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IN THIS ISSUE

OVERVIEW

The January 2017 issue of *Staying Current: Formulation of Biopharmaceuticals* contains a new feature, a focus section at the end. This one contains articles on protein oxidation and associated analytical methods. Please consider these five papers as a group on this important topic. In addition, we have a wide range of articles, including many on protein aggregation. Some of the articles this month challenge our current thinking about various mechanisms of stabilization or possible improper usage of a common method, so please pay close attention to those papers and read them carefully. As always, there is a diverse group of articles from around the world for you to consider.

Staying Current
Formulation of Biopharmaceuticals

SILICONE OIL MIGRATION

Funke et al., Silicone Migration from Baked-On Silicone Layers. Particle Characterization in Placebo and Protein Solutions. *J. Pharm. Sci.* **2016**, 105: 3520-3531.

Abstract: The degree of sloughing of silicone oil particles from syringes where the silicone oil was baked-on was examined using resonant mass measurement, micro-flow imaging, and light obscuration. The degree of droplet formation after agitation correlated with the amount of silicone oil applied to the barrel and the levels were comparable in placebo and IgG1-containing solutions both with 0.04% PS 20 present. Headspace substantially increased the formation of silicone oil droplets during agitation. Overall, the particle levels were quite low compared to syringes where the silicone oil was sprayed-on. It appears that baked-on silicone oil at levels of ≤ 100 µg limits silicone oil migration into protein products.

Analysis: This work was a collaboration between Roche and Ludwig-Maximilians Universität in Munich. It describes the degree to which baked-on silicone oil migrates into drug products and finds that the protein does not affect the migration and that the silicone oil was much more stable to agitation than with sprayed-on silicone syringes.

AGGREGATION OF p53

Cino et al., Aggregation Tendencies in the p53 Family Are Modulated by Backbone Hydrogen Bonds. *Sci. Rep.* **2016**, 6: 32535.

Abstract: The p53 family is composed of p53, p63, and p73. It was found that the DNA-binding domain (DBD) of p53 aggregates more quickly and to a greater extent than for the other two proteins. The aggregation propensity was strongly correlated to thermal stability. Molecular dynamics (MD) simulations indicate that the structural heterogeneity that leads to lower conformational stability is due to the increased incidence of exposed backbone hydrogen bonds.

Analysis: This article from Brazil finds that aggregation tendency correlates with conformational stability, but using MD simulations identifies the specific structural feature that is likely the root of the lower stability, the prevalence of exposed backbone hydrogen bonds.

AGGREGATE QUANTITATION USING AUC

Wafer et al., Quantifying Trace Amounts of Aggregates in Biopharmaceuticals Using Analytical Ultracentrifugation Sedimentation Velocity: Bayesian Analyses and *F* Statistics. *AAPS J.* **2016**, 18: 849-860.

Abstract: When estimating the aggregate content of samples using analytical ultracentrifugation sedimentation velocity (AUC-SV), a software package called SEDFIT is often used. This article examines the bases for the SEDFIT algorithm and how those may affect quantitation of the aggregate levels of a protein solution. An explicit Bayesian approach, as implemented by SEDFIT, was found to be largely correct. Guidelines are provided for proper utilization of SEDFIT, especially for heterogeneous samples (as with different glycoforms). Numerous examples using a monoclonal antibody are provided to illustrate the strengths and weaknesses of these approaches.

Analysis: This work comes from Pfizer and offers a critical examination of the most common software package used to analyze AUC-SV data. This will aid in guiding anyone involved in the use of AUC for aggregation characterization, especially if the aggregate levels are low or if the sample displays microheterogeneity.

WATER REARRANGEMENT IN AMYLOID FIBRILS

Arya et al., Water Rearrangements upon Disorder-to-Order Amyloid Transition. *J. Phys. Chem. Lett.* **2016**, 7: 4105-4110.

Abstract: Monomeric κ-casein adopts a disordered, but compact, state under physiological conditions. This form is capable of spontaneously forming fibrils. It was determined that some water "biological water") was restrained during amyloid formation. Furthermore, there was ordering of trapped water as the fibrils underwent a disorder-to-order transition. These results illustrate the role that water confinement and chain desolvation play in fibril formation.

