

Protein Aggregation A Review

ABSTRACT

Protein aggregation is arguably the most common and troubling manifestation of protein instability, encountered in almost all stage of protein drug development. Protein generally will tend to aggregate under variety of condition. The extent of aggregation depends on many factors that can be classified as intrinsic (primary, secondary, tertiary or quaternary structure) and extrinsic (environment in which protein present or processed). These protein aggregate may exhibit less desirable characteristics like reduce or no biological activity. Although a Variety of method have been used/ designed to inhibit protein aggregation. It involved structural modification (internal) and environmental modification (external).

Keywords: [protein aggregation, protein instability, no biological activity]

1. INTRODUCTION

Protein is a molecule composed of polymers of amino acids joined together by peptide bonds. It can be distinguished from fats and carbohydrates by containing nitrogen. Other components include carbon, hydrogen, oxygen, sulphur, and sometimes phosphorus.

A protein is a linear polymer built from about 20 different amino acids. The type and the sequence of amino acids in a protein are specified by the DNA in the cell that produces them. This sequence of amino acids is essential since it determines the overall structure and function of a protein.

A protein has several functions. It may serve as a structural material (e.g. keratin), as enzymes, as transporters (e.g. hemoglobin), as antibodies, or as regulators of gene expression.

A protein may be classified based on its form and main functions: it can be a globular protein like most enzymes, fibrous protein which are for structural role; and membrane proteins that serve as receptors or channels for polar or charged molecule to pass through the cell membrane (1).

1.1 Functions of Protein in the Human Body

Protein has a large number of important functions in the human body and in fact, the human body is about 45% protein. It's an essential macromolecule without which our bodies would be unable to repair, regulate, or protect themselves.

Protein has a range of essential functions in the body, including the following:

- Required for building and repair of body tissues (including muscle).
- Enzymes, hormones, and many immune molecules are proteins.
- Essential body processes such as water balancing, nutrient transport, and muscle contractions require protein to function.
- Protein is a source of energy.
- Protein helps keep skin, hair, and nails healthy.
- Protein, like most other essential nutrients, is absolutely crucial for overall good health.

Proteins are the main actioners in cells and in an entire organism. Without proteins the most basic functions of life could not be carried out. Respiration, for example, requires muscle contractions, and muscle contractions require proteins.

In last three decades therapeutics peptide and protein have raised in prominence a potential drug of the future. Biotechnology play a key role in development of peptide and protein drug. A new series of peptide and protein based pharmaceutical have discover method to clone and express the cDNA encoding. The resent advances in large scale fermentation and purification processes and analytical characterization has further widen. The protein is available at much purer form in significant quantities, at reasonable coast. The problem of immunogenicity and antigenicity has also been considerably reduced. The peptide and protein drug produced by recombinant DNA technology are the exact replicas of that obtained from natural sources. Endogenously the protein is synthesized in small amount, normally act in tracent fashion and are stabilized by the cellular and extracellular milieu. In contrast, the protein drug must be highly purified and concentrated and have a shelf life at least two year. Oral administration is limited due to enzymatic degradation. Only after some viable novel delivery system develop to improve their systemic bioavailability, these peptide and protein drug would be of therapeutic importance (1,2).

64 **Table no;1** Some representative peptidal and proteinceous drug with their potential function
 65 and biomedical application (1).
 66

Peptide or protein drug (s)	Function or biomedical application
Cardiovascular active Angiotensin II antagonist Bradykinin Captopril Tissue plasminogen activator	Lowering blood pressure Improving peripheral circulation Heart failure management Dissolution of blood clot
CNS active Cholecystokinin B-endorphine Neuropeptide γ Nerve growth factore	Suppressing appetite Relieving pain Controlling feeding and drinking behavior Stimulating nerve growth and repair
Gastri intestinal active Somatostatine Pancreatic enzyme	Reducing bleeding of gastric ulcer Digestive supplement
Immunomodulating Interferon Tumer necroising factor	Enhancing activity of killer cell

