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Review Article

A REVIEW ON THERAPEUTIC PROTEINS: STABILITY ASPECTS AND CLINICAL IMPLICATIONS

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ABSTRACT

Therapeutics proteins require protection against several sources of chemical and /or physical instabilities. One such physical instability is the high tendency of protein molecules to aggregate under a wide range of processing and storage conditions. Aggregates of the protein drug may enhance the product's immunogenicity and could compromise its efficacy. There are two major pathways of protein aggregation; these are aggregation of native protein conformations (colloidal instability) or aggregation of partially denatured proteins (non-native protein aggregation). Certain solution conditions, which reduce aggregation through one pathway, may lead to an increase in aggregation through the other pathway; therefore, a logical balanced formulation procedure should be implemented in order to reduce aggregation due to both pathways. For certain protein molecules, optimizing solution conditions might not result in the required reduction in protein aggregation; in this case, alteration in the protein structure might be required. This alteration can be, either in vivo through protein coding gene manipulation or synthetic such as protein PEGylation. There are two aggregation pathways for protein in liquid formulations, aggregation due to conformational stability and aggregation due to colloidal instability. Protein melting temperature (Tm) and time-dependent rate of thermal unfolding can assess conformational stability of a protein while measurement of second virial coefficients from static light scattering or protein precipitation in the presence of a salting out salt such as ammonium sulphate can be used to assess colloidal stability of a protein.

KEYWORDS: Aggregation, colloidal instability, conformational instability, long-term stability, clinical implications.

INTRODUCTION

Therapeutic proteins are used in medical treatment of various conditions including rheumatoid arthritis,[1] Crohn's disease,[2] breast cancer,[3] psoriasis[4] and others. Protein drugs are currently available as liquid and/or freeze-dried solid dosage forms. Manufacturers and users usually prefer liquid protein dosage forms.[5] However, for some protein therapeutics, chemical or physical instability issues are difficult to control adequately in the liquid state.

Chemical instabilities involve processes that make or break covalent bonds, generating new chemical entities, factors influencing chemical instability of a protein molecule are similar to those responsible for chemical degradation of conventional small molecule drugs.

Physical instabilities for proteins in which the chemical composition is unaltered but the physical state of the protein does change. Physical instabilities include denaturation, aggregation, precipitation, and adsorption. Precipitation may or may not be connected with aggregation. Protein precipitation may simply be due to conditions whereby the protein has exceeded its solubility limit.

Protein aggregates are assemblies of native or partially denatured (partially unfolded) protein molecules. These protein aggregates are product-related impurities (degradation products).[6] The presence of protein aggregates could affect the protein drug efficacy,[7,8] but a primary concern is that aggregates of the protein drug may enhance the product's immunogenicity.[9] Aggregation is therefore a fundamental attribute for assessing the quality of protein drugs.[6]

The initial protein aggregates are soluble oligomers but gradually become insoluble as they exceed certain size and solubility limits resulting in precipitation of different forms such as amorphous precipitates (disordered protein aggregates) or fibrils (ordered protein aggregates), depending on the protein structures and experimental conditions.[10]

There are two major pathways for protein aggregation; these are aggregation of native protein conformations (colloidal instability) or aggregation of partially denatured proteins (non-native aggregation).[6,11] Both aggregation pathways may occur for a single protein[12] such as the aggregation of recombinant human granulocyte colony-stimulating factor (rhGCSF).[13]

In order to prevent or minimize protein aggregation to an acceptable level, both of these two aggregation pathways are targeted.[14] Factors affecting the process of protein aggregation can be divided into two major types; these are internal factors (protein structure related) and external factors (solution conditions related).

Solution condition factors include surface adsorption, temperature, pH and excipients in the liquid formulation. Internal and external factors are not separate from each other, for example, one protein molecule might aggregate under one set of external factors while another protein is stable under these same external factors. Manipulation of external factors is usually the first choice by a formulation scientist. This approach involves changing the

formulation or process-related factors. It consists of two steps. First, the conformational stability of the native state must be increased. Second, the energetics of the intermolecular interactions between native protein molecules must be manipulated in order to maximize intermolecular repulsion, that is, to maximize the colloidal stability for the protein molecule.[11]

If a protein drug candidate has a very high tendency to aggregate, changing the formulation or process-related factors may not be sufficient to minimize protein aggregation to an acceptable level. In this case, a more drastic approach may be required, which is structural modification of the protein either genetically or chemically. A major limitation of this approach is the possible reduction or even complete loss in protein activity; one popular synthetic method is PEGylation.

