International Journal of Pharmaceutics 390 (2010) 89–99

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Review

Protein aggregation—Pathways and influencing factors

Wei Wang*, Sandeep Nema, Dirk Teagarden

Pfizer Inc., Global Biologics, 700 Chesterfield Parkway West, Chesterfield, MO 63017, United States

ARTICLE INFO

Article history:
Received 25 August 2009
Received in revised form 8 January 2010
Accepted 17 February 2010
Available online 24 February 2010

Keywords:
Aggregation
Oligomerization
Melting
Formulation
Excipient
Stabilization

ABSTRACT

Proteins generally will tend to aggregate under a variety of environmental conditions in comparison with small drug molecules. 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. Protein aggregation remains one of the major challenges in the development and commercialization of biotechnology products. This article is intended to review and discuss the latest understandings in protein aggregation pathways and the possible extrinsic factors that affect or control the protein aggregation process.

© 2010 Elsevier B.V. All rights reserved.

Contents

* Corresponding author. Tel.: +1 636 247 2111; fax: +1 636 247 5030.
E-mail address: wei.2.wang@pfizer.com (W. Wang).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.02.025

1. Introduction

Proteins generally will tend to aggregate under a variety of environmental conditions in comparison with small drug molecules. 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. A significant number of review articles have been published in recent years emphasizing different aspects of protein aggregation, such as physical aggregation-driving forces (Chi et al., 2003), aggregation mechanisms (Philo and Arakawa, 2009), kinetics (Morris et al., 2009), prediction (Weiss et al., 2009), theoretical considerations (Gsponer and Vendruscolo, 2006), inhibition (Wang, 2005; Hamada et al., 2009), and its relation to diseases (Wang et al., 2008a). This article is intended to review/discuss protein aggregation pathways and the possible extrinsic factors that affect the protein aggregation process.

2. 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).

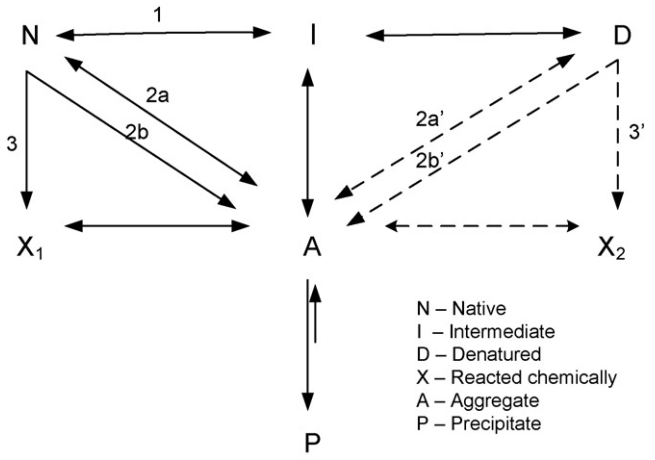

Fig. 1. Graphic illustration of major protein aggregation pathways.

2.1. 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.

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 (Zhang et al., 2008).

Interaction (attraction) of these intermediates leads to formation of protein aggregates (A state in Fig. 1). 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. Dimers, trimers, which maintain the native-like state will not fall under the definition of aggregates. Another similar term is oligomers or critical oligomers (Modler et al., 2003). These terms are often used for the description of early protein or peptide fibrillation process, also referred to as fibrillogenesis or amyloidogenesis. Based on our proposed definition of aggregates, all non-native protein oligomers can be termed as protein aggregates. They can be the nuclei both for amorphous aggregation or fibrillation.

The initial protein aggregates are soluble but gradually become insoluble as they exceed certain size and solubility limits (precipitates; P state in Fig. 1). The P state can take on different forms such as amorphous precipitates or ordered structures such as fibrils. The amorphous precipitation and fibril formation have been described, respectively, as disordered and ordered aggregation pathways. Because of the possible co-existence of the two aggregation outcomes, initial oligomerization may not be an obligate intermediate step in the process of fibril formation as demonstrated for $A\beta_{42}$.

2.2. 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 oligomers/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 (Weiss et al., 2009). Multiple sites can participate in a single protein in the association process (Kanai et al., 2008).

A key parameter for the measurement of the tendency of protein-protein self-association is the osmotic second virial coefficient. A positive B_{22} value indicates protein–protein repulsion while a negative value indicates protein–protein attraction. In the latter case, protein–protein interactions are favored over protein–solvent interactions, resulting in colloidal instability of proteins which could potentially lead to protein aggregation. The B_{22} values can be significantly different depending on the solution pH and ionic strength since both affect the charge density/distribution of proteins. Denaturants and even non-electrolyte excipients could have a strong influence on the B_{22} value. It should be noted that a protein with a large and positive B_{22} value can still aggregate if the measured B_{22} value does not reflect the true value of the monomer form (Alford et al., 2008a).

It is known that protein self-association is mainly related to colloidal stability and formation of intermediates is mainly related to conformational stability. Either conformational or colloidal stability could potentially be rate-limiting, depending on the solution conditions. It is sometimes difficult, in reality, to differentiate between these two pathways. Partly because of this, the relative B_{22} values do not necessarily indicate the relative aggregation tendency of a protein. In general, minimization of protein surface charge (reduction of B_{22}) often leads to increased aggregation.

2.3. Aggregation through direct chemical linkages

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 (Cabra et al., 2008). It is obvious that surface-located cysteines are more readily involved than internally located cysteines in participation of disulfide bond formation/exchanges. Disulfide-bonded proteins without any free cysteines can still undergo aggregation through disulfide exchanges via β-elimination. The formation of disulfide-bonded aggregates remains a key challenge in the development of protein drug products.

Other non-disulfide cross-linking pathways have also been reported, which lead to protein aggregation, including formaldehyde-mediated cross-linking, dityrosine formation, oxidation, and Maillard type reactions. These degradation pathways do not, in general, seem to occur as frequently as the disulfide bond formation/exchange.

2.4. 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. Such examples include auto-oxidation/oxidation (Rosenfeld et al., 2009), dimerization (Roostaee et al., 2009), deamidation (Takata et al., 2008), hydrolysis (Van Buren et al., 2008), and glycation (Wei et al., 2009). 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. Therefore, it is essential to analyze the composition/structure of the protein aggregates and determine the true cause of protein aggregation before designing a strategy for aggregation inhibition/prevention.

2.5. Other aggregation pathways and multiplicity

Theoretically, the denatured state (D) of a protein can form aggregates directly (pathway 2a′ in Fig. 1). This is true for many proteins that have been shown to be largely in the unfolded state naturally or to possess only two apparent states, i.e. native and denatured states, such as the microtubule-associated protein tau, and α-synuclein. The denatured state can also undergo chemical degradation and form aggregates either directly (pathway 2b′ in Fig. 1) or indirectly (pathway 3′ in Fig. 1). However, most protein drug products, in reality, are in folded states and aggregation contribution from the unfolded states is likely not significant. It is worth noting that multiple aggregation pathways can often occur to a single protein. In such cases, one aggregation pathway often dominates and should be controlled first.

2.6. Aggregation nucleation and kinetics

Several types of aggregation kinetics or models have recently been discussed in detail. Analysis of the kinetic data could help to identify the potential aggregation mechanisms. If protein unfolding is the rate-limiting step in protein aggregation, the aggregation kinetics would be considered as unfolding-limited aggregation and the observed aggregation rate constant (k_{obs}) should be equal to the unfolding rate constant (k_{unf}) (Weiss et al., 2009).

It appears that majority of protein aggregation events are initiated by formation of an aggregation nucleus and hence is nucleation-dependent aggregation. The most widely studied example of nucleation-dependent protein/peptide aggregation is the fibrillation of β-amyloid peptides. The process from N or D to A in Fig. 1 can be considered as the nucleation step. Therefore, several aggregation pathways may lead to formation of a nucleus. The definition of an aggregation nucleus has not been described consistently in the literature. Such a nucleus has been considered as small as the size of a dimer (Krishnan et al., 2003), or multimer/oligomer (Baynes et al., 2005). The next phase after nucleation is growth in aggregate size. Several terms have been exchangeably used in the literature to describe the growth phase. These include aggregation, elongation, fibrillation, and polymerization.

