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OVERVIEW

This month's issue contains a mixture of articles, where we cover topics related to chemical instability, aggregation, and lyophilization. There are also some helpful articles on drug delivery devices as well as some interesting analytical methodologies. We hope you find this selection useful and interesting.

Staying Current

Formulation of Biopharmaceuticals

MODIFICATION OF MONOCLONAL ANTIBODIES BY CITRATE

Chumsae et al., Discovery of a Chemical Modification by Citric Acid in a Recombinant Monoclonal Antibody. *Analytical Chem.* **2014**, 86: 8932-8936.

Abstract: It was discovered that citric acid can covalently modify a recombinant monoclonal antibody (MAB) in a citrate/phosphate buffered solution at pH 5.2. The resulting acidic species showed mass increases of 174 and 156 Da, respectively. Mass spectrometry revealed that the N-terminus of the light chain has been modified. Three other antibodies showed the same reactivity, but to different extents. The two products appear to arise from nucleophilic attack of the N-terminal amine on the anhydride form of citric acid.

Analysis: This study from AbbVie, in collaboration with colleagues at Northeastern University, has identified a novel chemical modification arising from the reaction of MAbs with citrate under acidic conditions. The reactivity is such that the same type of reaction could occur with other carboxylate buffers, like succinate.

DENATURATION AND AGGREGATION OF AN IgG1

Cerasoli et al., Temperature Denaturation and Aggregation of a Multi-Domain Protein (IgG1) Investigated with an Array of Complementary Biophysical Methods. *Analytical Bioanalytical Chem.* **2014**, 406: 6577-6586.

Abstract: A variety of biophysical techniques (DSC, FTIR, CD, fluorescence, DLS) were used to follow the thermal denaturation and subsequent aggregation of an IgG1. This information was used to identify the conformational changes leading to aggregation. In the case of this IgG1, unfolding of the C_{H2} domain appears to lead to formation of soluble oligomers. The thermal denaturation of the second domain leads to assembly of these nuclei into larger aggregates.

Analysis: This work from groups in the UK examines the conformational changes associated with progressive unfolding and aggregation of a monoclonal antibody. There are some details here one might find helpful when considering how conformational changes are related to aggregate formation.

EPIMERIZATION BY TANDEM LC-MS

Tao and Julian, Identification of Amino Acid Epimerization and Isomerization in Crystallin Proteins by Tandem LC-MS. *Analytical Chem.* **2014**, 86: 9733-9741.

Abstract: While isomerization of an amino acid does not produce mass shift, it is possible to identify racemized or epimerized amino acids using mass spectrometry. Epimerization of aspartate groups in crystallins from sheep eye lens was detected using tandem mass spectrometry after LC separation. Numerous sites of isomerization were identified,

including ones never previously reported. For α -crystallins, the greatest degree of isomerization appears to occur within disordered regions of the protein.

Analysis: This article from UC-Riverside reveals how tandem LC-MS using a combination of radical directed dissociation and collision induced dissociation can be employed to identify isomerized or epimerized amino acids within large proteins. Moreover, the correlation between reactivity and chain flexibility is worth noting.

Asp ISOMERIZATION AND PROTEIN DISSOCIATION

Sakaue et al., Alpha B- and β A3-Crystallins Containing D-Aspartic Acid Exist in a Monomeric State. *Biochim. Biophys. Acta* **2015**, 1854: 1-9.

Abstract: Isomerization of Asp residues in crystallins from eye lens have been investigated for some time. In this study, there was relatively little isomerization on γ -crystallins, but Asp isomerization in both α - and β -crystallins leads to a lower apparent molecular weight. Therefore, it appears that formation of D-Asp residues leads to dissociation of oligomeric states and production of monomeric species. This is the first report that stereoinversion can alter protein assembly state in aged human lens.

Analysis: The second article this month on isomerization/epimerization indicates that this particular chemical modification can lead to dissociation of a protein that normally forms oligomers or polymers. This study from Japan illustrates how chemical modification can affect physical stability and association state.

ANTIBODY-LOADED NANOPARTICLES

Patel et al., A Novel Approach for Antibody Nanocarriers Development through Hydrophobic Ion-Pairing Complexation. *J. Microencapsulation* **2014**, *31*: 542-550.

Abstract: A Fab fragment of an IgG was complexed with three different sulfated ion-pairing agents. The formation of hydrophobic ion-paired (HIP) complexes was dependent on pH and the molar ratio of the ion-pairing agent. All of the HIP complexes exhibited reduced solubility in water. The dextran sulfate complex has the lowest aqueous solubility. Encapsulation into nanoparticles (NPs) was more efficient with the HIP complexes than with the protein itself. This demonstrates that HIP complexation may be an effective approach for preparation of antibody-loaded NPs.