One of the main factor that may influence the stability of therapeutic proteins is temperature. Most often, extracted proteins are stored for an extended period to maintain their activity and original structural integrity. Usually, proteins are best stored at 2-8 °C. Storage at room temperature often lead to the degradation of therapeutic proteins. Furthermore, protein instability during sustained delivery can result in the formation of protein particles during in vivo sustained release, which is considered another factor of protein instability. This may induce an immune response in patients treated with sustained release formulations of protein therapeutics.[15]

Size-exclusion chromatography (SEC) with either UV or light scattering detection has been used for detecting and quantifying protein aggregation and is a common quality control/quality assurance method. Another method used for aggregate analysis is Native polyacrylamide gel electrophoresis (PAGE) as aggregates stay intact during PAGE.[16]

This review is intended to cover therapeutic proteins aggregation, their long-term storage stability prediction as well as their clinical implications.

AGGREGATION PATHWAYS

Native globular protein conformation is spontaneously achieved in aqueous solution due to burial of hydrophobic moieties inside the globular protein core, this is energetically favorable, and thus the driving force for protein folding in aqueous environment is primarily burial of a polar (non-polar) surface area.[17] Although most of the protein core is composed of hydrophobic moieties, structures in the Protein Data Bank (PDB) show that acidic and basic side chains are also buried in the protein core, in addition to the presence of acidic and basic side chains on the globular protein surface.[18]

The aqueous solvent has a major influence on protein folding, in addition various types of weak intramolecular interactions such as hydrogen bonds, salt bridges and weak Vander Waal's bonds are responsible for contribution towards the conformational stability of the protein molecule. The exact overall contribution of the various weak intramolecular bonds towards the conformational stability is dependent on the amino acid sequence of the protein and solution conditions. This mean precise contribution of different intramolecular bonds towards overall conformational stability for a protein molecule is normally different from one protein molecule to another and it is different under different solution conditions even for the same protein molecule.

Proteins are prone to denature (alter their native secondary or tertiary structure or both) on exposure to various stress factors, these stress factors include temperature, chemical degradation, surface adsorption, shaking, solution conditions such as pH, salts and other additives.[19]

Most proteins unfold as the temperature is elevated, and at temperatures below their unfolding transition (T_m), where only partial unfolding occur, aggregates begin to form. The resistance to unfolding, also known as thermodynamic stability varies among different proteins and depends on a combination of various forces that contribute to the stability of the protein's globular conformation.[20] The transition midpoint (T_m) is the temperature where 50% of the protein is in its native conformation and the other 50% is denatured, the T_m for proteins generally represent their relative thermal stability.[21]

Thermodynamic methods such as differential scanning calorimetry (DSC) can be useful in determining the effect of temperature on protein's conformational stability by measuring T_m.[22]

This technique can be used to identify optimal pH, buffer species, stabilizer, etc., where protein conformation is most stable.[23]

In certain proteins, aggregation appear to occur with only minor conformational changes. An example of this is the self-association of insulin; these minor changes can be detected using spectroscopic methods, particularly far-UV (180–260 nm) circular dichroism rather than DSC.[24]

In addition to high temperature, low temperatures below the freezing point can also promote protein aggregation by changing the physical properties of the frozen solutions. In the presence of a polymeric excipient, freezing can cause phase separation, promoting protein aggregation.[25,26] In order to reduce aggregation due to thermal instability and freezing temperatures, protein drugs are commonly kept at cold temperatures (2-8 ºC) to restrict their conformational flexibility and to preserve their structural integrity.

The rate-limiting step in non-native protein aggregation is associated with unfolding or partial unfolding of the protein from its native conformation.[27,28] Thermally induced protein unfolding is often followed or accompanied by immediate aggregation due to exposure of the hydrophobic residues to the protein surface, from the globular protein core.[29,30]

Chemical degradation may also lead to protein aggregation; however, it is difficult to predict the effect of a particular structural change on protein aggregation tendency, as the site at which chemical modification occurs may or may not influence protein aggregation. For example, oxidation of Met[4] residue in the variable domain of an IgG light chain induced noticeable secondary and tertiary structural changes and made this protein more sensitive to stirring-induced aggregation.[31]

Some proteins tend to expose hydrophobic patches, normally present in the core of the native protein structure when a hydrophobic interface is present. These adsorbed, partially unfolded protein molecules form aggregates, leave the surface, return to the aqueous phase, form larger aggregates and precipitate.[32] This is the proposed mechanism for aggregation of insulin in aqueous media through contact with a hydrophobic surface or water–air interface.[33] Many studies have demonstrated that the magnitude and duration of shear exposure per se does not cause protein aggregation.[34] A probable reason for the frequent association of protein aggregation with processes that exert shear forces on fluids is the concomitant presence of interfaces in high-shear process equipment rather than the stress of shearing.[35]

