Staying Current

Formulation of Biopharmaceuticals

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OVERVIEW

This issue of *Staying Current: Formulation of Biopharmaceuticals* emphasizes three important areas: particle formation and characterization, behavior of the lyophilized materials, and aggregation kinetics. Two new articles in each area are discussed. Please take time to consider them carefully, as they challenge our current thinking and call for renewed efforts to identify and develop improved methods and algorithms.

SUBVISIBLE PARTICULATES IN BIOPHARMACEUTICAL PRODUCTS

Carpenter et al., Overlooking Subvisible Particles in Therapeutic Products: Gaps That May Compromise Product Quality. *J. Pharm. Sci.* **2009**, 98: 1201-1205.

Abstract: For decades, quantitation of visible particles using USP <788> has been the accepted standard for determining particle contamination in injectable products, including proteins. At the same time, characterization of subvisible particles has been largely ignored. There is a growing concern that these particles may, in fact, be more immunogenic and toxic than the relatively small number of large particles. This commentary calls for increased attention to subvisible particles, including a call for improved analytical methodology to characterize and quantify these species. Moreover, the current USP method was not designed to control the risks from large protein aggregates and new testing is needed to replace it for biopharmaceutical products.

Analysis: This commentary, drafted by professors as well as researchers at the US FDA, highlights an important issue for injectable biopharmaceutical products. For some time, the methodology for particulate quantitation has relied on visual inspection and measurement of visible particulates. All the while, little or no attention has been paid to subvisible particles. In fact, these may contribute much more to immunogenicity and toxicity effects, making them a real concern for safety. While there are many unanswered questions, it is clear that this will become an active area of study. There will need to be new analytical methods developed. Each of these new methods will need to be compared carefully, to allow regulatory agencies to evaluate which ones provide the best information regarding product quality and safety. In addition, formulation strategies will need to be adjusted to minimize subvisible particle formation as well.

SILICONE OIL AND PROTEIN AGGREGATION

Sharma et al., Silicone Microdroplets in Protein Formulations: Detection and Enumeration. *Pharm. Technol.* 2009, 33 (4): 74+

Abstract: Release of silicone oil droplets from the walls of syringes is a persistent problem in the biopharmaceutical industry. Often these microdroplets are similar in size to subvisible protein aggregates. In this study, micro-flow imaging (MFI) was used to distinguish the two species. In general, silicone oil droplets have an aspect ratio near 1 (indicating a circular shape). In addition, the droplets in this study were ~ 2 mm in size, much smaller than many of the aggregated protein particles. Finally, MFI is able to capture images of individual protein aggregates, showing them to be fibrillar in nature. This stands in marked contrast to the regular, circular nature of the microdroplets.

Analysis: Following the commentary described above, we have this excellent technical note detailing efforts to distinguish silicone microdroplets from protein aggregates formed by freeze-thaw cycling. Using MFI, the size and shape of the particles appear to be distinct and different. Given the call in the previous article for new technologies to be applied to subvisible particles, here we have one clear example in MFI that shows some real promise.

GLYCOSYLATION AND PROTEIN STABILITY

Solá and Griebenow, Effects of Glycosylation on the Stability of Protein Pharmaceuticals. *J. Pharm. Sci.* **2009**, 98: 1223-1245.

Abstract: This review describes the literature surrounding stabilization, broadly defined, by glycosylation of proteins of pharmaceutical interest. The possible mechanisms by which glycans induce stability are discussed. Given the well-established effect of glycans on protein stability, rational manipulation of glycosylation could improve stability both *in vitro* and *in vivo*.

Analysis: Here we have an exhaustive review on the effects of glycosylation on protein stability. The review is comprehensive and very well written. In addition, the authors seek to identify the basis for the general observation that addition of glycans typically improves stability, solubility, etc. For anyone working with glycosylated proteins, especially where the extent and nature of glycosylation varies (and can be varied), this is required reading.

MODEL DISCRIMINATION IN PROTEIN AGGREGATION KINETICS

Bernacki and Murphy, Model Discrimination and Mechanistic Interpretation of Kinetic Data in Protein Aggregation Studies. *Biophys. J.* **2009**, 96: 2871-2887.

Abstract: There has been an increased interest in protein aggregation kinetics and the ability of these models to provide mechanistic insight. Most are based on monomer loss data. This study demonstrates that many kinetic schemes provide similar fits to such data. Only by including information on fibril growth data (using molar fibril concentration not just mass information) can some mechanistic distinction be achieved. Therefore, the type and quality of data needed to achieve more definitive conclusions regarding aggregation mechanisms can be defined. In particular direct measurement of fibril size is critical.

