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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.

**REFERENCES**

1. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, Stevens RM, Shaw T. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis, N. Engl. J. Med. 2004; 350, 2572–2581.
2. Wilhelm SM, and Love BL. Management of patients with inflammatory bowel disease: current and future treatments. Clinical Pharmacist. 2017; 3(9):83-92.
3. Baselga J, Cortes J, Kim SB, Im SA, Hegg R, Im YH, Roman L, Pedrini JL, Pienkowski T, Knott A, Clark E, Benyunes MC, Ross G, Swain SM. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer, N. Engl. J. Med. 2012; 366 (2), 109–119.
4. Tausend W, Downing C, Tyring S. Systematic review of interleukin-12, interleukin-17, and interleukin-23 pathway inhibitors for the treatment of moderate-to-severe chronic plaque psoriasis: ustekinumab, briakinumab, tildrakizumab, guselkumab, secukinumab, ixekizumab, and brodalumab. J Cutan Med Surg. 2014; 18:156–169.
5. Wang YJ, Hanson MA. Parenteral formulations of proteins and peptides: stability and stabilizers, J. Parent. Sci. Technol. Technol. Rep. 1988; 10 (Suppl. 42) 42S.
6. Mahler HC, Friess W Grauschopf U, Kiese S. Protein aggregation: Pathways, induction factors and analysis . J Pharm Sci, 2009; 98 (9): 2909-2934.
7. Basu A, Yang K, Wang M, Liu S, Chintala R, Palm T, Zhao H, Peng P, Wu D,
Zhang Z, Hua J, Hsieh MC, Zhou J, Petti G, Li X, Janjua A, Mendez M, Liu J,
Longley C, Zhang Z, Mehlig M, Borowski V, Viswanathan M, Filpula D. Structure-function engineering of interferon - beta - 1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site – selective mono-PEGylation. Bioconjug Chem, 2006; 17: 618 – 630.
8. Wang W, Kelner DN. Correlation of rFVIII inactivation with aggregation in solution . Pharm Res, 2003; 20 (4): 693-700.
9. Rosenberg AS. Effects of protein aggregates: An immunologic perspective. AAPS J,
2006; 8 (3): E501–E507.
10. Ecroyd H, Carver JA. The effect of small molecules in modulating the chaperone activity of alpha B-crystallin against ordered and disordered protein aggregation. FEBS J, 2008; 275 (5): 935-947.
11. Chen BL, Arakawa T, Morris, CF, Kenney WC, Wells CM, Pitt CG.
Aggregation pathway of recombinant human keratinocyte growth factor and its stabilization, Pharm, Res. 1994; 11, 1581–1587.
12. Sun Y, Hayakawa S. Heat-induced gels of egg white/ovalbumin from five avian species: Thermal aggregation, molecular forces involved and rheological properties. J Agric Food Chem, 2002; 50 (6): 1636-1642.
13. Rousseau F, Schymkowitz J, Serrano L. Protein aggregation and amyloidosis:
Confusion of the kinds? Curr Opin Struct Biol., 2006; 16 (1): 118-126.
14. Chi EY, Krishnan S, Kendrick BS, Chang BS, Carpenter JF, Randolph TW. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony - stimulating factor. Protein Sci, 2003; 12 (5): 903-913.
15. Jiskoot W, van Schie RM, Carstens MG, Schellekens H. Immunological risk of injectable drug delivery systems. Pharm Res, 2009; 26: 1303–1314.
16. Herman AC, Boone TC, and Lu HS. Characterization, formulation, and stability of Neupogen (Filgrastim), a recombinant human granulocyte colony-stimulating factor. In formulation, characterization and stability of protein drugs (ed. R. Pearlman and Y.J. Wang), Plenum Press, New York: 1996; pp. 303–328.

17. Anfinsen CB. Principles that govern the folding of protein chains. Science, 1973; 181(4096): 223-230.
18. Onuchic JN, LutheySchulten Z, Wolynes PG. Theory of protein folding: The energy landscape perspective. Annu Rev Phys Chem, 1997; 48: 545-600.
19. Tanford C. Protein denaturation. Adv. Protein Chem., 1968; 23: 121-275.
20. Von Hippel PH, Wong KY. On the conformational stability of globular proteins. J. Biol. Chem. 1965; 240:3909-3923.
21. Pace CN, Scholtz JM. Measuring the conformational stability of a protein. In Protein structure: a practical approach (ed. T. E. Creighton): 1997; pp. 299–321. Oxford: IRL Press.
22. Remmele Jr RL, Nightlinger NS, Srinivasan S, Gombotz WR. Interleukin-1 receptor (IL-1R) liquid formulation development using differential scanning calorimetry, Pharm. Res, 1998;15, 200–208.
