use in containment systems for the manufacture of parenteral drugs to ensure that sterile conditions are maintained; however, hydrogen peroxide presents strong oxidising properties that can affect the quality of biopharmaceutical products (51).

8.2.1 Vapour phase hydrogen peroxide sterilisation cycle

The process for an effective sterilisation cycle comprises several steps (Figure 12). First, the necessary equipment is prepared and tubing is set-up inside the isolator (Figures 13 A and B). A previously validated cycle is then initiated, followed by an aeration step designed to reduce the hydrogen peroxide concentration inside the isolator. Clean in place (CIP) and steam in place (SIP) are then carried out to ensure tubing sterilisation, which is subsequently

dried. After the aeration step has been completed, filling can be undertaken or scheduled for a later time, depending on production availability (51).

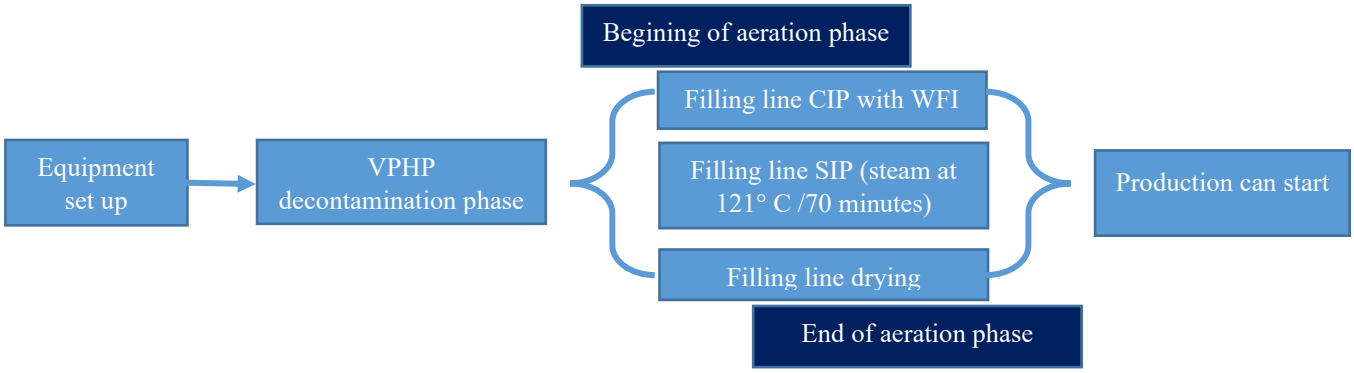


Figure 12. Process flow of vapour phase hydrogen peroxide (VPHP) sterilization. (VPHP – Vapour phase hydrogen peroxide; WFI – Water for injection; CIP –Clean in place; SIP – Steam in place)

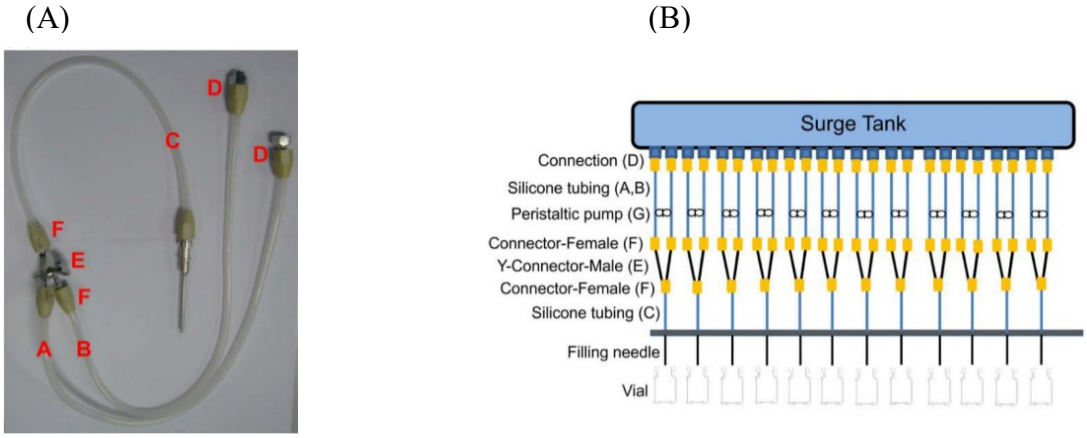


Figure 13. (A) Tubing assembly; (B) Filling line set up (51)

During the sterilisation cycle, hydrogen peroxide reaches all surfaces within the isolator, where it may be adsorbed or absorbed. Even after the aeration phase, residual vapour hydrogen peroxide (VHP) can be found in the isolator's atmosphere, which represents a critical aspect of its use that requires close monitoring as it can be reliased into protein solutions and trigger oxidative pathways resulting in the oxidation of methionine residues to methionine sulfoxide. Additionally, the presence of transition metals catalyses the formation of hydroxyl radicals that may lead to the oxidation of aromatic residues, such as tryptophan, tyrosine, and histidine (54).