Analysis: This large article is worth noting for a number of reasons. First, it provides an excellent summary of some of the general mechanisms that exist to describe protein aggregation. Second, it is a critical examination of the ability of various kinetic models to provide definitive mechanistic information. Finally, it challenges our thinking about aggregation kinetics. For example, increased aggregation by seeding is usually taken as definitive proof of a nucleation-dependent mechanism. These authors demonstrate that this might not necessarily be the case. Furthermore, they raise important questions regarding the meaning of lag times measurements (as a function of concentration and temperature) and the relationship between reaction order and stoichiometry. All in all, this article addresses many important issues and raises significant questions for us all to consider carefully.

PREDICTING SHELF LIFE AND PROTEIN AGGREGATION RATES

Weiss et al., Principles, Approaches, and Challenges for Predicting Protein Aggregation Rates and Shelf Life. *J. Pharm. Sci.* **2009**, 98: 1246-1277.

Abstract: This review describes the current state of the art regarding experimental and theoretical approaches to predicting aggregation rates. The principles and assumption behind the four basis models are discussed, along with advantages and disadvantages of each. The importance of appropriate experimental methods and models is stressed. A summary of the current methods to predict aggregation rates from sequence or composition is included.

Analysis: Here we have an extensive review from Professor Roberts' group, appearing at the same time as the detailed study just discussed from Professor Murphy's laboratory. While they cover different aspects of protein aggregation kinetics, together they emphasize how complex kinetic modeling can be. Given the potential to gather important mechanistic insight, it is essential that such modeling be done correctly and lead to unique answers that discriminate one pathway from another. Both articles seek to do that. In my view, these two articles are complementary, providing the formulation scientist with different views on how to conduct experiments correctly and apply the appropriate mathematical model. In doing so, one should be able to obtain useful mechanistic insight for their particular system.

THERMAL DENATURATION OF PROTEINS IN THE SOLID STATE

Pikal et al., Solid State Chemistry of Proteins. IV. What is the Meaning of Thermal Denaturation in Freeze Dried Proteins? *J. Pharm. Sci.* **2009**, 98: 1387-1399.

Abstract: The thermal denaturation of human growth hormone (hGH) in lyophilized formulations was examined using DSC. Differences in glass transition temperature (T_g), molecular mobility, and denaturation temperatures indicate that unfolding is under partial thermodynamic control. Unfolding of hGH was simulated using a three-state kinetic model. Free energy vs. temperature curves were calculated. These curves indicate that even in saccharide-based formulations, proteins are conformationally unstable near ambient temperature, where significant denaturation is prevented by low mobility.

Analysis: The observation of protein denaturation in dried solids is relatively rare. Here we have a detailed study on hGH across a number of formulations. The results are surprising and demand careful consideration. They show that the denaturation temperature is linked to T_g, indicating that denaturation is not under purely kinetic control. The calculations provided here indicate that proteins are conformationally unstable in lyophilized cakes (analogous to cold denaturation in solution), where denaturation is prevented only due to the lack of mobility well below T_g. As with other studies from Professor Pikal, this one requires thought and consideration for the implications of this study on the design sugar-based formulations and how proteins behave in the solid state.

MOBILITY IN AMORPHOUS SUCROSE AND TREHALOSE

Dranca et al., Implications of Global and Local Mobility in Amorphous Sucrose and Trehalose as Determined by Differential Scanning Calorimetry. *Pharm. Res.* **2009**, 26: 1064-1072.

Abstract: The global and local mobility of amorphous trehalose and sucrose were examined using differential scanning calorimetry (DSC). The α-relaxations in trehalose were characterized by larger activation energy barriers than for sucrose, likely reflecting the more compact structure of trehalose. Meanwhile, the activation energy of β-relaxations increased with annealing temperature due to increasing cooperative motions. The increase was larger for sucrose than for trehalose. This suggests that even small temperature excursions could have a significant impact on local mobility in sucrose glasses.

Analysis: There continues to be both a practical and academic interest in the differences in behavior of sucrose and trehalose in the solid state. This study provides some detailed information on mobility within glassy matrices of these two widely used sugars. The data suggest that local mobility, that is, b-relaxations, which have been implicated as being important in long-term stability of lyophilized proteins, are quite sensitive to thermal history of the sample. This aspect needs to be explored further. If correct, it could have a significant impact on the stability of a sucrose-based protein formulation.