Cyclosporine	Controlling polymorphonuclear function Inhibiting function of T lymphocyte
Metabolic modulating	
Insulin	Treating diabetes mellitus
Vasopressine	Treating diabetes insipidus
Human growth hormone	Treating hypopituitary dwarfism

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68 1.2 Stability of protein

69 protein stability primarily in terms of the thermodynamic stability of a protein that unfolds and
70 refolds rapidly, reversibly, cooperatively, and with a simple, two-state mechanism:



72 Where K_u , is the equilibrium constant for unfolding.

73 The stability of the protein is simply the difference in Gibbs free energy, ΔG , between the folded
74 and the unfolded states. The only factors affecting stability are the relative free energies of the
75 folded (G_f) and the unfolded (G_u) states. The larger and more positive ΔG_u , the more stable is
76 the protein to denaturation.

77
$$\Delta G_u = G_u - G_f$$

78 The Gibbs free energy, G , is made up the two terms enthalpy (H) and entropy (S), related by the
79 equation:

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81

82
$$G = H - TS$$

83 Where T is the temperature in Kelvin (3, 4, 5).

2 Protein Aggregation

Protein aggregation means two or more protein molecule come together and form small mass like structure call aggregate. The definition of newly formed aggregate is still debatable. We propose that protein aggregates be defined as any protein species in non-native states and whose sizes are at least twice as that of the native protein. Proteins generally will tend to aggregate under a variety of environmental conditions in comparison with small drug molecules(2). The extent of aggregation is dependent on many factors that can be broadly classified as intrinsic (primary, secondary, tertiary or quaternary structure) or extrinsic (environment in which protein is present, processing conditions, etc). These protein aggregates may exhibit less desirable characteristics like reduced or no biological activity, potential for immunogenicity or other side effects. The scientific and regulatory challenges in overcoming protein aggregation issues remain one of the major hurdles in commercialization of such drug products. Thus, it has been one of the most important areas of intensive research and development in both academia and biopharmaceutical companies to identify the cause(s) of protein aggregation and to control protein aggregation to an acceptable level. Among these physical and chemical deterioration pathways, protein aggregation is arguably the most common and troubling manifestation of protein instability, almost in all phases of protein drug development. Presence of any insoluble aggregates in a protein pharmaceutical is generally unacceptable for product release. Aggregation is a general term that encompasses several types of interactions or characteristics. Aggregates of proteins may arise from several mechanisms and may be classified in numerous ways, including soluble/insoluble, covalent/non covalent, reversible/irreversible, and native/denatured. For protein therapeutics, the presence of aggregates of any type is typically considered to be undesirable because of the concern that the aggregates may lead to an immunogenic reaction (small aggregates) or may cause adverse events on administration (3, 4, 5).

2.1 Protein aggregation pathways and characteristics

Proteins aggregate through different mechanisms/pathways .The major pathways are shown in Fig. 1 and can be roughly divided into: (1) aggregation through unfolding intermediates and unfolded states (pathway 1); (2) aggregation through protein self association (pathway 2a) or chemical linkages (pathway 2b); and (3) aggregation through chemical degradations (pathway 3).

2.1.1 Aggregation through unfolding intermediates and unfolded States

Under normal conditions, native proteins in solution are in equilibrium with a small amount of unfolding intermediates—I states, which are further in equilibrium with the completely unfolded/denatured states—D states in Fig. 1. Depending on the degree of protein unfolding, the unfolding intermediates may be further divided into more native-like intermediates and more unfolded-like intermediates. The latter are also referred to as molten globules for globular proteins, commonly used in the description of the protein folding/unfolding process