8.2.2 Uptake of hydrogen peroxide

To avoid subjective evaluations, it is important to consider several operating conditions when dealing with the adsorption or absorption of hydrogen peroxide. These factors include relative humidity, exposure time, and residual concentration. The uptake mechanism of hydrogen peroxide is dependent on the material exposed. Adsorption is typically observed in empty glass vials, stoppers, and syringes, whereas absorption is more commonly observed in polymeric materials, such as tubings and bags (54).

When analysing which factors – VHP concentration, relative humidity, and exposure time – have the greatest influence on hydrogen peroxide adsorption to glass vials and rubber stopper surfaces, Kushwah et al. (2020) concluded that VHP concentration and relative humidity have the greatest impact. They observed that at elevated humidity, the extent of adsorption of hidrogen peroxide is diminished, indicating a competitive co-adsorption mechanism (55).

In a study conducted by Eisner et al. (2019), it was found that hydrogen peroxide is absorbed by surfaces and that this absorption correlates with atmospheric concentration in the isolator. Adsorption varies with changes in atmospheric concentration until equilibrium is reached (52).

In another study, four possible causes of hydrogen peroxide uptake were identified: glass surfaces (*e.g.*, vials or syringes), solution retained at the filling needle, exposed filled vials / syringes, and filling line tubing (51).

The findings of these three studies contributed to establish the concentration of hydrogen peroxide and the efficacy of the aeration phase leading to an acceptable residual level of hydrogen peroxide that can be tolerated in the final product. Factors such as low exposure of filled vials (*i.e.*, the vials should be closed as soon as they are filled to avoid contact with hydrogen peroxide residues in the isolator atmosphere), and adequate choice of tubing material, as well as suitable, validated CIP and SIP procedures, can all reduce the adsorption/absorption of hydrogen peroxide to surfaces and possible resorption to the solution, avoiding possible oxidation and loss of final product quality.

8.3 Elemental impurities

Low levels of elemental impurities, even when below toxicity levels, can impact on the quality attributes of the product. As the elemental impurities do not exert any role in the formulation and are not acting as the active ingredient, they are considered to be a contaminant since there is no justification for their presence in the final product. Although, in recent years, we have come to comply with certain daily exposure limits for these contaminants, in the cases where the permissible daily exposure (PDE) is elevated, other limits (lower then the PDE) might be considered to avoid quality issues, and other guidelines should be consulted (56).

Potential sources of elemental impurities to be considered for risk assessment include drug substance, excipients, manufacturing equipment, container-closure system, and utilities (57).

Drug substances and excipients are the greatest contributors of elemental impurities during production of drug products (57). Therefore, it is essential to buy high quality excipients from qualified, reputable suppliers. In the scope of the present work, the drug substance does not represent a great source of elemental impurities because it is provenient from biological production (more details on section 8.3.3).

To ensure the quality attributes of the final product and its safety, a risk assessment is necessary. The assessment is carried out in the drug product, and all the steps of production are taken into consideration to ensure that the levels of elemental impurities (when present) are below the PDE and do not compromise the quality of the medicine.

The assessment begins with the identification of all potential sources of contaminants, followed by the experimental or predicted levels of elemental impurities from each of the contributors and cross-checking results with the reference amount for daily exposure as per Appendix 2 of the ICH Q3D guideline; the final step is the implementation of control strategies, when necessary (57).

8.3.1 Elemental impurity classification

The elemental impurities have been divided into three classes, based on their toxicity (PDE) and likelihood of occurrence, described in Table 3.

