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Physical Stability of Protein Pharmaceuticals

Byeong S. Chang, Ph.D. and Bernice Yeung, Ph.D.

Symyx Technologies, Inc.

820 Calle Plano, Camarillo, CA 93012

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1. Introduction

The unique three dimensional structures of proteins are not only critical for specific interactions with related molecules for precise biochemical reactions but also important for other supportive functions like feedback mechanism, transport, and solubility in physiological environments (Valente et al., 2006; Luo et al., 2007; Goodey and Benkovic, 2008). For these reasons, the native structures of proteins are designed to adapt to environmental changes, e.g., pH, ionic strength, hydrophobicity, surface, ligands, other biological molecules including proteins, etc (Shahrokh et al, 1997; Carpenter and Manning, 2000; McNalley, 2000). While the flexibility of structure is required for necessary biological function of proteins, it also presents unique challenges in the development of proteins for pharmaceutical applications by causing conformational changes, aggregation, and precipitation, which often are related to loss of biological activity as well as immunogenicity of the proteins (Schellekens, 2008; Rosenberg, 2006; Bennett et al., 2004; Hermeling et al., 2006, 2004). In order to address this particular issue, various platforms for manufacturing process and formulation development have been introduced to the pharmaceutical industry. In practice, research focuses on comprehending the nature of protein structures, understanding the conditions that can cause the alteration from native structures and their contribution to the degradation of proteins, developing novel analytical tools that examine these processes and end results of degradation pathways, and designing approaches to utilize this information for maintaining the nativity of each protein for its safe pharmaceutical application (for related review articles, see Carpenter et al., 1996; Carpenter et al., 1999; Wang, 1999; Carpenter et al, 2002; Chang and Hershenson, 2002; Kendrick et al., 2002; Patro et al, 2002; Chi et al., 2003; Frokjaer and Otzen, 2005).

Although fully understanding all the possible combinations of structural variants, especially under stressed conditions, would be virtually impossible, several decades worth of research by both academic and pharmaceutical institutions has established general approaches that can be applied for the successful development of most biopharmaceutical products (Cleland et al, 1993; Nail and Akers, 2002; Cromwell et al., 2006; Patro et al., 2002; Chang and Hershenson, 2002). Among these, key accomplishments relevant for pharmaceutical development, e.g., identification of key stress factors, understanding major degradation products, and developing analytical methods to determine the degradation products, will be discussed in this chapter.

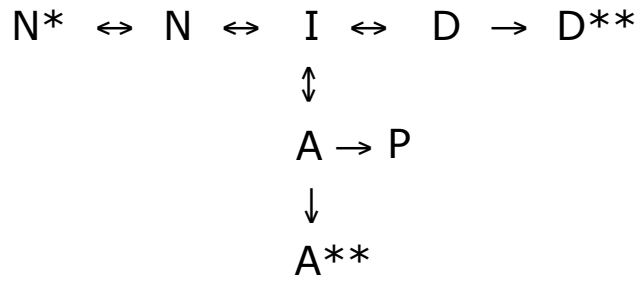
This chapter will specifically focus on the physical changes that occur in proteins and their contribution to the overall stability relevant to product development, so readers are referred to other chapters in this book for chemical degradations and the like.

2. Physical stability of protein pharmaceuticals

2.1 Physical forms of proteins found in practice

Unlike conventional small molecular pharmaceuticals, proteins present unique challenges related to the proper folding of their native state and their appropriate structural

maintenance. The classic discussions of the primary, secondary, tertiary, and quaternary structure of proteins can be found in most basic biochemistry textbooks, so they will not be repeated here (Branden and Tooze, 1999; Lesk, 2000) . It is generally agreed that proteins will spontaneously fold to assume their native structures to fit the environment in which they are naturally found, e.g., exposing hydrophilic residues and hiding hydrophobic residues in the core in an aqueous environment. When proteins are purified and kept in non-physiological environments and exposed to physicochemical stresses in particular, proteins undergo structural changes to adapt to their changing environments. The structural changes can result in combinations of structural variants during the handling and processing of these molecules (Santuicci et al., 2008, Ferreira et al., 2006). The structural changes invariably introduce different physical properties to the molecule, e.g., solubility, tendency to aggregate, surface activity, which ultimately results in the formation of undesirable degradations such as soluble aggregates, insoluble precipitates, losses due to surface adsorption, etc. Various physical forms of proteins that have been routinely observed are summarized in Figure 1. The native structure of a protein (N) assumes a correctly folded form with the lowest free energy state. In reality, their native conformations are in thermodynamic equilibrium with their environment. N* represents a rare state when the structure of protein becomes more compact due to changes in the environment (Kendrick et al., 1997). The probability of unfolding is smaller in this compact structural state of the molecule as evidenced by reduced rates of H-D exchange and/or exposure of varied residues. The native structure can also undergo partial structural changes, when either stress is induced or is found naturally in solution, and can convert into various forms of folding intermediate structures (I) which have been routinely observed during the refolding processes of proteins (Santucci et al., 2008; Tang et al., 1998). Typically, subtle structural changes, e.g., changes in tertiary structure, are involved in this process even though the whole spectrum of folding intermediates can fit in the species. Among the intermediate species, certain species will have a greater tendency to interact with other protein molecules and form soluble or insoluble aggregates (A) or precipitates (P) (Roberts, 2007). These soluble aggregates may be reversible to a monomeric intermediate form, but they can turn into irreversible aggregates as the molecules undergo further structural changes for stable aggregate conformations (A**) (Calamai et al., 2005). From the intermediate forms, the proteins can further lose their crucial structural elements, e.g., secondary structures, resulting in a denatured form (D). This transition does not routinely occur in reality unless strong physical stresses, e.g., heating above thermal unfolding temperature, or high concentrations of chaeotropes, e.g., guanidine hydrochloride, are introduced to the solution. Although the denaturation can be reversible, other physicochemical changes to the denatured form can render the process irreversible (D**). Stable proteins should be able to maintain the native form (N) despite the presence of some reversible forms like N*, I, D, A, although, this may be inevitable in practice.



Where,

N:	*Native structure*
I:	*Intermediate folding structure*
A:	*Aggregated form*
D:	*Denatured form*
P:	*Precipitated form*

2.1 Measure of the physical stability of proteins

2.1.1 Structural stability of proteins

Proteins become soluble in aqueous solutions based on the interaction of surface exposed residues of their native structure with water, molecules, and other surfaces. The solubility of a protein may change as the structure undergoes changes due to its exposure to different residues. Therefore, the physical stability of most proteins can be expressed as their resistance to unfolding forces because aggregation and/or precipitation can occur when the structural change results in a less soluble conformational state. This has been also expressed in thermodynamic terms, e.g., thermal unfolding temperature or free energy of unfolding (Tanford, 1968; Pace, 1975). Essentially, proteins can undergo conformational changes from their native and biologically active forms to non-native and/or inactive conformations. 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 folding of proteins. These forces originate from covalent bonds like disulfide bonds, electrostatic interactions, hydrophobic interactions, hydrogen bonds, and Vander Waals interactions (Dill, 1990). The majority of information available from literature refers to the complete loss of structure, e.g., loss of secondary structure. However, in practice, most degradation products observed in the development of protein therapeutics are derived from more subtle changes, e.g., quaternary or tertiary structural changes (Brent et al., 1997; Raso et al, 2005). For example, thermal analysis of precipitated protein paste with microcalorimetry often shows a full endothermic unfolding curve suggesting that proteins with intact secondary structure can still precipitate (unpublished data). This model of differential solubility of various structural ensembles is better understood from protein refolding research (King et al., 1996; Fink et al., 1993; Dill and Chan, 1997; Eaton et al., 1996).

There have been enormous research efforts to understand the structural stability of proteins. These include structural analyses of thermophilic proteins ((Kumar et al., 2000; Trevedi et al., 2006; Argos et al., 1979; etc). Furthermore, site-specific mutation studies have been conducted to find key stabilizing forces (Van den Berg et al., 1998; Querol et al., 1996).

Generally speaking, the intrinsic physical stability of proteins at their native structure can be expressed as a resistance to the formation of transitional intermediate states which are prone to convert to irreversible conformations.

2.1 2 Colloidal stability

Alternative measurements of a protein's structural stability can be derived from the protein's ability to remain as a monomeric native form. A protein's colloidal stability is influenced by interactions with other molecules (proteins, excipients, preservatives, metal ions, salts, ligands, leachables, nuclei, etc) as well as other external factors like surfaces and solvents (Valente et al, 2005; Ruckenstein and Shulgin, 2006; Winzor et al., 2007)

In an ideal environment, proteins would always refold back to their native, active, and soluble form. However, proteins possess the flexibility to assume structures other than their native forms, e.g., soluble aggregates or insoluble forms, if lower free energy states can be achieved in different environments, e.g., purified into simpler buffered systems (Leandro and Gomes, 2008). These transitions to non-native structures can be reversible, but some structural changes bear a significant activation energy barrier and for all practical purposes become irreversible under normal operating conditions (Gianni et al., 2007). Thus, it is important to understand the protein molecules as colloidal particles and their adaptations to the surrounding environment.