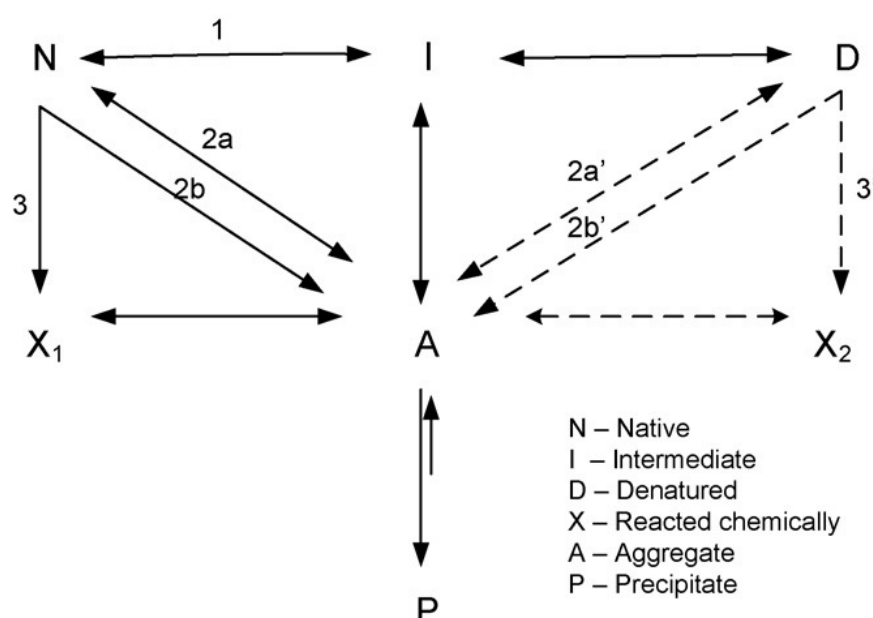


Figure 1. Graphic illustration of protein aggregation

In terms of the physical aggregation process, significant evidence suggests that the poorly populated protein folding/unfolding intermediates are precursors of the aggregation process. This is because these intermediates expose more hydrophobic patches and have higher flexibility relative to the folded state. Completely folded or unfolded proteins, in contrast, do not aggregate easily since the hydrophobic side chains are either mostly buried out of contact with water or randomly scattered. Recent simulation studies indicate that aggregation of native proteins with accessible hydrophobic areas on its surface is intensified when the solution conditions favor the partially unfolded conformation as opposed to either the native or fully unfolded conformations. Interaction (attraction) of these intermediates leads to formation of protein aggregates. Dimmers, trimmers, which maintain the native-like state, will not fall under

the definition of aggregates. Another similar term is oligomers or critical oligomers. These terms are often used for the description of early protein or peptide fibrillation process, also referred to as fibrillogenesis or amyloidogenesis (6).

2.1.2 Aggregation through self association

Many proteins can directly associate into protein aggregates physically from the native (folded) state (pathway 2a in Fig. 1) without going through the intermediate state (I). Such associations can be simply electrostatic or both electrostatic and hydrophobic depending on the experimental conditions. Other weaker forces (Van der Waals forces) can also initiate the association process. Protein self-association may or may not be accompanied by subtle conformational changes. Such association often leads to formation of reversible aggregates which can be considered the precursors of irreversible aggregates. If protein self-association is the rate-limiting step, protein aggregation resulting from self association would be association-limited (7).

2.1.3 Aggregation through chemical linkage

Many chemical degradations directly crosslink protein chains and thereby lead to aggregation (pathway 2b in Fig. 1). The most common cross-linking reaction is the intermolecular disulfide bond formation/exchange. Formation of disulfide linkages can further promote physical aggregation of proteins (8).

2.1.4 Aggregation through chemical degradation

The last major aggregation pathway (pathway 3 in Fig. 1) is the chemical degradation-induced protein aggregation. Many chemical degradation pathways have been shown to increase the aggregation tendency of proteins. Chemical degradation often changes the physical properties of a protein, such as protein hydrophobicity or association tendency, secondary/tertiary structures, and the thermodynamic/kinetic barrier to protein unfolding (9).

3 Type of protein aggregation

Protein molecule may aggregate by means of physical and chemically (Finke et al., 2000).

3.1 Physical aggregation

In these aggregations protein may aggregate simply by physical association with one another without any change in primary structure or by formation of a new covalent bond.

3.2 Chemical aggregation

In these aggregation formation of bond by directly crosslink protein or indirectly alter aggregation tendency of the original protein.