Analysis: We are seeing more studies examine the role of hydration and water ordering in protein aggregation. This study from groups in India examines the changing mobility of water as fibrillation of casein proceeds.

QSAR EXCIPIENT SCREENING

Oyetayo et al., Diversity Selection, Screening and Quantitative Structure-Activity Relationships of Osmolyte-Like Additive Effects on the Thermal Stability of a Monoclonal Antibody. *Eur. J. Pharm. Sci.* **2017**, 97: 151-157.

Abstract: A library of 79 diverse compounds was screened for their ability to increase the thermal stability of a monoclonal antibody (MAb) at three distinct pH values (3.5, 5.2, 6.5). Quantitative structure-activity relationships (QSAR) were derived for individual compound classes. Excipients containing a phenyl ring tended to reduce Tm, while polar compounds capable of forming at least two hydrogen bonds were stabilizers. Polyols were found to stabilize at all pH values, while the effect of amino acids was pH-dependent. This kind of a QSAR approach could serve to direct future research on protein stabilization by additives.

Analysis: This paper is a collaboration between the University of Cambridge in the UK and Biberach University in Germany. It evaluated a large number of excipients and tried to identify what structural properties would lead to conformational stability of a MAb, which could be advantageous in the search for novel protein stabilizers.

X-RAY SCATTERING OF MAbs

Inouye et al., Utility of Solution X-Ray Scattering for the Development of Antibody Biopharmaceuticals. *J. Pharm. Sci.* **2016**, 105: 3278-3289.

Abstract: X-ray scattering was measured for solutions of three monoclonal antibodies (MAbs), one of which was available as glycosylated and deglycosylated forms. Samples were evaluated over a concentration range from 0.5 mg/ml to > 168 mg/ml. Not only does x-ray scattering provide structural information, but it can be used to determine colloidal stability, providing A_2 values (and higher order virial coefficients) as a function of protein concentration. It was observed that A2 values exhibited a non-linear dependence, meaning that measurements made in dilute solution may not reflect colloidal stability at higher concentrations.

Analysis: This study from Biogen and Northeastern University demonstrates the utility of solution x-ray scattering in gathering information on colloidal stability directly in high concentration formulations of MAbs, making it one of the few techniques that can easily measure A_2 values at >100 mg/ml.

INSULIN AMYLOID AT INJECTION SITES

Nilsson, Insulin Amyloid at Injection Sites of Patients with Diabetes. *Amyloid* **2016**, 23: 139-147.

Abstract: The formation of amyloid fibrils at the injection site of patients receiving repeated injections of insulin is reviewed. The number of reported cases has increased markedly since 2002. The reasons for this increase are not known, but may be due to increased awareness, changes in delivery systems, or a wider range of insulin products.

Analysis: This review comes from McDaniel College in Maryland. It examines the formation of insulin aggregates at the injection site in diabetic patients. The possible underlying causes for an increase in amyloid formation are discussed in detail.

STABILIZATION OF β-D-GALACTOSIDASE

Wahba, Thermostabilization of *Aspergillus oryzae* β-D-Galactosidase. *Biotechnol. Appl. Biochem.* **2016**, 63: 546-552.

Abstract: The thermal stability of β-galactosidase from *A. oryzae* was found to be highest at pH 6 in 20 mM phosphate buffer. Addition of various sugars yielded greater retention of activity when the samples were heated at 60 C for 65 minutes. Of all of the sugars evaluated, galactose had the greatest effect, presumably due to string binding to the native state of the protein. Raffinose was also stabilizing, but it was unclear if this was due to preferential exclusion or direct ligand binding to the enzyme.

Analysis: This study comes from two groups in Cairo, Egypt. They found that addition of galactose, a substrate for the enzyme, provides increased thermal stability, even in an optimized buffer. Ligand binding to the native state is one mechanism for conformational stabilization of a protein, as illustrated here.

PROTEIN-PROTEIN INTERACTIONS FROM DIFFUSIVITY

Sorret et al., Challenges in Predicting Protein-Protein Interactions from Measurements of Molecular Diffusivity. *Biophys. J.* **2016**, 111: 1831-1842.