Various physical properties of proteins, including the collective interactive forces of exposed amino acid residues as well as the stability of the native structure, will contribute in keeping the native protein molecules from aggregating or unfolding (Guo et al., 2006). These intermolecular interactions should include charge interactions, hydrophobic interactions, hydrogen bonds, Vander Waals interactions, etc.

The colloidal stability of protein molecules contributes to important physical properties like solubility, viscosity, surface interaction, foaming, thermal stability, resistance to shear stress, etc (Valente et al., 2005; Chi et al., 2005). All possible transitional state conformations should be considered when the colloidal stability is concerned as certain unstable structures may become limiting factors.

Proteins colloidal stability relies on a number of factors:

- Intrinsic structure of native forms
- Formation of transitional conformations under stress
- External factors such as solvents, solutes, concentration, etc.

The colloidal stability of protein molecules has been expressed as a number of useful parameters like preferential interaction parameters and excluded volume effects (Timasheff, 1993, 1998) as well as the second virial coefficient (Neal et al., 1999; Valente et al., 2005; Alford et al., 2008). The preferential hydration has been useful in understanding the physical stability of proteins in solution in terms of cosolvent induced changes. The second virial coefficient is another thermodynamic parameter that characterizes two body interactions in dilute solutions by reflecting the magnitude and sign of interaction (Neal, 1999). Using these parameters, the physical stability of various proteins has been successfully correlated with different formulation conditions (Chi e al., 2003; Valente et al., 2005).

2.1.3 Physical Degradation Products

Protein physical stability has been measured via a couple different methods: (i) analyzing the structural changes under stresses; (ii) quantifying the changes resulting from the stress (see Analytical Methods section below).

The former approach is useful in understanding both reversible and irreversible structural changes that contribute to the change in protein physical properties. Analytical methods useful for the former approach include various spectrometric analyses equipped with means to introduce various stresses to the samples as well as thermal analyses (Privalov, 2009). Monitoring the degree of unfolding by measuring the kinetics of association with external molecules, e.g., probes that exert unique signals upon interaction with proteins or specific residues, chemical reagents that bind to specific residues, hydrogen to deuterium exchange, is another approach for measuring the exposure of specific residues or surfaces (Yan et al., 2004). This approach is useful for understanding all the relevant structural changes that need to occur before critical degradation takes place. Often, there are multiple structural changes involved before the final degradation products are formed. Any of them can be a rate limiting process. While this approach is useful for various structural changes that occur under stress, it also provides information regarding irrelevant structural changes, e.g., reversible changes or the reactions that do not occur at real-life situation, which can potentially interfere with desirable signals (see Section 4.1).

The latter approach is routinely used during quality control of protein pharmaceuticals as it determines specific degradation products that result from specific stresses (Piedmonte and Treuheit, 2008; Herman et al., 1996). First, proteins are exposed to various stresses, e.g., forced degradation studies and/or accelerated stability studies, followed by analysis of the degradation products that are generated by irreversible reactions, or kinetic traps (see Section 4). Analytical methods useful for this approach include routine chromatographic analyses, particle analyses, electrophoresis, biological activity assays, mass spectrometry, visual observation, etc (see Section 3). Chromatographic analyses, primarily HPLC or UPLC methods, have been successfully utilized to determine changes in size, shape, surface charge, hydrophobicity, etc. Particle analyses have been also useful in charactering both soluble as well as insoluble particles with light scattering methods and analytical ultracentrifuge. The limitation of this approach is a consequence of treating

the whole degradation process as a black box: it lacks the ability to identify and understand the rate limiting structural changes.

In theory, proteins should be able to recover their native structure upon removal of stresses unless there is irreversible transition involved. Therefore, the following reactions that make the structural changes effectively irreversible will be considered as relevant degradation pathways. In general, the degradation pathways can be classified roughly into the following criteria:

- Formation of stable misfolded species
- Aggregation or precipitation of misfolded species
- Aggregation-induced structural changes
- Chemical modification of critical residues
- Interaction with other solutes (metal, preservatives)
- Surface induced structural changes/aggregation
- Surface adsorption

(1) Formation of stable misfolded species

This is based on the assumption that proteins may assume structures other than the native structure during the course of refolding (King et al., 1996; Fink et al., 1993). In theory, the native structure of each protein is the most thermodynamically stable state with the lowest free energy. However, different conformations have been observed during process development of refolding proteins from inclusion bodies (Leandro and Gomes, 2008; Gianni et al., 2007). In this metastable structural state, the misfolded proteins can remain reasonably stable until additional energy is introduced to overcome its activation energy barrier before refolding to the native structure.

(2) Aggregation or precipitation of misfolded species

Proteins aggregate and/or precipitate in their native structures and/or partially unfolded transitional ensembles, e.g., both intermediate state ensembles, denatured state ensembles, and/or transitional state ensembles, when formation of aggregates is feasible (Tobler and Fernandez, 2002; Fawzi et al., 2005). Underlying principles for both soluble and insoluble aggregates are not significantly different from a practical point of view, so both forms of aggregates will be discussed interchangeably in this discussion. Aggregated forms of proteins can be classified into two important groups: reversible aggregates and irreversible aggregates.

<u>Reversible aggregates or precipitates</u>

The best example of reversible aggregates can be found in the aggregation, or precipitation, due to the equilibrium solubility of proteins (Shulgin and Ruckenstein, 2006; Keeler et al., 2004; Dannies, 2003; Shih et al., 1992). If the concentrations of proteins increase above their critical concentrations or solubility limits, proteins will naturally form aggregates or insoluble precipitates. Unless further structural changes

beyond the solubility-induced aggregation occur, the proteins will revert back to their native monomeric forms as soon as the concentrations fall below the solubility limit or the environment changes to permit greater solubility. This reversible aggregation process is considered as an equilibrium process similar to the solubility phenomena of other molecules (Liu et al., 2005).

Irreversible aggregates or precipitates

This is one of the most common as well as critical degradation products as the smallest quantity of aggregated forms can routinely induce undesirable immune responses (Rosenberg, 2006). Moreover, it is one of major problems associated with the quality control of protein pharmaceuticals as small quantities of protein, e.g., 1-10 μg of protein, can form large numbers of visible particles. If aggregated forms of protein do not dissociate into native monomeric molecules when diluted into lower concentrations with the same formulation, then they are considered to be irreversible aggregates. Often, an aggregate is deemed irreversible if it fails to dissociate when the product is administered to its targeted delivery site, e.g., injection site. Irreversible aggregates are generally formed during intermediate state ensembles (King et al., 1996) or molten globule states (Safar et al., 1994) through hydrophobic interactions.

Irreversibly aggregated forms of proteins can be dissociated into monomeric forms in many different ways depending on the nature and strength of its intermolecular forces. Some weakly associated non-covalent aggregates can be dissociated with a simple addition of surfactant(s) into the solution, adjustment of temperature, adjustment of pH to induce charge repulsion among molecules, and/or adjustment of ionic strength. Formulation strategies to stabilize proteins by introducing dissociation factors will be discussed in better detail later in this chapter.

Stronger non-covalent aggregates can be dissociated by exposing the aggregates to harsher non-physiological conditions such as extreme pH, strong detergents like SDS, and/or high concentrations of chaeotropes like guanidine hydrochloride or urea. As most proteins adapt to the harsh environment by changing to their non-native structures, these approaches are not generally recommended for pharmaceutical formulations.

Covalent aggregates, mostly with intermolecular disulfide bonds, currently can only be dissociated into monomers with the help of reducing agents like mercaptoethanol, dithiothreitol, tris-[2-carboxyethyl] phosphine (TCEP), or glutathione. These covalent aggregates are usually formed directly from free cysteine residues found in native protein molecules but can also be formed from intermolecular disulfide scrambling of pre-existing intramolecular disulfide bonds (Mamathambika and Bardwell, 2008; Chang et al., 2005).

Soluble forms that precipitate when introduced to physiological conditions

Proteins can maintain their integrity in their molten globule state or transitional state under certain solution conditions (Herman et al., 1996). When these formulations are

delivered into physiological conditions, the proteins may precipitate or aggregate during the course of refolding back to their native structures. For example, if some proteins have limited solubility or are prone to aggregation and/or precipitation during storage at neutral pHs, the proteins may be formulated in an acidic environment. Alternatively, additional solubilizing agents could be added. If these formulations are introduced parenterally into a physiological environment, a fraction of the proteins can result in an aggregated or precipitated form at the delivery site.