IMMUNOTOXICITY OF ANTIBODIES

Descotes, Immunotoxicity of Monoclonal Antibodies. *mAbs* **2009**, 1: 104-111.

Abstract: The accumulated clinical experience on the immunotoxicity of monoclonal antibodies (MAbs) is reviewed. These effects include immunosuppression, immunostimulation and hypersensitivity (immunogenicity). So far, preclincal studies have been unable to explain or predict all of the observed clinical responses. In addition, clinical studies rarely deal with validated end points, making predictions of immunotoxicity even more difficult. These points emphasize the pressing need to improve our understanding of MAb-induced toxicity effects and identification of methods for assessing their safety.

Analysis: Here we have a review article from a new journal, mAbs. The immunogenicity and related toxicities are well summarized here. The review also discusses the lack of suitable animal models to predict clinical responses in humans. Coupled with the difficulties in quantifying toxicity end points, the review rightly emphasizes the marked need for better understanding and methodology in this area, especially given the large number of MAb products under development.

SOLUBILITY DIFFERENCES IN E. COLI PROTEINS

Niwa et al., Bimodal Protein Solubility Distribution Revealed by an Aggregation Analysis of the Entire Ensemble of *Escherichia coli* Proteins. *Proc. Natl. Acad. Sci. USA* **2009**, 106: 4201-4206.

Abstract: The entire ensemble of *E.coli* proteins was individually synthesized using an in vitro translation system. Analysis of the aggregation propensity revealed a bimodal distribution of solubilities. In other words, there appears to be two populations: soluble proteins and aggregation-prone proteins. Aggregation propensity did not appear to correlate strongly with physical properties, such as hydrophobicity, pI, or composition. Conversely, using Structural Classification of Proteins (SCOP) groupings, aggregation propensity appears to be correlated with structure.

Analysis: Here we have a remarkable study that examines the solubility profiles of over 3000 proteins. The results are that there seem to be a group of soluble proteins and those that are much more likely to aggregate and be insoluble. Furthermore, the strongest correlations are not to physical properties, as one might expect, but to the structural classification or protein fold. While there are still many unanswered questions here, it is a study that challenges our thinking and could have implications for the manufacturability of certain structural classes of proteins.

POSSIBLE MECHANISM OF PROTEIN STABILIZATION BY TMAO

Rezus and Bakker, Destabilization of the Hydrogen Bond Structure of Water by the Osmolyte Trimethylamine N-oxide. *J. Phys. Chem. B* **2009**, 113: 4038-4044.

Abstract: Using femtosecond pump-probe spectroscopy, the structural dynamics of water were investigated in the presence of various compounds. It was found that trimethylamine N-oxide (TMAO) had a profound ability to orient water molecules. Meanwhile, urea has the opposite effect. These two effects are additive. It is likely that the water-ordering seen with TMAO is the basis for the stabilization effect it has on proteins.

Analysis: While this study does not address protein stabilization *per se*, it is important to be aware of the detailed biophysical work being done (both experimental as well as theoretical) on the behavior of common additives (stabilizers as well as chaotropes) in water. Many of these studies have been discussed here in *Staying Current: Formulation of Biopharmaceuticals*. The ability to order water has been implicated for trehalose as part of its remarkable stabilization behavior, and now TMAO appears to behave similarly. The fact that the TMAO effect and urea appear to balance each other has interesting implications for both the formulation scientist but also for the biologist where these two compounds can exist in high concentrations simultaneously in some organisms.

Met OXIDATION DURING LONG-TERM STORAGE

Takenawa et al., Protein Oxidation during Long Storage: Identification of Oxidation Sites in Dihydrofolate Reductase from *Escherichia coli* by LC-MS and Fragment Studies. *J. Biochem.* **2009**, 145: 517-523.

Abstract: Samples of dihydrofolate reductase (DHFR) were stored for up to 18 months. During storage, some oxidation occurs, corresponding to oxidation at one, two or three sites. Peptide mapping and LC-MS analysis reveals that methionines at positions 1, 16, and 20 undergo oxidation to some extent, while Met^{42} and Met^{92} do not oxidize under these conditions. The relative reactivity appears to correlate with the degree of accessible surface area, as Met^{42} and Met^{92} are completely buried. One of the oxidized species exhibits activity greater than the wild type, while the others display reduced activity.