Analysis: Delivery systems for antibodies are an area of great interest. In this study from UMKC, we see how HIP modulates the solubility of the protein to allow it to be more efficiently incorporated into a drug delivery system.

FIBRIL POLYMORPHISM

Tycko, Physical and Structural Basis for Polymorphism in Amyloid Fibrils. *Protein Sci.* **2014**, *23*: 1528-1539.

Abstract: While all amyloid fibrils do have common structural features, they can vary leading to the observation of fibril polymorphism. This review describes the current understanding about what molecular properties underlie fibril polymorphism and how solution conditions can lead to differences in fibrillar structure. While the article focuses primarily on fibrils formed by

A β peptides, the same principles apply to other amyloid-forming peptides and proteins.

Analysis: This review from Dr. Tycko at NIH summarizes the growing field of fibril polymorphism. While this is usually observed in peptides, it has been seen with proteins as well. In some cases, multiple forms of fibrils from the same polypeptide can be observed in solution. It is helpful for us to have a review on what we currently understand about this aspect of aggregation behavior.

MAB AGGREGATION IN CELL CULTURE

Paul et al., Direct Analysis of mAb Aggregates in Mammalian Cell Culture Supernatant. *BMC Biotechnology* **2014**, *14*: article 99.

Abstract: Currently, the extent of protein aggregation in cell culture is measured by using SEC after a previous affinity chromatography step. Yet, it is unknown if this treatment alters the measured degree of aggregation. In this work, it is shown that SEC can be used directly and will separate MAb monomer and aggregate from cell culture components. This method was used for a MAb-producing CHO cell line. It was found that over 75% of the MAb was present as a dimer and larger oligomers, much higher than what had been previously reported. This suggests that aggregate formation needs to be addressed directly in cell culture and not downstream.

Analysis: This study from Biberach University in Germany sheds some light on an important issue in drug substance preparation, namely the extent to which proteins aggregate in culture. Not only is this a helpful analytical article, it illustrates there is much to learn about MAb aggregation during upstream processing.

LYOPHILIZATION OF hEGF

Santana et al., Stabilization of a Recombinant Human Epidermal Growth Factor Parenteral Formulation through Freeze-Drying. *Biologicals* **2014**, 42: 322-333.

Abstract: Development of a stable lyophilized formulation of human epidermal growth factor (hEGF) is described. Freeze-drying microscopy was employed in the course of excipient selection. The highest stability was achieved by formulation with sucrose, dextran, raffinose, or trehalose. Lyophilization cycles run with and without annealing were evaluated. It was determined that the most stable formulation was comprised of a mixture of sucrose and dextran. The degradation rate at 37° C was about 100-fold less for the lyophilized formulation than for the corresponding formulation in aqueous solution.

Analysis: Here we have a case study from groups in Cuba describing the development of a stable freeze-dried formulation of hEGF.

HIC ANALYSIS OF MABs

Haverick et al., Separation of mAbs Molecular Variants by Analytical Hydrophobic Interaction Chromatography HPLC- Overview and Applications. *mAbs* **2014**, 6: 852-858.

Abstract: Hydrophobic interaction chromatography (HIC) can resolve a wide variety of species, including chemically modified versions, misfolded structures, and aggregates. This is true for monoclonal antibodies (MABs) as well as Fc fusion proteins. A number of examples are provided illustrating how HIC can resolve degradation products due to Trp oxidation, Asp isomerization, and serine fucosylation, to name a few. A summary of the critical

method parameters for HIC are provided as well.

Analysis: This work from Gilead is a very helpful article on HIC as it applies to MABs and fusion proteins. Not only are there helpful case studies, but this is a great resource on how to develop and optimize HIC methods.

PROTECTED HINGE IN IgG2-A₂

Liu et al., Protected Hinge in the Immunoglobulin G2-A₂ Disulfide Isoform. *Protein Sci.* **2014**, 23: 1753-1764.

Abstract: There are three predominant disulfide isoforms in IgG2 monoclonal antibodies (MABs). The structural basis for the differences between the A₁ and A₂ isoform subtypes was investigated. While A₁ converts into the A/B and B isoforms, the A₂ subtype does not. While these two subtypes appear to have the same disulfide linkages, the disulfide associated with the upper hinge in the A₂ form is more restricted and protected against reduction. By comparison, the same disulfide is rapidly reduced in the A₁ subtype. The flexibility of the upper hinge region in the IgG2-A₂ isoform is more similar to the interior of a globular protein than the flexible hinge region of a MAB.

Analysis: This study from Amgen demonstrates how there is still much to be discovered about the behavior of monoclonal antibodies. A particular isoform subtype of an IgG2 is described that has a more rugged stability profile than the closely related A₁ form. Understanding these subtle differences should lead us to more robust MABs as drug candidates.