A lag time before aggregation often signifies the nucleation step. Depending on the solution conditions, the observable lag phase can take from a few minutes to several days. A recent study demonstrated multiple lag phases with multiple steps in the aggregation of IL-1ra (Krishnan and Raibekas, 2009). Needless to say, the apparent aggregate rate of proteins can be significantly different depending on the duration of lag phase and the time of measurement. Although protein aggregation requires participation of at least two molecules, an apparent first-order reaction kinetics was often observed, especially when protein unfolding is the rate-limiting step in the aggregation process.

2.7. Reversibility of protein aggregation

The reversibility of protein aggregation means the ability of protein aggregates to dissociate (disaggregate) in an equilibrium or upon reversal of the solution condition when aggregation is induced—such as pH, temperature, or concentration of excipients (e.g. salts). A dilution test can be used conveniently to examine the

reversibility of concentration-dependent protein aggregation. The reversibility of protein aggregation mainly depends on the stage of the aggregation process. While early aggregation process tends to be reversible (N to A in Fig. 1), late-stage aggregation or precipitation tends to be irreversible. The reversibility obviously depends on the time scale at which protein aggregation is evaluated and also the size of the aggregates. Thermally induced protein aggregation is often irreversible. Understanding aggregation reversibility is important since this may relate to the biological consequence of protein aggregates.

2.8. Pathway-dependent aggregate morphology

Protein aggregates come in a variety of forms such as fibrils (ordered aggregates), particulates (irregular or spherical), skin, gels, or a combination of these. Even fibrils may have different types with different secondary structures and intermolecular interactions (Natalello et al., 2008). The final form of aggregates seems to depend on the aggregation pathway (Giurleo et al., 2008).

The solution pH is a key parameter that determines the dominant form of aggregates, since the solution pH affects the type and density of surface charge and the degree of protein structural disruption. It was demonstrated that seven widely different, non-related proteins (bovine insulin, bovine β-lactoglobulin, human transthyretin, horse heart myoglobin, hen egg white lysozyme, BSA, and human α-synuclein) could all form fibrils under pH conditions favoring high net charge, however, under conditions of low net charge, particulates form (Krebs et al., 2007). Similar results were also obtained in other studies (Krebs et al., 2009). These results suggest that the formation of particulates in the regime where charge on the molecules is minimal is a common property of all proteins. Therefore, protein misfolding into clearly defined aggregates is apparently a generic process which depends solely on the general properties of the protein state during aggregation rather than the specific amino acid sequence.

Protein gelation is another form of aggregation and can often occur when the solution condition favors weak interactions among protein molecules or particulates. A typical condition would be when the solution pH is close to the pI of a protein (Krebs et al., 2007). The formation of incipient gels can take place quickly, e.g. within a minute, depending the experimental conditions (Chodankar et al., 2009) and continue to grow in multiple phases (Mercade-Prieto and Gunasekaran, 2009). The strength of the resulting gels depends on the solution temperature, pH, ionic strength, protein concentration, and formation of chemical linkages. The gel appearance can be translucent or turbid. Effective prevention or inhibition of protein aggregation may mean multiple interventions against different aggregation mechanisms in different stages of the aggregation process.

3. Effect of processing steps on protein aggregation

Protein aggregation can be induced by various processing steps. Such processing steps include fermentation/expression, unfolding/refolding, purification, freeze–thaw, shaking and shearing, pressurization, drying, preparation of protein drug delivery systems, and administration, etc.

3.1. Fermentation/expression

Fermentation or cell culturing is the first step in large-scale protein production. Proteins can be expressed in a variety of cell systems under a wide range of process conditions. Many proteins easily aggregate into so-called inclusion bodies during expression in bacterial systems (Espargaro et al., 2008). Formation of aggregates competes with the proper folding in these systems. Inhibition of protein aggregation during fermentation/expression can be achieved by adjusting the fermentation/expression conditions such as fermentation temperature (Hao et al., 2007), use of surfactants (Bahrami et al., 2009), or other additives (Bahrami et al., 2009). The host systems have a strong influence on the glycosylation state/pattern of expressed proteins, leading to various behavior of protein aggregation.

3.2. Unfolding/refolding

Unfolding/refolding is a key step after protein expression in a microbial expression system during large-scale protein production. Unfolded proteins can easily aggregate during the refolding process as they go through the various intermediate states (Yazdanparast et al., 2007). Protein aggregation can therefore significantly reduce the yield of correctly folded proteins during refolding. Many factors have been found to affect protein aggregation during refolding. These include temperature, protein concentration, type and concentration of denaturant, pH, ionic strength, refolding catalysts, thiol/disulfide agents, and miscellaneous additives. These factors are not discussed here, as a vast body of literature is available (Jungbauer and Kaar, 2007; Mannall et al., 2007; Burgess, 2009).

3.3. Purification

Purification is another key step in protein production where residual host cell proteins and other contaminants are removed. This process could potentially induce protein aggregation, if the purification conditions are too harsh. Protein A affinity chromatography is a highly efficient method for protein purification and generally requires low pH for protein elution. The low pH during elution could induce significant protein aggregation (Shukla et al., 2007). Protein aggregation can also occur even under mild purification conditions. Processing of Eprex bulk drug substance through an ion exchange HiTrap Q HP column (with 10 mM Tris buffer at pH 6.9 and a step gradient of NaCl) increased the aggregate level from 1.4% to 3.6% presumably due to an unfavorable solution condition (Heavner et al., 2007). Protein aggregation during purification could be minimized by adjusting the detailed purification conditions. Use of proper additives could significantly reduce the aggregation of a mAb during the UF/DF process (Cromwell et al., 2006).

3.4. Freeze–thaw

Freezing a protein solution is often required for preservation of protein activity/stability during protein production processes. The freezing process could potentially cause protein aggregation, depending on the type/concentration of proteins and presence of other formulation additives. Freezing-induced protein aggregation is likely due to one or more of the following stresses—low temperature, solute concentration, formation of ice–water interfaces, potential pH changes, and phase separation. The rate of freezing could affect aggregation of proteins as it affects the size of the ice crystals and therefore, the surface area of ice–water interface. It also influences crystallization of other solution components. Freezing-induced pH change is another major cause for protein aggregation due to selective crystallization of certain buffering species. Other factors also influence the aggregation of proteins such as freezing/thawing time, which varies significantly depending on the configuration of containers (Pivovarova et al., 2007), and the solution pH before freeze/thaw (Kueltzo et al., 2008).

Use of proper formulation additives could prevent or inhibit freeze/thaw-induced protein aggregation. These additives may inhibit protein aggregation by minimization of protein–ice interactions, suppression of pH changes, preferential interaction and/or

other mechanisms such as increased solution viscosity, and steric hindrance of protein–protein interactions. Among the possible additives, surfactants seem to be the most commonly used since they are effective in minimizing protein–ice interactions. It should be mentioned that concentrated protein solutions are often more resistant to freezing-induced protein aggregation, likely due to one or more of the following factors—limited area of ice–water interface for interfacial denaturation, protein's self stabilization, and conversion of monomers to active and more stable dimers or multimers.

3.5. Shaking and shearing

Shaking and shearing are often encountered during protein production or shipping. Both shaking and shearing could potentially induce protein aggregation although the extent of impact depends both on the intensity and duration of exposure to such stresses. Shaking creates air/water interfaces. The hydrophobic property of air relative to water induces protein alignment at the interface, maximizes exposure of the hydrophobic residues to the air, and initiates aggregation. Shearing could also potentially expose the hydrophobic areas of proteins and causes aggregation. However, few studies have been conducted to separate the impact of shear and air–liquid/solid interface. Moderate shear generally would not induce protein aggregation but very high shear ($\sim 10^7\ s^{-1}$) could (Bee et al., 2009a). Presence of a solid–liquid interface generally facilitates the impact of shear. Surfactants are often used in reducing shaking/shearing-induced protein aggregation likely by competing with the protein molecules for the hydrophobic surfaces/interfaces, by binding directly to proteins or by increasing the viscosity of a protein solution, restraining the motion of proteins. Alternative additives such as HP-β-CD may also work by similar mechanisms (Serno et al., 2009).