The colloidal stability of a protein against native protein aggregation depends on the net charge of the protein and the hydrophobicity of the external surface of the globular protein.[36,37] In this aggregation route, the protein molecules retain their correctly folded native conformation, but have aggregation-competent regions on their surface, such as localized charged regions or hydrophobic patches that cause the proteins to stick together and aggregate.[38] Native protein aggregation or colloidal instability often leads to formation of reversible oligomers/aggregates,[39] and these can be considered, the precursors of irreversible aggregates/precipitates.[40]

The solution pH can influence protein in several ways such as type and distribution of surface charges on proteins, the nature of intramolecular folding interactions[41,42] and chemical degradation of a protein.[43,44]

Proteins aggregate at a neutral pH mainly because protein–protein interactions or surface hydrophobicity are favored.[12] The isoelectric point (pl) of a protein is the pH at which its net charge is zero. At a pH below the pI, the protein net charge is positive, and then negative when the pH is above the PI.

A key parameter to measure the tendency of protein–protein self–association due to surface charge is the second virial coefficient B 22. A positive value indicates protein–protein net repulsion while a negative value indicates protein–protein net attraction. In the latter case, protein–protein interactions are favored over protein–solvent interactions, potentially dominating protein aggregation.[23,36] A limitation in the use of the second viral coefficient as a screening tool is that the relative B22 values do not predict aggregation tendency as a result of conformational instability.[45,46]

There is a strong correlation between protein solubility and protein-protein interactions: protein solubility decreases when the protein-protein interactions become less repulsive or more attractive.[47-49] The pI of a protein to some extent determines the solubility of a protein at a given pH, with the lowest solubility theoretically occurring at the pH equivalent to the pI. Titration of the pH away from the pI to more either basic or more acidic conditions often improves solubility within the pH limit of protein chemical structure and native conformation retention.[50]

EFFECT OF SOLUTION CONDITIONS ON PROTEIN AGGREGATION

Although freshly isolated proteins normally fold into a distinct three-dimensional globular structures in water, this folded structure is not stable enough without appropriate additives, protein molecules in water must be stabilized by certain compounds and stored under appropriate solution conditions in order to maintain their stability.[51] These solution conditions/factors could potentially influence protein aggregation directly or could indirectly contribute to the overall rate of protein aggregation in solution. The possible effects of several formulation parameters on protein aggregation are described here. These parameters can be varied sequentially, or in combinations.

A-The solution pH

Solution pH can affect protein aggregation through one or more of the following three possible mechanisms:

1- Proteins aggregate at a neutral pH mainly because protein–protein interactions or surface hydrophobicity is favored. For example, Giger et al [50] studied the aggregation of insulin in the pH range of 3–9 and found that aggregation (turbidity) of insulin at room temperature was fastest at pH 5.6 (pI 5.5) in a 10 mM NaCl solution.

2- The number, density, and location of charged residues on the globular protein surface determine solubility and also influence the colloidal stability of proteins.[52,53] However, solution pH values above PI for a protein result in ionization of the acidic functional groups (glutamic and aspartic acids) and appear to contribute more towards increasing protein's solubility, compared to pH values lower than the PI for a protein which result in the protonation of basic functional groups (lysine and arginine). This is best explained by the stronger water-binding properties of the carboxylate anions in the ionized form of the acids, compared to lower water binding ability of the ammonium cations in lysine and arginine.[54]

3- pH-induced protein destabilization/partial unfolding, resulting in non-native aggregation.

For example Bajaj et al[53] found, that aggregation of a mAb was accelerated at pH 4.0 compared to pH 7.4, apparently due to partial unfolding of the protein at pH 4.0.

4-pH change may result in altered chemical degradation rates and pathways, which could lead to protein aggregation. The Deamidation reaction for a single reactive Asparagine displays a V-shaped pH-rate profile, with the minimum being between pH 3 and 6 [55], direct deamidation involve the hydrolysis of Asparagine and Glutamine side chain amides at acidic pH or deamidation via the succinamide intermediate at more basic pH.[56]

From above, it appears that the appropriate solution pH for each protein must be determined based on several instabilities (both chemical and physical) simultaneously. If slight variation in the solution pH affects the rate of protein aggregation or degradation, then the use of a buffering agent would be required in order to maintain the solution at an optimum pH. The aggregation behavior of proteins can be significantly different in different buffer systems[35], and/or at different buffer concentrations .[57,58]