23. Won CM, Molnar TE, McKean RE, Spenlehauer GA. Stabilizers against heat - induced aggregation of RPR 114849, an acidic fibroblast growth factor (AFGF). Int J Pharm, 1998; 167:25-36.
24. Raso SW, Abel J, Barnes JM, Maloney KM, Pipes G, Treuheit MJ, King J, Brems DN. Aggregation of granulocyte-colony-stimulating factor in vitro involves a conformationally altered monomeric state. Protein Sci, 2005; 14 ( 9 ): 2246 – 2257.
25. Schellman JA. The thermodynamic stability of proteins. Ann. Rev. Biophys. Biophys. Chem. 1987; 16:115-137.
26. Heller MC, Carpenter JF, Randolph TW. Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation. Biotechnol Bioeng, 1999; 63 (2): 166 – 174
27. Gazit E. The "correctly folded" state of proteins: is it a metastable state. Angew Chem Int Ed Engl. 2002; 41 (2): 257-9.
28. Andrew J, Baldwin T, Knowles PJ, Tartaglia GG, Fitzpatrick AW, Devlin GL, Shammas SL, Waudby CA, Mossuto MF, Meehan S, Gras SL, Christodoulou J, Anthony-Cahill SJ, Barker PD, Vendruscolo M, Dobson CM. Metastability of native proteins and the phenomenon of amyloid formation. J. Am. Chem. Soc. 2011; 133 (36), 14160–14163.
29. Yan YB, Wang Q, He HW, Zhou HM. Protein thermal aggregation involves distinct regions: Sequential events in the heat - induced unfolding and aggregation of hemoglobin. Biophys J. 2004; 86 (3): 1682-1690.
30. Brange J, Andersen L, Laursen ED, Meyn G, Rasmussen E. Toward understanding insulin fibrillation. J Pharm Sci. 1997; 86 (5): 517-525.
31. Hu D, Qin Z, Xue B, Fink AL, Uversky VN. Effect of methionine oxidation
on the structural properties, conformational stability, and aggregation of immunoglobulin
light chain LEN. Biochemistry (Mosc) 2008; 47 (33): 8665-8677.
32. Thurow H, Geisen K. Stabilization of dissolved proteins against denaturation at hydrophobic interfaces. Diabetologica. 1984; 27:212–8.
33. Sluzky V, Tamada JA, Klibanov AM, Langer R. Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces, Proc Natl Acad Sci U S A. 1991; 88:9377-81.
34. Jaspe J, Hagen SJ. Do protein molecules unfold in a simple shear flow? Biophys J, 2006; 91 (9): 3415-3424.
35. Tzannis ST, Hrushesky WJM, Wood PA, Przybycien TM. Adsorption of a
formulated protein on a drug delivery device surface. J Colloid Interface Sci. 1997;
189 (2): 216-228.
36. Chiti F, Stefani M, Taddei N, Ramponi G, Dobson CM. Rationalization of the effects of mutations on peptide and protein aggregation rates. Nature. 2003; 424 (6950): 805-808.
37. Shaw KL, Grimsley GR, Yakovlev GI, Makarov AA, Pace CN. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. Protein Sci. 2001; 10, 1206–1215.
38. Liu J, Nguyen MD, Andya JD , Shire SJ. Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. J Pharm Sci. 2005; 94 (9): 1928-1940.
39. Kanai S, Liu J, Patapoff TW, Shire SJ. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab-Fab interaction that impacts solution viscosity. J Pharm Sci. 2008; 97(10): 4219-27.
40. Saluja A, Kalonia DS. Nature and consequences of protein-protein interactions in high protein concentration solutions . Int J Pharm. 2008; 358 (1-2): 1-15.
41. Schein CH. Solubility as a function of protein structure and solvent components. Biotechnology. 1990; 8: 308-317.
42. Kohn WD , Kay CM , Hodges RS. Salt effects on protein stability: Two-stranded alpha - helical coiled - coils containing inter-or intrahelical ion pairs. J Mol Biol. 1997; 267 (4): 1039-1052.
43. Vrkljan M, Foster TM, Powers ME, Henkin J, Porter WR, Staack H, Carpenter
JF, Manning MC. Thermal stability of low molecular weight urokinase during heat treatment. II. Effect of polymeric additives. Pharm Res. 1994; 11 (7): 1004-1008.
44. Fatouros A, Osterberg T, Mikaelsson M. Recombinant factor VIII SQ-Influence of oxygen, metal ions, pH and ionic strength on its stability in aqueous solution. Int J Pharm, 1997; 155: 121-131.
45. Bajaj H, Sharma VK, Badkar A, Zeng D, Nema S, Kalonia DS. Protein structural conformation and not second virial coefficient relates to long-term irreversible aggregation of a monoclonal antibody and ovalbumin in solution. Pharm Res. 2006; 23 (6): 1382-1394.