3.6. Pressurization

It is expected that low to moderate pressures (e.g. during filtration process) would not exert any significant effect on the aggregation behavior of proteins. However, high pressure could cause protein unfolding and facilitate protein aggregation due to increased hydrophobic interactions. This has been observed for rhIL-1ra at pH 7 above 180 MPa (Seefeldt et al., 2005) and β-lactoglobulin B at pH 7.2 above 500 MPa (Considine et al., 2007). Such aggregation could be inhibited by lowering the operation temperature. Pressure treatment can also generate chemically linked aggregates since high pressure can expose reactive groups which are normally well covered under atmospheric pressure (Huppertz et al., 2006). In contrast, in the presence of a moderate concentration of chaotropic agents, high pressure treatment (100–200 MPa) could actually foster disaggregation of proteins such as GH and lysozyme by solubilizing the aggregates (Crisman and Randolph, 2009). Release of pressure can then allow the proteins to refold properly leading to a reduction in aggregation.

3.7. Drying

Several drying methods have been used for preparation of solid protein products, including vacuum drying, freeze-drying, spray-drying and spray freeze-drying. All of these processes could potentially cause protein aggregation to a different degree, as the drying process removes part of a protein's hydration layer, disrupting its native state (Mukherjee et al., 2009). Vacuum drying is the simplest method for rapid dehydration of proteins and can induce protein aggregation (Kumar et al., 2009). Freeze-drying process is the most commonly used method for preparation of solid protein products and could promote protein aggregation (both non-covalent and covalent) due to impact of both freezing and drying stresses. Protein aggregation during freeze-drying can be effectively inhibited by addition of a protein stabilizer(s) such as sugars. The protective effect of formulation excipients is strongly dependent on the excipient concentration, specifically the relative amount of sugar and protein. High initial protein concentration could potentially saturate ice–water interfaces and thereby limits the relative extent of protein aggregation. However, antibodies have been shown to be less stable both during lyophilization and storage at high concentrations (Hagiwara et al., 2000).

Spray-drying is another widely examined method for dehydration of proteins. During this process proteins are exposed to an elevated temperature and a significant increase in the air–water interface for a short period of time during atomization. Both stresses may cause protein destabilization and aggregation. Since the main stress during spray-drying is the exposure of proteins to the air–water interface, use of surfactants is often effective in inhibiting protein aggregation (Abdul-Fattah et al., 2008). Spray freeze-drying eliminates the use of elevated temperature but the air–water interfaces are still created for possible protein aggregation (Yu et al., 2006). This process could be more damaging to a protein than the simple spray-drying process. Supercritical fluid (SCF) drying is another drying method that has been evaluated and found to induce protein aggregation (Jovanovic et al., 2008). Reconstitution of a dried protein product could also lead to a variable degree of protein aggregation, depending on the reconstitution procedure, reconstitution medium, and presence of other additives.

3.8. Analytical methodologies

The level of protein aggregates measured in samples can be significantly different depending on the sample treatment and analytical procedures utilized. Limitations of several key analytical methodologies have been reviewed recently in the characterization of non-particulate aggregates (Philo, 2009). For example, the aggregates of a therapeutic IgG formed at pH of approximately 7 can be easily disrupted during field flow fractionation (FFF) analysis (Demeule et al., 2007). The rate and extent of thermal protein denaturation/aggregation can be significantly different depending on the heating rate (Golub et al., 2008). Therefore, the result from one particular analytical method may not reflect the true aggregation behavior of a protein and partly for this reason, multiple analytical methods are often used in charactering protein aggregates.

3.9. Miscellaneous processes

Other processes were also reported to destabilize a protein and cause protein aggregation. Such processes include filtration (Wang et al., 2008b), filling (Cromwell et al., 2006), nebulization (Webb et al., 2002), and protein labeling (Holmberg et al., 2007), and preparation of protein delivery systems (Castellanos et al., 2003).

4. Effect of temperature on protein aggregation

Temperature is a measure of the average kinetic energy of a certain kind of vibrational motion of the constituent particles (i.e. molecules, atoms, and subatomic particles). In a liquid state, the Stokes–Einstein equation relate the diffusion coefficient of a species (D) directly to the absolute temperature (T) (Macchioni et al., 2008). Therefore, changing the temperature of a protein solution or solid would alter the degree of vibrational motion and diffusion of protein molecules, which is a necessary step for physical protein aggregation. Temperature is arguably the most critical environmental factor for consideration when proteins are handled during the entire development and commercialization processes.

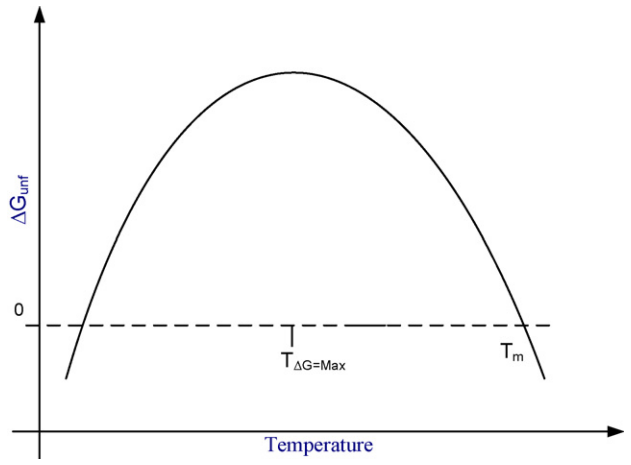

Fig. 2. Relationship between temperature and protein thermodynamic stability.

4.1. Temperature change vs protein aggregation

The relationship between ΔG_{unf} and temperature (T) has been described under certain pressure and solution conditions (Talla-Singh and Stites, 2008). This relationship can also be graphically illustrated in Fig. 2 (Rees and Robertson, 2001). It is clear that the maximum ΔG_{unf} resides in a narrow temperature range. Decreasing or increasing temperature will destabilize the protein and can potentially promote protein aggregation. Some proteins can aggregate easily at room temperature. Increasing temperature may have several additional effects leading to accelerated formation of aggregates. These effects include reduction of activation energy, enhancement of hydrophobic interaction, increased diffusion of proteins, and increased frequency of molecular collisions. The activation energy for aggregation pathway 1 (Fig. 1) is the energy required for the formation of I states and facilitated formation of I states may lead to increased protein aggregation. Reduction of the activation energy for chemical reactions could lead to increased protein aggregation via pathway 2b (formation of chemically linked aggregates) and pathway 3 (formation of chemical and aggregation-prone degradants).

When the aggregation process is composed of two distinct steps, i.e. nucleation and growth, temperature may affect each step to a different degree (Andrews et al., 2008). Due to the multiple aggregation steps and/or co-existence of multiple protein aggregation pathways, temperature may change the kinetic regime of protein aggregation. Temperature can also affect protein aggregation indirectly by changing the relative composition of secondary structures and, thereby, result in different aggregation behavior. Thus, high temperature is a common parameter to be selected for accelerating protein aggregation, albeit frequent non-Arrhenius temperature dependency.

As indicated in Fig. 2, decreasing temperatures also destabilize or even denature proteins. RNase undergoes cold denaturation at −22 °C under 3000 bar (to prevent freezing) (Zhang et al., 1995) and so does serum albumin at a temperature lower than 0 °C (Kosa et al., 1998). Destabilization at low temperature may facilitate generation of unfolding intermediates for protein aggregation. However, aggregation may not occur easily at low temperatures due to weaker hydrophobic interactions. Probably due to similar reasons, low temperature-induced protein aggregation is often reversible in contrast to high temperature-induced protein aggregation. The well-known low temperature-induced aggregation (below 37 °C) of serum cryoglobulins is reversible. Human IgM cryoglobulin preparations easily precipitate or gel at temperatures below 10–12 °C but the process can be reversible at a higher temperature. Although the low temperature-induced protein aggregation does not seem to present a critical issue, it should be well understood and controlled, especially before drug administration.

Low temperature storage below the freezing point can still promote protein aggregation by changing the physical properties of the frozen solutions. A frequent observation is the crystallization of an excipient(s) which leads to phase separation, loss of protein protection, and protein aggregation during low temperature storage above the glass transition temperature (T_g') of the maximally freeze-concentrated solution. Freezing can easily cause phase separation in the presence of a polymeric excipient and, thereby, promote protein aggregation. Such aggregates are typically not reversible. In summary, temperature can have a complex effect on protein aggregation, either directly or indirectly for both physical and chemically induced aggregation processes.