(3) Aggregation-induced structural changes

Proteins typically maintain their native structures in their natural environments in order to be functional. Furthermore, they are flexible so as to adapt to changing environments for their biological functions. For instance, most proteins undergo conformational changes when they interact with ligands or receptors for their activities. This implies that proteins may sacrifice the original structure and rearrange to different conformations when the environment changes, e.g., contact surface, temperature, concentration, solution condition. A similar scenario occurs when protein molecules associate with each other while forming aggregates. Once the association begins, loosely linked native molecules can further squeeze into irreversibly aggregated forms through additional structural rearrangements like the formation of β-sheets and domain swapping (Janowski et al., 2001; Liu and Eisenberg, 2002). This is well illustrated by the formation of amyloid fibrils from soluble protein to prefibrillar species followed by protofilaments and ultimately branched fibrils (Dobson, 2003).

(4) Chemical modifications of critical residues

During the formation of transitional state ensembles, some amino acid residues susceptible to chemical modifications can become exposed. Due to the change in physicochemical properties around the modified residues, the affected protein molecules may not be able to refold back into their native structures. The structure-induced exposure and modification of buried residues as well as the irreversible changes in the resulting structures of proteins are well illustrated by the oxidation of methionine residues (Gao et al., 1998; Li et al., 1995). Change in other residues by other chemical modifications can also affect the transitional state ensembles' ability to refold back to their native structures. For example, the rate of deamidation of asparagines residues can be influenced by the flexibility of the peptide backbone (Robinson and Robinson, 2001). At the same time, the resulting changes in charge from amino residue of asparagines to the carboxylic residues of aspartic acid and/or iso-aspartic acid may also alter the way the proteins will be refolded (Gupta and Srivastava, 2004).

(5) Interaction with other solutes (metal, preservatives)

Proteins interact very closely with other solutes and can integrate into other stable structural states. The principle of preferential hydration for stabilizing solutes, e.g., sugars, salts, and amino acid residues has been well discussed in the literature (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Timasheff, 1993; Kendrick et al., 1997).

The stabilizing solutes have a tendency to be excluded from the hydration surface of proteins, thus leaving the protein molecules preferentially hydrated. At the preferentially hydrated state, the void that the protein molecules occupy is thermodynamically unfavorable and keeps the proteins with a minimal exposed surface area. As the denatured state of protein molecules would have a much larger exposed surface area, the larger difference in the free energy of unfolding in the presence of these cosolvents is the major driving force for stabilization. As the preferential hydration effect comes at the cost of a relatively high concentration of the cosolvents, e.g., 1 M or greater for sucrose, these cosolvents have not been widely used in pharmaceutical applications due to the hyperosmolality of resulting formulations.

Another group of stabilizing cosolvents works more specifically with the exposed chemical nature of proteins. The effectiveness of these cosolvents, e.g., charged polymers, is rather difficult to predict as the stabilizing effect comes from a very specific interaction with proteins (Chen et al., 1994; Volkin et al., 1993).

Another group of excipients that showed direct physical interaction with proteins and contributed to the aggregation and/or precipitation of proteins is phenolic compounds. More relevant to pharmaceutical applications, these antimicrobial preservatives have shown greater impact on the physical stability of proteins (. These parenterally approved preservatives include benzyl alcohol, cresol, phenol, parabenes, benzalkonium chloride, etc. The preservatives interact with proteins and keep the transitional state ensembles from folding back to their native structure (Krishnan et al., 2006; Chi et al 2005; Zhang et al., 2004).

Metal ions contribute to the intrinsic structural stability of proteins by increasing resistance to unfolding or directing their folding (DeGrado et al., 1999). Removal of the metal ions from these proteins often results in a loss of the native structure and functionality. However, metal ions also foster intermolecular interactions and cause aggregation and/or precipitation. Zinc is typically added to promote association to hexamers and to increase chemical stability (Brange, 1987, 1992).

Additionally, metal impurities facilitate undesirable aggregation or precipitation. Uverski et al., (2001) showed metal induced conformational changes followed by the formation of fibrillar forms of α-Synuclein. There have been presentations discussing the aggregation of therapeutic proteins in prefilled syringes caused by tungsten oxide leaching out of staked needles (Rosenberg, 2006).

(6) Surface induced structural changes/aggregation

During product shipping and handling, proteins in aqueous solution formulations are subjected to agitation or shaking, which is one of the most common physical stresses that can cause protein aggregation (Henson et al., 1970; Maa & Hsu, 1997). Agitation induced protein denaturation and/or aggregation is presumably the consequence of protein interactions with surfaces; e.g., the air-water and vial-water interfaces (Charman et al., 1993; Henson et al., 1970; Kreilgaard et al., 1998; MacRitchie, 1986; Sluzky et al.,

1991). It is generally believed that protein unfolding at air-liquid interfaces may irreversibly expose the interior hydrophobic core, which may in turn lead to intermolecular associations of non-polar residues. It has been observed that agitation induced protein aggregation decreases at higher protein concentrations. Small amounts of surfactants are usually effective in stabilizing proteins against this type of stress (Chang et al., 1996b; Kreilgaard et al., 1998).

A widely accepted argument seen in previous studies attributes protein aggregation during processing to its increased exposure to various surfaces, most commonly the air-water interfaces (Cumper and Alexander, 1950; Thomas and Dunnill, 1979; Virkar et al., 1981). Proteins are known to form films of a non-native conformation at such boundaries. It has been argued that turbulence caused by agitation can create additional surface area and facilitate an exchange between the native population in the bulk and the denatured species on the surface. Presumably, accumulation of these denatured species eventually leads to precipitation. By the same trend of thought, solid-liquid interfaces were ascribed for the aggregation seen during filtration (Vikar et al., 1981; Truskey et al., 1987) and pumping (Narendranathan and Dunnill, 1982). However, direct proof of these pathways is scarce and little is known about the details of how such proteins form aggregates when exposed to these stresses.

These results challenge the "surface denaturation" hypothesis because the native forms of the most labile proteins do not adsorb to the surface fast enough to account for its rapid precipitation during agitation. It is based on the assumption that the adsorption of native protein to the surface is an instantaneous process. Instead, they suggest that the decrease in surface tension is a result of the proteins' need to be unfolded into a more hydrophobic configuration to adsorb to the air-water interface. This implication is consistent with an alternate hypothesis that claims shear stress is the primary source of protein denaturation during stirring, pumping, and filtration (Charm and Wong, 1970 and 1981; Charm and Lai, 1971; Tirrel and Middleman, 1979; Lencki et al., 1993). They discovered that the product of shear rate and time, Gt, correlates with the kinetics of enzyme inactivation. Residual protein activity with respect to Gt commonly indicates that a protein is approximately stable up to a certain Gt value but above this point the decrease in activity is directly proportional to ln(Gt).

The reversibility of surface adsorption has been debated in literature. Earlier works support that the adsorption process, especially with additional denaturation at the interface, is irreversible (Langmuir and Schaefer, 1930; Bull, 1947). This is further supported by the formation of insoluble proteins at the interface due to the changed conformation (Bull and Neurath, 1937; Kaplan and Frazer, 1953).

(7) Surface adsorption to solid surfaces

The presence of surfaces and interfaces can have a profound effect on aggregate development and that the growth rates and final aggregate morphologies are sensitive to the details of the surface chemistry of an interface, e.g., hydrophobicity, charge (Sharp et al., 2002; Adams et al., 2002; Zhu et al., 2002; Sluzky et al., 1991). Among more relevant

therapeutic applications, extensive research has been conducted on the surface adsorption of insulin pump systems (Sefton, 1982) and inactivation/surface adsorption of IL-2 to tubing materials (Vlasveld et al., 1993; Miles et al., 1990; Tzannis et al., 1997)

The degree of structural and activity loss depends on the individual protein. While extensive loss is expected for labile proteins like insulin and IL-2, a significantly smaller loss, about the quantity for monolayer adsorption on the order of a few micrograms per 3 mL vial/stopper configuration, has been observed for more stable proteins.

The reversibility of proteins adsorbed to solid surface has been well summarized by Norde (1986) and MacRitchie (1998). Main points are summarized below:

- Only a small fraction of surface adsorbed proteins desorb upon diluting the system with additional solvent.
- The degree of adsorption depends on multiple factors like solution condition and temperature.
- Protein molecules may be exchanged between surface and bulk solutions.
- Adsorbed proteins may be displaced from the surface by molecules of other species.
- Protein molecules may not refold back to their original structures once they are unfolded at the interface.

It is generally agreed that surface adsorbed protein molecules are difficult to desorb from the surface unless the solution condition is changed, e.g., additional surface active agents.

3. Analytical methods for physical changes of proteins

To characterize conformational changes in a protein, caused either by changes in its solution environment or by physical degradation in the protein itself, biophysical techniques are often employed to examine the secondary or tertiary structures of the molecule (Jiskoot and Crommelin, 2005). Results generated from these techniques not only lend insight into the understanding of protein structures, but may also provide guidance in their formulation development. The common techniques used in formulation development include spectroscopic analyses (second derivative ultraviolet spectroscopy, circular dichroism, Fourier Transform infrared spectroscopy, Raman spectroscopy, fluorescence and phosphorescence spectroscopy), thermal analysis (differential scanning calorimetry), and size based analysis (analytical ultracentrifuge, light scattering). In addition, the three dimensional structure of proteins may also be studied to gain a better understanding of their structure-function relationship as well as their interaction with various factors in their environment. Techniques for this characterization include hydrogen/deuterium exchange mass spectrometry, nuclear magnetic resonance spectroscopy, x-ray crystallography and electron paramagnetic spin resonance spectroscopy.