Abstract: It has become routine to use dynamic light scattering (DLS) to determine k_D values, which indicate the colloidal stability of a protein in a particular formulation. A theoretical assessment, described herein, indicates that the diffusivity, the basis for calculating k_D values, cannot be reliably determined if (a) the ionic strength is low or (b) if the protein concentration is too high. Consequently, k_D values measured under these conditions may not accurately reflect the actual extent of protein-protein interactions.

Analysis: This work comes from the University of Colorado. It provides us with a critical examination of the use of DLS to determine k_D values, which indicate colloidal stability. As they show, the k_D values become suspect when the ionic strength is low or when the protein concentration is relatively high. Thus, this work serves as a guideline for the proper use of DLS for measuring colloidal stability.

MEASURING PROTEIN UNFOLDING AT THE AIR-LIQUID INTERFACE

Leiske et al., A Method to Measure Protein Unfolding at an Air-Liquid Interface. *Langmuir* **2016**, 32: 9930-9937.

Abstract: Few techniques exist to measure conformational changes of proteins at the air-liquid interface. It was discovered that the dye, Nile Red, sows low background fluorescence of monoclonal antibodies (MAbs) in solution and at the glass-liquid interface. However, the fluorescence intensity increases substantially after adsorption to the air-liquid interface. It appears that the hydrophobicity at the interface affects the fluorescence behavior of the dye. Interfacial hydrophobicity may indicate native or folded protein, while a time evolution of the fluorescence appears to be consistent with unfolding and subsequent aggregation. Thus, this technique may provide an alternative methodology for measuring hydrophobicity changes of proteins, one that requires very little material and minimal sample preparation.

Analysis: This method was developed at Genentech. It may end up providing a

convenient approach to evaluating behavior of MAbs at the air-liquid interface, even allowing one to rank order the sensitivity of a protein or formulation to interfacial unfolding and aggregation.

PROTEIN STABILIZATION BY CHARGED AMINO ACIDS

Platts et al., Control of Globular Protein Thermal Stability in Aqueous Formulations by the Positively Charged Amino Acid Excipients. *J. Pharm. Sci.* **2016**, 105: 3532-3536.

Abstract: The effects of charged amino acids on the thermal stability of three model proteins (myoglobin, BSA, lysozyme) were evaluated. Both lysine (Lys) and histidine (His) were found to affect stability in a concentration-dependent manner by mechanisms that appear to similar to what is seen with arginine (Arg). Interaction with glycine residues appears to be an important facet of the effects of these compounds with proteins. Overall, all three charged amino acids (Arg, Lys, His) were found to reduce thermal stability (i.e., lower T_m) in a protein- and concentration-dependent fashion.

Analysis: This study from the University of Sheffield in the UK seeks to identify the molecular mechanisms of stabilization or destabilization of protein by charged amino acids and related compounds, like imidazole. It may be that one would select a molar ratio of the amino acid to protein rather than a specific bulk concentration to achieve the desired effect, but more work on protein-excipient interactions is required for these important excipients.

OSMOLYTE STABILIZATION OF PROTEINS

Panuszko et al, General Mechanism of Osmolytes' Influence on Protein Stability Irrespective of the Type of Osmolyte Cosolvent. *J. Phys. Chem. B* **2016**, 120: 11159-11169.

Abstract: The hydration sphere of a stabilizing osmolyte is similar to that of a protein, while those of destabilizing osmolytes are markedly different. This study finds that no relationship exist between stabilization by an osmolyte and its ability to make or break water structure. Moreover, their accumulation at the protein surface also does not seem to determine the degree of stabilization. The explanation appears to reside in the similarity of water properties around the protein and the osmolyte.

Analysis: This article from Gdansk University of Technology in Poland is worth careful consideration, as it calls into question a long-standing hypothesis on protein stabilization, namely stabilization due to preferential exclusion. Furthermore, it departs from the ksomotrope/chaotrope language of the Hofmeister effect. Rather the focus is on water structure and the similarity between proteins and stabilizing osmolytes. It will be most interesting to see if this hypothesis proves to be the basis for stabilizing of proteins by a wide range of excipients, which is at the heart of protein formulation.

