Review Paper

Use of excipients to control aggregation in peptide and protein formulations.

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ABSTRACT

Aggregation is rapidly emerging as a key issue underlying multiple deleterious effects for peptide and protein-based therapeutics, including loss of efficacy, altered pharmacokinetics, reduced stability or product shelf life, and induction of unwanted immunogenicity. In addition, bioavailability and pharmacokinetics of a self-associating peptide can be influenced by aggregate size and the ease of disruption of the non-covalent intermolecular interactions at the subcutaneous site. This review highlights the various types of aggregates encountered in peptide and protein formulation, methods useful in detecting aggregates, and recent developments in the use of excipients to prevent or reduce peptide and protein aggregation.

KEY WORDS: Aggregation, peptide or protein-based therapeutics, protein stability, shelf life, immunogenicity, self-association, light scattering, size exclusion chromatography

INTRODUCTION

In addition to the usual routes of degradation that are common to all molecules, such as oxidation, hydrolysis etc, protein and peptide therapeutics may also undergo conformational changes such as denaturation or unfolding. These involve changes in the secondary, tertiary or quaternary structure of such protein or peptide molecules and are usually followed by aggregation. It is well known that aggregation of proteins may cause reduced efficacy, bioavailability, stability and the mobilization of an unwanted host immune response toward the protein therapeutic.

Today, there is a pronounced trend among major companies in the pharmaceutical industry toward increased emphasis on protein drugs, particularly monoclonal antibodies, cytokines, fusion proteins, biologically active peptides and peptide fragments, many of which require relatively high concentration to allow for practical administration volumes. High concentration is a key contributor to aggregation, thus elevating the importance of addressing the aggregation problem. Dimers or higher order aggregates may be produced as a consequence of aggregation. This phenomenon is especially difficult to prevent as it may be caused by changes in several seemingly

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innocuous formulation conditions such as pH, ionic strength, counter-ion composition, concentration or the addition of excipients such as certain antimicrobial preservatives (1).

Perhaps most significant are the actual physical processes used to concentrate proteins during manufacturing or in creating a final formulation. Since proteins are usually expressed in fermentation media at concentrations that are significantly lesser than those required for practical clinical use, they need to be concentrated before fill-and-finish operations. A variety of methods are used to concentrate proteins such as filtration, chromatography, lyophilization, dialysis or precipitation. All of these methods can potentially affect denaturation and aggregation of protein therapeutics. In addition, high shear manufacturing operations can also cause denaturation and aggregation.

Aggregation can also cause membrane fouling during the sterile filtration process leading to dramatic losses of the valuable protein active pharmaceutical ingredient (2).

Aggregation is frequently increased at higher temperatures. Protein therapeutics thus needs to be shipped and stored under refrigeration which adds a significant cost to the product. Such so-called "cold chain" cost may be significantly reduced if methods can be found to reduce the influence of temperature on the aggregative or denaturation processes.

IMPACT OF AGGREGATION ON BIO-AVAILABILITY AND BIOLOGICAL FUNCTION

A particularly egregious example, which illustrates the dramatic effect that aggregation can have on the subcutaneous bioavailability and pharmacokinetics, is illustrated by a chemically modified GLP-1 analog (4). In this instance, the absorption of the peptide was totally blocked due to such aggregation.

Since protein aggregation may also adversely impact biological product process yield and potency it has become a major issue for pharmaceutical formulators. Additionally, regulatory oversight of aggregation events by the FDA and other regulatory agencies has significantly increased in recent years. Thus biopharmaceutical companies have increased their efforts to understand them. Of particular concern is the induction of unwanted immunogenicity, a well accepted sequella of aggregation (5). Aggregative phenomena that occur due to changes in the 'native' quaternary non covalent interactions, or by the artificial creation of new and mismatched ones frequently induce the mobilization of a host immunogenic response toward the (aggregated) peptide therapeutic. Such immunogenic responses may result in a decrease in the therapeutic efficacy of the peptide. As an example, neutralizing antibodies to interferon beta (6) result in a higher relapse rate and more disease activity, as measured by brain MRI scans (7). A further example is provided by erythropoietin (EPO) with which a life-threatening antibody response, "pure red cell aplasia" has been seen in some patients (8).