B-Salts

At low salt concentrations, (<0.2M for a simple salt like NaCl) salts increase the solubility of the protein, this effect is known as salting-in. The salting in effect is due to the charge screening effects of salts in reducing electrostatic interactions between protein molecules.[59]

At salt concentration higher than the concentration needed for the charge screening effect, some salts interact preferentially with the solvent (salting out salts or kosmotropes) while other salts interact preferentially with the protein (salting in salts or chaotropes). Salting out salts stabilize protein conformations through the preferential exclusion mechanism.[60]

The preferential exclusion of the salt from the protein surface lead to decrease in the protein's solubility in the solvent because of decreased interaction of the protein with the solvent. The decreased interaction of the protein with the solvent result in a more compact globular protein and increased thermal stability of the protein. The continued increase in the concentration of the salting out salt leads to protein precipitation. Different salts cause precipitation at different concentrations. The ranking in effectiveness of precipitation and stabilization follows the well-known Hofmeister series.[61]

Foster et al. reported an example of kosmotropic stabilization, where they showed that the aggregation levels of heat-treated low molecular-weight urokinase (LMW-UK) is reduced in the presence of up to 0.19 M ammonium sulfate. Little additional benefit was observed above 0.19 M, and at concentrations above 1.7 M, the LMW-UK protein precipitates.[62]

In addition to general preferential interaction of salts with either the solvent or the protein, some salts interact specifically with particular proteins, e.g., metal-binding proteins, or calmodulin and tubulin that strongly bind Ca^{2+} and Mg^{2+}, respectively.[60]

C-Polyols

Polyols are a class of excipients that include sugars (e.g., mannitol, sucrose, trehalose and sorbitol and other polyhydric alcohols (e.g., glycerol and propylene glycol). In aqueous protein solutions, polyols interact preferentially with water, and are excluded from protein structure, thus they are preferentially excluded from protein surface, resulting in increase in the thermodynamic stability of the native globular protein conformation; this effect is similar to the effect of salting out salts.

The rate of aggregation of Interferon-tau (IFN-tau) at 50 ° C was reduced gradually with increasing sucrose concentrations at 0.25, 0.5, 0.75, and 1.0 M.[63] Arakawa and Timasheff proposed that preferential exclusion is a common element in aqueous sugar systems at the concentrations typically utilized, and therefore, it is reasonable to assume that other simple sugars would have similar stabilizing effects as sucrose.[64] In addition to increasing thermodynamic stability for proteins at certain polyols concentration, the preferential exclusion effect of polyols result in protein precipitation at higher polyols concentration.

D-Non-ionic surfactants

Certain non-ionic surfactants such as polysorbate 80 and polysorbate 20 are incorporated in marketed protein formulations.[65] The mechanism of action for non-ionic surfactants results from the binding of their hydrophobic tail to hydrophobic patches on protein surfaces and exposure of their hydrophilic heads to the solvent, such an interaction would block or partially block the aggregation-prone hydrophobic sites on the protein surface, preventing protein-protein interactions and increasing the protein solubility.[66]

The optimal amount of surfactants required, is the amount needed to saturate the hydrophobic patches on the globular protein surface.[67,68] Due to their preferential accumulation at water-air interface, non-ionic surfactants could also be useful against shaking/shipping/mixing-induced aggregation of proteins.

PREDICTING LONG-TERM STORAGE STABILITY OF THERAPEUTIC PROTEINS

Approaches normally used for improving physical stability of liquid protein formulations are adjustment of solution composition. Chemical alteration of aggregation prone protein molecules is considered if the adjustment of solution composition does not result in the required physical stability.

The use of rapid method/s for assessing the effect of different solution composition on the physical stability of protein formulations, reduce the required time for pre-formulation studies.

As stated previously in this review, there are two pathways by which protein molecules can aggregate. Susceptibility of a protein for aggregation through each pathway require a separate test/s. The assessment for susceptibility of protein to aggregation through both pathways is needed in order to get complete understanding of the tendency of protein aggregation under certain solution conditions.

Protein melting temperature as a predictive tool

The determination of protein melting temperature (Tm), which is the temperature at which protein unfolds, is a commonly used method for assessing physical stability of proteins.[69-72] Tm can be a useful prescreening tool to identify particularly conformational stable or unstable molecules or formulations, but may not, on its own, be predictive of long-term storage stability for all samples.