3.1 Spectroscopic Techniques

3.1.1 Second Derivative Ultraviolet (UV) Spectroscopy

The environment of aromatic residues in a protein can be monitored using second derivative UV spectroscopy. When the tertiary structure of a protein is perturbed as a function of pH, heat or other factors, shifts in the UV peak positions can be observed in its second derivative UV spectrum. An example of the application of second derivative UV spectroscopy includes the study of aggregation behavior of an IgG_2 antibody as a function of pH and temperature (Kueltzo et al., 2008). By monitoring the shifts in peak maxima of five selected peaks in their second derivative UV spectra, stable pH and temperature ranges were determined which gave insight into the aggregation behavior of the antibody during freeze-thawing.

3.1.2. Circular Dichroism (CD)

CD measures the difference in absorbance for the left-handed and right-handed circularly polarized light from a protein sample. This difference is contributed to the interaction of chromophores in a protein that are located in a chiral (asymmetrical) environment. In CD analysis, both near-UV CD (310-255 nm) and far-UV CD (below 250 nm) regions of the spectrum can be measured. Aromatic amino acids (tryptophan, tyrosine, phenylalanine) and cystine contribute to the near-UV CD signals, which reflect the tertiary structure of the protein. The peptide bonds (amide) contribute to the far-UV CD signals, which represent the secondary structure of the protein (i.e. α-helix, β-sheet, β-turn structures).

Typical applications with CD analysis include (a) estimation of the content of protein secondary structures; (b) detection of conformational changes in a protein due to changes in its solution environment (pH, excipients, addition of denaturants) and temperature. In a recent study, an *E.coli*-expressed IgG1 Fc was studied by CD before and after forced oxidation by H_2O_2, and the data indicates perturbation in the tertiary structure of the molecule, whereas its secondary structure was largely unchanged (Liu et al., 2008). In another case, MAb samples at over 100 mg/mL concentration were directly analyzed with CD, in which two MAb molecules were compared by CD as a function of temperature. The results revealed a 7°C stabilization of one MAb over the other at the same protein concentration (Harn et al., 2007).

3.1.3. Fourier Transform Infrared Spectroscopy (FTIR)

In infrared spectroscopy, a molecule absorbs infrared radiation as it undergoes a net change in dipole moment resulted from its vibrational or rotational motion. In terms of wavenumber, the infrared spectrum is divided into near- (12800 to 4000 cm^{-1}), middle- (4000 to 200 cm^{-1}), and far-IR regions (200 to 10 cm^{-1}), with 1700 to 1600 cm^{-1} being the most commonly used region for protein analysis. This region, commonly referred to as the amide I region, corresponds to the carbonyl (C=O) stretching vibration of a peptide bond. Hydrogen bonding of the C=O group to the amide group yields information on the secondary structures of a protein with the following known maxima (Barth, 2007): α-

helix (1654 cm^{-1}), β-sheet (1633 and 1684 cm^{-1}), γ-turn (1672 cm^{-1}), and random coil (1654 cm^{-1}). A comparison of the band intensities at these wavenumbers may indicate differences in secondary structures between protein samples.

Modern infrared spectrometers are usually Fourier transform infrared (FTIR) spectrometers, due to the fact that the detector signal is related by Fourier transformation into the measured spectrum. To avoid issues caused by short pathlength, in which interferences from water in the aqueous sample overlaps with the amide I band, the attenuated total reflectance (ATR) technique may be employed. In ATR, a thin film of the sample is placed on a crystal, and the infrared light is reflected several times through the crystal and the sample before measurements are made.

FTIR has been widely applied to study changes in the secondary structures of proteins in formulation studies, because formulations that produce secondary structures closest to the native protein structure often lead to longer storage stability. Recent examples of FTIR applications have included the effects of mannitol content in spray-dried antibody formulations (Schule et al., 2007), effects of sucrose and sorbitol contents in lyophilized antibody formulations (Chang et al., 2005), and a comparison of various sucrose contents for an antibody in different presentations (freeze-dried, foam and spray dried) (Abdul-Fattah et al., 2007). In an interesting application of FTIR, the structural integrity of rhBMP-2 microparticles prepared from controlled precipitation was compared to that of a reference formulation in solution. It was found that between 20 to 70°C, the FTIR spectra of both the rhBMP-2 microparticles and the resolubilized microparticles showed largely the same pattern as that of the liquid formulation. Moreover, after 6 months of storage, the microparticle suspension maintained its secondary structure as those of the unstressed and stressed reference formulations in liquid (Schwartz et al., 2006).

3.1.5. Fourier Transform (FT) Raman Spectroscopy

The Raman Effect arises when a beam of intense radiation, typically from a laser, passes through a sample where the molecules undergo a change in molecular polarizability as they vibrate. This change in polarizability results in information regarding the peptide backbones and secondary structures of protein samples. Information obtained from FT-Raman is often complementary to results from FTIR. One advantage of FT-Raman over FTIR is that water happens to be a weak Raman scatter. Consequently, proteins in their native (aqueous) state can be studied in addition to their solid state. Typical regions in FT-Raman spectra that are characteristic for proteins include the amide I region for α-helical structure (at ~1660 cm^{-1}) and the amide III region (1250 to 1350 cm^{-1}).

Recently, three model proteins differing in size and structure were compared in terms of their native structures before and after lyophilization and spray-drying. The results from their FT-Raman analysis showed good correlation to data from biological (enzyme activity) assays (Elkordy et al., 2008), which indicate that among the proteins studied, lysozyme and deoxyribonuclease I show better folding reversibility in their lyophilized form than their spray dried form.

3.1.6. Fluorescence Spectroscopy

In fluorescence measurements, the protein is excited at a wavelength corresponding to its excitation maximum, and light is emitted during the protein's transition from the excited state to the ground state. Aromatic amino acids including tryptophan, tyrosine, phenylalanine, and histidine provide intrinsic fluorescence signals that can be measured. The information obtained is related to the environment surrounding these residues, hence, the integrity of the tertiary structure of the molecule can be interpreted. For these reasons, fluorescence spectroscopy is a useful tool for studying the unfolding of a protein in different environments (pH, temperature, buffer excipients, etc). A recent publication demonstrates the application of fluorescence spectroscopy towards the direct analysis of lyophilized drug products in accelerated degradation. A close correlation was found between the diminishing fluorescence intensities of the lyophilized samples with the SE-HPLC results obtained from the reconstituted samples (Ramachander et al., 2008).

Besides intrinsic fluorescence, proteins may also be derivitized with fluorescent probes to enhance the sensitivity of the measurement at wavelengths that are characteristic of the probes used. Probes such as 1-anilino-8-napthalene sulfonate (ANS), Nile Red, and thioflavin T have been used for the detection of protein aggregates. The probes bind to the hydrophobic surface of proteins that are exposed during fibril formation. Once bound, their fluorescence maxima are shifted and their intensities can be measured. Recently, aggregation in an antibody at high concentration (193 mg/mL) in different buffers was characterized without dilution using Nile Red binding and the results between fluorescence spectroscopy and fluorescence microscopy analyses were well correlated (Demeule et al., 2007).

3.1.7 Phosphorescence Spectroscopy

Similar to fluorescence spectroscopy, a molecule is excited from its lowest vibrational state to an excited state in phosphorescence spectroscopy. While in its excited state, a change in electron spin must occur in order for the molecule to phosphoresce. This means that a phosphorescence event is less probable than other spectroscopic events and the rate is also slow. Phosphorescence lifetimes are typically on the order of 10^{-4} to 10 sec, in contrast to a fluorescence lifetime of 10^{-9} to 10^{-6} sec.

Conventionally, phosphorescence measurements needed to be performed at liquid nitrogen temperature in order to prevent degradation of the phosphorescence signal due to collisional deactivation. Nowadays, room temperature phosphorescence may be performed in solution, as long as the sample is purged of all traces of oxygen. A recent example discusses the use of this technique to access differences in the microenvironment of two tryptophan residues in a glutamine binding protein. The difference in phosphorescence lifetimes of Trp^{220} and Trp^{32} is on the order of several hundred milliseconds, which is sufficient for determining Trp^{32} to be in a well ordered environment (Auria et al., 2008). The work illustrates the potential of using phosphorescence measurement to probe changes in the tertiary structure of proteins caused by ligand binding or other environmental changes.