It has been demonstrated that 15%-30% of hemophilic patients treated with recombinant human factor VIII (rFVIII) develop inhibitory antibodies toward this multidomain glycoprotein therapeutic.

The presence of aggregated protein in formulations is generally believed to enhance the antibody development response (9). In the case of hemophilia A, neutralizing antibodies to Factor VIII can cause life-threatening bleeding episodes, resulting in significant morbidity and necessitating treatment with a prolonged course of a tolerance-inducing therapy to reverse immunity (10, 11).

When stored at temperatures greater than those encountered under refrigeration, the aggregation of insulin causes it to rapidly lose its activity (12, 13).

Aggregation of recombinant AAV2 results in reduced yield during purification and has deleterious effects on immunogenicity following *in vivo* administration (14). Monoclonal antibody based therapeutics have also been shown to be subject to inactivation as a result of protein aggregation (15). The number of approved monoclonal antibody drugs at present is 26 with many more in human clinical trials and literally hundreds in preclinical development (16). Monoclonal antibodies usually require a high dose (1 to 2 mg/kg). They need to be sufficiently concentrated so that they can be administered in a variety of settings such as in a hospital by intravenous injection or infusion or at home by subcutaneous injection. Development of antibody formulations at high concentrations pose stability, manufacturing, and delivery challenges related to the propensity of antibodies to aggregate at higher concentrations.

As with monoclonal antibodies, many other peptide and protein therapeutics are frequently formulated at high concentration so that the volume of the formulation administered can be kept small, thereby minimizing patient discomfort. In addition, protein aggregation can be induced by necessary excipients such as the antimicrobial preservative benzyl alcohol, which is sometimes used to maintain product sterility (1).

The pancreatic hormone, Amylin, is a 37 amino acid peptide that is co-secreted with insulin. The amyloid deposits that occur in the islets of Langerhans in Type II diabetic patients have been demonstrated to be composed, in large part, of aggregated Amylin. The native form of the peptide shows a tendency to form fibrils in solution and consequently exhibits low solubility and stability characteristics (17, 18). In an attempt to circumvent the significant aggregative propensity of the native peptide, various sequence modified synthetic analogs have been prepared. Such analogs, including pramlintide acetate, while demonstrating a lesser propensity to aggregate; unfortunately also exhibit significantly less bioactivity than the native enzyme. Sequence modified analogs usually induce a greater immunogenic host response compared with the native protein. It may be hypothesized that a stabilized non-aggregating form of native amylin would potentially exhibit increased activity, reduced aggregation, and reduced immunogenicity, compared to amylin analogs. Enzymes are also known to lose activity as a result of aggregation. For example thermal inactivation of urokinase occurs via aggregation (19).

The more common (and frequently water mediated) degradation pathways of oxidation and hydrolysis may be circumvented by lyophilization. However, during formulation, attention must be paid to the reconstitution of lyophilates because this process frequently results in protein aggregate formation or precipitation.

Several excipients included in reconstitution media have been found to result in a significant reduction of aggregation. These include sulfated polysaccharides, polyphosphates, amino acids and various surfactants (20). In some cases, a combination of alcohols, organic solvents, such as in Fortical (Unigene), a nasally delivered calcitonin product, may be used. Roccatano *et al.* (21) have used trifluoroethanol mixtures to stabilize various polypeptides. Unfortunately, such agents may be harsh on mucosal tissue causing patient discomfort or local toxicity.

THE PROTEIN AGGREGATION PROBLEM IN FORMULATION

A main objective of protein formulation is development of manufacturable and stable dosage forms. Physical stability properties, critical for processing and handling, are often poorly characterized and a broad range of physical instability phenomena may be unpredictably encountered, for example association, aggregation, crystallization and precipitation. This poses a significant analytical challenge. The types of aggregates that can be

encountered in biopharmaceutical solutions can vary with respect to general characteristics as well as size. For example, aggregates may be soluble, insoluble, covalent, noncovalent, reversible, or irreversible. They may be present as visible or sub-visible particles. They may be present as low order aggregates, for example dimers, trimers, tetramers, or in the case of insulin, as hexamers, having dimensions in the nanometer range, all the way up to aggregates containing millions of subunits and having dimensions in the micron range directly exhibiting visible cloudiness (a light scattering phenomenon), or visible precipitates. In all cases, it is well accepted that protein aggregates can induce an immune response.