High-temperature aggregation kinetics as a predictive tool

An additional approach to the use of Tm as a predictor of storage stability is to observe the kinetics of aggregation when the sample is held at a temperature that accelerates the formation of aggregates. A number of studies has demonstrated that measuring aggregation rates at elevated temperatures for rapid formulation and candidate screening, is important in predicting behavior at lower temperatures and longer times. Protein intrinsic fluorescence technique can be used to monitor the time-dependent rate of thermally induced unfolding of protein, while the corresponding rate of aggregation can be obtained using light scattering technique.[73-75]

Protein-protein interactions as a predictive tool

The attractive interactions between native proteins in solution can potentially lead to the formation of aggregates, and it is therefore essential to measure the strength and nature (attractive or repulsive) of these interactions for candidate proteins or formulations.

Measurement of the second virial coefficient is an important method for assessing colloidal stability for a protein. The sign of this value indicates whether the protein-protein interactions are attractive (a negative value) or repulsive (a positive value) while the magnitude of the value indicates the strength of the interaction.[76-78] It appears that protein solubility is a useful predictive tool for improving long-term aggregation resistance at 4 °C. [79] This is probably the case for proteins for which dominant aggregation pathway is colloidal instability.

CLINICAL IMPLICATIONS OF THERAPEUTIC PROTEINS

Therapeutic proteins have revolutionized the treatment of many diseases. In the near future, many more proteins that are therapeutic are likely to become available for an increasingly wide range of indications. It has been recognized that these proteins may induce humoral and cellular immune responses.

The consequences of an immune reaction to a therapeutic protein range from transient appearance of antibodies without any clinical significance to severe life threatening complications such as anaphylaxis or decrease in efficacy and induction of autoimmunity, including antibodies to the endogenous form of the protein.

Many factors may influence the immunogenicity of therapeutic proteins, these factors could be product related or patient related factors.[80]

The reported incidence of antibody formation with therapeutic proteins varies widely between proteins and between studies (depending on the assay techniques used). The clinical consequences of antibody formation vary with the type of antibody present; for example, neutralizing antibodies are more likely to cause loss of efficacy than non-neutralizing antibodies.

Addressing protein drugs Immunogenicity

In addition to selecting appropriate solution conditions and composition as described above, several techniques have been developed to try to address the issue of immunogenicity from therapeutic proteins by which therapeutic proteins might be structurally modified in order to reduce their immunogenicity, including PEGylation, site-specific mutagenesis, exon shuffling, and humanization of monoclonal antibodies.[81]

Among synthetic methods, the most common method of modifying protein structure is by the addition of polyethylene glycol (PEG) molecules to protein[82] in a process referred to as PEGylation. In PEGylation, monofunctional PEGs are used to PEGylate proteins with a functional group attached to one end of the PEG polymer allowing reaction with N-terminal amine, lysine, cysteine, and other amino acids.[83]

While the initial intent of PEGylation was to extend the half-life of a protein in vivo [84], it is now known that PEGylation can improve the conformational and physical stability of a protein. For example, interferon-α1b was stabilized by the addition of PEG groups in a site-specific manner.[85] PEGylation indirectly diminish protein immunogenicity by minimizing protein aggregation, as well as by shielding immunogenic protein epitopes from the immune system.[86,87]

CONCLUSION

Long-term storage stability against aggregation of proteins can be achieved through understanding protein aggregation pathways and how excipients interact with proteins. For a given protein, in some instances, the critical control of the aggregation rate will be due to conformational stability, and in others, colloidal stability will dominate. Therefore, optimal mechanisms for reducing aggregation of proteins depend on the cause/s of aggregation.

If protein aggregation were due to conformational instability then increasing thermodynamic stability of the native state as conferred by protein stabilizers in addition to optimum pH and salt type and concentration would help in controlling aggregation caused by conformational instability of the tertiary structure of the protein.

Aggregation of native conformations or colloidal instability of the protein could be reduced by reducing intermolecular hydrophobic interactions between the native conformation using aggregation suppressors such as non-ionic surfactants or very low concentration of simple salts such as NaCl. The use of anti-adhesion agents such as serum albumin or non-ionic surfactants would help in controlling aggregation caused by surface adsorption.

Protein aggregates through two major pathways, currently available tests for evaluating susceptibility to aggregation in a protein, can only predict if aggregation is likely to occur through one pathway not both pathways. These mean separate different assessments are required in order to predict aggregation potential from each aggregation pathway. Once the aggregation pathway is determined for a particular protein under certain solution conditions is determined, then various suitable approaches to reduce aggregation are evaluated.

Immune responses to therapeutic proteins are usually of clinical significance if they are associated with the development of treatment resistance. Although various means to reduce the immunogenicity of therapeutic proteins have been suggested, monitoring for antibodies during clinical trials and post-marketing surveillance remains an important issue for all therapeutic proteins.

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