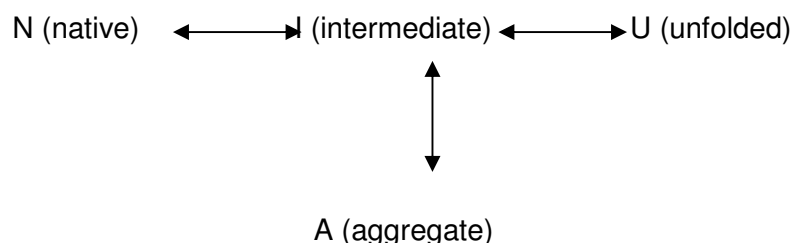
Both above mechanism can occur simultaneously to a protein and may lead to formation of either soluble or insoluble aggregates, depending on the protein, environmental condition, and stage of the aggregation process. For example, insulin can undergo both physical aggregation process, leading to formation of either soluble hexamers or insoluble fibrils and chemical aggregation process, leading to formation of either soluble dimer via cyclic anhydride intermediate or insoluble disulfide-bonded aggregates (10, 11, 12)

4 Mechanism of physical protein aggregation

4.1 Folding/unfolding intermediates and Protein aggregation

Traditional view of protein aggregation is the association of the unfolded state(s) of proteins (13,14). However, there is over whelming evidence that protein folding/unfolding intermediates are precursors in protein aggregation (15), even though the intermediates are usually not stable and poorly populate. In contrast, completely folded or unfolded proteins do not aggregate easily as the hydrophobic side chains are either mostly buried out of contact with water, or randomly scattered. It is the patches of continuous hydrophobic groups in the folding/unfolding intermediates that initiate the aggregation process (16).

The aggregation process can be described as scheme (1), where proteins form reversible unfolding intermediates, which then form reversible unfolded proteins or irreversible/reversible aggregates,



206 The intermediate state (I) is equivalent to the aggregation-competent state (A) or transition state
207 (17).

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210 **4.2 Mechanism of chemical protein aggregation**

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212 There are many chemical reactions that can directly crosslink protein chains or change the
213 hydrophobicity of a protein, indirectly changing its aggregation behavior. Chemical reactions that
214 cause aggregation involve Disulfide bond formation/exchange, Non-disulfide cross linking
215 pathways, Oxidation, Millard reaction (18).

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217 **5 Characterization of protein aggregation**

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219 **5.1 Morphological method**

220 There is no consistent definition of what is meant by a “soluble” aggregate, so working
221 definitions are often employed. Soluble aggregates refer to those that are not visible as discrete
222 particles and that may not be removed by a filter with a pore size of 0.22 μm . Conversely,
223 insoluble aggregates may be removed by filtration and are often visible to the unaided eye. Both
224 types of aggregates may be problematic for the development of a therapeutic protein. There are
225 clear guidelines and limitations on the number of particles $\geq 10 \mu\text{m}$ and $\geq 25 \mu\text{m}$ in size that may
226 be present in pharmaceutical preparations. However, the levels of soluble aggregates such as
227 dimers and trimers that are acceptable are not well defined.

228 Protein aggregates come in different shapes and sizes, even for aggregates of a single protein.
229 In most cases, protein aggregates are amorphous. For many small proteins or peptides, fibrils
230 are a common form of aggregates such as insulin (19), β -amyloid peptide and its fragments
231 (20). Many factors may influence the morphology of protein aggregates. Major ones include the
232 primary sequence of the protein. A single mutation in a protein or peptide can change the
233 morphology of aggregates.