EXTRACTABLES IN PREFILLED SYRINGES

Jenke, Extractables and Leachables Considerations for Prefilled Syringes. *Exp. Opin. Drug Deliv.* **2014**, 11: 1591-1600.

Abstract: Incompatibilities between drug products and prefilled syringes are reviewed, as they can impact product stability, safety, and efficacy. The impact on protein products is highlighted, as the traditional approaches to leachables and extractables must be accompanied by rigorous compatibility testing.

Analysis: Prefilled syringes (PFS) have become the preferred option for many biopharmaceutical products, so it is important to understand their performance and how that might affect the stability profile of your product. This review from Dr. Jenke at Baxter provides us with quite a bit of information on issues associated with leachables and extractables.

IMPROVING PROTEIN SOLUBILITY

Johnson et al., Protein Solubilization: A Novel Approach. *J. Chrom. B* **2014**, 971: 99-106.

Abstract: Protein-protein interactions affect solubility, aggregation behavior, and viscosity. The thermodynamic parameter measuring protein-protein interactions is the osmotic second virial coefficient (B value). In this study, B values were determined using self-interaction chromatography (SIC). The first phase involved looking at individual excipients, while a smaller second phase examined combinations of additives. From these data, an artificial neural network (ANN) was developed and trained that was able to make predictions for more than 4000 new formulations. Optimization of B values was

shown to increase the solubility and reduce aggregation of an IgG with poor solubility.

Analysis: This work from the University of Alabama-Birmingham and Mississippi State University uses SIC to measure B values for various compositions. The trained ANN was then employed to identify the optimal conditions for increased solubility. While some of the optimal compositions are not viable for pharmaceutical products, the approach is worth noting, as protein-protein interactions govern many critical physical properties of proteins in aqueous solution.

AGGREGATION OF α 1-ACID GLYCOPROTEIN

Iwura et al., Conformational Stability, Reversibility and Heat-Induced Aggregation of α -1-Acid Glycoprotein. *J. Biochem.* **2014**, 156: 345-352.

Abstract: The conformation, thermal unfolding, and aggregation of α 1-acid glycoprotein (AGP) was investigated. The conformational stability of AGP is reduced under more acidic conditions, whereas the reversibility of denaturation is decreased at high pH values. Large aggregates are formed at lower pH, while the dimer predominates when AGP is heated to 50° C at higher pH. Thus, it appears that lower conformational stability is related to the production of larger soluble aggregates, while reversibility is related to dimer formation.

Analysis: This case study from Japan emphasizes the importance of conformational stability in controlling aggregate formation, but also indicates the role of the reversibility of unfolding in oligomerization as well.

QUANTITATION OF SUCCINIMIDE

Klaene et al., *Detection and Quantitation of Succinimide in Intact Protein via Hydrazine Trapping and Chemical Derivatization. J. Pharm. Sci.* **2014**, 103: 3033-3042.

Abstract: Formation of succinimide is a common post-translational modification that occurs under acidic conditions. A method for detection and quantitation of succinimide in intact proteins is described involving trapping with hydrazine and direct analysis by mass spectrometry. The resultant hydrazine can also be derivatized to allow detection by UV absorbance or fluorescence spectroscopy. Fluorescence labeling of the hydrazide allows one to detect less 0.5% of succinimide in an intact protein.

Analysis: This collaboration between Washington State University and Northeastern University provides us with a new methodology to quantify succinimide formation, which can be difficult given its propensity to convert to other degradation products. The ability to label the hydrazine and then detect succinimide formation using spectrophotometric methods is quite advantageous as well.

MICROWAVE-ASSISTED DIGESTION

Formolo et al., *Analysis of Deamidation Artifacts Induced by Microwave-Assisted Tryptic Digestion of a Monoclonal Antibody. Analytical Bioanalytical Chem.* **2014**, 406: 6587-6598.

Abstract: Peptide mapping of a biopharmaceutical protein is a powerful tool, both for identification, but also to detect chemical modifications. Deamidation of asparagines (Asn) residues during tryptic digestion has been reported. Since microwave-assisted procedures are often used to shorten digestion times, the impact of this

approach on deamidation artifacts in a MAB was examined. It was discovered that the microwave process did not introduce any additional artifacts. In addition, it appears that using higher temperatures (with or without microwave assistance) did lead to an increase in the number of missed cleavage sites compared to overnight digestion at room temperature.

Analysis: As peptide mapping is such a common procedure for determining product quality and stability, this article from NIST is helpful in determining whether certain tryptic digestion conditions are actual improvements over the usual overnight digestion process.