Analysis: Once again, the degree of solvent exposure appears to control the rate of Met oxidation, reinforcing this important concept. Furthermore, this article demonstrates that not every chemical modification has an adverse effect on function or activity, making activity measurements questionable stability-indicating assays. These lessons are true not just for DHFR, but for biopharmaceutical products as well.

POST-TRANSLATIONAL MODIFICATIONS OF HUMAN SERUM ALBUMIN

Otagiri and Chuang, Pharmaceutically Important Pre- and Post-Translational Modifications on Human Serum Albumin. *Biol. Pharm. Bull.* **2009**, 32: 527-534.

Abstract: Human serum albumin is an important pharmaceutical agent, both as an active ingredient and as a stabilizer. Multiple genetic variations of HSA are known, most of which do not affect the overall conformation of the protein. *In vivo*, it is an effective antioxidant in addition to its well known ability to bind and transport small molecules. This review examines the effects of three modification processes that occur in the body: oxidation, glycation and S-nitrosylation. This review provides aspects to consider when designing HSA mutants with desirable properties.

Analysis: This is a valuable summary of what is known about the stability of HSA from a variety of viewpoints. In addition to being a valuable pharmaceutical product, it is also a great example of natural variation providing insight into factors governing stability and activity. The discussion on oxidation and glycation are particularly useful for the formulation scientist.

STABILIZATION OF SAVINASE BY COSOLVENTS

Nasiripourdiri et al., Co-solvent Effects on Structure and Function Properties of Savinase: Co-solvent Induced Thermal Stabilization. *Intl. J. Biol. Macromol.* **2009**, 44: 311-315.

Abstract: Savinase is a subtilisin-like enzyme used in detergent formulations. At elevated temperature, it is inactivated by autolysis. Addition of the co-solvents, sorbitol and trehalose, lead to decreased autolysis and marked increase in half-life. Both co-solvents appear to work equally effective on a weight basis.

Analysis: This study reminds us that enzymes can be inactivated by autolytic degradation. Here we see that the excluded solutes, sorbitol and trehalose, are both effective at slowing autolysis, in addition to their ability to reverse other inactivation processes, such as chemical and thermal denaturation. While this study did not compare the excipients on an equal molar basis, they appear to work equally well on a weight basis. Such studies show us that work on industrial enzymes have parallels to the stabilization and formulation work done in the biopharmaceutical industry.

PREDICTION OF AGGREGATION PROPENSITY

Bellesia and Shea, Effect of β-Sheet Propensity on Peptide Aggregation. *J. Chem. Phys*. **2009**, 130: 145103.

Abstract: Using a coarse-grained lattice model, the effect of β-sheet propensity on phase formation was examined. Highly rigid peptides (having high β-sheet propensity) assemble into fibrillar structures, whereas peptides with lower β-sheet propensity form a variety of structures, including amorphous aggregates. These simulations suggest that decreasing β-sheet propensity will lead to reduced fibrillation and increased flexibility. Increased flexibility has been associated with increased cellular toxicity.

Analysis: Lattice models use simplified descriptions of a polymeric chain to allow for facile simulations of folding behavior. Despite their simplicity, one can vary the physical properties of each unit and obtain useful information on solution behavior. Many such studies exist in the literature. Using this methodology, this study provides some additional insight into the relative importance of β-sheet propensity in governing aggregation behavior.

SALT AND PROTEIN CONCENTRATION EFFECTS ON SPHERULITE FORMATION

Domike and Donald, Kinetics of Spherulite Formation and Growth: Salt and Protein Concentration Dependence on Proteins β-Lactoglobulin and Insulin. *Int. J. Biol. Macromol.* **2009**, 44: 301-310.

Abstract: Fibrils can aggregate into larger structures termed spherulites. The conditions favoring spherulite formation are not currently known. Monitoring spherulite growth by optical microscopy, the effect of salt and protein concentration was examined. The two model systems behaved similarly, suggesting that there is a common mechanism. The two proteins showed maximal growth rates at the same salt concentration, indicating that the salt affects colloidal properties and does not operate by a protein specific mechanism, such as anion binding. Second, spherulite growth rate increases as protein concentration increases. Lag times decrease as well. The data suggest that spherulite formation is not dependent on the spatial concentration of protein but on the ability of the protein to join the existing spherulite structure.

Analysis: Formation of supramolecular structures is a less often discussed aspect of protein aggregation. In this case, fibrils are known to assemble into larger structures called spherulites. The growth behavior of this process appears to obey similar rules for growth of soluble protein aggregates, as seen by the importance of colloidal stability and protein concentration.