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235 **5.2 Analytical method**

236 The amount, type, and size of the aggregates in biopharmaceuticals can have important
237 consequences for the safety and efficacy of biopharmaceuticals

238 The primary tools we use for aggregation analysis are:

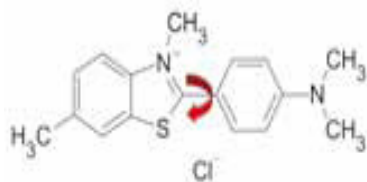
- analytical ultracentrifugation (AUC), including both sedimentation velocity and sedimentation equilibrium
- both static light scattering (SEC-MALS) and dynamic light scattering (DLS)
- native gel electrophoresis
- Proteostat protein aggregation analysis

5.2.1 ProteoStat™ Protein Aggregation Assay (21)

ProteoStat™ Protein aggregation assay provides a simple, homogenous assay format for monitoring peptide and protein aggregation. The assay can be employed to streamline protein processing and optimize formulation procedures during from protein expression through manufacturing. Relative to conventional protein aggregation dyes, such as Thioflavin T. ProteoStat™ detection reagent can detect a broader range of different protein. The assay yields a much brighter, provides at least 2 orders of magnitude linear dynamic, and offers superior performance across a broad range of values (4~10) and buffer compositions. Sensitivity of this assay is in the sub-micro molar so that less than 1% protein aggregate is detectable in a protein solution. The assay Z'factor scores greater than 0.5 providing quantitative analysis of protein aggregation in a robust and high-throughput fashion. Lyophilized native and aggregated proteins are included in the kit as negative and positive controls for monitoring changes in protein aggregation status.

Assay Mechanism

Proteins misfold and aggregate under stress from pH levels, temperature, buffers, light, oxygen, mechanical agitation and freeze/thaw cycles. Applications performed with the ProteoStat™ Protein aggregation dye can quantify aggregates in protein samples. ProteoStat™ is a molecular rotor dye that rotates like a propeller in the absence of protein aggregates and does not fluoresce. When the dye binds to the aggregate, it is immobilized – slowing down the rotational movement and causing the dye to fluoresce. Unlike other environmentally sensitive dyes that measure unfolding and exposure of hydrophobic regions of proteins, there is little interference from hydrophobic compounds or detergents present at normal concentrations.



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272 Thioflavin T: early prototype dye in the design of ProteoStat™ assay which also rotates around
273 a single bond (red arrow) in the absence of protein aggregates.



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275 Dye Thioflavin T immobilized
276 Dye is immobilized when bound to the aggregate and begins to fluoresce. The ProteoStat™
277 Protein aggregation assay is suitable for detecting protein aggregation caused by different
278 environments to which a protein product might be exposed, such as, temperature, light, oxygen,
279 mechanical and freeze/thaw cycle.

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282 **Table no: 2** Analytical Technique For Determination Of Protein Aggregation

Categories	Individual techniques	Applications	Protein examples
Calorimetry	DSC	Thermal protein unfolding/aggregation	FVIII SQ
Centrifugation Chromatography	Analytical centrifugation SEC-HPLC	Size and shape estimation Size estimation and Quantitation	Insulin Factor IX
Electrophoresis	SDS-PAGE Native PAGE	Size estimation and mechanistic probing Aggregation process	IL-2

		and mechanistic probing	
Light scattering	Static light scattering Dynamic light scattering Light scattering/obscuration	Size and shape estimation Size distribution of soluble Aggregates Size estimation and relative Distribution	Amyloid peptide, 1–40 Insulin Deoxy hemoglobin Deoxy hemoglobin

6 Inhibition of protein aggregation

Protein aggregation can be inhibited either by modifying proteins' structure (internally) or by changing proteins' environmental properties (externally). Modification can be made by site-directed mutagenesis or by chemical reactions. The key issue in structural modification for stabilization is to preserve the protein activity, as the modified protein can easily lose activity (22). Many structurally modified proteins have been shown to have reduced tendency to aggregate, including single amino acid mutants of human macrophage inflammatory protein (hMIP)-1, thymidylate synthase, monosubstituted, disubstituted, and trisubstituted insulins with *p*-succinamidophenylglucopyranoside(SAPG) (23).

Changing proteins' environmental properties often lead to inhibition of protein aggregation. A common method is to add excipients/additives in the protein preparation. A variety of excipients/additives are available for use, such as sugars and polyols, amino acids, amines, salts, polymers, and surfactants. These additives stabilize proteins by preferential interactions, a widely accepted concept of protein stabilization. Other mechanisms are also operable, such as

increased rate of protein folding, reduction of solvent accessibility and conformational mobility, and increase in solvent viscosities (23).