Table 3. Classification of elemental impurities – Adapted from (56)

Class I	Arsenic Cadmium Mercury Lead	• Human toxicants; • Limited or no use in the manufacturing of pharmaceuticals; • Test is only required when the risk assessment identifies that appropriate control is needed. Testing should be performed for all potential sources and all routes of administration.
Class II	**Class II A** Cobalt Nickel Vanadium	• High probability of occurrence in the drug product; • Risk assessment of all potential sources and routes of administration.
	Class II B Silver Gold Iridium Osmium Palladium Platinum Rhodium Ruthenium Selenium Titanium	• Reduced probability of occurrence in the drug product; • No risk assessment is needed unless the element/elements are intentionally added during the manufacturing of DS, excipients, or other components of the drug product.
Class III	Barium Chromium Copper Lithium Molybdenum Antimony Tin	• Low toxicities by the oral route of administration; • Unless the element is intentionally added, it does not need to be considered in the risk assessment; • For parenteral and inhalation: The potential for inclusion of these products should be evaluated during risk assessment, unless the route-specific PDE is above 500 µg/day.
Other elements	Aluminum Boron Calcium Iron Potassium Magnesium Manganese Sodium Tungsten Zinc	• Elements that do not have an established PDE; • In the case of inclusion in the drug product, regional regulations should be applicable to particular elements.

8.3.2 Potential sources of elemental impurities

Elemental impurities can come from several sources and can be added to the drug substance (e.g. catalysts); in this case, the risk assessment should address the specific testing of the added substance. Elemental impurities can be incorporated into the drug product through the addition of contaminated DS, water, or excipients used, or through contact with contaminated manufacturing equipment. It could also be leached into the DS and DP from container closure systems (56, 57), as presented in Figure 14.

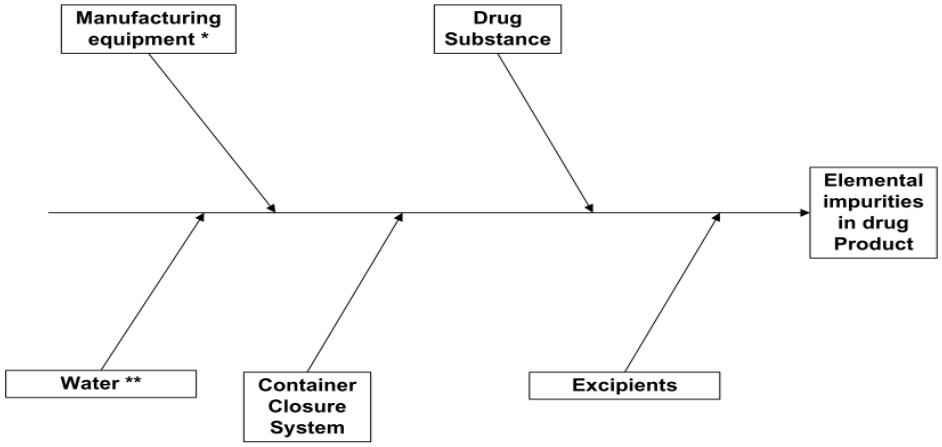


Figure 14. Sources of elemental impurities (56)

8.3.3 Mitigation actions

Elemental impurities can be identified through risk assessment and reduced by implementing risk minimising measures throughout the processes.

A thorough understanding of the processes, detailed equipment selection, qualification, and GMP are good strategies to reduce the risk of elemental impurities from equipment (56).

Adherence to the water quality requirements described in the compendial literature (*e.g.,* European Pharmacopoeia, Japanese Pharmacopoeia, US Pharmacopoeia Convention) during manufacturing processes can also help to reduce or even eliminate contamination (56).

On the excipient side, the selection of reputable suppliers and their qualification can help to avoid the problems associated with elemental impurity contamination.

The potential introduction of elemental impurities from the container closure system needs to be carefully assessed based on potential interactions between the drug product and its packaging. If the possibility of elemental leaching is detected, bearing in mind that for liquid and semi-solid drug products there is a higher probability of leaching during shelf-life, further studies should be conducted to assess the potential risk of this leaching throughout the shelf-life of the drug product (56).

The probability of elemental impurities arising from biopharmaceutical production of drug substance is unlikely, since these elements are not typically used as catalysts or reagents, and when present, they are typically in trace levels with no significant accumulation; furthermore, the purification processes (*i.e.*, chromatography, ultrafiltration- diafiltration) applied during upstream production are effective in removing elemental impurities from DS (56).

With regard to small molecules (as they can be conjugated with antibodies, for example), elemental impurities may be present in the drug substance even if not intentionally added, and following the ICH Q3D guidelines for risk assessment of elemental impurities becomes essential to identify, quantify, and comply with established limits for daily intake of these impurities (56).