46. Broersen K, Weijers M, de Groot J, Hamer RJ, de Jongh HHJ. Effect of protein charge on the generation of aggregation-prone conformers. Biomacromolecules. 2007; 8 (5): 1648-1656.
47. Schein CH. Solubility as a function of protein structure and solvent components. Biotechnology 1990; 8: 308 – 317.
48. Middaugh CR, Volkin DB. Protein solubility, in: Stability of protein pharmaceuticals T. J. Ahern and M. C. Manning, eds., vol. 2 Plenum Press, New York: 1992 pp. 109-134.
49. Wang YJ , Shahrokh Z , Vemuri S , Eberlein G , Beylin I , Busch M. Characterization, stability, and formulations of basic fibroblast growth factor. Pharm Biotechnol. 1996; 9: 141–180.
50. Giger K, Vanam RP, Seyrek E, Dubin PL. Suppression of insulin aggregation
by heparin . Biomacromolecules. 2008; 9 ( 9 ): 2338-2344.
51. Nema S, Washkuhn RJ, Brendel RJ. Excipients and their use in injectable products, J. Pharm. Sci. Technol. 1997; 51, 166–171.
52. Glyakina AV, Garbuzynskiy SO, Lobanov MY, Galzitskaya OV. Different packing of external residues can explain differences in the thermostability of proteins from thermophilic and mesophilic organisms. Bioinformatics. 2007; 23: 2231 – 2238.
53. Dominy BN, Minoux H, Brooks CLI. An electrostatic basis for the stability of thermophilic proteins. Proteins. 2004; 57: 128 – 141.
54. Kramer RM, Shends VR, Motl N, Pace CP, Schlotz JM. Towards molecular understanding of protein solubility: Increased negative charge correlates with increased solubility. Biophysical Journal. 2012; 102: 1907-1915.
55. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of Protein Pharmaceuticals: An Update. Pharmaceutical Research; 2010; 27, (4): 544-575
56. Kenley RA, Warne NW. Acid-catalyzed peptide bond hydrolysis of recombinant human interleukin 11. Pharm Res, 1994; 11 (1): 72-76.

57. Paborji M, Pochopin NL, Coppola WP, Bogardus JB. Chemical and physical stability of chimeric L6, a mouse-human monoclonal antibody. Pharm Res. 1994; 11, (5):764–771
58. Eberlein GA, Stratton PR, Wang YJ. Stability of rhbFGF as determined by UV spectroscopic measurements of turbidity. PDA J Pharm Sci Technol. 1994; 48 (5): 224–230.
59. Arnaudov LN, de Vries R. Strong impact of ionic strength on the kinetics of fibrilar aggregation of bovine beta - lactoglobulin. Bio macromolecules. 2006; 7 (12): 3490–3498.
60. The Interactions of Proteins with Salts, Amino Acids, and Sugars at High Concentration T. Arakawa and SN. Timashef. Advances in Comparative and Environmental Physiology
Vol. 9, Edited by Gilles et al. © Springer Verlag Berlin Heidelberg. 1991; pp 226-243.
61. General Principles and Strategies for Salting-Out Informed by the Hofmeister Series Alan M. Hyde, Susan L. Zultanski, Jacob H. Waldman, Yong-Li Zhong, Michael Shevlin, and Feng Peng Organic Process Research & Development. 2017; 21 (9), 1355-1370.
62. Foster TM, Dormish JJ, Narahari U, Meyer JD, Vrkljan M, Henkin J, Porter WR,
Staack H, Carpenter JF, Manning MC. Thermal stability of low molecular weight urokinase during heat treatment. III. Effect of salts, sugars and Tween 80, J Pharm. 1996; 134 (1-2): 193 – 201.
63. Kendrick BS, Chang BS, Arakawa T, Peterson B, Randolph TW, Manning MC, Carpenter J F. Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: Role in restricted conformational mobility and compaction of native state. Proc. Natl. Acad. Sci. 1997; 94:11917-11922.
64. Arakawa T, Timasheff SN. Stabilization of protein structure by sugars, Biochemistry. 1982; 21 (25): 6536 – 6544.
65. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein bio therapeutics: structure and degradation pathways, J. Pharm. Sci. 2008; 97, 2924–2935.
66. Bam NB, Randolph TW, Cleland JL. Stability of protein formulations: Investigation of surfactant effects by a novel EPR spectroscopic technique. Pharm Res. 1995; 12 (1): 2-11.
67. Randolph TW, Jones LS. Surfactant-Protein interactions. Pharm Biotechnology. 2002; 13: 159-175.
68. Bam NB, Randolph TW, Cleland JL. Stability of protein formulations: Investigation of surfactant effects by a novel EPR spectroscopic technique. Pharm Res. 1995; 12 (1): 2-11.