6.1 Denature concentration

In close relation to protein concentration, the denaturant concentration strongly affects both the rate and extent of on-pathway folding and off-pathway aggregation during refolding. This is because denaturants at different concentrations affect protein solubility to different degrees. Low concentrations of denaturants may decrease the solubility of a protein as the denaturants convert native to denatured and aggregation-prone species, while high denaturant concentrations should solubilize the protein as it weakens the protein–protein attractions in water. It seems that protein aggregation under such conditions results from the favorable formation of misfolded intermediates (24).

6.2 Use of additives

The well-known function of molecular chaperones in bacteria led to the use of such molecular systems (such as DnaK-DnaJ-GrpE and GroEL-GroES) to inhibit protein aggregation during protein refolding (25). They suppress protein aggregation by alternate binding and releasing of the folding intermediates (26). However, two major limitations seem to be associated with a routine use of these folding modulators—production cost and the extra purification burden. Therefore, other additives have been explored to achieve the same goal during refolding. The term “artificial chaperone” has been used to describe such non-protein additives, which have chaperone-like effect. Such additives include detergents and cyclodextrins. Detergents were found to suppress formation of protein aggregates (rather than dissolve aggregates) during protein refolding (27). Similarly, cyclodextrins were shown to prevent formation of protein aggregates during renaturation of carbonic anhydrase . To increase the efficiency, a combination of such additives was used for the refolding of lysozyme (28, 29).

6.3 Processing of proteins

Many routine processing steps can lead to protein aggregation to various degrees, such as heat-treatment, filtration, shaking/shearing, atomization, freezing, drying, and reconstitution. Proper care in that step can help to control protein aggregation.

6.4 Heat treatment

One of the viral inactivation methods in protein purification is heat treatment, which is usually performed at 60 °C. Unfortunately; many proteins aggregate at this temperature and thus, need proper protection. To protect a protein, the first step is to choose a stable solution pH, as proteins are usually stable in a narrow pH range (30). Although many buffering agents are available for pH adjustment, the aggregation behavior of proteins can be significantly different in different buffer systems and at different buffer concentrations (31).

Another two critical parameters in controlling protein aggregation during thermal treatment are protein concentration and ionic strength. Generally, increasing protein concentration increases the rate of protein aggregation due to the increased chances of intermolecular interactions (32). The effect of ionic strength is very much protein dependent. The salt type can also make a significant difference in protein aggregation. In addition, a stabilizer(s) is often added into a protein preparation to inhibit protein aggregation under thermal treatment. Polymers may inhibit protein aggregation through one or more of these properties: surface activity, preferential exclusion, steric hindrance of protein–protein interactions, and increased viscosity limiting protein structural movement. PEGs of different molecular weights were found to inhibit aggregation of recombinant human keratinocyte growth factor (rhKGF) at 45 °C and to inhibit thermally induced aggregation (33).

6.5 Shaking and shearing

Shaking creates air/water interface. The hydrophobic property of air relative to water induces protein alignment at the interface, maximizing exposure of the hydrophobic residues to the air and initiating aggregation (34). Shearing also exposes the hydrophobic areas of proteins, causing aggregation. Many proteins easily aggregate during shaking or shearing, such as rFXIII (35,36), hGH (37), and insulin (38).

To protect proteins from shaking/shearing-induced aggregation, surfactants are most commonly used. They inhibit protein aggregation by accumulating, competitively with proteins, at hydrophobic surfaces/ interfaces and/or by binding directly to proteins. Certain surfactants such as poloxamers may also increase the viscosity of a protein solution, restraining the motion of protein backbone to inhibit protein aggregation (39).