8.3.4 Metal-induced particle formation

Polysorbate is extensively used in the manufacture of biopharmaceuticals because of its stabilising properties. However, due to enzymatic activity, commonly triggered by host cell proteins (HCPs), such as lysosomal phospholipase A2 (LPLA2), which can be purified together with the desired protein, can later catalyze the hydrolysis of polysorbate during long-term storage (58-60).

Allmedinger et al. (2021) found that the presence of elemental impurities from the "other elements" group, such as aluminium, calcium, and magnesium, were detected in visible particles sourced from a solution containing PS below the theoretical solubility limit and typically used for biopharmaceutical manufacturing. The origin of the leachbles was found to be the glass container (primary packaging), and the authors concluded that multivalent

cations, such as aluminium (Al^{3+}), can interact with free fatty acids (FFA), leading to insoluble complexes, which can act as nucleation seeds (61).

Gregorritza et al. (2022) conducted experiments to characterise the mechanism behind metal-induced FFA formation and the use of chelators as a mitigation option. The results corroborate the preliminary finding from Allmendinger, *i.e.*, an increase in Al^{3+} concentration led to faster FFA metal nucleation and earlier onset of visible particles. A follow-up study with bivalent (Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}) and trivalent (Al^{3+}, Fe^{3+}) cations showed the influence of trivalent cations on particle formation, while divalent cations did not influence the formation of particles. For the aluminium samples, there was a direct correlation between the concentration of the metal and the size and velocity of particle formation. For iron, an increase in concentration resulted in instant particle formation but no size growth over time. Furthermore, chelators, such as Ethylenediaminetetraacetic acid (EDTA) and Diethylenetriamine pentaacetate (DTPA), were found to prevent metal-induced FFA nucleation and subsequent formation and growth of FFA particles (58).

During the manufacturing process, there are numerous potential sources of elemental impurities. To prevent contamination, it is necessary to conduct a detailed risk assessment, considering the factors outlined in section 8.3.1. Adequate measures aforementioned (8.3.3), being the most significant one the assessment of closing systems, can reduce the risk of contamination. Beyond potential toxicity (when exceeding the PDE), metals can act as nucleation seeds and trigger the formation of particles throughout the shelf-life of a biopharmaceutical. This increases the possibility of batch recall and has a negative impact on manufacturing. Therefore, controlling elemental impurities, even when they are below the PDE, is crucial for long-term stability. After considering the measures mentioned above, it is crucial to evaluate the need for additional steps because even small amounts of elemental impurities in the presence of FFA can have severe consequences.

8.4 Enzimatic degradation

The interaction between proteins and various interfaces, liquid-solid, liquid-air, solid-air, and liquid-air, throughout the production stages, as well as their interaction with surrounding

surfaces such as stainless steel vessels, diverse tubing materials, SUT materials, glass, and rubber, may result in conformational changes leading to protein degradation and particle formation (62).

To mitigate the impact of various interfaces and material surfaces on proteins, the use of surfactants such as PS 20, PS 80, and Poloxamer 188 is necessary (63).

Surfactants are molecules that facilitate contact between proteins and their surroundings. However, surfactants are susceptible to degradation by enzymatic hydrolysis, which results in FFAs, and consequently visible particles throughout the shelf-life of biopharmaceuticals (58, 63, 64), leading to a potential batch recall and adverse impact on the manufacturer. It is important to note that certain measures can be taken to prevent these issues.

8.4.1 Polyssorbate

Surfactants are added to the formulation of biopharmaceuticals to promote its stability, lowering the chances of interfacial stresses and adsorption to surfaces that can potentially instigate conformational changes that can lead to particle formation.

Surfactants possess amphiphilic properties due to their hydrophilic head, in the case of PS sorbitol or isosorbide (64), and hydrophobic tail, resulting in a stabilisation effect on proteins. These surfactants can be anionic, cationic, or non-ionic[2] and interact with proteins, providing a protective effect. They also create a layer between the protein and the interface, saturating it (63).

Polysorbates 20 and 80 are the most extensively used surfactants currently used for the formulation of biopharmaceuticals. They have great stabilizing properties and recognised safety profiles and are highly versatile as they can be used for different routes of administration (*i.e.*, IV, IM, SC) and in solutions, suspensions, and lyophilisates. They were employed in more then 90% of EU approved mAbs in 2018 (62).