4.2. Melting temperature (T_m) and protein aggregation

The melting temperature (T_m) of proteins generally represents its relative thermal stability. It is usually expressed as the peak temperature where protein molecules (assuming homogeneous and pure sample) are half way through the unfolding process. The higher the T_m the less likely the protein will unfold. A general positive relationship was found between protein thermodynamic stability and T_m with a stability increase of approximately 0.008 kJ/mole/degree in T_m (Rees and Robertson, 2001). Thermally induced protein unfolding is often followed/accompanied by immediate aggregation due to exposure of the hydrophobic residues. Exceptions do exist possibly because of one or more of the following reasons—low aggregation tendency of the protein sequence, positive B_{22} value, or unfavorable solution conditions for aggregation (e.g. extreme pHs, low protein concentration and low salt condition, etc.). In these cases, a higher temperature above T_m may be required for protein aggregation. Therefore, a protein with a higher T_m does not necessarily possess a lower tendency to aggregate. Very often, it is the actual solution conditions that control protein aggregation.

4.3. Linearity of temperature-induced protein aggregation

A major force in controlling protein aggregation is the intermolecular hydrophobic interactions. Since such interactions are temperature-dependent, protein aggregation is generally not expected to follow the Arrhenius relationship. This is because the Arrhenius relationship is mainly based on the effect of temperature on molecular diffusion. Therefore, predication of protein aggregation based on accelerated stability studies is generally not accurate. In reality, protein aggregation may follow the Arrhenius relationship in a narrow temperature range (Wang et al., 2008b). The temperature-induced protein aggregation in a solid state appeared to be better predicted than in a liquid state due to limited hydrophobic interactions.

5. Effect of environmental factors on protein aggregation

A variety of environmental factors can significantly impact the aggregation behavior of proteins. These major factors include general solution conditions, protein concentration, container/closure system and surfaces, and light and irradiation.

5.1. General solution conditions

Several solution conditions play a critical role in controlling protein aggregation. Among these, the solution pH is arguably the most critical one. The solution pH dictates the type and distribution

of surface charges on proteins, which not only affect intramolecular folding interactions but also intermolecular protein–protein interactions. Therefore, the solution pH, along with sequence hydrophobicity and propensity of forming secondary structures are key parameters in determining the rate of protein aggregation. At extreme pHs, proteins are heavily charged. The dense charges on the protein surface would significantly increase repulsive intramolecular and intermolecular interactions, which lead to at least partial protein unfolding. Proteins may or may not aggregate depending on the relative contribution of intermolecular hydrophobic attraction and electrostatic repulsion. Because of the multiple effects of pH, several types of relationships between protein aggregation and solution pH have been reported such as well-shaped, bell-shaped, sigmoidal, and roughly linear.

Theoretically, protein solubility is minimal at its pI due to minimal protein charge–charge repulsions. Recent results suggest that significant protein–protein interactions can still exist from the marked charge anisotropy of protein around its pI as observed for insulin (Giger et al., 2008). Therefore, many proteins actually aggregate at a lower rate around their pIs (Majhi et al., 2006). Therefore, selection of a solution pH away from the pI of a protein may not be always successful in minimizing protein aggregation. Lastly, changing the solution pH may indirectly change the aggregation rate-limiting step/mechanisms and thus, the morphology of protein aggregates (Olsen et al., 2009).

Since pH strongly affects the behavior of protein aggregation, a buffering agent is usually used to maintain an optimum pH for stability. Although many buffering agents are available for pH adjustment, the stability and aggregation behavior of proteins can be significantly different in different buffer systems and concentrations. The different effect has been attributed to different binding effects of buffers.

In close relation to solution pH, the ionic strength is another key solution condition, which potentially affects protein aggregation. Both positive and negative ions in a solution can potentially bind to or interact electrostatically with proteins. Such interactions can lead to altered charge–charge interactions and even different conformational states, which may result in different aggregation behaviors. Some aggregation processes contain two clear stages—nucleation and growth, and ionic strength may have different effects in these stages (Alford et al., 2008b). The salt type can also make a significant difference in protein aggregation. The overall effect of ionic strength on protein aggregation is very much protein-dependent. If neutralization of protein surface charges favors protein folding/stability, reduction of such interactions by increasing ionic strength would destabilize the protein, partially expose the hydrophobic patches, and lead to increased protein aggregation. Otherwise, protein aggregation may be inhibited. In reality, more complex relationships are often seen between protein aggregation and ionic strength (Saluja and Kalonia, 2008). The effect of ionic strength on protein aggregation may also be dependent on the solution pH (Saluja et al., 2007) and glycosylation state of a protein (Hoiberg-Nielsen et al., 2006). Ionic strength can indirectly affect protein aggregation by affecting the rate of chemical reactions (Livney et al., 2003) or the effect of other aggregation-inducing agents (Rezaei-Ghaleh et al., 2007).

In the development of protein pharmaceutical products, many types of formulation excipients/additives are used, which could affect their aggregation behavior. Some additives destabilize proteins and promote aggregation while others stabilize proteins. Protein stabilization can be achieved through preferential exclusion between excipients/additives and proteins, a widely accepted concept of protein stabilization. Other stabilization mechanisms are also postulated, including increased rate of protein folding, reduction of solvent accessibility and conformational mobility, and increase in solvent viscosities. Recent results indicate that protein stabilization can be achieved through binding between these additives and aggregation intermediates (Necula et al., 2007). Typical formulation excipients/additives include small neutral additives like sugars and polyols, amino acids, amines, polymers, surfactants, and preservatives. Many small neutral compounds have a profound effect on protein stability or aggregation (Hamada et al., 2009). A major category of these neutral compounds is sugars/polyols. These sugars/polyols normally have a stabilization effect and inhibit protein aggregation under different experimental conditions. They can maintain the correct conformation of proteins and are widely referred to as osmolytes or sometimes chemical chaperons (Xia et al., 2007). In a few cases, these sugars/polyols have been shown to destabilize proteins and promote protein degradation, or even aggregation (Schule et al., 2008). Several types of small charged organic additives have been shown to affect the aggregation of proteins. They influence the aggregation behavior of proteins as a result of ionic strength and/or specific interactions with proteins. Additional effects include destabilization of the partially folded states and early aggregate formation; or solubilization of the native state (Ghosh et al., 2009). Some of these compounds may behave like chemical chaperones. Particularly, some positively charged amino acids are very effective in inhibiting protein aggregation, likely by weakening protein–protein interactions/associations such as histidine, arginine, proline, hydroxyproline, glycine, lysine, putrescine and spermidine, imidazole, betaine and sulfobetaine. Negative effect was also reported in a few cases, such as histidine (Lam et al., 2000), glycine or trimethylamine-N-oxide (Eronina et al., 2005), and dopamine (Moussa et al., 2008). The dual effects of these compounds on protein aggregation can occur to the same protein simply by changing their concentrations. Other additives include organic acids, polyions and polymers, and amphoteric polymers. A strong hydrophobic/electrostatic interaction between the polymer and protein, however, may cause denaturation or even aggregation.

Surfactants are often added into protein solutions to inhibit protein aggregation in different processes, such as shaking/shearing and thermal treatment. Their stabilization effect is likely due to their weak binding to proteins blocking or partially blocking the aggregation-prone hydrophobic sites on the protein surface (Hoffmann et al., 2009). However, a negative effect of non-ionic surfactants has also been observed on protein aggregation in several accelerated or real-time stability studies (Wang et al., 2008c). The increased protein aggregation in the presence of a non-ionic surfactant could also be a result of binding between surfactants and proteins. A strong binding could possibly induce partial denaturation of proteins, leading to increased protein aggregation (Panyukov et al., 2007).

Other formulation excipients/additives, that potentially affect protein aggregation, include preservatives, antioxidants, reducing agents, and organic solvents. Other residual components from raw materials or process equipment are also critical in controlling the rate of protein aggregation, such as multivalent metal ions (Long et al., 2008), denaturants (Hamada et al., 2008), contaminant enzymes (Creusot and Gruppen, 2007), lipids (Tsiroulnikov et al., 2009), and protein aggregates (Andrews et al., 2008).

