Physical Instability of Macromolecules

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Objective

To strengthen the understanding in relation to the physical stability of proteins

Introduction

Instability of drug does not impact only the potency, purity and safety of the protein formulation, but can also lead to formation of toxic impurities. Stability of the protein formulations is the critical quantity, playing an essential role as performance attributes, that needs to be evaluated during formulation development, shipping, handling, and marketing. Thus, stability assessment of proteins since early phase of product development throughout the post-marketing is significant in providing the quality and safety evidence.

Similar to the small molecules, instability of macromolecules can be separated into two common types: chemical and physical instability. Chemical stability relates to the processes of forming or breaking the covalent bonds, which create new compounds. Various chemical degradation processes are such as deamidation, hydrolysis, oxidation, proteolysis, racemization, beta-elimination, disulfide exchange, and DKP formation. While physical instability means the process that changes physical state of the protein but the chemical composition does not. This article will focus on only physical instability pathways including four major processes: denaturation, aggregation, precipitation, and adsorption.

TYPES OF PHYSICAL INSTABILITY

Denaturation

Denaturation refers that the globular (native state of the macromolecules) or three-dimensional structure of proteins become damaged. This also involves the unfolding of secondary and/or tertiary structure. The most general stress of globular denaturation is temperature. Protein, even in the solid state, losses its structure at elevated temperature. As mostly reported, thermally protein denaturation induces the irreversible form since the unfolded protein molecules rapidly form the aggregates. Temperature of melting (T_m) associates with the unfolded state of protein. T_m exhibits the conformational stability of protein, for example a protein formulation with high T_m is more stable than the one with lower T_m. On the other hand, it has also been realized that at low temperature, a cold denaturation process of many proteins can occur at below the freezing

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point of water. The cold denaturation related to glass transition temperature for maximally freeze concentrated solution (T_g'). At temperature below T_g', the maximally freeze concentrated solution becomes glass, while at temperature above T_g', protein increases mobility similar to that in liquid phase.

Pressure-Induced Denaturation

Unlike other stresses, which cause protein unfolding, the unfolded proteins induce by high pressure are generally completely reversible. Pressures of 2,000-4,000 bar can lead to protein unfolding while, intermediate pressures of 1,000–1,500 bar can be used to dissociate aggregates and allow the aggregated protein to refold.

Aggregation

Protein immunogenicity is one of the serious advance effects of using the therapeutic biotech products. The unwanted immunogenicity limits efficacy of the drug and negatively impacts its safety profile. There are several factors causing immunogenicity. The presence of aggregates is undesirable and considered as a significant product-related factor playing a role in immunogenicity by triggering the immune system. Therefore, it is important to minimize opportunities of aggregates formation throughout the product development cycle. Aggregates are assumed to be easily recognized by the immune system as compared with the native protein. Therefore, protein aggregation has become a big challenge in the manufacturing and development of protein therapeutics. A great number of studies on protein aggregation have been published.

Aggregates of proteins may result from several mechanisms and may be classified in different ways, such as soluble/insoluble, covalent/noncovalent, reversible/irreversible, and native/denatured. Protein aggregation may also be induced by chemical degradation/modification. More than one mechanism can occur for the same product. Mary E.M. and coworkers summarized the five common mechanisms for protein aggregation as follows; 1) reversible association of native monomers 2) aggregation of conformationally-altered monomer, 3) aggregation of chemically-modified monomers, 4) nucleation-controlled aggregation and 5) surface-induced aggregation. However, this article would focus on only physical aggregation.

Definition of aggregates has not been consistent, however, for the purpose of this article, soluble aggregates is defined as the invisible aggregates which may not be removed by filtration. On the other hand, insoluble aggregates are often visible and may be removed by filtration. Both types of aggregates cause problems for the development of a therapeutic protein. Size of aggregates

presented in protein products can be ranged from small (dimers) to large assemblies (subvisible or even visible particles).

Aggregation of protein can occur during production, storage, shipment or delivery to the patient. Various types of stresses during each step of bioprocessing such as protein concentration, temperature, freezing, light, shaking, surfaces, interactions with metal surfaces, or pH adjustments may result in surface denaturation and lead to protein aggregation. Moreover, interfacial stress during development, manufacturing, and clinical administration of protein products is one of the most concerns of aggregation.

Surface-induced aggregation: proteins can expose to air-liquid, liquid-solid, and liquid-liquid surfaces during mixing, freezing, filling and shipping, during reconstitution of lyophilized products, or during contact with chromatographic columns, pipes, vessels, filters, etc. These interfaces can significantly impact the protein product quality including formation of aggregates. Protein aggregation at interfaces is often come with conformation changes since proteins modify their higher order structure in response to interfacial stresses such as hydrophobicity, charge, and mechanical stress. For example, some studies reported that aggregation of proteins occurred when solution contacted with ice during freezing.