[2] Only non-ionic surfactants are used in pharmaceuticals, due to its safety profile in the presence of electrolytes.

Polysorbate is produced from sorbitol (Figure 15 A) dehydration and sterification, originating sorbitan (Figue 15 B) and, on further dehydration, isosorbide (Figure 15 C), the fatty acid chain is added through esterification (Figure 16) and finally an ethoxylation with ethylene oxide. The ethoxylated moiety is partially esterified with lauric or oleic acid, originating PS20 (Figure 17 A) and PS80 (Figure 17 B) respectively (64, 65).

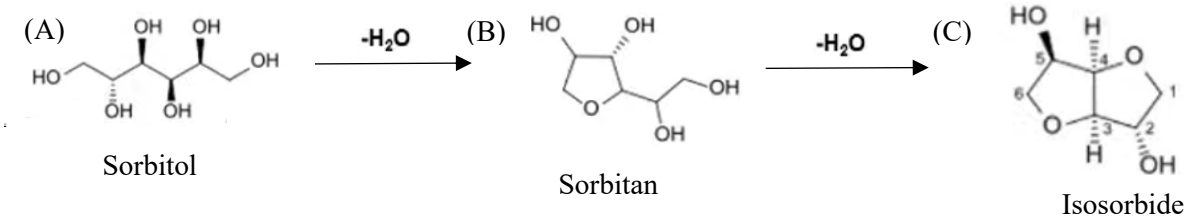


Figure 15. Dehydration of sorbitol – (A) Sorbitol, (B) Sorbitan and (C) Isosorbide

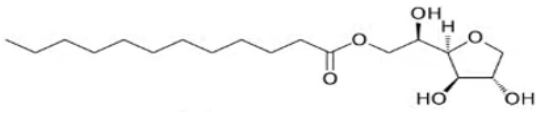


Figure 16. Addition of fatty acid to Sorbitan through esterification

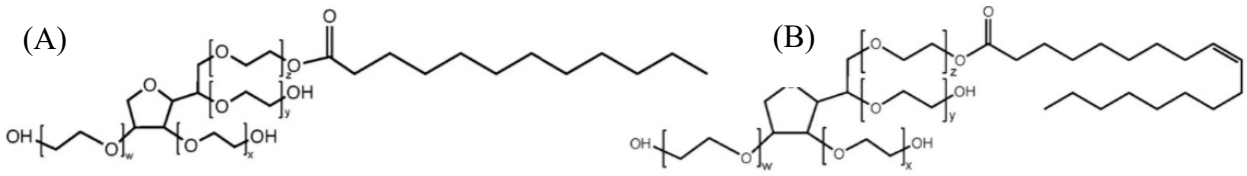


Figure 17. Ethoxylation - (A) PS20, (B) PS80

8.4.2 Enzymatic degradation of polysorbate

Polysorbates can be easily degraded via oxidative and enzymatic routes. Oxidative degradation occurs through temperature, light, or catalysing agents such as transition metals. Enzymatic degradation occurs through the hydrolysis of ester bonds, followed by the

cleavage and release of FFA (62). Hydrolysis has been attributed to HCPs, lipase, that can be co-purified with the desired protein through downstream processing. The lipase then promotes the cleavage of ester bonds of PS, thereby accumulating FFA throughout the biopharmaceutical shelf-life (Figure 18) (58, 62).

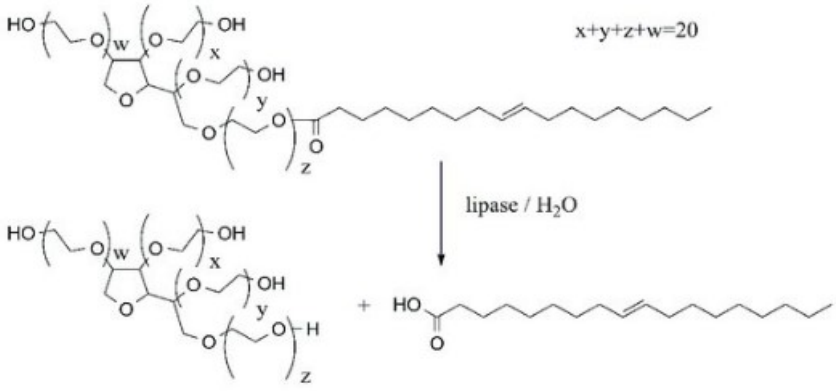


Figure 18. Enzymatic degradation of Polysorbate – Adapted from (66)

Besides the FFA that are released into the solution as a result of hydrolysis, other concerns also arise from that, such as the loss of function of the degraded surfactant and particle formation throughout the shelf-life (63).