PULMONARY DELIVERY OF BIOLOGICS

Hoe et al., *Manufacturing and Device Options for the Delivery of Biotherapeutics. J. Aerosol Med. Pulmon. Drug Deliv.* **2014**, 27: 315-328.

Abstract: Aerosol delivery to the lungs can be used for both systemic as well as local drug delivery. For biotherapeutics, manufacturing and device selection are critical. Four case studies are presented, primarily focusing on spray drying of bacteriophages. The focus is on stresses that can occur during spray drying, modeling droplet behavior and processing, formulation development, and activity of the final dried powder.

Analysis: This overview from the University of Alberta in Canada examines the production of spray-dried biotherapeutics for delivery to the lung. Inhalation is an alternative to parenteral administration and this article provides some helpful insights into manufacturing issues as well as device selection.

TRANSDERMAL PEPTIDE DELIVERY

Namjoshi et al., Enhanced Transdermal Peptide Delivery and Stability by Lipid Conjugation: Epidermal Permeation, Stereoselectivity and Mechanistic Insights. *Pharm. Res.* **2014**, 31: 3304-3312.

Abstract: Using the tetrapeptide, AAPV, an elastase inhibitor, as a model, the effect of coupling to short chain lipoamino acids (Laa: C6-C10) on transdermal delivery was investigated. The Laas increased permeation into the skin. In addition, the D-diastereomer was favored for permeation enhancement. The Laa-peptides were found to be surface active. Overall, the Laa conjugation approach appears to be useful for enhancing the permeation of moderately sized peptides, especially for treatment of skin disorders.

Analysis: This work on using lipid conjugation to modify peptides to enhance skin permeation was done in Australia. It provides us with another option for developing peptide drugs, especially for local delivery in the skin, but possibly also to enhance systemic delivery.

FREEZE-DRYING OF HSA NANOPARTICLES

Dadparvar et al., Freeze-Drying of HI-6-Loaded Recombinant Human Serum Albumin Nanoparticles for Improved Storage Stability. *Eur. J. Pharm. Biopharm.* **2014**, 88: 510-517.

Abstract: HI-6 is an acetyl cholinesterase reactivator used to treat organophosphate intoxication. HI-6-loaded nanoparticles comprised of HSA allow this drug to increase transport across the blood-brain barrier. As HI-6 is moisture sensitive, the formulation was freeze-dried. Trehalose and sucrose proved to be superior to mannitol in protecting the nanoparticles. Trehalose-based

formulations were placed on stability at storage temperatures between -20° C and 40° C. The shelf-life at -20° C was determined to be > 18 months.

Analysis: This case study from Germany provides some guidance on how to formulate drug-loaded HSA nanoparticles that are prepared as dried powders. Protein-based nanoparticles being explored more for delivery of small molecules, as in this case, and proper formulation is critical.

MONITORING MAb AGGREGATION USING RAMAN SPECTROSCOPY

de la Cuesta et al., Monitoring Antibody Aggregation in Early Drug Development using Raman Spectroscopy and Perturbation-Correlation Moving Windows. *Analytical Chem.* **2014**, 86: 11133-11140.

Abstract: Five antibodies with different aggregation propensities were examined using two-dimensional Raman spectroscopy as a function of temperature. In particular, the perturbation-correlation moving window (PCMW) technique allows one to identify specific spectral features that correlate with variations in temperature. From this analysis, specific conformational transitions could be identified in these antibodies, both for changes in secondary structure and in terms of tertiary structure, as indicated by variations in vibrations associated with Trp residues.

Analysis: While separation techniques can quantify changes in aggregation state, it is more difficult to determine specific structural changes associated with thermal aggregation. In this case study from Lonza and the University of Manchester, 2-D Raman spectroscopy is used to examine changes that occur from 58° C to 78° C in antibodies with widely varying proclivities to unfold and aggregate.

SUPERSTRUCTURES IN PROTEIN AGGREGATION

Foderà et al., Observation of the Early Structural Changes Leading to the Formation of Protein Superstructures. *J. Phys. Chem. Lett.* **2014**, 5: 3254-3258.

Abstract: The formation of superstructures in the protein aggregation process is still incompletely understood. In this study, spectroscopic methods and microscopy were used to investigate the formation of particulates in equine lysozyme (EL). It was discovered that this particular superstructure (i.e., particulates) arises from a partially unfolded intermediate of EL where the α -domain structure remains highly native-like and the C-helix adopts a more flexible conformation, which destabilizes the neighboring β -domain.

Analysis: This work from groups in Sweden, the UK, Italy, Belgium, and Denmark gives us molecular-level details on how a partially unfolded intermediate leads to aggregation and ultimately to assembly into larger superstructures, which, in this case, is a spherical particulate.