ANALYTICAL METHODS FOR AGGREGATION DETECTION AND CHARACTERIZATION

A variety of analytical techniques have been developed to characterize the broad range of aggregation possibilities shown in Table 1.

Table 1 Analytical techniques for characterizing aggregation possibilities

Right angle light scattering
Dynamic light scattering
Multi-angle light laser scattering
Forward light scatter
Size exclusion chromatography
Analytical ultracentrifugation

The most commonly utilized methodologies are light scattering and size exclusion chromatography. Light scattering measurements offer a range of non-destructive techniques routinely used to examine or characterize the aggregation state and solution properties of macromolecules. The intensity of the scattered light can be measured as a continuous function of scatter angle or can be measured at fixed angles. In its simplest form, right angle light scattering can be performed using a fluorometer in which the excitation light path is set at 90 degrees to the detection light path, and in which the excitation and emission wavelengths are set to the same value to allow passage of light of a single wavelength. This method is only suitable for very large aggregates. Dynamic light scattering and multi-angle light scattering, utilizing two quite different physical phenomena, allow the determination of absolute molecular mass and the average size or size distribution for particles in solution, as small as dimers and trimers in many cases. Forward light scattering can also be used for rapid determination of *in situ* particle size distributions.

Size exclusion chromatography and analytical ultracentrifugation are physical separation methods that permit the direct observation of aggregates. The simpler of these two methods is size exclusion HPLC or SE-HPLC. While this is a generally accepted and frequently used methodology, it is subject to certain limitations such as protein absorption, in particular, preferential absorption of aggregates onto the column media, and dissociation of aggregates during the chromatographic run.

Analytical ultracentrifugation is increasingly being used as a confirmatory methodology even though it is more complex, has lower throughput, and the instrumentation is not as broadly available as SE–HPLC. The protein is analyzed directly in the sample solution and closely sized aggregates can be clearly resolved. Analytical ultracentrifugation has its own limitations. A principal concern is sedimentation of solution components other than the protein which can give rise to concentration gradients across the centrifugation cell (22).

STABILIZATION OF PROTEINS BY EXCIPIENTS

Protein-stabilizing excipients can be broadly divided at least into the following types: buffers, sugars and other polyols, polymers, surfactants, amino acids, amines, and salts (see Table 2). The excipients should be soluble and nontoxic. Their stabilizing effects are usually concentration- and protein-dependent,

although higher concentrations of excipients may not be necessarily more effective than lower concentrations. Additionally, an excipient found to be stabilizing in the case of one protein may be inactive or destabilizing in the case of a different protein. Selection and optimization of the best excipient(s) for each specific protein should be explored in a typical matrix fashion.

Table 2 Classes of Protein-Stabilizing Excipients

Buffers
Sugars and Polyols
Salts
Polymers
Cyclodextrins
Metal ions
Amino acids
Surfactants

Buffers and pH

Typically, a protein is stable only over a very narrow pH range. Accurate pH control is fundamental to protein stabilization, but unfortunately there are no general rules for specific buffer selection. Typical protein formulation buffers include acetate, phosphate, glycine, and citrate. Examples of stabilization or destabilization of different proteins with each buffer type abound in the literature.

Sugars and polyols

Sugars and polyols are often-used stabilizers. Among various sugars, sucrose and trehalose appear to be the most commonly used stabilizers for formulation although examples of glucose, sorbitol, lactose, ascorbic acid or maltose may also be found. The stabilizing effect of sugars depends on its concentration. A concentration of 0.3 M (or 5%) sugar or polyols has been suggested to be the minimum to achieve significant protein stabilization (23). Higher concentrations (e.g., 1 M sucrose or 10% glycerol are often used in preformulation processing. Sugars and polyols can also offer inhibition of oxidative degradation (24, 25).

