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IN THIS ISSUE

OVERVIEW

In the January 2023 issue of *Staying Current: Formulation of Biopharmaceuticals*, the attention turns to drug delivery and excipient usage, including some novel additives. In addition, there are articles covering oxidation, aggregation, and solubility. As always, we hope coverage of these various and varied topics will assist you in your own work.

If your article is featured, we invite you, if you have not done so, to subscribe. We really appreciate everyone's support year after year, as we continue to bring you the varied aspects of the stabilization and formulation of biotherapeutics.

Staying Current
Formulation of Biopharmaceuticals

IONIC LIQUID-BASED METHOD FOR PREDICTING AGGREGATION

Shmool et al., Ionic Liquid-Based Strategy for Predicting Protein Aggregation Propensity and Thermodynamic Stability. *JACS Au* **2022**, 2: 2068-2080.

Abstract: The stability of an IgG4 monoclonal antibody (mAb) was studied in detail at varying levels of choline chloride-based ionic liquids (ILs) including after one year of storage at 4º C. In addition, molecular dynamics (MD) simulations were conducted to examine aggregation propensity in these systems. It was found that increasing the choline chloride concentration reduced aggregation propensity, both initially and after storage. Such a system could be used to determine to predict and improve the storage stability of proteins.

Analysis: This article from Imperial College London and University of Oxford uses ILs to assess the structural and conformational stabilities of an IgG4, in an effort to devise a strategy for predicting the propensity of a protein to aggregate.

DEVELOPABILITY ASSESSMENT OF mAbs

Whitaker et al., Developability Assessments of Monoclonal Antibody Candidates to Minimize Aggregation During Large-Scale Ultrafiltration and Diafiltration (UF-DF) Processing. *J. Pharm. Sci.* **2022**, 111: 2998-3008.

Abstract: A suite of biophysical methods were employed to assess the physical stability of mAb candidates in PBS. In addition, the interfacial stability of these proteins was evaluated in a Langmuir trough as well. Following these studies, stability was assessed during agitation, lab-scale UF-DF processing, and dilational stress. These were done in buffers commonly used during UF-DF processing. Together, these methods can be used to predict mAb stability during larger scale UF-DF operations.

Analysis: Researchers at the University of Kansas and BMS collaborated to investigate the use of biophysical methods and tools to predict stability profiles of mAb candidates during UF-DF processing. In particular, these studies illustrate the value of Langmuir-trough methodologies.

POLYSORBATES VS. CYCLODEXTRIN

Zhang et al., Polysorbates versus Hydroxypropyl Beta-Cyclodextrin (HPβCD): Comparative Study on Excipient Stability and Stabilization Benefits on Monoclonal Antibodies. *Molecules* **2022**, 27: 6497.

Abstract: The stability of HPβCD was compared to that of polysorbates under various stress conditions. While heat stress degraded both PS 20 and PS 80, HPβCD remained mostly (> 90%) intact. The same was true for exposure to light and oxidative stresses. The stability of adalimumab under light stress in the presence of HPβCD was found to be superior to mAb samples containing PS 80. Furthermore, HPβCD was found to reduce aggregation and particle formation for mAbs subjected to agitation and thermal stress.

Analysis: This work from Roquette examined the stability of HPβCD compared to polysorbates. In addition, they examined its ability to protect a mAb from thermal, light and interfacial stress.

USE OF POLYSORBATES

Wuchner et al., Industry Perspective on the Use and Characterization of Polysorbates for Biopharmaceutical Products Part 2: Survey Report on Control Strategy Preparing for the Future. *J. Pharm. Sci.* **2022**, 111: 2955-2967.

Abstract: Various aspects of polysorbate degradation (mechanisms, monitoring, mitigation) are reviewed in an effort to devise an appropriate control strategy.

Analysis: In the August 2022 issue of *Staying Current*, we highlighted Part 1 of this series, where representatives of sixteen different biopharmaceutical companies examined the issues surrounding the use of polysorbates in protein products. In Part 2, the literature is reviewed regarding polysorbate degradation, with the intent on devising appropriate control strategies for ensuring their integrity when being used in biopharmaceutical products.