8.4.3 Poloxamer 188

Poloxamers are co-polymers consisting of 2 blocks of hydrophilic polymer ethylene oxide linked to a hydrophobic chain of the polymer propylene oxide. Figure 19 depicts the structure of poloxamer 188.

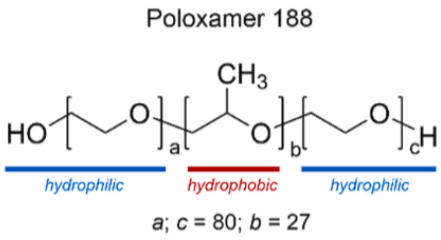


Figure 19. Structure of Poloxamer 188- Adapted from (62)

Poloxamer 188 was added to the formulation of approximately 10% of the EU-approved mAbs in 2018. Besides PS, poloxamer 188 is the only other surfactant currently used in the manufacturing of biopharmaceuticals. With the problems arising from the use of PS (listed on section 8.4.2), poloxamer 188 might become more commonly employed in the formulation of biopharmaceuticals in the next years (62).

To find a suitable substitute for PS, more investigation needs to be conducted on new surfactants. Studies on animals and humans have shown that poloxamer 188 is well tolerated through the intravenous route, even in high concentrations. The mechanism of stabilisation used by the three polymers in the discussion so far is still not quite clear (62).

PS has its performance well stablished due to its extensive use, while poloxamer 188 use has not been nearly as frequent; therefore, more investigation is needed to come to a final conclusion if PS can be substituted by poloxamer 188.

8.4.4 FFA and metal interaction

FFA can interact with multivalent cations (elemental impurities), such as aluminium (Al^{3+}), present in the solution, forming insoluble complexes. As the solubility of the FFA becomes lower than in its non-complexed form, these complexes act like nucleation seeds and can grow steadily through the biopharmaceutical shelf life (58).

As mentioned before (in chapter 8.3), there are several sources that can transfer elemental impurities to the solution. Stainless steel is a commonly used material in production, shipping, and manufacture of biopharmaceuticals and is a common source of iron, chrome, and nickel. Polymeric materials, on the other hand, can leach magnesium, calcium, iron, manganese, and zinc (58).

To investigate the formation of visible and subvisible particles derived from FFA-metal interaction as well as the use of a chelator to mitigate the negative impact, Gregoritza et al. (2022) conducted a study with lauric acid (due to be the most abundant residue of PS20 hydrolysis). The method chosen to analyse the formation of the nucleation seeds was DLS,

it allows the detection of the early onset of particle formation. The mesurements were conducted for three days during this first assessment.

In the first part of the study, different concentrations of Al^{3+} (20, 40, 60 and 80 ppb) were spiked in FFA-containing solution, and nanoparticles were detected in all formulations, even below the solubility limit of FFA in aqueous solution. The result was that particle formation and growth is time-dependent, and the quantity and size of particles increased over time, whereas the concentration of Al^{3+} shows to influence solely the growth of particles. Even trace levels of Al^{3+} (40-60 ppb) significantly influenced particle growth; however, a further increase in concentration (80 ppb) did not show a significant increase in particle kinetics (Table 4) (58).

Table 4. Al^{3}+ Concentration effect on particle growth – Adapted from (58)

[Al^{3+}]	**Observations**
20 ppb	No complex formed before 65 hours
40 - 60 ppb	Significant particle growth
80 ppb	No increase in particle growth

Similarities with previous research (Allmendinger et al., 2021) were observed, indicating that an increase in Al^{3+} concentration resulted in faster FFA metal nucleation and earlier particle formation.

In a subsequent investigation, bivalent and trivalent metal ions were evaluated, with only the latter exhibiting particle formation and growth (58). Please refer to section 8.3.4 of chapter 8.3 for further details.

9. Conclusions, current trends and future directions

Manufacturing biopharmaceuticals continues to present a considerable challenge even with the exponential technological improvements of the last century. In fact, there are still hurdles to be overcome in the production of biopharmaceuticals, particularly in maintaining their stability, safety and efficacy throughout shelf life. This review explored some of the challenges faced in this process and the potential solutions that scientists around the world have put to the test.