3.2 Thermal Analysis

3.2.1. Differential Scanning Calorimetry (DSC)

In DSC, the heat flow (or heat capacity) of a sample is measured as a function of temperature. The technique has been useful for studying the unfolding of secondary structures of protein molecules and for characterizing the conformational stability of proteins in different conditions, in both solid and liquid states. While protein unfolding produces endothermic peaks as measured by DSC, protein aggregation is detected as an exothermic event. With DSC, the melting or denaturation temperatures (T_m) of different protein samples can be compared, giving insight to the differences in their secondary structures. Another application of DSC has been its use in optimizing lyophilization parameters. Protein formulations may be analyzed by DSC to determine the optimal freezing and annealing temperatures during the lyophilization cycle, which would in turn lead to a more elegant cake appearance. A recent example of applying DSC in formulation development shows the effects of sucrose on rHSA, which clearly shows sucrose adding protection to the protein by shifting the protein's melting point from 123°C to 147°C, whereas rHSA formulated with mannitol alone did not show the same benefits (Han et al., 2007). Other applications have included the comparison of two MAb molecules by DSC and the effects of various excipients on the thermal stability of recombinant adenoviruses (Ihnat et al., 2005).

3.3. Size Based Analysis

3.3.1. Analytical Ultracentrifugation (AUC)

For routine, high-throughput analysis of protein aggregates, SE-HPLC is unquestionably the most sensitive and reproducible method available. However, SE-HPLC suffers a few drawbacks, due to its reliance on the use of a column which can lead to poor mass recovery of aggregated species (Gagrielson et al., 2007). The non-specific interaction between the proteins and the column matrix may necessitate the addition of salt and organic solvents that would in turn disrupt the aggregated species. On the contrary, AUC does not have the same issues as SE-HPLC. Although the technique is not as sensitive as SE-HPLC and requires highly trained expertise for data analysis, it is a valuable technique for studying protein aggregation in solution and is often used to validate results obtained by SE-HPLC.

The basic AUC instrumentation includes a centrifuge, a rotor with sample compartments, and an optical system than can measure the protein concentration gradients under centrifugal force. Two basic modes of operation exist in AUC: sedimentation velocity and sedimentation equilibrium. Sedimentation velocity, which is conducted at high centrifugal speeds (40-60k rpm), yields information related to the sedimentation coefficient, diffusion coefficient, and molecular weight. Sedimentation equilibrium, which is performed at lower rotor speeds (10-30k rpm), yields information on molecular weight, stoichiometry, and binding affinity of self- or hetero-associating molecules.

3.3.2 Light Scattering

The principle of light scattering is based on the fact that the intensity of light scattered by a sample is proportional to the particle size and the concentration of that sample. For protein applications, two types of light scattering techniques are commonly used: Dynamic light scattering (DLS) and multiangle laser light scattering (MALLS), also referred to as static light scattering. With DLS, a hydrodynamic diameter of a sample in the range of 1 nm to 1 μm can be measured and results reported in a volume-based distribution. A cytokine in liquid formulations at various pH was studied by DLS and the results indicate an increase in % volume of aggregated species as a function of increasing pH (Hawe and Friess, 2008). Other examples of DLS applications include the study of effects of excipients on an immunoglobulin G both in terms of aggregate prevention and thermodynamic stability (Alrer et al., 2006) and the investigation into the opalescent appearance of an IgG1 formulation at high concentrations (Sukumar et al., 2004).

In MALLS, the weight-average molecular weight (Mw) and number-average molecular weight (Mn) are determined as a function of concentration. MALLS can be used as a standalone detector for batch analysis or it can be used as an on-line detector in conjunction with refractive index detection for SE-HPLC analysis. Most commonly, the 90° signal from a MALLS measurement is used for the determination of Mw and Mn. Provided that the d*n*/d*c* ratio of an analyte is known, MALLS measurements can provide the absolute determination of Mw and Mn that is independent of the hydrodynamic size of the analyte (i.e. its elution position on SE-HPLC relative to molecular weight standards). The stability samples of two antibody drug products are analyzed by SE-HPLC with MALLS detection and the MW of the various aggregated species can be determined (Ye, 2006).

3.4. Three-dimensional Structural Characterization of Proteins

3.4.1. Hydrogen/Deuterium Exchange Mass Spectrometry

Hydrogen/deuterium exchange has been a powerful tool for studying the surface properties of proteins. Surface exposed hydrogens in OH, NH, and SH functional groups can be replaced with deuteriums upon exposure to D_2O-containing buffers. The only exception is proline, which does not contain amide hydrogen. H/D exchange may take place either in properly folded proteins, in which only localized exchanges occur, or in partially or completely unfolded proteins. The H/D exchange reaction occurs readily and the reaction can be followed by a variety of techniques including mass spectrometry, FTIR, electron paramagnetic resonance spectroscopy, and nuclear magnetic resonance spectroscopy. Recently, H/D mass spectrometry (MS) utilizing electrospray ionization has by far had the widest applications. Since mass information is obtained in MS, analysis of H/D exchange by MS yields information on the structure of the entire protein, rather than just a few specific residues as in the case with spectroscopic based techniques.

To conduct MS analysis of an H/D exchange reaction in a protein, samples at fixed time intervals during the reaction can be quenched at low pH and low temperature prior to

injection onto the MS system. Whole mass measurements are performed and the mass shift due to H/D exchange can be followed as a function of reaction time. To gain a better understanding of local H/D exchange rates, quenched samples may be digested using pepsin at low pH (2.5) and low temperature (0°C) for a short time, followed by peptide mapping using an HPLC coupled to the MS and tandem mass spectrometric analysis (MS/MS).

Recently, H/D MS analysis has been applied to the characterization of protein conformation in lyophilized solids (Li et al., 2008). The model protein Calmodulin was lyophilized in the presence of various carbohydrate excipients. The lyophilized cakes were then exposed to D_2O vapor under controlled humidity and temperature and samples at specific time intervals were digested with pepsin and analyzed by LC-MS/MS. The results demonstrate the best protective effects by raffinose, dextran, and trehalose, while mannitol and sucrose show greater uptake of deuterium than the other carbohydrates. Furthermore, the peptide map results show that different carbohydrates protect different regions of the protein.

3.4.2. X-Ray Crystallography

In X-ray crystallography, a beam of X-ray strikes a single crystal and scatters into different angles. The intensities and angles the X-ray scatters allow for the determination of electron density within the crystal, which in turn leads to the deduction of a three-dimensional structure of the crystal. A variety of molecules have been successfully analyzed by X-ray crystallography, including small molecule drugs, proteins, and nucleic acids. In a recent example, the structure in insulin NPH (neutral protamine hagedorn) formulations was studied. Protamine is an arginine-rich small peptide that is co-crystallized with insulin in the presence of zinc in the formulations. Characterization of the insulin NPH crystals indicates that protamine adopts a disordered crystal structure, which suggests its primary function to be the balancing of overall charges of insulin in the formulations rather than any kind of specific binding to insulin (Norman et al., 2007).

3.4.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

In NMR, an analyte is studied in the presence of a constant magnetic field while an orthogonal magnetic field is applied. The response to the perturbing magnetic field forms the basis of NMR, in which all nuclei with an odd number of protons and/or neutrons give an intrinsic magnetic moment that can be measured. ^{1}H and ^{13}C NMR are the most commonly used nuclei in NMR. In general, large magnetic fields are preferred since they offer increased sensitivity. In recent years, solid state NMR has been useful in providing structural information of proteins that include membrane proteins and lyophilized proteins. One recent example demonstrates that the stability offered by arginine in freeze-dried antibody products is due to non-covalent interactions between the arginine side chain and the protein (Tien et al., 2007). Results from this work suggest that charged amino acids may provide greater stabilizing effects than the non-charged ones, perhaps due to the preferential binding of the stabilizer with the protein during freezing.

3.4.4. Electron Paramagnetic Resonance Spectroscopy (EPR)

EPR, or electron spin resonance (ESR), is similar in principle to NMR, except that it is the magnetic moment of an unpaired electron in an analyte that is measured, rather than that of an atomic nucleus. EPR is less commonly used than NMR because most stable molecules have their electrons paired. An example of application of EPR can be found in a report by Kempe et al., 2008, in which chitosan has been characterized as a potential biopolymer for drug delivery in the presence of glycerol-2-phosphate. EPR has been utilized to monitor the dynamics of insulin, the drug candidate used in this study, inside the chitosan gel and during its release. From this study, the optimal percentage of glycerol-2-phosphate and pH were determined for gel formation at room temperature, and the release of insulin was found to be sustainable for a period of up to 2 weeks under optimal conditions.

3.5. Stability Indicating Assays for Detection of Physical Degradation in Protein Therapeutics

Analytical assays used for the stability testing of protein therapeutics need to encompass all possible physical degradations that result from normal stresses during a typical stability program. Common biochemical degradations include aggregation, deamidation, oxidation, and fragmentation (clipping). In the case of glycoproteins, additional degradation may include some degree of deglycosylation or desialylation. Recently, cyclization of N-terminal glutamine has been reported in recombinant monoclonal antibodies after prolonged heat stress (Liu et al., 2006; Yu et al., 2006). All of these degradations can usually be detected as product impurities by HPLC and electrophoresis based assays, with separation principles based on size, charge, or hydrophobicity. The following are techniques typically used in the analysis of physical degradation in stability testing.