OSMOLYTES AND MOLTEN GLOBULE STATES

Rahamtullah et al., Polyol and Sugar Osmolytes Stabilize the Molten Globule State of α-Lactalbumin and Inhibit Amyloid Fibril Formation. *Biochim. Biophys. Acta* **2022**, 1870: 140853.

Abstract: Select osmolytes (glycerol, sorbitol, trehalose) were found to inhibit fibril formation in α-lactalbumin under acidic conditions in a concentration-dependent manner, resulting in a delay in the lag phase. This delay was attributed to stabilization of a molten state, thereby hindering progression on to fibrils.

Analysis: In this study from Jawaharlal Nehru University, the ability of these excluded solutes to stabilize an intermediate conformation for α-lactalbumin provides us with a different perspective in how sugars and polyols could be effective at slowing aggregation processes.

pH EFFECTS ON INSULIN AGGREGATION

Thorlaksen et al., Subtle pH Variation around pH 4.0 Affects Aggregation Kinetics and Aggregate Characteristics of Recombinant Human Insulin. *Eur. J. Pharm. Biopharm.* **2022**, 179: 166-172.

Abstract: Subtle changes in pH from 4.1 to 4.3 has a profound effect on the particulate formation kinetics of human insulin. Moreover, these small changes in pH affects particle sizes as well. Differences in secondary structure were also noted. These studies illustrate the potential impact of pH on the stability of protein drug products and its role in quality control.

Analysis: Some systems are exquisitely sensitive to small variations in pH, such as this case study from Novo Nordisk, University of Copenhagen, and Leiden University on insulin aggregation.

HSA MITIGATES FFA PARTICLE FORMATION

Kim et al., Human Serum Albumin Mitigates Formation of Fatty Acid Particles in Polysorbate-Containing Solutions. *J. Pharm. Sci.* **2022**, 111: 3185-3188.

Abstract: Degradation of polysorbates leads to production of free fatty acids (FFAs), which can form particulates in protein formulations. In this study, HSA was added and found to prevent particle formation of FFAs. Moreover, it was determined that HSA can solubilize pre-formed particles as well. These observations are consistent with previous studies on the fate of FFAs in human plasma.

Analysis: This work from Regeneron examines the role that HSA can and does play in mitigating particles formed by the FFAs that arise from polysorbate degradation. These observations indicate that low levels of particles in the final drug product should be of little concern, given the ability of HSA to solubilize these species.

NOVEL VACCINE FORMULATION

Li et al., Enhanced Stability of Foot-and-Mouth Disease Vaccine Antigens with a Novel Formulation. *Pharm. Dev. Technol.* **2022**, 27: 759-765.

Abstract: It was determined that the 146S vaccine against foot-and-mouth disease (FMD) was most stable in phosphate-buffered saline (PBS) buffer. A number of excipients were evaluated for their ability to stabilize 146S even more. A novel composition (5% trehalose, 5% sucrose, 0.05 M arginine, 0.01 M cysteine, 0.01 M calcium chloride, 1% HSA, 0.001 M ascorbic acid) was found to provide maximal stability, resulting in retention of 14% activity after storage of the solution at 4 C for 14 months.

Analysis: This work from groups in Langzhou, China describes the use of a sugar-based formulation that avoids the use of frozen product. It uses both trehalose and sucrose as stabilizers.

NASAL DELIVERY OF PEPTIDES

Alabsi et al., Nose-to-Brain Delivery of Therapeutic Peptides as Nasal Aerosols. *Pharmaceutics* **2022**, 14: 1870.

Abstract: Nose-to-brain delivery can be a convenient approach for transport of drugs into the central nervous system (CNS). This review examines this route of administration for peptides to target the CNS. The focus is on clinical studies, nasal delivery devices, and the advantages and limitations of this approach.

Analysis: This review from the University of Arizona and Florida International University examines nasal administration as a mode of delivering peptides to the CNS. It provides a helpful overview of a route of delivery for peptide drugs.

