Introduction

Protein-based therapeutics has found a prominent place in the biopharmaceutical industry and is one of the most effective clinical methods to treat a wide spectrum of diseases. The protein therapeutics market holds tremendous growth potential and is estimated to reach 1.4 billion USD by 2015 ("Global Protein Therapeutics Market Forecast to 2015" report by RNCOS, Published April 2012). However, the proper stabilization of protein therapeutics remains a major challenge for the industry. Proteins are only marginally stable and are prone to physical degradations. A wide variety of excipients are often added to formulation to stabilize proteins by suppressing aggregation and surface adsorption, or to provide physiological osmolality. The purpose of this mini-review is to give an overview of the different types of excipients used, with a focus on providing a fundamental understanding of excipient-protein interactions and the mechanisms by which excipients stabilize protein therapeutics in the solution state.

Challenges of Protein Therapeutics Formulation

Proteins are large molecules and their functional properties depend on their three-dimensional structures. The thermodynamic stability of the native protein conformation is only about 20-80 kJ/mol in free energy more stable than unfolded, biologically inactive conformations under physiological conditions (Jaenicke 1988; Jaenicke and Rudolph 1989; Dill 1990; Jaenicke 1991; Pace et al. 1996), which is much weaker than covalent or ionic bonds. Proteins also exhibit the typical behavior of soft materials, cooperativity, where the folding and unfolding transition is a cooperative, two state process. As a result of these characteristics, marginal stability and cooperative folding, microscopic stimuli can trigger macroscopic changes in protein structure. For protein therapeutics, relatively small changes of external variables (e.g., temperature, pH, salt content, etc.) in the protein-solvent system, and/or the proteins themselves (e.g., chemical modifications, mutations, etc.), can destabilize the protein structure, leading to denaturation, aggregation, and precipitation (Chi et al. 2003b). In fact, protein aggregation is routinely encountered during all stages of the life time of a protein therapeutic,
including expression, refolding, purification, sterilization, shipping, storage and delivery processes, which leads to low yield, unstable product, and possibly immunogenicity in patients. Although our knowledge of protein stability is increasing, optimizing formulation conditions to completely suppress aggregation and ensure 18-24 months of shelf life remains a major challenge. We briefly review major protein degradation pathways and how excipients stabilize proteins and affect the quality of biologics in the next few sections.

**Protein Degradation Pathways**

Proteins can degrade via chemical and physical pathways. Chemical degradations include hydrolysis or cyclic imide mediated deamidation of Asp and Gly residues. Deamidation can occur rapidly under physiological conditions, with half-lives ranging from 6 to over 3000 days. As the rate of deamidation is affected by pH, temperature, and salt content, proper selection of formulation and storage conditions can minimize deamidation. Removal or substitution of putative residues is also effective at reducing deamidation. Oxidation of Met, Cys, His, Trp, and Tyr residues can also occur via auto- or catalyzed oxidation pathways. Typical oxidants include hydrogen peroxide, UV light, metal ions, dimethyl sulfoxide, and periodate. Thus, low temperatures, protection from light, and the addition of antioxidants can reduce the rate of protein oxidation.

More problematic are protein physical degradations, including aggregation, subunit dissociation, denaturation and unfolding, coagulation, particulate formation, and opalescence. Aggregation, the assembly of native, folded proteins to form aggregates containing non-native structures, is the primary pathway of protein physical degradation. Aggregation can occur even under physiological, non-denaturing conditions where the native protein conformation is greatly favored. Aggregation is often irreversible, resulting in non-native aggregates that are inactive, and sometimes immunogenic and toxic. Empirically, protein aggregation is affected by solution conditions such as temperature, pH, ligands and co-solutes, salt type and concentration, and the presence of preservatives and surfactants. Mechanistically, we have shown that protein aggregation can be fundamentally linked to conformational stability of the protein native state, colloidal stability of the protein solution, and interfacial stability of the protein.

The aggregation of proteins from a folded native conformation to non-native aggregates involves at least two critical steps, conformational changes to the native state and assembly reactions to form large aggregates. The energy barriers of the two steps are reflected in experimentally measurable
values of free energy of unfolding ($\Delta G_{\text{unf}}$) and osmotic second virial coefficient ($B_{22}$), respectively. $\Delta G_{\text{unf}}$ is defined as the free energy difference between the unfolded state and the native, folded state. Increasing $\Delta G_{\text{unf}}$ values correspond to higher conformational stability and decreased aggregation propensity. $B_{22}$ is a measure of two-body interactions in solution, in this case, protein-protein interactions. Negative $B_{22}$ values indicate attractive protein-protein interactions while positive values indicate repulsive protein-protein interactions. A protein solution is “colloidal unstable” when the $B_{22}$ value is negative. Increasing the $B_{22}$ value, or increasing the repulsive interactions between proteins, reduces the propensity of proteins to assemble or aggregate. In addition to conformational and colloidal stability, protein surface activity and interfacial behaviors can emerge to be an important factor controlling aggregation. High surface activity coupled with favorable protein-surface contacts can drive aggregation by inducing denaturation and heterogeneous nucleation. Removing exogenous seeds and/or decreasing the extent or affinity of protein adsorption to exogenous surfaces or interfaces have been shown to be effective at reducing aggregation.