Salts

Salts may stabilize, destabilize, or have no effect on protein stability depending on the protein and its concentration (26). NaCl, a freqeuntly used salt, has been found to play a critical role in stabilizing certain proteins such as IL-1R (27). KCl can also be valuable as a protein stabilizer. The separate contribution of anions and cations in salts to the stability of a protein can be significantly different or even act in opposite directions (28), so reiterating the caveat expressed above, selection and optimization of the best excipient(s) for each specific protein should be explored in a matrix fashion.

Polymers

Various kinds of polymers have been shown to stabilize proteins by non-covalent interactions. Polythylene glycols (PEGs) are perhaps the most often used polymers in this regard (note that such non-covalent stabilization methods are different from covalent PEGylation which is primarily carried out to change *in vivo* protein disposition). PEGs are available in different molecular weights ranging from hundreds to thousands of Daltons and stabilization seems to be dependent on the protein and the size of PEGs in an idiosyncratic manner. Other useful polymers include dextrans and dextran sulfates of various molecular weights, heparin and low molecular weight heparin, gelatins type A and B, hydroxyethyl starch, poly-L-glutamic acid, poly-L-lysine, fucoidan, pentosan polysulfate, polyvinyl sulfate, keratan sulfate, poly-Asp and poly-Glu, and hydroxyethylcellulose.

Cyclodextrins

A variety of cyclodextrins have been investigated for their potential as protein stabilization excipients. Hydroxypropyl-β-cyclodextrin (HP-β-CD) appears to be a particularly valuable stabilizing excipient because it is both a good solubilizing agent and it is considered safe for parenteral administration. Serno *et al.* (29) have described

the stabilization of monoclonal antibodies using hydroxy propyl-β-cyclodextrin demonstrating the superiority of HP-β-CD to other excipients frequently used in protein formulations such as sugars, sugar alcohols, or polysorbate 80 in agitation-induced aggregation of IgG in aqueous solution using size exclusion chromatography. In earlier studies it had been shown to be effective in stabilizing a number of other structurally divergent proteins including growth hormone (30), and IL-2 and insulin (31).

Metal ions

Certain metal ions, particularly calcium, magnesium, and zinc have been found to be potent protein stabilizers, presumably by binding to a protein and making the overall protein structure more rigid and compact (32). Once again, stabilization appears to be idiosyncratic with respect to the specific cation and protein.

Amino acids

Certain amino acids, either alone or in combination with other excipients, stabilize proteins most likely by preferential exclusion (33). Protein aggregation has been inhibited for a number of proteins in the presence of the following amino acids: histidine, glycine, lysine, aspartic acid, glutamic acid and arginine (20).

Surfactants

Surfactants may be classified as either nonionic (including amphoteric) and ionic (cationic and anionic). Low concentrations of nonionic surfactants are often sufficient to prevent or reduce protein surface adsorption or aggregation due to their relatively low critical micelle concentrations (CMC) (34). Examples of useful non-ionic detergents include Tween 20, Tween 80, Triton X-100, Polysorbate 20, Polysorbate 80, Pluronic F68, Pluronic F88, Pluoronic F-127, Brij 35 (polyoxyethylene alkyl ether). At the same time, some of these surfactants may be contaminated with alkyl peroxides, particularly the polysorbates, which can accelerate oxidation of proteins (35).

Surfactants that are most commonly used to prevent aggregation are the polysorbates Tween 20 and Tween 80 (36). These are used for stabilization of certain monoclonal antibody products, for example, Rituxan, Remicade, ReoPro and Humira. They are also used to stabilize Enbrel, a dimeric fusion protein comprised of the extracellular ligand-binding portion of the human tumor necrosis factor receptor linked to the Fc portion of human IgG1. Pluronic F127 has been shown to stabilize recombinant human growth hormone (rhGH) by preventing aggregation (37). It has also been used to prevent aggregation during the encapsulation process required for the preparation of an extended action poly (lactic-co-glycolic acid) (PLGA) microsphere formulation of rhGH (38). Some of these surfactants may be contaminated with alkyl peroxides arising from the ether linkages incorporated in their structures, as is particularly the case with polysorbates, which can accelerate the oxidation of proteins (39). The use of methionine and tryptophan mentioned above (34) in preventing oxidation from polysorbate degradation products is a significant finding. Each of these surfactants has proven utility and will undoubtedly see increased use in development in future formulations now that the aggregation issue has been highlighted.