3.5.1. Size Based Determination

<u>Size Exclusion Chromatography (SE-HPLC)</u>

In this technique, protein species are separated based on their hydrodynamic size under native (aqueous) conditions. Although primarily intended for detection of soluble aggregates, SE-HPLC may also be applied for analysis of clipped species in a protein sample. Popular choices of columns used for protein analysis have included the Tosoh TSK gel and Shodex KW-800 series, both of which are silica based with high recovery and resolution. Proteins in the range of ~10 kD (cytokines) up to 150 kD (monoclonal antibodies) have been successfully analyzed on these columns. For hydrophobic proteins that tend to have poor recovery, polymer based columns may be utilized at the cost of resolution compared to their silica based counterparts.

Typical mobile phase used for SE-HPLC includes phosphate based buffers with 100 to 500 mM of sodium chloride at pH close to neutral (i.e. 6.5 to 7.5). As an alternative, sodium sulfate can be used as a substitute for sodium chloride at similar concentrations

(Qian et al., 2008). For proteins that tend to have excessive interaction with the silica columns, leading to tailing or poor recovery, arginine at 0.2 to 0.75 M may be used to minimize adsorption (Ejima et al., 2005).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS)

SDS-PAGE has been the conventional size-based technique for analysis of proteins. Unlike SE-HPLC, SDS-PAGE is performed in the presence of SDS and therefore, offers complementary information on aggregates that are covalent in nature. Generally, SDS-PAGE offers better resolution than SE-HPLC as both aggregated and clipped species can be separated within the same gel at high efficiency. For stability and lot release testing, Coomassie blue staining has been the method of choice for visualization and densitometry may be applied for a semi-quantitative measurement of the separated bands. If greater sensitivity is required, silver or Sypro staining may be applied, though the results are typically not as quantitative as Coomassie staining and the techniques are not as QC friendly.

In recent years, CE-SDS has been gaining acceptance as a substitute for SDS-PAGE in both the stability and lot release testing of protein therapeutics. Unlike SDS-PAGE, in CE-SDS the analyte does not sieve through a solid polyacrylamide matrix; rather, a polymer liquid matrix is used for size based separation in a fused silica capillary with a small internal diameter (50 to 100μm). The polymer liquid gel includes polyethylene glycol/polyethylene oxide, dextran, hydroxyalkyl cellulose, etc., at various concentrations depending on the molecular weight range of interest. The exact makeup of commercially available CE-SDS gel is proprietary information; however, a gel kit optimized specifically for MAb analysis is available from Beckman Coulter.

CE-SDS may be performed using a conventional CE instrument where a physical capillary is used for separation (i.e. P/ACE system made by Beckman Coulter, or HPCE system by Agilent) or a chip based CE instrument where microfluidic channels are utilized for analysis (i.e. Bioanalyzer made by Agilent, or the Experion made by Bio-Rad). In either case, the analyte is mixed with a SDS-containing sample buffer much like in SDS-PAGE, with the option of adding a reducing agent if reduced analysis is desired. The sample is then injected electrokinetically into the capillary for separation, under an applied voltage of 15 to 30kV. Due to effective heat dissipation in a capillary or microfludic channel, much higher voltages can be applied than in SDS-PAGE with little generated current, leading to superior resolution in CE-SDS. In addition, CE-SDS data is quantitative and can be analyzed much like HPLC data, which is an added benefit over conventional SDS-PAGE analysis. Typical electropherograms are shown from the CE-SDS analysis of antibody molecules using a conventional CE instrument (Han et al., 2006) and a chip-based CE system (Vasilveva et al., 2004).

CE-SDS analysis is usually performed using UV detection, which yields sensitivity equivalent to that of Coomassie blue staining. For added sensitivity, the analyte may be derivatized with a suitable fluorescent tag during sample preparation and detected using

laser-induced fluorescence (LIF). Recently, a CE-SDS assay utilizing LIF detection has been validated for use in a QC environment to support the stability monitoring of a MAb molecule (Salas-Solano et al., 2006). The sensitivity obtained by this approach is similar to that of silver staining.

3.5.2. Charge Based Determination

Ion Exchange Chromatography

For lot release and stability testing of protein therapeutics, a charge based purity method is typically expected from the regulatory agencies as part of the standard panel of test methods. Under conditions used ion exchange chromatography (IEX), surface charges on a protein are exposed and they interact with the ionic functional groups in the column. By choosing a mobile phase with an appropriate pH that is suitably above or below the *p*I of the protein, either anion or cation exchange can be performed. In either case, the separation mechanism in IEX is based on charge, which allows for the separation of deamidated, deglycosylated (Gaza-Bulseco et al., 2008) or other charge based variants (Han et al., 2006; Vasilveva et al., 2004; Johnson et al., 2007; Lyubarskaya et al., 2006) from the main product peak in stability monitoring.

Capillary Isoelectric Focusing (cIEF) and Capillary Zone Electrophoresis (CZE)

cIEF separates charged based variants on the same principle as conventional IEF, whereas the separation mechanism in CZE is based on both charge and mass. cIEF performed in a conventional CE instrument has been widely applied for the characterization of *p*I values for protein therapeutics, and is gaining acceptance for use in lot release and stability testing. More recently, cIEF performed with whole column imaging detection ("imaged cIEF") is gaining attention for its advantage over conventional cIEF, in which the focused protein bands are imaged without the need for band mobilization (Bo and Pawilszyn, 2006) that can lead to peak broadening.

3.5.3 Hydrophobicity Based Determination

Separation in reversed phase (RP) and hydrophobic interaction chromatography (HIC) is based on hydrophobicity. These techniques are often used for the detection of product impurities that include oxidation (at either methionine or tryptophan), fragmentation (clipping), and sometimes deamidation. Recently, RP-HPLC has been applied to the detection of pyroglutamic acid formation at the N-terminal of the heavy chain in an IgG1 monoclonal antibody (Han et al., 2007). Though less utilized than RP-HPLC, HIC-HPLC has been applied in the stability monitoring of protein therapeutics, and in one case, the isomerization of an aspartic acid residue was detected by HIC-HPLC along with RP-HPLC (Wakankar et al., 2007).

3.5.4 Other Techniques

Purity analysis by HPLC and electrophoresis based methods is typically sufficient for the stability monitoring of protein therapeutics. By characterizing and identifying the minor forms detected in these purity methods, they can be applied towards direct stability monitoring without the need for more detailed structural characterization such as peptide mapping and oligosaccharide mapping. While the data obtained from these detailed analysis is valuable, peptide and oligosaccharide mappings are time consuming and can be highly variable depending on the analyst. Therefore, these techniques are typically not included in stability monitoring programs and stability specifications; rather, they are reserved for characterization use only. For additional information about this field, readers are referred to the following articles (Peter-Katalinić, 2005; Wuhrer et al., 2007).

4. Stresses that cause physical degradation of proteins

Among the physical stresses, the effects of heat, agitation, surfaces, and pressure are relatively well characterized and plenty of examples can be found in literature (Manning et al., 1989; Carpenter et al., 1993). Other chemical stresses that are known to affect the structure of proteins include pH, metal ions, chaeotropic salts, detergents, preservatives, and organic solvents. While all of these can clearly induce structural changes of the proteins, one must seek the most relevant stresses to individual proteins as some proteins are more susceptible to certain stresses than others. In this section, a brief list of common stresses and experimental approaches to stress induction are discussed.

4.1 Heat

Like most reactions, the rates of most protein degradation pathways are accelerated with the increase of temperature. This is especially true for structural changes in proteins as the temperature dependency, or activation energy, of structural changes is in the order of 25-150 kcal/mole, much greater than the reactions of small molecules. At elevated temperatures, the solution conditions as well as the intrinsic flexibility of proteins alters so that the population of transitional state ensembles increases. This is the basis for incubating products at higher temperatures to accelerate degradation reactions so that expected degradation products and better formulations can be found in much smaller time scales than actual storage conditions. At the same time, relevant stability indicating assays can be developed using the stressed samples. While the benefit of utilizing the accelerated reactions at elevated temperatures is large, one must always remember the importance of observations in real life situations.

A common mistake found in the development of protein formulations is the use of thermal analyses for screening purposes. This can be achieved by differential scanning calorimetry and/or any other device that can determine the denaturation of proteins while equipped with a temperature control system. Information obtained from such instruments includes thermal unfolding temperatures, enthalpies of unfolding, change of specific heat during thermal unfolding, etc (Spink, 2008; Privalov and Dragan, 2007). While these results are relatively quick to obtain and can very effectively differentiate testing samples,

the information regarding the unfolding of proteins has little to do with most degradation products that are routinely observed during the refrigerated storage of protein products. While the activation energies for thermal unfolding of proteins are on the order of 100 kcal/mole (Privalov, 2009), the activation energies for most pharmaceutically relevant degradation products are on the order of 30 kcal/mole (unpublished general observations). This is because most physical degradations of proteins result from subtle structural changes, i.e., transitional state ensembles, rather than a complete loss of secondary structure. Therefore, the information obtained from thermal analyses should be used for formulation development with discretion.