5.2. Protein concentration

The effect of protein concentration on protein aggregation has been evaluated extensively. Because of the existence of different aggregation pathways (e.g. partial unfolding-induced vs simple association-initiated aggregation), increasing protein concentration could potentially result in the following consequences—(1) decreased aggregation due to crowding effect; (2) increased aggregation due to increased chance of association; and (3) precipitation due to solubility limit. The ultimate effect is protein-dependent and the net result of these factors. The concept of macromolecu-

lar crowding has been proposed elsewhere and is used to describe the crowded environment in intracellular compartments. According to this theory, crowding should favor formation of compact conformations over extended conformations of flexible macromolecules and thereby reduce the aggregation tendency. On the other hand, increasing protein concentrations would increase the chances of protein–protein association, in most cases, facilitating protein aggregation. Lastly, each protein has a solubility limit, which is strongly dependent on the solution conditions. Proteins can readily aggregate, precipitate, or in few cases crystallize when protein concentration exceeds this limit (Adachi et al., 2009). In many cases, protein concentrations are well below its solubility limit under normal experimental conditions and solubility-limited protein aggregation is not a common concern.

5.3. Contact surfaces and container/closure systems

Proteins have a tendency to be adsorbed onto a variety of surfaces. Such adsorption may cause loss of proteins and/or protein aggregation with or without structural changes. In any case, the energy barrier to the formation of aggregate seeds is likely reduced upon protein adsorption. Adsorption of recombinant factor VIII (rFVIII) to hydrophobic silica surface (silanized) caused changes in tertiary structure while a less degree of structural change was seen on negatively charged hydrophilic surfaces (Joshi et al., 2008). The reversible adsorption of rhPAF-AH (pI = 6.7) to nano-sized hydrophilic silica particles induced no detectable change in protein secondary/tertiary structures but resulted in rapid and extensive aggregation of rhPAF-AH at pH 6.5 (Chi et al., 2005). Protein adsorption can form multiple layers, facilitating aggregation such as IgG on the surface of polyethylene terephthalate (PET) (Holmberg and Hou, 2009).

Proteins may have different affinities to different types of surfaces, and adsorption is strongly affected by solution pH, salt concentration and temperature. For example, recombinant factor VIII at 50 μg/mL can be adsorbed at 0.13 $\mu g/cm^2$ on hydrophobic silica surface (silanized) in the presence of 8 ppm Tween 80 mainly through hydrophobic interactions and at 0.3 $\mu g/cm^2$ on negatively charged hydrophilic surface mainly through electrostatic attractions (Joshi et al., 2008). Surfactants may or may not affect protein adsorption depending on the type of surface and the protein (Joshi and McGuire, 2008). Reduction in protein adsorption in the presence of a surfactant can be attributable to several possibilities—(1) faster movement of the surfactant to the interface; (2) higher affinity of the surfactant at the interface than the protein; or formation of surfactant–protein complex.

The container/closure systems can certainly induce surface-related protein aggregation. Metal containers/surfaces have been shown to cause surface-induced protein aggregation (Bee et al., 2009b; Zhang et al., 2009). The effect of surface material on protein aggregation could be amplified under stressed processing conditions (Kueltzo et al., 2008). In addition, indirect effect on protein aggregation was reported through container/closure leachables or residual processing aids. Rubber stoppers or syringes are often siliconized for ease of processing or administration. Trace amount of silicone may facilitate protein aggregation due to its hydrophobic nature (Thirumangalathu et al., 2009). Residual tungsten extract in syringe manufacturing process could induce significant protein aggregation (Bee et al., 2009c; Jiang et al., 2009).

Table 1
Major factors affecting protein aggregation.

Category	Individual factors	Effects on protein and aggregation
General factors	Temperature	Potentially induces partial/complete protein unfolding, protein–protein association, and chemical degradations, enhancing protein aggregation
	Light/irradiation	Potentially induces chemical degradation, which may enhances protein aggregation or directly crosslink proteins
	Container/closure systems	Generally induce surface protein adsorption, partial protein unfolding, release of stability-influencing leachables, enhancing protein aggregation
Solution factors	pH	Potentially induces partial/complete protein unfolding and alters colloidal stability, which may enhance protein aggregation
	Buffering agent and concentration	Variable effects, depending on the specificity and strength of ion–protein interaction (chaotropic vs kosmotropic effect) and effect of ionic strength (see below)
	Ionic strength	Variable effects, balancing on general charge-screening effect, ion–protein interactions, and interference with protein–protein interactions
	Additives (excipients) or residual components	Variable effects, depending on the additive/component type and concentration
	Protein concentration	High protein concentration generally enhances protein aggregation through enhanced protein–protein interactions, and solubility limitations
Processing factors	Fermentation/expression	Variable effects, depending on the expression host system, and fermentation/expression conditions
	Refolding	Generally lead to protein aggregation to a variable level, depending on refolding reconditions, such as temperature, protein concentration, type/concentration of denaturant and redox pair, and other additives
	Purification	Variable effects, depending on the purification conditions
	Freeze-thawing	Induces ice–protein interaction, interfacial adsorption and unfolding, cryoconcentration, cold denaturation, and changes in pH and ionic strength, leading to enhanced protein aggregation
	Shaking	Induces air–liquid interfacial protein adsorption and unfolding, enhancing protein aggregation
	Shearing	Potentially expose hydrophobic patches/regions, have either no effect or in some cases enhancing protein aggregation
	Pressurization	No effect under low to moderate pressures encountered in the protein drug product manufacturing. Potentially (>100 MPa) induces loss of structure, affecting protein aggregation
	Drying	Loss of hydration layer leads to disruption of protein structure, enhancing protein aggregation
	Analytical methodologies	Potentially induce/eliminate protein aggregation during sample preparation and analysis

5.4. Light and irradiation

Proteins can be sensitive to light/UV exposure. Light/UV irradiation has been shown to promote protein unfolding, leading to protein aggregation (Redecke et al., 2009), or induce photo cross-linking reaction(s), leading to covalent aggregation (Qi et al., 2008). It has been found that UV irradiation can induce cross-linking reactions between Cys and Trp residues and photolysis of native disulfide bonds for new disulfide bond formation/exchange (Davies, 2003). UV irradiation can oxidize Trp residues, leading to formation of thiol radicals and subsequent formation of new disulfide bonds (Roy et al., 2009). Gamma irradiation was also shown to cause aggregation of ceruloplasmin and haemoglobin (Assemand et al., 2003). Therefore, light protection is necessary for such light-sensitive protein products.

6. Summary

Aggregation is a common but complex mode of protein instability. Several distinctive aggregation pathways have been discussed and illustrated in Fig. 1. The major aggregation pathways include: (1) physical aggregation through unfolding intermediates and unfolded states; (2) aggregation through protein self-association or chemical linkages; and (3) aggregation through chemical degradations. These aggregation pathways may change depending on the experimental conditions. It is not uncommon that a protein can aggregate by multiple pathways, making it difficult to elucidate the mechanism. All these aggregation pathways can eventually lead to formation of physical and/or chemically linked aggregates with or without visible precipitates.

A variety of factors have been identified affecting the rate and extent of protein aggregation, as summarized in Table 1. These major factors can be divided into several categories: (1) general environmental factors (temperature, light, and container/closure system); (2) solution factors (pH, buffer type and concentration, ionic strength, excipients and level, protein concentration, etc.); (3) processing factors (fermentation/expression, unfolding/refolding, purification, freeze–thaw, shaking and shearing, pressurization, formulation/filling, drying, preparation of modified protein or delivery systems, and analytical methodologies, etc.).

Among these factors, temperature is the most critical and commonly encountered variable that needs to be controlled. The next critical factor is arguably the solution pH. All these factors need to be considered during processing or storing proteins. Since the aggregation pathways and ultimate aggregate composition/structure are different under different experimental conditions, it is critical to identify the major stresses that cause aggregation before designing an effective way to inhibit/prevent protein aggregation. Inhibition of protein aggregation is frequently achieved by changing the immediate environment of the protein through use of a compatible additive(s). The commonly used protein stabilizing additives include sugars, polyols, surfactants, salts, metal ions, and amino acids. These additives may not always have a positive effect depending on the protein and additive concentration.