Given these mechanistic insights into the driving forces and energetics of protein aggregation, general formulation strategies for stabilizing and increasing the shelf-lives of protein therapeutics are: 1. eliminate chemical instabilities, 2. increase conformational stability, 3. increase colloidal stability, and 4. optimize interfacial stability. Excipients are an important tool to achieving these protein stabilization goals, as well as improving other qualities of biologics.

**Excipient Categories and Examples**

A variety of excipients have been added to formulations to stabilize proteins, act as antimicrobials, aid in the manufacture of the dosage form, control or target drug delivery, and minimize pain upon injection. Examples include buffering agents to control pH, surfactants to inhibit protein adsorption to interfaces, preservatives to prevent microbial growth, carbohydrates as bulking agents for lyophilization, polymers to increase solution viscosity, and salts or sugars to stabilize proteins and to obtain physiological tonicity and osmolality. Excipients can be broadly divided into five categories based on their modes of action:

1. **Protein stabilizers.** These excipients stabilize the protein native conformation. Examples include polyols, sugars, amino acids, amines, and salting out salts. Sucrose and trehalose are the most frequently used sugars and large polyols are in general better stabilizers than smaller polyols. The stabilizers act by four inter-related mechanisms and are all preferentially excluded from the protein surface driven
by thermodynamically unfavorable excipient-protein interactions.

2. Polymers and proteins. Hydrophilic polymers, such as polyethylene glycols (PEGs), polysaccharides, and inert proteins, are used to non-specifically to stabilize proteins and enhance protein assembly. Examples include dextran, hydroxyl ethyl starch (HETA), PEG-4000, and gelatin. Smaller PEGs have been found to be less effective than larger ones. Additionally, non-polar moieties on certain polymers such as PEGs and Pluronics can decrease water surface tension rendering them as surfactants that suppress surface adsorption induced aggregation.

3. Surfactants. Non-ionic surfactants are widely used to stabilize proteins, suppress aggregation, and assist in protein refolding. Polysorbate 80 and Polysorbate 20, also known as Tween 80 and Tween 20, respectively, have been widely incorporated in marketed protein pharmaceuticals at 0.0003-0.3% range. Other examples include Brij 35, Triton X-10, Pluronic F127, and sodium doceyl sulfate (SDS).

4. Amino acids. These excipients stabilize proteins by a variety of mechanisms. Examples include histidine, arginine, and glycine. Other amino acids used as formulation excipients include methionine, proline, lysine, glutamic acid, and arginine mixtures.

5. Preservatives. These compounds are included in formulations to prevent microbial growth, particularly for multi-dose, single container formulations. Examples include benzyl alcohol, m-cresol, and phenol. However, preservatives can oftentimes cause protein aggregation.

Excipient-Protein Interactions and Mechanisms of Excipient-Induced Protein Stabilization

The effects of excipients on protein therapeutics are mainly caused by their interaction with the protein, the solvent (i.e., water), container surfaces and other interfaces. Protein stabilizing excipients (Category 1) stabilize the protein native structure at moderate (0.1 M) to high (1 M) concentrations, that is, these co-solvents increase the $\Delta G_{unf}$ of proteins in solution. These excipients exert their protective effect via four inter-related mechanisms, all of which are involved in different interactions with water. First, the excipients, mostly salts, can increase the surface tension of water, that is, they exert a cohesive force on water. As a result, these salts are preferentially excluded from the protein surface. Second, excipients larger than water are excluded from the protein surface, resulting in a layer of excess water surrounding the protein. This exclusion effect is more pronounced for larger co-solutes,
such as polymers. Third, excipients such as amino acids may unfavorably interact with the peptide bond on the protein, causing the excipients to remain in bulk water. Lastly, many sugars, polyols and certain salts exhibit repulsive interaction with the protein and are preferentially excluded from the protein surface. Regardless of the specific mechanism of action, it is evident that all excipients in this category are preferentially excluded, that is, they are depleted from the protein surface. As a result, water is enriched at the protein surface. Preferential exclusion can also be interpreted as negative binding or thermodynamically unfavorable excipient-protein interactions. The degree of exclusion is proportional to the solvent exposed surface area of the protein. During unfolding, protein surface area increases. Thus, the net effect of the excipients is to favor the native state, thus increasing the $\Delta G_{\text{unf}}$ of the protein and decreasing aggregation propensity and rate.