Recent efforts to increase the transmucosal absorption of protein therapeutics resulted in the discovery of a new class of alkyl saccharide excipients (ProTek® Excipients, Aegis Therapeutics, San Diego, CA). These patented, non-toxic compounds are considered to be GRAS substances. They have been demonstrated to significantly increase the transmucosal absorption of proteins below 30 kDa (40-43). Coincidental with this absorption enhancement, these excipients (ProTek®) have also been found to stabilize proteins against aggregation. These alkylsaccharides consisting of disacchrides coupled to a long chain fatty

acid or a corresponding alcohol undergo *in vivo* metabolisis to CO_2 and water. They are soluble in water and consequently may be used to manufacture a variety of dosage forms and are compatible with a variety of different routes of administration.

Examples of the dramatic stabilization of some currently marketed protein therapeutics at low concentrations of ProTek® excipients are shown in Figures 1-5. The stabilization of recombinant human insulin (Humulin®, Eli Lilly) by a ProTek® alkyl saccharide (a glycoside) at two concentrations is shown in Figure 1 (40). Insulin was subjected to accelerated stress by continuous shaking at 150 RPM at 37°C over the course of 90 days. The insulin to which the ProTek® excipients were added, did not demonstrate any aggregation as measured by increased right angle light scatter for the full 90 day period of continuous agitation in the study. In contrast, the untreated insulin started to denature within one day of constant agitation.

The corresponding non-ProTek alkyl saccharide, an isomer having the same sugar moiety and same alkyl chain length, is seen to behave very differently, providing some protection for a short period of time but failing between 2 and 3 weeks into the study. ProTek® similarly increased the stability of human growth hormone (44) while preventing membrane fouling and protein loss during

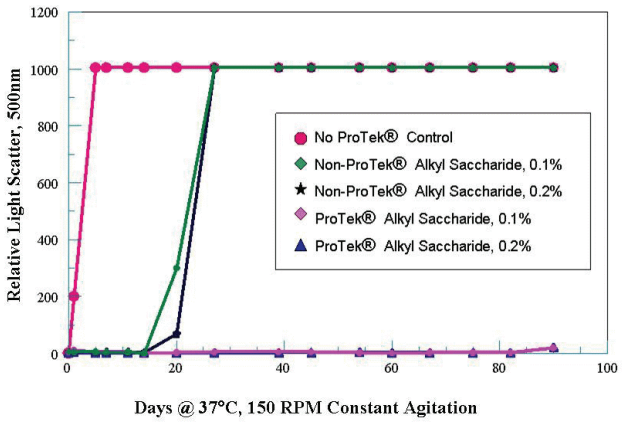

Figure 1 Stabilization of Humulin® R - Recombinant Human Insulin.

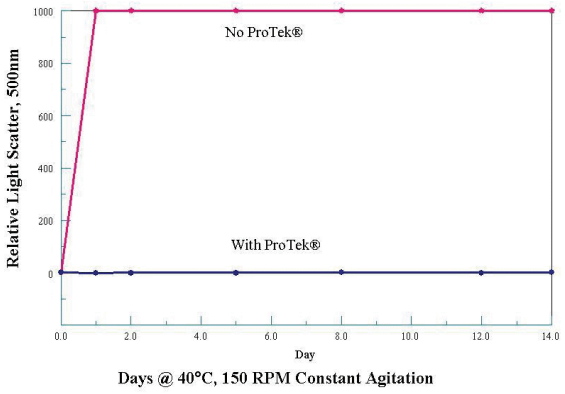

Figure 2 Stabilization of ZT-031 PTH Analog.

filtration. It also prevented the aggregation of the octapeptide CCR5 inhibitor D-Ala-Peptide T Amide, which is prone to rapid, severe aggregation and the formation of fibrils large enough to be viewed under the electron microscope (45).

Another example of aggregation prevention for a bioactive peptide is shown in Figure 2. ZT-031 is a patented PTH 1-31 cyclic peptide analog that provides rapid onset of clinically meaningful bone formation activity, increased bone mineral density which can potentially reduce fracture rates, and has a low potential for calcium-related toxicity. In a study conducted at 40°C to accelerate stress and heat induced aggregation, aggregation of ZT-031 measured by right angle light scattering was seen to be significant in the absence of ProTek® at day, but was stabilized for at least 5 days when continuously shaken at 150 rpm on an orbital shaker in the presence of ProTek®.