Proteins are structurally diverse and the relative effect of these factors on protein aggregation is very much protein-dependent. It is not unusual that a single factor can have a completely opposite effect on different proteins or even the same protein depending on the experimental conditions such as solution pH (Singh et al., 2009). Many exceptions do exist. Therefore, universal rules, which can be applied in prevention/inhibition of protein aggregation, have not yet been defined. The traditional, semi-empirical approach in controlling protein aggregation will continue to be adopted by scientists in the near future until a better understanding of the mechanism(s) of protein aggregation is achieved.

Acknowledgements

The authors greatly appreciate the critical review of the manuscript by Dr. Satish Singh, Prof. Ted Randolph and the journal reviewers. They made invaluable comments throughout the manuscript.

References

Abdul-Fattah, A.M., Lechuga-Ballesteros, D., Kalonia, D.S., Pikal, M.J., 2008. The impact of drying method and formulation on the physical properties and stability of methionyl human growth hormone in the amorphous solid state. J. Pharm. Sci. 97, 163–184.

Adachi, K., Ding, M., Asakura, T., Surrey, S., 2009. Relationship between beta4 hydrogen bond and beta6 hydrophobic interactions during aggregate, fiber or crystal formation in oversaturated solutions of hemoglobin A and S. Arch. Biochem. Biophys. 481, 137–144.

Alford, J.R., Kendrick, B.S., Carpenter, J.F., Randolph, T.W., 2008a. High concentration formulations of recombinant human interleukin-1 receptor antagonist. II. Aggregation kinetics. J. Pharm. Sci. 97, 3005–3021.

Alford, J.R., Kendrick, B.S., Carpenter, J.F., Randolph, T.W., 2008b. Measurement of the second osmotic virial coefficient for protein solutions exhibiting monomer–dimer equilibrium. Anal. Biochem. 377, 128–133.

Andrews, J.M., Weiss, W.F., Roberts, C.J., 2008. Nucleation, growth, and activation energies for seeded and unseeded aggregation of alpha-chymotrypsinogen A. Biochemistry 47, 2397–2403.

Assemand, E., Lacroix, M., Mateescu, M.A., 2003. L-Tyrosine prevents aggregation of therapeutic proteins by gamma-irradiation. Biotechnol. Appl. Biochem. 38, 151–156.

Bahrami, A., Shojaosadati, S.A., Khalilzadeh, R., Mohammadian, J., Farahani, E.V., Masoumian, M.R., 2009. Prevention of human granulocyte colony-stimulating factor protein aggregation in recombinant *Pichia pastoris* fed-batch fermentation using additives. Biotechnol. Appl. Biochem. 52, 141–148.

Baynes, B.M., Wang, D.I., Trout, B.L., 2005. Role of arginine in the stabilization of proteins against aggregation. Biochemistry 44, 4919–4925.

Bee, J.S., Chiu, D., Sawicki, S., Stevenson, J.L., Chatterjee, K., Freund, E., Carpenter, J.F., Randolph, T.W., 2009a. Monoclonal antibody interactions with micro- and nanoparticles: adsorption, aggregation, and accelerated stress studies. J. Pharm. Sci. 98 (9), 3218–3238.

Bee, J.S., Nelson, S.A., Freund, E., Carpenter, J.F., Randolph, T.W., 2009b. Precipitation of a monoclonal antibody by soluble tungsten. J. Pharm. Sci. 98 (9), 3290–3301.

Bee, J.S., Stevenson, J.L., Mehta, B., Svitel, J., Pollastrini, J., Platz, R., Freund, E., Carpenter, J.F., Randolph, T.W., 2009c. Response of a concentrated monoclonal antibody formulation to high shear. Biotechnol. Bioeng. 103, 936–943.

Burgess, R.R., 2009. Refolding solubilized inclusion body proteins. Methods Enzymol. 463, 259–282.

Cabra, V., Vazquez-Contreras, E., Moreno, A., Arreguin-Espinosa, R., 2008. The effect of sulfhydryl groups and disulphide linkage in the thermal aggregation of Z19 alpha-zein. Biochim. Biophys. Acta 1784, 1028–1036.

Castellanos, I.J., Crespo, R., Griebenow, K., 2003. Poly(ethylene glycol) as stabilizer and emulsifying agent: a novel stabilization approach preventing aggregation and inactivation of proteins upon encapsulation in bioerodible polyester microspheres. J. Control. Release 88, 135–145.

Chi, E.Y., Krishnan, S., Randolph, T.W., Carpenter, J.F., 2003. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharm. Res. 20, 1325–1336.

Chi, E.Y., Weickmann, J., Carpenter, J.F., Manning, M.C., Randolph, T.W., 2005. Heterogeneous nucleation-controlled particulate formation of recombinant human platelet-activating factor acetylhydrolase in pharmaceutical formulation. J. Pharm. Sci. 94, 256–274.

Chodankar, S., Aswal, V.K., Kohlbrecher, J., Vavrin, R., Wagh, A.G., 2009. Small-angle neutron scattering study of structure and kinetics of temperature-induced protein gelation. Phys. Rev. E: Stat. Nonlinear Soft Matter Phys. 79, 021912.

Considine, T., Patel, H.A., Singh, H., Creamer, L.K., 2007. Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of beta-lactoglobulin B. Food Chem. 102, 1270–1280.

Creusot, N., Gruppen, H., 2007. Enzyme-induced aggregation and gelation of proteins. Biotechnol. Adv. 25, 597–601.

Crisman, R.L., Randolph, T.W., 2009. Refolding of proteins from inclusion bodies is favored by a diminished hydrophobic effect at elevated pressures. Biotechnol. Bioeng. 102, 483–492.

Cromwell, M.E., Hilario, E., Jacobson, F., 2006. Protein aggregation and bioprocessing. AAPS J. 8, E572–E579.

Davies, M.J., 2003. Singlet oxygen-mediated damage to proteins and its consequences. Biochem. Biophys. Res. Commun. 305, 761–770.

Demeule, B., Lawrence, M.J., Drake, A.F., Gurny, R., Arvinte, T., 2007. Characterization of protein aggregation: the case of a therapeutic immunoglobulin. Biochim. Biophys. Acta 1774, 146–153.

Eronina, T.B., Chebotareva, N.A., Kurganov, B.I., 2005. Influence of osmolytes on inactivation and aggregation of muscle glycogen phosphorylase b by guanidine hydrochloride. Stimulation of protein aggregation under crowding conditions. Biochemistry (Mosc.) 70, 1020–1026.

Espargaro, A., Castillo, V., de Groot, N.S., Ventura, S., 2008. The in vivo and in vitro aggregation properties of globular proteins correlate with their conformational stability: the SH3 case. J. Mol. Biol. 378, 1116–1131.

Ghosh, R., Sharma, S., Chattopadhyay, K., 2009. Effect of arginine on protein aggregation studied by fluorescence correlation spectroscopy and other biophysical methods. Biochemistry 48, 1135–1143.

Giger, K., Vanam, R.P., Seyrek, E., Dubin, P.L., 2008. Suppression of insulin aggregation by heparin. Biomacromolecules 9, 2338–2344.

Giurleo, J.T., He, X., Talaga, D.S., 2008. Beta-lactoglobulin assembles into amyloid through sequential aggregated intermediates. J. Mol. Biol. 381, 1332–1348.

Golub, N.V., Markossian, K.A., Kasilovich, N.V., Sholukh, M.V., Orlov, V.N., Kurganov, B.I., 2008. Thermal inactivation, denaturation and aggregation of mitochondrial aspartate aminotransferase. Biophys. Chem. 135, 125–131.

Gsponer, J., Vendruscolo, M., 2006. Theoretical approaches to protein aggregation. Protein Pept. Lett. 13, 287–293.

Hagiwara, H., Yuasa H., Yamamoto, Y., 2000. Stabilized human monoclonal antibody preparation. US6165467.

Hamada, H., Arakawa, T., Shiraki, K., 2009. Effect of additives on protein aggregation. Curr. Pharm. Biotechnol. 10, 400–407.

Hamada, H., Takahashi, R., Noguchi, T., Shiraki, K., 2008. Differences in the effects of solution additives on heat- and refolding-induced aggregation. Biotechnol. Prog. 24, 436–443.

Hao, Y., Chu, J., Wang, Y., Zhuang, Y., Zhang, S., 2007. The inhibition of aggregation of recombinant human consensus interferon-alpha mutant during *Pichia pastoris* fermentation. Appl. Microbiol. and Biotechnol. 74, 578–584.