DECISION MAKING USING HOS DATA

Weiss et al., Technical Decision Making with Higher Order Structure Data: Perspectives on Higher Order Structure Characterization from the Biopharmaceutical Industry. *J. Pharm. Sci.* **2016**, 105: 3465-3470.

Abstract: Determination of higher order structure (HOS) has become an essential feature in the characterization of therapeutic proteins. This commentary examines the proper use of HOS information in the development cycle of biotherapeutics. Strategies need to be developed that are specific to the protein being examined. At the same time, consistent HOS approaches for all therapeutic proteins need to be developed and applied.

Analysis: This commentary article has authors from a number of large companies engaged in the development of biopharmaceuticals. It provides a current overview of HOS characterization and how this information can and should be used to further drug development activities.

OSMOLYTE EFFECTS ON FIBRIL GROWTH

Muttathukattil and Reddy, Osmolyte Effects on the Growth of Amyloid Fibrils. *J. Phys. Chem. B* **2016**, 120: 10979-10989.

Abstract: The formation of amyloid fibrils by the amyloidogenic peptide, NNQQNY, in the presence of trimethylamine N-oxide (TAMO) was studied using molecular dynamics (MD) simulations. It was determined that TMAO stabilizes the locked state of the peptide, where it is strongly bound to the fibril. In particular, there appears to be a stronger interaction with the Tyr residue via the methyl groups on the osmolyte. However, the overall increased stability of the protofibril in the presence of TMAO is due to entropic or indirect interactions with the backbone Gln and Asn residues.

Analysis: As we have stated many times before, theoretical methods, like MD, can provide us with molecular level understanding that may be difficult to obtain experimentally. This study from the Indian Institute of Science illustrates an important facet of excluded solutes (i.e., osmolytes). Excluded solutes, such as TMAO, push the system towards states with the smallest surface area, which, in this case, is the locked form bound to the fibril. Thus, osmolytes can facilitate aggregation, if that form possesses a smaller overall surface area than the native or folded state.

PHASE SEPARATION IN LYO-PHILIZED PROTEIN FORMULATIONS

Izutsu et al., Amorphous-Amorphous Phase Separation of Freeze-Concentrated Protein and Amino Acid Excipients for Lyophilized Formulations. *Chem. Pharm. Bull.* **2016**, 64: 1674-1680.

Abstract: Frozen solutions of a protein (HAS, gelatin) or a polymer (dextran) with an amino acid (various forms of arginine or sodium glutamate) were prepared at various mass ratios. While polymer rich formulations display only a single T_g' value, amino acid-rich compositions often showed two distinct T_g' values, indicative of phase separation. Annealing was able to induce phase separation in formulations with intermediate compositions. The exact behavior was dependent on thermal history, salt types and the presence of other solutes, such as NaCl. This information should be considered when preparing lyophilized protein formulations containing substantial amounts of an amino acid excipient.

Analysis: This study from the National Institute of Health Sciences in Japan is a detailed study of phase separation in the frozen state when using amino acids, especially arginine, in lyophilized protein formulations. As there has been increased interest in Arg-based formulations, a detailed study, such as this one, could be quite helpful in guiding the final composition.

FOCUS AREA: PROTEIN OXIDATION AND ANALYSIS

OXIDATION OF CDRs IN MAbs

Dashivets et al., Oxidation in the Complementarity-Determining Regions Differentially Influences the Properties of Therapeutic Antibodies. *MABS* **2016**, 8: 1525-1535.

Abstract: Oxidation of Trp residues within the CDRs was found to affect the bioactivity of two different monoclonal antibodies (MAbs) to varying degrees. Of the three MAbs evaluated in this study, there were differences in the stability, activity, and even in the primary degradation pathways for antibodies susceptible to oxidation.

Analysis: This study is from various groups within Roche as well as researchers at the Technische Universität in Munich. It describes how Trp residues within CDRs are susceptible to oxidation and that chemical modification can have a profound impact on antigen binding.

Met OXIDATION OF Aβ(1-40)

Gu and Viles, Methionine Oxidation Reduces Lag-Times for Amyloid-β(1-40) Fiber Formation but Generates Highly Fragmented Fibers. *Biochim. Biophys. Acta* **2016**, 1864: 1260-1269.