Prevention of aggregation of interferons β-1a and β-1b using ProTek® (Figure 3) was recently shown to reduce or eliminate immunogenicity upon injection or nasal administration (46). Figure 4 compares the stabilization of pramlintide, an amylin analog and calcitonin, both members of the calcitonin gene family of bioactive peptides, thus illustrating the differences in stabilization characteristics of

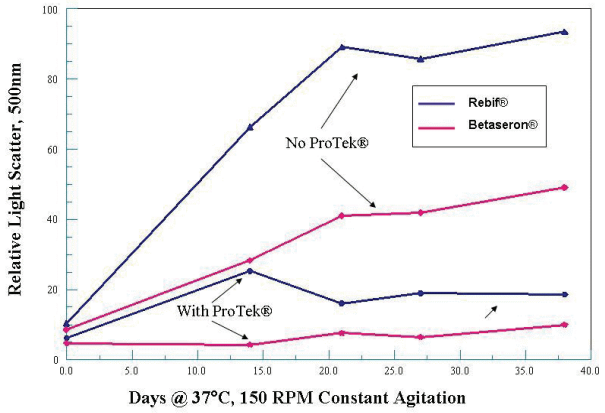

Figure 3 Stabilization of β-Interferons-1a (Rebif®) and -1b (Betaseron®).

proteins even within the same family. While pramlintide exhibits aggregation which is significantly prevented by ProTek®, calcitonin was found to be more or less completely stable under the stress conditions employed. In one of the most significant applications, owing to the high concentrations typically required for practical administration, monoclonal antibodies can also be efficiently stabilized. Figure 5 shows the stabilization of a monoclonal antibody under accelerated heat and agitation stress conditions. Aggregation, as measured by right angle light scattering was significant at day 1, but the protein was stabilized for at least 4 days of continuous shaking at 150 rpm on an orbital shaker showing no increase in light scatter in the presence of ProTek®.

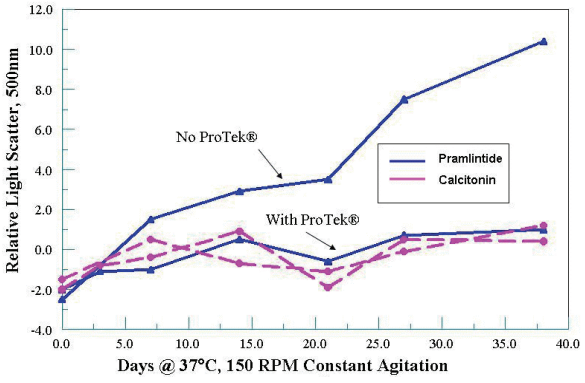

Figure 4 Stabilization of Pramlintide and Calcitron.

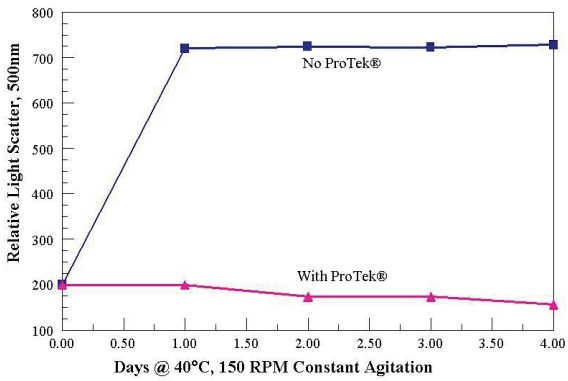

Figure 5 Stabilization of IgG Monoclonal Antibody.

CONCLUSION

While there may be obvious choices for selecting initial test conditions in seeking a stabilized protein or peptide formulations such as concentration, pH, and suitability for a particular administration route, choice of excipients is largely dependent upon he idiosyncratic properties of the particular protein or peptide. The scientific literature surrounding peptides of a similar structural class can often provide a good starting point for selecting stabilization conditions. To achieve an optimal formulation, however, requires systematic screening and optimization of the final excipient matrix most appropriate for each individual protein.

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