Heavner, G.A., Arakawa, T., Philo, J.S., Calmann, M.A., Labrenz, S., 2007. Protein isolated from biopharmaceutical formulations cannot be used for comparative studies: Follow-up to "a case study using Epoetin Alfa from Epogen and EPREX". J Pharm Sci 96 (12), 3214–3225.

Hoffmann, C., Blume, A., Miller, I., Garidel, P., 2009. Insights into protein–polysorbate interactions analysed by means of isothermal titration and differential scanning calorimetry. Eur. Biophys. J..

Hoiberg-Nielsen, R., Fuglsang, C.C., Arleth, L., Westh, P., 2006. Interrelationships of glycosylation and aggregation kinetics for *Peniophora lycii* phytase. Biochemistry 45, 5057–5066.

Holmberg, M., Hou, X., 2009. Competitive protein adsorption—multilayer adsorption and surface induced protein aggregation. Langmuir 25, 2081–2089.

Holmberg, M., Stibius, K.B., Ndoni, S., Larsen, N.B., Kingshott, P., Hou, X.L., 2007. Protein aggregation and degradation during iodine labelling and its consequences for protein adsorption to biomaterials. Anal. Biochem. 361, 120–125.

Huppertz, T., Fox, P.F., de Kruif, K.G., Kelly, A.L., 2006. High pressure-induced changes in bovine milk proteins: a review. Biochim. Biophys. Acta 1764, 593–598.

Jiang, Y., Nashed-Samuel, Y., Li, C., Liu, W., Pollastrini, J., Mallard, D., Wen, Z.Q., Fujimori, K., Pallitto, M., Donahue, L., Chu, G., Torraca, G., Vance, A., Mire-Sluis, T., Freund, E., Davis, J., Narhi, L., 2009. Tungsten-induced protein aggregation: solution behavior. J. Pharm. Sci. 98, 4695–4710.

Joshi, O., McGuire, J., 2008. adsorption behavior of lysozyme and Tween 80 at hydrophilic and hydrophobic silica–water interfaces. Appl. Biochem. Biotechnol., doi:10.1007/s12010-008-8246-8.

Joshi, O., McGuire, J., Wang, D.Q., 2008. Adsorption and function of recombinant factor VIII at solid–water interfaces in the presence of Tween-80. J. Pharm. Sci., doi:10.1002/jps.21333.

Jovanovic, N., Bouchard, A., Sutter, M., Van Speybroeck, M., Hofland, G.W., Witkamp, G.J., Crommelin, D.J., Jiskoot, W., 2008. Stable sugar-based protein formulations by supercritical fluid drying. Int. J. Pharm. 346, 102–108.

Jungbauer, A., Kaar, W., 2007. Current status of technical protein refolding. J. Biotechnol. 128, 587–596.

Kanai, S., Liu, J., Patapoff, T.W., Shire, S.J., 2008. Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab–Fab interaction that impacts solution viscosity. J. Pharm. Sci., 21322, doi:10.1002/jps.

Kosa, T., Maruyama, T., Otagiri, M., 1998. Species differences of serum albumins. II. Chemical and thermal stability. Pharm. Res. 15, 449–454.

Krebs, M.R., Devlin, G.L., Donald, A.M., 2007. Protein particulates: another generic form of protein aggregation? Biophys. J. 92, 1336–1342.

Krebs, M.R., Domike, K.R., Donald, A.M., 2009. Protein aggregation: more than just fibrils. Biochem. Soc. Trans. 37, 682–686.

Krishnan, S., Chi, E.Y., Wood, S.J., Kendrick, B.S., Li, C., Garzon-Rodriguez, W., Wypych, J., Randolph, T.W., Narhi, L.O., Biere, A.L., Citron, M., Carpenter, J.F., 2003. Oxidative dimer formation is the critical rate-limiting step for Parkinson's disease alpha-synuclein fibrillogenesis. Biochemistry 42, 829–837.

Krishnan, S., Raibekas, A.A., 2009. Multistep aggregation pathway of human interleukin-1 receptor antagonist: kinetic, structural, and morphological characterization. Biophys. J. 96, 199–208.

Kueltzo, L.A., Wang, W., Randolph, T.W., Carpenter, J.F., 2008. Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze-thawing. J. Pharm. Sci. 97, 1801–1812.

Kumar, V., Sharma, V.K., Kalonia, D.S., 2009. In situ precipitation and vacuum drying of interferon alpha-2a: development of a single-step process for obtaining dry, stable protein formulation. Int. J. Pharm. 366, 88–98.

Lam, X.M., Oeswein J.Q., Ongpipattanakul, B., Shahrokh, Z., Wang, S.X., Weissburg, R.P., Wong, R.L., 2000. Stabilized antibody formulation. US6991790 B1.

Livney, Y.D., Verespej, E., Dalgleish, D.G., 2003. Steric effects governing disulfide bond interchange during thermal aggregation in solutions of beta-lactoglobulin B and alpha-lactalbumin. J. Agric. Food Chem. 51, 8098–8106.

Long, X., Zhang, C., Cheng, J., Bi, S., 2008. A novel method for study of the aggregation of protein induced by metal ion aluminum(III) using resonance Rayleigh scattering technique. Spectrochim. Acta A: Mol. Biomol. Spectrosc. 69, 71–77.

Macchioni, A., Ciancaleoni, G., Zuccaccia, C., Zuccaccia, D., 2008. Determining accurate molecular sizes in solution through NMR diffusion spectroscopy. Chem. Soc. Rev. 37, 479–489.

Majhi, P.R., Ganta, R.R., Vanam, R.P., Seyrek, E., Giger, K., Dubin, P.L., 2006. Electrostatically driven protein aggregation: beta-lactoglobulin at low ionic strength. Langmuir 22, 9150–9159.

Mannall, G.J., Titchener-Hooker, N.J., Dalby, P.A., 2007. Factors affecting protein refolding yields in a fed-batch and batch-refolding system. Biotechnol. Bioeng. 97, 1523–1534.

Mercade-Prieto, R., Gunasekaran, S., 2009. Alkali cold gelation of whey proteins. Part II. Protein concentration. Langmuir 25, 5793–5801.

Modler, A.J., Gast, K., Lutsch, G., Damaschun, G., 2003. Assembly of amyloid protofibrils via critical oligomers—a novel pathway of amyloid formation. J. Mol. Biol. 325, 135–148.

Morris, A.M., Watzky, M.A., Finke, R.G., 2009. Protein aggregation kinetics, mechanism, and curve-fitting: a review of the literature. Biochim. Biophys. Acta 1794, 375–397.

Moussa, C.E., Mahmoodian, F., Tomita, Y., Sidhu, A., 2008. Dopamine differentially induces aggregation of A53T mutant and wild type alpha-synuclein: insights into the protein chemistry of Parkinson's disease. Biochem. Biophys. Res. Commun. 365, 833–839.

Mukherjee, S., Chowdhury, P., Gai, F., 2009. Effect of dehydration on the aggregation kinetics of two amyloid peptides. J. Phys. Chem. B 113, 531–535.

Natalello, A., Prokorov, V.V., Tagliavini, F., Morbin, M., Forloni, G., Beeg, M., Manzoni, C., Colombo, L., Gobbi, M., Salmona, M., Doglia, S.M., 2008. Conformational plasticity of the Gerstmann–Straussler–Scheinker disease peptide as indicated by its multiple aggregation pathways. J. Mol. Biol. 381, 1349–1361.

Necula, M., Kayed, R., Milton, S., Glabe, C.G., 2007. Small molecule inhibitors of aggregation indicate that amyloid beta oligomerization and fibrillization pathways are independent and distinct. J. Biol. Chem. 282, 10311–10324.

Olsen, S.N., Andersen, K.B., Randolph, T.W., Carpenter, J.F., Westh, P., 2009. Role of electrostatic repulsion on colloidal stability of *Bacillus halmapalus* alpha-amylase. Biochim. Biophys. Acta, doi:10.1016/j.bbapap.2009.02.010, S1570-9639(09)00031-4 [pii].

Panyukov, Y.V., Nemykh, M.A., Dobrov, E.N., Drachev, V.A., 2007. Surfactant-induced amorphous aggregation of tobacco mosaic virus coat protein: a physical methods approach. Macromol. Biosci..