6.6 Care in freezing step

Freezing of a protein solution may cause protein aggregation due to one or more of the following freezing induced stresses: low temperature, solute concentration, formation of ice-

water interfaces, and potential pH changes or phase separation (40). Understanding the cause(s) of protein aggregation is the first step in aggregation prevention/inhibition during freezing. For proteins that denature during freezing due to their interaction with the ice-water interface, a slower rate of freezing should inhibit aggregation of these proteins as a smaller ice-water interface will be generated (41,42,43). On the other hand, a lower freezing rate may facilitate crystallization of other solution components, promoting potential Crystallization-induced protein aggregation (44). A frequent cause of protein aggregation during freezing is the buffer-induced pH change during the freezing process. In this case, selection of a suitable buffer would prevent/inhibit protein aggregation. Many additives can be used to inhibit freezing induced protein aggregation. They may inhibit protein aggregation by preferential interaction and/or other mechanisms such as increased solution viscosity, steric hindrance of protein-protein interactions or suppression of pH changes during freezing (45).

6.7 Proper care in drying step

The amount of water covering the surface of a protein in a fully hydrated state is around 0.3 g/g protein (46), while the water content of a dried protein product is usually less than 10%. Therefore, the drying process removes part of the hydration layer, which may disrupt the native state of a protein and cause protein aggregation. To protect the protein from dehydration induced aggregation, excipients, capable of forming hydrogen bonds with the protein, are added to replace water so that the hydrogen bonding requirements of polar groups on the protein surface can be satisfied (47). These excipients serve as water substitutes. The formation of hydrogen bonding has been demonstrated by IR spectroscopy between carbohydrates and many freeze-dried proteins. These excipients are preferably amorphous. In fact, the formation of an amorphous glass during dehydration has been considered as a prerequisite for protein stabilization (48).

The commonly used stabilizing agents during drying are sugars. Both trehalose and sucrose inhibit aggregation of many proteins during lyophilization such as IL-6 and human factor XIII. Some polymers are also effective, as they can form a glass with a high transition temperature. Examples include hydroxyethyl cellulose at 1% in the complete inhibition of a FGF aggregation and PEG in the inhibition of human FXIII aggregation during lyophilization. Probably due to polymer-associated properties, many proteins protect themselves from aggregation during drying (49,50,51).

6.8 Other techniques

To prevent protein aggregation during refolding, a variety of novel techniques or methods have been tried. These include refolding of proteins on a size-exclusion column during elution , use of high hydrostatic pressure (52,53).

7 Conclusions

A common phenomenon of protein instability is the formation of aggregates, which can be soluble or insoluble, non-covalent or covalent, and reversible or irreversible. Although protein sequence determines the behavior of protein aggregation, many external factors play a critical role in controlling/affecting protein aggregation, including temperature, pH, ionic strength, surface adsorption, shearing, shaking, presence of metal ions, organic solvents and additives, protein concentration, purity and morphism, pressure, freezing and drying. Chemical transformations can lead to direct or indirect protein aggregation, such as disulfide bond formation/exchange, non-disulfide crosslinking, transamidation, and oxidation. Although many analytical techniques are available in monitoring protein aggregation, their differences in quantitation, accuracy, sensitivity, and ease of operation require careful selection of some of these methods in monitoring all aspects of protein aggregation.

Protein aggregation occurs readily in almost all the biopharmaceutical processes, especially during fermentation, refolding, purification, formulation, and storage. Recognition of different causes of protein aggregation in these processes is the basis for selection of different techniques/methods for aggregation inhibition. Although protein aggregation can be inhibited effectively by modifying the primary sequence of a protein, this method is seldom used mainly because of the unpredictable consequences of structural modifications. Therefore, inhibition of protein aggregation is commonly achieved by changing the immediate environment of the protein. One particularly useful and simple method is the use of a compatible excipient(s). The often-used protein stabilizing excipients include sugars, polyols, surfactants, salts, PEGs, polymers, metal ions, and amino acids.

Although there are a variety of methods and a large pool of excipients for use to inhibit protein aggregation, a satisfactory level of aggregation inhibition has not been achieved for many proteins. Trial-and error has been the main stay in the prevention or inhibition of protein aggregation in most biopharmaceutical processes. This is partly due to our lack of a clear understanding of the protein aggregation process. Therefore, intensive and rigorous

investigation of mechanisms of protein aggregation is urgently needed for an ultimate solution of protein aggregation in biopharmaceutics.

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