The predominant mechanism by which hydrophilic polymers (Category 2) stabilize proteins is the excluded volume effect, which favors the more compact, or native, conformation of proteins. As such, the stabilizing effect of these compounds increase with increasing polymer size and concentration. However, because the excluded volume effect also favors protein association even in the absence of any structural changes, hydrophilic polymers can also favor protein-protein interactions that result in precipitation. Hydrophobic moieties on polymers can also bind to proteins through hydrophobic interactions (e.g., PEG with aromatic groups), these polymers can also destabilize the native protein conformation by stabilizing unfolded protein conformations. Thus, the effect of hydrophilic polymers is a balance between stabilizing effect due to excluded volume and destabilizing effect due to hydrophobic interactions. Charged polymers stabilize proteins by protein-specific effects. For example, anionic polymers have been found to stabilize proteins with highly positively charged surface (REF) and cationic polyethyleneimine prevents protein aggregation by chelating metal ions and suppressing protein oxidation (REF). Protein excipients, for example human serum albumin (HSA), stabilize biopharmaceuticals by competitively adsorbing to surfaces and interfaces and preventing interface induced aggregation of the drug product.

However, concerns about the infectious agents in human derived product have led to the use of recombinant versions of HSA and increased usage of non-ionic surfactants.

Non-ionic surfactants (Category 3) protect proteins from surface (e.g., agitation or shaking) and stress induced aggregation (e.g., freezing, lyophilization, and reconstitution). Surfactants acts by competing with proteins for contain surface, air/water interface, ice/water interface, or any other solid surfaces and prevent non-specific adsorption and adsorption induced denaturation and subsequent aggregation. In some
cases, surfactants also prevent aggregation by serving as chaperones and foster protein folding and refolding (e.g., induction of folding of membrane proteins by surfactants). However, the commonly used polysorbates may degrade by oxidation or hydrolysis, and their degradation products may exert varying effects on protein stability. Additionally, it can be difficult to control the level of surfactants in the formulation due to complex behaviors during membrane filtration steps.

Amino acids (Category 4) stabilize proteins by a variety of mechanisms, including preferential exclusion, direct protein binding, buffering capacity, and antioxidant properties. Histidine has been used as a buffering agent for antibody formulations and as an antioxidant that scavenges hydroxyl radicals in solution. Glycine has also been used as a buffering agent, but also as a bulking agent during lyophilization. Arginine has been widely used as a solubilizing agent during purification steps, as a mobile phase component in analytical HPLC, and as an excipient. Arginine has been shown to be highly effective at suppressing aggregation. During refolding processes, arginine increases recovery by suppressing the aggregation of folding intermediates. Arginine also reduces protein aggregation during heat- or urea-induced denaturation, but it does not increase protein thermal stability. In addition, arginine has been found to increase protein solubility and reduce the viscosity of high concentration antibody formulations, thus allowing for shorter administration time. The exact mechanism by which arginine affects protein solutions is still unclear. Arginine is not a protein stabilizing co-solute. It does not strongly bind to proteins, nor is it strongly excluded from protein surface. Arginine has been found to demonstrate weak affinity for protein side-chains, especially charged and aromatic side chains via cation-π type interactions that can stabilize protein conformational sub-states and folding intermediates (REF). Despite a lack of understanding of the multiple mechanism by which arginine interacts with affected protein solutions, due to its aggregation suppression ability and its safety in humans, the application of arginine in biologics is rapidly growing.

Preservatives (Category 5) are used to ensure sterility of multi-dose, single container formulations. However, preservatives can often time cause protein aggregation in a concentration, temperature, and time dependent manner. Benzyl alcohol has been shown to rapidly induce the aggregation of recombinant interlukin-1 receptor antagonist. This preservative is found to weakly bind to hydrophobic contacts on the protein, thus shifting the protein population towards partially unfolded species that are more aggregation prone. Stabilizing co-solute such as sucrose was found to be effective at suppressing benzyl-alcohol induced aggregation.
Conclusions

As proteins continue to gain prominence as therapies to treat human diseases and disorders, the need to rationally design formulation to stabilize and improve the quality of protein-based therapeutics will grow. The challenge is that proteins are only marginally stable and are prone to chemical and physical degradations. The aggregation of proteins is fundamentally driven by conformational, colloidal, and interfacial instabilities. A number of excipients can be utilized to enhance protein stability and increase shelf-live. Understanding the predominant pathways of the degradation of a protein therapeutic and the mechanisms by which excipients interact with and stabilize proteins will guide the development of an optimal formation with desired biologics qualities.

References

This review extensively used the following three references. Please refer to these references and citations wherein for more information.