Philo, J.S., 2009. A critical review of methods for size characterization of non-particulate protein aggregates. Curr. Pharm. Biotechnol. 10, 359–372.

Philo, J.S., Arakawa, T., 2009. Mechanisms of protein aggregation. Curr. Pharm. Biotechnol. 10, 348–351.

Pivovarova, A.V., Chebotareva, N.A., Chernik, I.S., Gusev, N.B., Levitsky, D.I., 2007. Small heat shock protein Hsp27 prevents heat-induced aggregation of F-actin by forming soluble complexes with denatured actin. FEBS J. 274, 5937–5948.

Qi, P., Volkin, D.B., Zhao, H., Nedved, M.L., Hughes, R., Bass, R., Yi, S.C., Panek, M.E., Wang, D., Dalmonte, P., Bond, M.D., 2008. Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form. J. Pharm. Sci., doi:10.1002/jps.21617.

Redecke, L., Binder, S., Elmallah, M.I., Broadbent, R., Tilkorn, C., Schulz, B., May, P., Goos, A., Eich, A., Rubhausen, M., Betzel, C., 2009. UV-light-induced conversion and aggregation of prion proteins. Free Radic. Biol. Med. 46, 1353–1361.

Rees, D.C., Robertson, A.D., 2001. Some thermodynamic implications for the thermostability of proteins. Protein Sci. 10, 1187–1194.

Rezaei-Ghaleh, N., Ebrahim-Habibi, A., Moosavi-Movahedi, A.A., Nemat-Gorgani, M., 2007. Role of electrostatic interactions in 2,2,2-trifluoroethanol-induced structural changes and aggregation of alpha-chymotrypsin. Arch. Biochem. Biophys. 457, 160–169.

Roostaee, A., Cote, S., Roucou, X., 2009. Aggregation and amyloid fibril formation induced by chemical dimerization of recombinant prion protein in physiological-like conditions. J. Biol. Chem. 284, 30907–30916.

Rosenfeld, M.A., Leonova, V.B., Konstantinova, M.L., Razumovskii, S.D., 2009. Self-assembly of fibrin monomers and fibrinogen aggregation during ozone oxidation. Biochemistry (Mosc.) 74, 41–46.

Roy, S., Mason, B.D., Schoneich, C.S., Carpenter, J.F., Boone, T.C., Kerwin, B.A., 2009. Light-induced aggregation of type I soluble tumor necrosis factor receptor. J. Pharm. Sci. 98, 3182–3199.

Saluja, A., Badkar, A.V., Zeng, D.L., Kalonia, D.S., 2007. Ultrasonic rheology of a monoclonal antibody (IgG2) solution: implications for physical stability of proteins in high concentration formulations. J. Pharm. Sci. 96, 3181–3195.

Saluja, A., Kalonia, D.S., 2008. Nature and consequences of protein–protein interactions in high protein concentration solutions. Int. J. Pharm. 358, 1–15.

Schule, S., Schulz-Fademrecht, T., Garidel, P., Bechtold-Peters, K., Friess, W., 2008. Stabilization of IgG1 in spray-dried powders for inhalation. Eur. J. Pharm. Biopharm. 69, 793–807.

Seefeldt, M.B., Kim, Y.S., Tolley, K.P., Seely, J., Carpenter, J.F., Randolph, T.W., 2005. High-pressure studies of aggregation of recombinant human interleukin-1 receptor antagonist: thermodynamics, kinetics, and application to accelerated formulation studies. Protein Sci. 14, 2258–2266.

Serno, T., Carpenter, J.F., Randolph, T.W., Winter, G., 2009. Inhibition of agitation-induced aggregation of an IgG-antibody by hydroxypropyl-beta-cyclodextrin. J. Pharm. Sci. 99 (3), 1193–1206.
Shukla, A.A., Gupta, P., Han, X., 2007. Protein aggregation kinetics during Protein A chromatography: case study for an Fc fusion protein. J. Chromatogr. A 1171, 22–28.
Singh, L.R., Dar, T.A., Rahman, S., Jamal, S., Ahmad, F., 2009. Glycine betaine may have opposite effects on protein stability at high and low pH values. Biochim. Biophys. Acta.
Takata, T., Oxford, J.T., Demeler, B., Lampi, K.J., 2008. Deamidation destabilizes and triggers aggregation of a lens protein, {beta}A3-crystallin. Protein Sci., doi:10.1110/ps.035410.108.
Talla-Singh, D., Stites, W.E., 2008. Refinement of noncalorimetric determination of the change in heat capacity. DeltaC(p), of protein unfolding and validation across a wide temperature range. Proteins 71, 1607–1616.
Thirumangalathu, R., Krishnan, S., Ricci, M.S., Brems, D.N., Randolph, T.W., Carpenter, J.F., 2009. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J. Pharm. Sci. 98, 3167–3181.
Tsiroulnikov, K., Shchutskaya, Y., Muronetz, V., Chobert, J.M., Haertle, T., 2009. Phospholipids influence the aggregation of recombinant ovine prions: from rapid extensive aggregation to amyloidogenic conversion. Biochim. Biophys. Acta 1794, 506–511.
Van Buren, N., Rehder, D., Gadgil, H., Matsumura, M., Jacob, J., 2008. Elucidation of two major aggregation pathways in an IgG2 antibody. J. Pharm. Sci., doi:10.1002/jps.21514.
Wang, B., Tchessalov, S., Cicerone, M.T., Warne, N.W., Pikal, M.J., 2008a. Impact of sucrose level on storage stability of proteins in freeze-dried solids. II. Correlation of aggregation rate with protein structure and molecular mobility. J. Pharm. Sci., doi:10.1002/jps.21622.
Wang, S.S., Wu, J.W., Yamamoto, S., Liu, H.S., 2008b. Diseases of protein aggregation and the hunt for potential pharmacological agents. Biotechnol. J. 3, 165–192.
Wang, W., 2005. Protein aggregation and its inhibition in biopharmaceutics. Int. J. Pharm. 289, 1–30.
Wang, W., Wang, Y.J., Wang, D.Q., 2008c. Dual effects of Tween 80 on protein stability. Int. J. Pharm. 347, 31–38.
Webb, S.D., Golledge, S.L., Cleland, J.L., Carpenter, J.F., Randolph, T.W., 2002. Surface adsorption of recombinant human interferon-gamma in lyophilized and spray-lyophilized formulations. J. Pharm. Sci. 91, 1474–1487.
Wei, Y., Chen, L., Chen, J., Ge, L., He, R.Q., 2009. Rapid glycation with D-ribose induces globular amyloid-like aggregations of BSA with high cytotoxicity to SH-SY5Y cells. BMC Cell. Biol. 10, 10.
Weiss, W.F., Young, T.M., Roberts, C.J., 2009. Principles, approaches, and challenges for predicting protein aggregation rates and shelf life. J. Pharm. Sci. 98, 1246–1277.
Xia, Y., Park, Y.D., Mu, H., Zhou, H.M., Wang, X.Y., Meng, F.G., 2007. The protective effects of osmolytes on arginine kinase unfolding and aggregation. Int. J. Biol. Macromol. 40, 437–443.
Yazdanparast, R., Esmaeili, M.A., Khodagholi, F., 2007. Control of aggregation in protein refolding: cooperative effects of artificial chaperone and cold temperature. Int. J. Biol. Macromol. 40, 126–133.
Yu, Z., Johnston, K.P., Williams III, R.O., 2006. Spray freezing into liquid versus spray-freeze drying: influence of atomization on protein aggregation and biological activity. Eur. J. Pharm. Sci. 27, 9–18.
Zhang, D., Neumann, O., Wang, H., Yuwono, V.M., Barhoumi, A., Perham, M., Hartgerink, J.D., Wittung-Stafshede, P., Halas, N.J., 2009. Gold nanoparticles can induce the formation of protein-based aggregates at physiological pH. Nano Lett. 9, 666–671.
Zhang, J., Peng, X., Jonas, A., Jonas, J., 1995. NMR study of the cold, heat, and pressure unfolding of ribonuclease A. Biochemistry 34, 8631–8641.
Zhang, L., Lu, D., Liu, Z., 2008. How native proteins aggregate in solution: a dynamic Monte Carlo simulation. Biophys. Chem. 133, 71–80.