Proteins may have an inherent tendency towards aggregation or may be prompted to do so through various stressors such as interfacial tension, adsorption to surfaces or exposure to extrinsic particles that serve as nucleation seeds.

The process of freezing DS increases flexibility, enabling storage and shipping across the globe. The time rates of freezing and thawing can influence protein aggregation due to the duration of contact with different physical interfaces: liquid, liquid-solid, solid, and the reverse process during thawing. Slow freezing and fast thawing together have effectively avoided particle aggregation resulting from the process. A decrease in the sodium chloride concentration, coupled with an increase in protein concentration, may lessen the likelihood of aggregation caused by interfacial stress. However, high protein concentration may represents an increased risk for aggregation throughout other stages of manufacturing.

Mixing is a crucial process to ensure homogeneity throughout biopharmaceutical production. Since gas is introduced into the solution during mixing, this can lead to protein aggregation caused by air-liquid interfacial stress. Additionally, mechanical shear caused by the mixer is also associated with the potential for aggregation. However, a reliable formulation is the key to reducing the risk of aggregation from these sources. Usually, particles generated during mixing can be easily removed through filtration. However, when dealing with high particle load formulations, there is a risk of filter clogging. In such cases, human intervention may be necessary, which can pose an unnecessary risk of breaking the aseptic barrier and causing contamination. In this scenario, an additional filtration step could be a potential solution to prevent filter clogs. However, it is important to note that small particles (under 0.22µm) have the ability to pass through filter membranes and enter the solution. Such particles can

potentially become nucleation seeds, which may grow in size throughout the shelf-life and potentially result in a product recall.

Different mixers can impact a solution in various ways. Bottom-mounted mixers, designed with a narrow gap between the impeller and the drive unit, can trigger SvP formation due to a high shear zone, whereas mixers designed with a wider gap are less prone to SvP formation.

Proteins can adhere to and interact with filter membranes, resulting in conformational changes. Absorbed proteins have the potential to detach from filter membranes, migrate to the filtrate and aggregate. The appropriate filter selection, taking into account both filter and solution properties, is critical to reduce adsorption to a minimum and prevent any potential conformational changes.

Adequate formulation of the DP is crucial to minimize protein adsorption to filter membranes. Essential components of DP formulation include surfactants such as PS20, PS80, and poloxamer 188. In combination with careful filter selection, these components have demonstrated effectiveness in preventing protein adsorption and consequent denaturation, which can act as nucleation seeds and affect the filtrate quality.

Aseptic filling is a critical process in the production of biopharmaceuticals. It can be performed alongside sterile filtration or, in some cases, after the sterile filtration is completed. Factors such as adsorption, friction, cavitation, and shear stress contribute to protein aggregation by causing denaturation. However, during filling, these factors do not appear to be the primary source of protein denaturation. A combination of factors is responsible for the potential formation of particles during the filling process. Adsorption of proteins to pump surfaces, combined with the aforementioned factors, may create suitable conditions for the formation of particles.

Pump material and design can reduce protein adsorption to surfaces and alleviate pumping-induced stress. Piston pumps, made of ceramic and stainless steel, appear to be more likely to induce aggregation. In contrast, peristaltic pumps, diaphragm pumps, and time-pressure filling exhibit reduced propensity to generate aggregates. Despite this, piston pumps are preferred due to their accuracy in filling small volumes, as required for ophthalmic use for

example. A thorough consideration of the materials and design of the pump is necessary, in accordance with the fill volume, to evaluate the risks and benefits of the filling systems.

Exposure to light can lead to photo-oxidation, colour changes and aggregation, and it is almost impossible to avoid light exposure completely. Light exposure can pose a problem for both proteins present in the drug product and excipients, such as PS20 and PS80.

To prevent photo-oxidation, viable mitigation strategies include employing light sources that have restricted output in blue and UV wavelengths, using selective filters/shields, implementing time-based exposure control, utilizing robust packaging, and incorporating molecules that act like sacrificial antioxidants. Adding methionine to the DP formulation has demonstrated the ability to protect antibodies from photo-oxidation.

The containment systems, such as Restricted Access Barrier System (RABS), utilized for fill-finish in aseptic conditions, are commonly sterilized with hydrogen peroxide due to its effectiveness in reducing microbial load and environmentally friendly nature. However, it is susceptible to adsorption and absorption into materials, which could later migrate to solutions and compromise their quality because of its oxidizing properties. Thus, caution should be taken while using it.