Similar consideration should be given when extrapolating the results obtained from higher temperatures to realistic storage conditions, e.g., refrigerated storage. Each protein degradation pathway may involve multiple species of structural ensembles as well as multiple processes of structural changes. Therefore, it is difficult to assume a simple two-state model. In addition, multiple degradations will have to be considered when optimizing formulation and/or process conditions. Each pathway will have its own unique reaction order and activation energy, so it is difficult to assume the same major degradation products will occur from storage at both 4 °C and 37°C. In reality, empirical thermodynamic parameters obtained from actual stability studies may come from several different rate limiting processes at different temperatures.

4.2 Surface and/or shear

As discussed in earlier sections, proteins are sensitive to surface-induced denaturations. Most proteins form soluble aggregates and/or precipitates, and can unfold/adsorb to solid surfaces when sufficient agitation or shear forces are introduced (Henson et al., 1970; Charm and Wong, 1970; Trusley et al., 1987; MacRitchie, 1989; Sluzky et al., 1991). This phenomenon is observed during routine pharmaceutical processing or handling like pumping, mixing, ultrafiltration/diafiltration, stirring, membrane filtration, filling, transportation, etc. With the exception of very sensitive proteins, the problem can be easily resolved by including a small quantity of surface active agents, as will be discussed in the formulation section below.

Experimental results that unequivocally explain the underlying principles of the surface induced denaturation of proteins are difficult to find in the literature. A couple of leading hypotheses are: (1) Surface induced denaturation which represents surface adsorption, denaturation/coagulation, recycling back to bulk solution, aggregation among the transitional state species, and/or adsorption to other solid surfaces (MacRitchie, 1989; Sluzky et al., 1991); (2) Shear-induced denaturation which represents the structural alteration of proteins in bulk solution state by shear force, aggregation among the transitional state species, and/or adsorption to solid surfaces (Charm and Wong, 1981; Penarrubia and Moreno, 1987; Lee at al, 1989; Elias and Joshi, 1998). For practical purposes, the process of these structural changes followed by irreversible aggregation or precipitation of protein molecules is considered identical. Due to the ambiguity, it has been difficult to design instrumentation to introduce quantitative stress. The most common approaches which introduce surface denaturation are vortexing and stirring.

Vortexing with head space air or additional beads has been successfully used to understand the susceptibility of surface denaturation as well as to identify necessary surface active agents (Bam et al., 1998; Kreilgaard et al., 1998; Chou et al., 2005). Stirring with a magnetic bar has also been used with equal effectiveness (Kiese et al., 2008).

The simple agitation or stirring experiments for understanding each protein's susceptibility to surface induced aggregation is equally important as other incubation experiments, as incubation at elevated temperatures does not introduce an equivalent stress to mechanical stimulation. The agitation experiment only takes few hours and it is recommended that this study be performed prior to other accelerated stability studies. Once stable formulation is identified against the surface induced denaturation, the protein should remain stable during various stressful processes like pumping, filtration, filling, shipping, and shaking.

Relevant surfaces should include air-liquid interfaces, solid-liquid interfaces, and liquid-liquid interfaces. Proteins adsorb to air-liquid interfaces, concentrate at the interface, undergo structural changes, then self associate as well as aggregate (Meinders et al., 2001; Kudryashova et al., 2005). Formation of insoluble films at interfaces has been observed for some labile proteins. The agitation of protein solutions with head space air is also a routine source of aggregation. Likewise, proteins are known as good foaming agents because of their structural flexibility to adsorb to the air-liquid interface and rearrange their structure to form a strong cohesive viscoelastic film, which is required for stability of foam (Horiuchi and Fukushima, 1978; Townsend and Nakai, 1983).

Proteins are also known to stabilize liquid-liquid interfaces via similar principles. Proteins stabilize these interfaces by rearranging their hydrophobic and hydrophilic residues to reduce free energy at these interfaces (Kato and Nakai, 1980; Kinsella, 1981; Halling, 1981). Good examples of protein degradations at the liquid-liquid interface include aggregation of proteins by silicon oil (Jones et al, 2005), oil-in water emulsion (Kim et al., 2002), and water-lipid bilayer (Gorbenko et al., 2007).

Similar structural changes were also observed at solid-liquid interfaces. Protein aggregations resulting from interaction with container/closure systems, membranes, tubing, and pumps are well documented in the literature (Ruiz et al, 2005; Tzannis et al., 1996, 1997; Cromwell et al., 2006; Tyagi et al., 2009). Most articles describe the effect of the solid-liquid interface in terms of process of adsorption, structural changes at the interface, irreversible adsorption, and/or possible recycling to bulk solution, etc. Such interactions with solid surfaces may result in irreversible adsorption, and/or decrease in recovery, aggregation, as well as loss of biological activity of the proteins.

The effect of smaller solid particles on nucleation of protein aggregation has been extensively studied in the area of amyloid aggregation (Jarrett and Lansbury, 1993; Lazol et al, 2005; Haass and Steiner 2001; Klein et al., 2001, 2004; Kirkitadze et al., 2002; Walsh et al., 2002). The formation of small nuclei with the complimenting surface is a rate limiting process for the growth of aggregate. This phenomenon is well illustrated by

the particle formation of an antibody product when it was pumped through a piston pump fostering stainless particles (Tyagi et al, 2009).

4.3 Light

Exposure to light introduces various stresses to proteins (Kerwin and Rimmele, 2007; Qi et al, 2009). Light induced denaturation proteins can be largely divided into two areas: intrinsic and extrinsic. Intrinsic changes are induced as direct structural changes to proteins. Various amino acid residues including tryptophan, tyrosine, phenylalanine, methionine, histidine, and cysteine/cystine are oxidized during exposure to light. This may result in physical changes in proteins such as aggregation, denaturation, and inactivation.

Extrinsic changes result from light induced changes in the surrounding solution environment, e.g., change in solvent and or excipients. It is well known that light generates reactive oxygen species that further react with proteins and cause various oxidative damages (Berlett and Stadtman, 1997; Swallow, 1960; Garrison, 1987; Garrison et al., 1962; and Scheussler, 1984; Stadtman and Levine 2000). The excipients that are prone to the oxidative damages and foster the reactive oxygen species or free radicals include metals, polyethylene glycols, polysorbates, ascorbic acid, lipids, and amino acids like tryptophan, histidine, and tyrosine.

4.4 Dehydration

The native structures of proteins in aqueous solution are defined by their balancing forces primarily between the hydrophobic and hydrophilic interactions. Therefore, the presence of bulk water around proteins is required for maintaining their native structure (Franks, 2002). When powder forms of proteins are preferred, the water molecules will have to be removed from the surface of proteins. It appears that the native structures of proteins are relatively well preserved until the water molecules from the hydration layer, or monolayer, are removed from the surface of proteins (Hsu et al., 1992). This phenomenon is better illustrated by the effective stabilization of native structure by carbohydrates during drying processes (Carpenter and Crow, 1988; Prestrelski et al, 1993). This will be further discussed in the formulation section below, but the sugars protect the native structure of proteins during drying processes by substituting the molecular interactions from water molecules, e.g., hydrogen bonds.

4.5 Freezing

Proteins experience various physicochemical stresses during the freezing process (Chang et al., 1996; Pikal-Cleland et al., 2002; Cao et al., 2003). In addition to the decrease in temperature and subsequent phase separation of water into ice crystals, the changes in the concentration of protein and other unfrozen solutes can become rather complicated. In the freeze-concentrated unfrozen fractions, solutes may undergo further phase separations into insoluble precipitates, crystals, and/or liquid-liquid phase separations. It is well known that the selective precipitation of buffer components may also result in pH

changes, e.g., decrease in pH when disodium phosphate precipitates while monosodium phosphate remains amorphous (van den Berg, 1959; van den Berg and Rose, 1959).

Phase separation of stabilizing additives can also result in undesirable structural changes of proteins. Various excipients, e.g., sugars, amino acids, salts, polyols, etc., have been used to effectively stabilize proteins during the freeze-thaw process (Timasheff, 1998; Carpenter and Crow, 1988; Carpenter and Chang, 1996). Some of these stabilizers may lose efficacy as they precipitate or crystallize into a frozen state. A detailed discussion of the phase separation of excipient in frozen state is available in the literature (Chatterjee et al., 2005a,b).

The surface induced structural changes are also experienced during the freezing and freeze-drying process. Proteins undergo structural changes at the surface of ice crystals and/or other crystalline excipients, which may result in aggregation or precipitation (Chang et al., 1996). A substantial body of literature is available demonstrating the positive correlation between the surface area of crystals and the degree of protein degradation (Jiang and Nail, 1998; Sarciaux et al., 1999; Eckhardt et al., 1991; Heller et al., 1997, 1999; Lueckel, 1998; Izutsu and Kojima, 2002).