Abstract: Oxidation of Met^{35} in Aβ(1-40) reduces the lag times for fibrillation but extends it for Aβ(1-42). There is an overall reduction in fiber length, leading to a more fragmented assembly of fibrils. Met oxidation does not affect the morphology of preformed fibrils.

Analysis: The impact of Met oxidation on the fibrillation of Aβ(1-40) appears to be different than the effect on the 1-42 variant. In addition, the fibers are highly fragmented (i.e., shorter in length). This work from the University of London shows how Met oxidation can have different effects on aggregation behavior, even for highly related polypeptides.

METAL-CATALYZED OXIDATION OF Aβ

Cheignon et al., Metal-Catalyzed Oxidation of Aβ and the Resulting Reorganization of Cu Binding Sites Promote ROS Production. *Metallomics* **2016**, 8: 1081-1089.

Abstract: The metal-catalyzed oxidation (MCO) of Aβ peptide targets residues involved in binding of copper ions, such as His, resulting in •OH production in the presence of ascorbate. Oxidative damage then alters copper coordination. In turn, this leads to increased oxidation of the peptide once the Aβ peptide is sufficiently oxidized.

Analysis: This study from France describes Cu-catalyzed MCO of Aβ in the presence of ascorbate. As oxidation proceeds, the coordination sites change and the oxidation increases even more. The article illustrates how free radical scavengers, like ascorbate, can actually facilitate and enhance MCO. It also teaches that the degree of oxidation depends on the nature of the metal binding site.

OXIDATION OF ETANERCEPT

Huang et al., Characterization and Comparability of Stress-Induced Oxidation and Deamidation on Vulnerable Sites of Etanercept Products. *J. Chrom. B* **2016**, 1032: 189-197.

Abstract: The chemical stability of etanercept was studied by examining deamidation and oxidation of Enbrel and a biosimilar, TuNEX. The rates of chemical instability were the same despite a two amino acid difference in the sequence of the heavy chain (Fc). The Met residues had basal levels of oxidation between 0 and 7% in both proteins. These levels increase to 42-100% upon exposure to 5% t-butyl hydroperoxide (TBHP). Oxidation did not affect the bioactivity, but forced deamidation did reduce the potency of both forms of etanercept.

Analysis: The chemical stability of etanercept was studied by this group from Mycenax and National Cheng Kung University in Taiwan. The proteins are not identical, but the chemical stability profiles were comparable. It was determined that oxidation, even at very high levels, did not impact the potency.

RP-UPLC-MS METHOD FOR MAb ANALYSIS

Zhang et al., Development of a Rapid RP-UHPLC-MS Method for Analysis of Modifications in Therapeutic Monoclonal Antibodies. *J. Chrom. B* **2016**, 1032: 172-181.

Abstract: A rapid UPLC reversed phase (RP) method is presented following MAb digestion with the IdeS enzyme, which produces MAb fragments that are ~25 kD in size. Within 10 minutes, efficient separation of native protein form variants, including Fc oxidized species, is possible. The development of the method is described in detail and may serve as an alternative to peptide mapping.

Analysis: The final article in our focus section describes a new RP-UPLC method from Genentech that is compatible with mass spectrometry and may present an alternative to peptide mapping for monitoring oxidation of MAbs.

CORRECTION

In last month's issue, we reviewed a paper by Brückl et al., (Brückl et al., A Systematic Evaluation of Mechanisms, Material Effects, and Protein-Dependent Differences on Friction-Related Protein Particle Formation in Formulation and Filling Steps. *Int. J. Pharm.* **2016**, 511: 931-945). Dr. Brückl wrote to correct mistakes we had made in summarizing their work. In particular, the authors wish to note that "the use of a hydrophobic sliding material in combination with a surfactant did not lead to the greatest degree of particle formation, instead to the smallest. Also, PTFE bearing with a surfactant showed less particle than SiC." Also, there was a typographical error and the surfactant should have been listed as polysorbate 80 (PS 80).

We sincerely apologize for these shortcomings in the description of this study. We encourage our readers to read this paper for themselves, as we believe it is a noteworthy article.