Variables such as relative humidity, exposure time, and residual concentration, as well as materials properties, can have an impact on the absorption or adsorption of hydrogen peroxide on surfaces. Empty glass vials, stoppers and syringes are prone to adsorption, whilst polymeric materials, such as tubing and bags, commonly absorb hydrogen peroxide. Elevated levels of humidity reduce the potential for hydrogen peroxide to adsorb, due to a competitive co-adsorption mechanism.

To minimise exposure of biopharmaceuticals to hydrogen peroxide, certain mitigation actions can be taken. One such measure is to limit the exposure of opened, filled vials by closing them as soon as they are filled. This will prevent any contact with hydrogen peroxide residues in the isolator's atmosphere. Proper selection of tubing material and implementation of validated CIP and SIP procedures can minimise the adsorption and absorption of hydrogen peroxide on surfaces, thereby preventing its release into the solution and avoiding potential oxidation and loss of product quality.

Elemental impurities are classified as contaminants because they have no function in the formulation and may even be toxic, depending on the dose and route through which they enter the organism.

Permissible daily exposure is predetermined based on the class of the elemental impurity. The principal sources of elemental impurities in DP include the drug substance, excipients, manufacturing equipment, and container closure systems. Risk assessment should be carried out to evaluate the need for testing DP for specific elemental impurities.

Impurities can interact with FFA that may be present in the solution, acting as nucleation seeds throughout the DP shelf life. This can lead to quality issues that might only become apparent later, during storage.

After conducting a rigorous risk assessment, several measures can be considered to minimise the risk of elemental impurity contamination. These include a comprehensive understanding of the manufacturing processes; detailed equipment selection, qualification and good manufacturing practice; adherence to water quality requirements as described in the compendial literature; selection of reputable suppliers and supplier qualification; and careful evaluation of the materials used in container closure systems.

Enzymatic degradation poses as a major risk to the long-term stability of the DP. It is often insidious and may only be detected during storage. PS20 and PS80 are commonly used surfactants that provide stability to protein formulations. They are prone to hydrolysis, which results in the release of FFA. As more and more surfactant is degraded during the shelf-life of the biopharmaceutical, protein stability could be jeopardized. This gradual process of aggregation is one of the most challenging issues. It occurs slowly throughout the biopharmaceutical shelf-life and beyond batch recall, it can lead to the repetition of stability tests and reduction of shelf-life, which could impact the supply chain. These occurrences can be expensive for the manufacturer as they would need to reduce inventory in the warehouse and among affiliates. If the affiliates, wholesalers, and pharmacies still possess stock from the batch, it must be discarded.

Other surfactants have been tested to identify a suitable substitute for PS. Thus far, poloxamer 188 has shown the most promising results and has been used in a limited number of

formulations with some success. However, further knowledge about poloxamer or other potential substitutes is necessary as PS is extensively used in the pharmaceutical field and remains the most commonly used surfactant thus far.

Many challenges related to the formation of aggregates can be tackled through enhancing the formulation and refining of manufacturing processes. Optimizing freeze and thaw cycles, selecting suitable equipment (such as mixers, vessels, tubing, filters and fill-finishing systems), using light-blocking filters and appropriate packaging, and conducting preventative GMP tasks such as thorough risk assessments for possible contaminants, are all considered safe measures that can be taken to reduce quality issues in the final product. This enhances product shelf-life, reduces the risk of batch recalls and financial losses, and safeguards the reputation of the manufacturer.

The utilization of SUT is becoming a prevalent trend throughout the industry. It undoubtedly provides several advantages, including a streamlined manufacturing process, the elimination of cleaning and sterilization validation requirements, reduced cross-contamination, and time-saving benefits. It can be used for various processes, such as freeze-thawing and mixing. Its usage is increasing throughout the biopharmaceutical industries, and it has been proven that implementing SUT is associated with fewer quality issues in biopharmaceuticals and optimizes processes. However, there are some inconveniences associated with its implementation, such as the potential for protein adsorption and for extractables/leachables to migrate from the material to the biopharmaceutical. Furthermore, there may be environmental implications associated with the use of these technologies.

Quality issues seem to evolve alongside technology. Particle aggregation remains a constant possibility throughout manufacturing, and it seems almost impossible to completely eliminate it. However, measurements can be taken at every stage of development and production to ensure a final product that maintains high quality standards from production to patien administration.

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