These freeze-induced physical changes of proteins are directly relevant to the stability of freeze-dried products. Additional stresses induced by dehydration should apply for the freeze-dried formulation as previously discussed.

4.6 Cold denaturation

The denaturation of proteins at lower temperature, i.e., cold denaturation, has also been reported (Pace and Tanford, 1968; Privalov, 1990; Privalov, 2000; Franks, 1988; Tang and Pikal, 2005). As the solubility of hydrophobic residues increases, pKa for acid/base changes, strength for hydrogen bonds changes, as well as the hydrophobic interaction force decreases at lower temperature, some proteins may find lower free energy in other unfolded states or dissociated subunits (Franks and Hatley, 1991; Jaenicke et al., 1990). In general, cold denaturation appears to be a reversible process, partly due to the enhanced solubility of hydrophobic species, and does not have a practical impact on the quality of protein products.

4.7 pH

One of the most critical variables in the physical and chemical stability of proteins is pH. pH is important for maintaining the native structure of proteins by balancing interactions among amino acid residues in the molecule as well as interaction with external solution environment through ionization states of individual residues, electrostatic free energy states, charge interactions, charge repulsions, etc (Creighton et al., 1993). In addition, pH also affects the colloidal stability of various structural states by similar mechanisms. Dependent on the ionic strength as well as the nature of other ionic species in the solution, proteins can readily adapt to different structural states including aggregates and

precipitates. In practice, the best way to find the most favorable pH condition of each protein is to expose that protein to a matrix of pHs and ionic strengths.

Proteins undergo significant conformational changes in the acidic pH range, which often results in severe aggregation, precipitation, and/or adsorption to container surfaces. The acid induced conformational change sometimes results in the molten globular state of the proteins (Ikeguchi et al., 1986; Kuwajima, 1989; Goto et al., 1990; Sugawara et al., 1991; Fink et al., 1993). In this molten globular state, proteins have native-like secondary structures (Dolgikh et al., 1981) but with incomplete tertiary structures (Marmorino et al., 1998; Ptitsyn, 1992; Ohgushi and Wada, 1983). It appears that the colloidal stability of the molten globular state molecules determines the rate of aggregation at acidic pHs. For some proteins, the reversible transition to and from the molten globular state at acidic pHs is a stable formulation condition for proteins which have limited stability in the neutral pH range (Herman et al., 1996).

Proteins generally have limited solubility around their isoelectric points due to balanced charges from ionic amino acid residues. Other pH dependent chemical reactions, e.g., deamidation, formation of cyclic imide, disulfide formation, and scrambling, etc., can also affect the structure of resulting molecules.

4.8 Pressure

Proteins also undergo structural changes at high pressures (Hawley, 1971; Jaenicke, 1981; Hermans, 1995). The structural changes and related population of unfolded species also resulted in aggregation and precipitation of proteins (Seefeldt et al., 2005; Webb et al., 2001; Kim et al., 2002). The pressure induced aggregation has not been a major issue in the development of protein therapeutics as the pressure required to unfold proteins, e.g., greater than 100 MPa, is beyond the range of routine exposure.

However, aggregated forms of proteins can be dissociated under pressure, and/or the solubility of misfolded forms of proteins is greater under pressure. This technique has been effectively used to increase the refolding efficiency at higher concentrations (St John et al., 1999, 2001; Lefebvre et al., 2004)

5. Practical approaches to minimizing physical degradations

In practice, proteins are exposed to various process conditions which are not always ideal for maintaining their physical structures. More often than not, we have little idea about the natural condition where the proteins perform their biological functions. These issues have been overcome by thorough scientific investigation and accumulated experience during the course of commercializing protein therapeutics. In general, proteins can be protected from undesirable physical degradations by following a few well-chosen approaches.

5.1 Optimization of formulation for native structure

The native structure of a protein can be preserved by optimizing the solution conditions to minimize structural changes and to minimize intermolecular interactions. Identifying optimal pH and ionic strength is a priority as both intramolecular as well as intermolecular charge interactions of ionic residues are most critical for preserving native structure without aggregation (Takahashi, 1997; Nakamura, 1996).

Addition of sugars, e.g., sucrose, trehalose, sorbitol, mannitol, etc. (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a), amino acids (Arakawa and Timasheff, 1983), and/or salts (Arakawa and Timasheff, 1982b) stabilizes the native structure of proteins by the principle of preferential hydration (Timasheff, 1998). Even more compact native structure species, which have less flexibility to the transitional state ensemble, are observed in the presence of high concentration sugars due to the preferential hydration effect (Kendrick et al., 1997).

In a dried state, the native structure of proteins are preserved by including sugars, e.g., sucrose and trehalose, which can replace the hydrogen bonds at the proteins' exposed polar groups (Carpenter and Crow, 1988; Prestrelski et al., 1993; Allison et al., 1999). Preservation of the native structure in a dried state is also effective in stabilizing the proteins during subsequent storage (Chang et al., 1996; Andya et al., 2003). For identifying the ideal sugar stabilizer for dry powder formulations, one of the most effective analytical tools is FTIR (Carpenter and Crow, 1988; Prestrelski et al., 1993) because spectra from both liquid states and dried states can be measured and directly compared.

5.2 Improving the colloidal stability

Attempting to keep proteins in their native structure is not practical for most proteins due to routine manufacturing, handling, transportation, and storage processes that are required for commercialization. Alternatively, a more sensible approach is to identify the condition where the unstable species have better colloidal stability (Schlieker et al., 2003). As long as the unstable transitional state species are kept intact during the course of stress, they will ultimately refold back to their native structure when the stresses after the stresses subside. This approach can be universally applied to all proteins, from the ones with unstable intrinsic stability to others that form unstable species under stress.

The colloidal stability of proteins is characterized by the formation of irreversible physical degradation products. Among all ensembles of intermediate folding species, the ones that cannot refold back to their native structures will be identified as degradation products which include inactive forms, aggregated forms, precipitated forms, and surface adsorbed forms. Experimentally, testing formulations will be exposed to various relevant stresses followed by analysis specifically for the irreversibly modified species. Through this approach, relevant stresses, key degradation products, and stability indicating assays are established. Using this methodology, formulations for improved physical stability are screened and the stability of proteins in given formulations is demonstrated. Likewise,

this is the basis of routine pharmaceutical stability studies, e.g., forced degradation studies, accelerated stability studies, and real time stability studies.

Both pH and ionic strength are the most critical factors in colloidal stability of various structural variants of proteins. More often than not, a simple adjustment of pH and/or ionic strength is sufficient to provide a good colloidal stability of a critical species. Consequently, this makes the product more stable against relevant stresses. The aggregation of proteins induced by benzyl alcohol was reduced at lower pHs by stabilizing unstable species generated by the interaction of the protein with benzyl alcohol (Thirumangalathu et al., 2006; Zhang et al., 2004).

Additives can also enhance the colloidal stability of proteins. Surfactants, primarily non-ionic surfactants like polysorbates for formulation use, effectively stabilize proteins against agitation (Bam et al., 1996; Bam et al, 1998; Chou et al, 2005), freeze-thawing (Chang et al., 1996; Krealgaard et al., 1998), and/or surface adsorption (Joshi et al., 2008). Surfactants not only prevent undesirable adsorption of proteins to surfaces by preoccupying them but also associate with unstable species and prevent their aggregation (Joshi et al., 2009).

Ionic additives like heparin are also effective in improving the colloidal stability of proteins (Giger et al., 2008), dextran sulfate (Chung et al, 2007).

Specific amino acids have been also used to enhance the colloidal stability of proteins. Arginine is probably one the most widely used amino acids for inhibiting proteins from aggregation (Taneja and Ahmad, 1994; Arakawa and Tsutomu, 2003; Shiraki et al., 2004; Baynes et al., 2005; Arakawa et al., 2007).

Naturally occurring cheparones are known to enhance the colloidal stability of various transitional state intermediates and mediate the correct folding of proteins (Beissinger and Buchner, 1998; Frydman, 2001; Young et al., 2003; Wegele et al., 2004; Mogk and bukau, 2004).

Intrinsic colloidal stability of native forms as well as transitional state intermediates has been improved by covalent conjugation with soluble polymers or glycosylation. The decrease in the rate of protein aggregation by pegylation has been reported numerous times (Katre, 1990; Kim and Park, 2001, Rajan et al., 2006). Glycosylation also has been effective in enhancing the colloidal stability of proteins and preventing aggregation (Narhi et al., 1991; Endo et al., 1992; Ono, 1994; Sinclair and Elliott, 2005).

Other stabilizers or formulation conditions that contribute to improved colloidal stability of proteins are often difficult to predict from theoretical point of view. However, some effective stabilizers have been discovered empirically by exposing proteins to relevant stresses followed by analyzing the presence of irreversible aggregates.

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