69. Cheng W, Joshi SB, He F, Brems DN, He B, Kerwin BA. Comparison of high-throughput biophysical methods to identify stabilizing excipients for a model IgG2 monoclonal antibody: conformational stability and kinetic aggregation measurements. J Pharm Sci. 2012; 101(5):1701–20.
70. Goldberg DS, Bishop SM, Shah AU, Sathish HA. Formulation development of therapeutic monoclonal antibodies using high-throughput fluorescence and static light scattering techniques: role of conformational and colloidal stability. J Pharm Sci. 2011; 100 (4):1306-15.
71. He F, Woods CE, Becker GW, Narhi LO, Razinkov VI. High-throughput assessment of thermal and colloidal stability parameters for monoclonal antibody formulations. J Pharm Sci. 2011; 100 (12):5126-41.
72. Sahin E, Grillo AO, Perkins MD, Roberts CJ. Comparative effects of pH and ionic strength on protein-protein interactions, unfolding, and aggregation for IgG1 antibodies. J Pharm Sci. 2010; 99 (12): 4830-48.
73. Brummitt RK, Nesta DP, Roberts CJ. Predicting accelerated aggregation rates for monoclonal antibody formulations, and challenges for low-temperature predictions. J Pharm Sci. 2011; 100 (10):4234-43.
74. Brummitt RK, Nesta DP, Chang L, Chase SF, Laue TM, Roberts CJ. Nonnative aggregation of an IgG1 antibody in acidic conditions Part 1. Unfolding, colloidal interactions, and formation of high-molecular-weight aggregates. J. Pharm. Sci. 2011; 100: 2087-2103
75. Kayser V, Chennamsetty N, Voynov V, Helk B, Forrer K, Trout BL. Evaluation of a non-Arrhenius model for therapeutic monoclonal antibody aggregation. J Pharm Sci. 2011; 100 (7): 2526-42.
76. Valente JJ. Colloidal Behavior of Proteins: Effects of the Second Virial Coefficient on Solubility, Crystallization and Aggregation of Proteins in Aqueous Solution, Current Pharmaceutical Biotechnology. 2005; 6 (6) 427-436
77. Li Y1, Mach H, Blue JT. High throughput formulation screening for global aggregation behaviors of three monoclonal antibodies. J Pharm Sci. 2011; 100 (6):2120-35.
78. Le Brun V, Friess W, Bassarab S, Mühlau S, Garidel PA. Critical evaluation of self-interaction chromatography as a predictive tool for the assessment of protein–protein interactions in protein formulation development: a case study of a therapeutic monoclonal antibody. Euro J Pharm Biopharm. 2010; 75 (1):16–25.
79. Banks DD, Latypov RF, Ketchem RR, Woodard J, Scavezze JL, Siska CC, Razinkov VI.
Native-state solubility and transfer free energy as predictive tools for selecting excipients to include in protein formulation development studies, J Pharm Sci. 2012 Aug;101(8):2720-32.
80. Concept paper on guideline on immunogenicity assessment of therapeutic proteins. Committee for medical products for human use. European Medicines Agency. Pre-authorization Evaluation of Medicines for Human Use. London, 22 February 2006. Doc. Ref. EMEA/CHMP/BMWP/246511/2005. [Cited 2017 July 10]. Available from: https://www.ema.europa.eu/documents/scientific-guideline/concept-paper-guideline-immunogenicity-assessment-therapeutic-proteins-first-version_en.pdf.
81. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. Clin Ther. 2002; 24 (11):1720-1740.
82. Veronese FM, Mero A. The impact of PEGylation on biological therapies. BioDrugs. 2008;22:315–29.
83. Bentley MD, Bossard MJ, Burton K, Viegas T. Poly(ethylene) glycol conjugates of biopharmaceuticals in drug delivery. Advanced Application Routes for Biopharmaceuticals. New York: Wiley. 2005; 1393-1418.
84. Basu A, Yang K, Wang M, Liu S, Chintala R, Palm T, et al. Structure-function engineering of interferon-β-1b for improving stability, solubility, potentcy, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. Bioconjug Chem. 2006;17:618–30.
85. Inada Y, Furukawa M. "Biomedical and biotechnological applications of PEG- and PM-modified proteins." Trends Biotechnol. 1995; 13(3): 86-91.
86. Harris JM, Martin NE. "Pegylation: a novel process for modifying pharmacokinetics." Clin Pharmacokinet. 2001; 40(7): 539-551.
87. Nandhakumar S, Dhanaraju DM. Clinical Implications of Molecular PEGylation on Therapeutic Proteins. J Basic Clin Pharma. 2017; 8:87-90.

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