Throughout the production, protein solution is pumped, stirred, and filtered. In certain environments, protein aggregates may be induced by foreign particles in the systems including particles of stainless steel, rubber particles from stoppers, salt crystals, glass particles generated during heating of containers for depyrogenation, and silicone oil droplets from siliconized syringes or stoppers. Some ligands or specific ions presented may also enhance aggregation.
The solution contains in the containers made of different materials including stainless steel, glass, and plastic. All of these factors are critical and one needs to be concerned in order to avoid the formation of aggregates.

Precipitation

Precipitation or formation of particulates has become an important scientific focus in the development of protein therapeutics. Particle formation of the protein that can visually be seen by coming out of solution. It may be simply as the protein has just exceeded its solubility limit in some conditions. Protein precipitation may or may not be associated with protein aggregation. In case of related with aggregation, the soluble aggregate formation may continue until the aggregates are very large and they can no longer remain as soluble. This precipitation behavior

is irreversible since the protein is partially or completely unfolded. Consequently, a macroscopic appearance of aggregation can be observed as fogginess or cloudiness. Subvisible particulates have become more interested from researchers and regulatory agency since this kind of particles might be the most immunogenic of particulates found in protein products. The new analytical techniques, such as micro-flow imaging (MFI) have been developed to quantify particles size range and also capture images of the individual particles. This technique is possible to differentiate protein aggregates from foreign materials. Except particle formation arises from aggregation, protein can also be salted out. As far as the knowledge available, salted-out proteins retain its activity and native-like structure. This precipitation is fully reversible upon dilution.

Surface Adsorption

Adsorption is a physical instability as it changes the physical state of the protein. However, the interfacial stress causes more damage to the protein. Surfaces adsorption of proteins may occur during bioprocessing and in the final dosage form. The interfacial stability is an important factor that should be seriously considered. Proteins in aqueous solution are realized to adsorb to various surfaces. There are many studies reported about protein adsorption, for example, granulocyte-colony stimulating factor (G-CSF) has been found to adsorb to glass surface, IgG1 bound with plastic, and BSA bound with stainless steel.

Mechanism of surface-induced protein instability begins with the adsorption of native or partially unfolded protein on the surface. Owing to the exposure of hydrophobic amino acid side chains of the partially unfolded proteins to the surface, interaction of the unfolded proteins is usually more actively preferable than the native structure. After adsorption of the protein, surface tension forces at various interfaces (such as air–liquid and solid–liquid interface) arise aggregation. The nucleation and growth of aggregates in bulk solution results from the protein structural perturbation at the surface combining with desorption of partially unfolded proteins from the surface. So, various factors include surface tension, available surface area for adsorption, surface property of the protein molecule (i.e., hydrophobicity), and structural stability have an influence on the interfacial stability of the protein.

Air-liquid interface

Air-liquid interface is the most challenging issue of the interfacial damage. It might be because this interface is the most common interface for any product during production. If the product is an aqueous formulation, the opportunity for interfacial damage can occur during storage, shipping and handling. An important stress causing interface damage is an agitation (e.g. stirring, shaking,

or vortex mixing). One interesting report is that it has been found that stirring resulted in much more aggregation than shaking. Adding a nonionic surfactant such as polysorbate 20 effectively diminished the problem. Polysorbate 20 and 80 are frequently added to prevent or reduce unwanted adsorption and aggregation of protein solutions during filtration, purification, storage, and transportation. Some studies used propylene glycol or polyethylene glycol to reduce the interfacial damage as well. Other stabilizers such as sucrose have the property to increase surface tension at the interface so, it helps increase conformation stability. Surfactants seem to be the most effective stabilizer however, the use of nonionic surfactants might obtain the undesired outcomes. Additionally, the use of surfactants is concentration dependence. The improper concentration may promote formation of protein aggregation in bulk solution. To sum up, using suitable concentration of surfactants can minimize adsorption of the protein to the surface.

Ice–liquid Interface

There have been a number of studies of interfacial damage in frozen systems. It is possible that a suitable amount of surfactant for stabilization at room temperature may be insufficient at lower temperatures. The unfavorable effect of multiple freeze-thaw cycles has been researched. Almost all freeze-thaw studies are now performed using multiple cycles. It is strongly recommended to use the same cooling and warming methods since variations in each of these may impact the physical stability of the protein. Even the highly surface-active protein, aggregation can arise after repeated freeze-thaw cycles.

In conclusion, however, the knowledge on protein instability in some issues is now very limited and unclear. A numerous number of researches have been working and publishing. The more scientific data to come will establish the better understanding on protein stability.

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