S.C. PROTEIN DELIVERY

Lou et al., Advanced Formulations/Drug Delivery Systems for Subcutaneous Delivery of Protein-Based Biotherapeutics. *J. Pharm. Sci.* **2022**, 111: 2968-2982.

Abstract: A number of strategies have evolved over the years to facilitate delivery of proteins via subcutaneous injections. These advances are reviewed, both from a drug delivery as well as a formulation perspective. These approaches include high concentration liquid formulations, co-formulation of two or more proteins, large volume injection, and the use of alternative formulations, like complexes and particulates/suspensions. The process of selecting and developing such delivery systems is discussed, along with shortcomings and advantages.

Analysis: The review comes from the University of Kansas and provides us with a comprehensive perspective of this important topic. It guides the reader through possible strategies with an eye on what factors need to be considered before selecting a certain approach.

STABILIZATION OF BACTERIOPHAGES

Wdowiak et al., Enhancing the Stability of Bacteriophages Using Physical, Chemical, and Nano-Based Approaches: A Review. *Pharmaceutics* **2022**, 14: 1936.

Abstract: Bacteriophages are known to lose activity and undergo degradation when stored for extended periods of time. This article reviews formulation strategies that lead to an increase in the stability of phage preparations. The focus is primarily on polymer-based approaches, along with encapsulation, lyophilization, and nanoscale formulations.

Analysis: Given the level of interest in bacteriophages, this review article from the Polish Academy of Sciences is timely and beneficial, as it reviews the various approaches that have been taken to stabilize phages, especially for longer term storage.

FIBRIL INHIBITION BY Arg

Wang et al., The Effect of Arginine on Inhibiting Amyloid Fibril Derived from β-Casein and the Binding Studies with Multi-Spectroscopic Techniques. *Spectrochim. Acta A* **2022**, 282: 121681.

Abstract: Amyloid fibrils from β-casein have been reported *in vivo*. It was found that arginine (Arg) not only inhibits fibril formation, but also leads to depolymerization of mature fibrils as well. It appears that a complex between Arg and β-casein forms, with a single binding site. The thermodynamic parameters for ligand binding were determined. Finally, the conformation of β-casein is affected by addition of Arg.

Analysis: This study from Jilin University and the Chinese Academy of Sciences finds that Arg can effectively reduce the formation of amyloid fibrils in β-casein and that the mechanism involves direct binding to the protein, resulting in net stabilization and conformation changes. This work may have implication for how Arg stabilizes other proteins as well.

STABILITY OF NIVOLUMAB

Torrente-López et al., Comprehensive Analysis of Nivolumab, a Therapeutic anti-PD-1 Monoclonal Antibody: Impact of Handling and Stress. *Pharmaceutics* **2022**, 14: 692.

Abstract: Nivolumab is the mAb active ingredient in the product Opdivo®. The stability of nivolumab was evaluated under various stress conditions using a variety of analytical techniques. Exposure to light caused the greatest degree of damage, generating dimers and different isoforms. In contrast, it was stable at 60º C for one hour. Some degradation was seen about freeze-thaw cycling.

Analysis: This forced degradation study from groups in Grenada, Spain provides us with a case study on the sensitivity of a marketed mAb to various stress conditions. The study found flight to cause the greatest degree of degradation, causing both chemical and physical instability.

SOLUBILITY DETERMINATIONS

Wei et al., Development of a Method for Fast Assessment of Protein Solubility Based on Ultrasonic Dispersion and Differential Centrifugation Technology. *ACS Omega* **2022**, 7: 31338-31347.

Abstract: A rapid method for measuring protein solubility was developed using ultrasonic dispersion, differential centrifugation, and spectral measurements. Lysozyme solubilities in NaCl aqueous solutions were determined to verify the methodology. Measurements of zein in NaOH solution and casein solubility were used as test cases.

Analysis: Various academic institutions in China collaborated on the development of this method for rapidly measuring protein solubility under different conditions. The technique was verified using a well known protein system (lysozyme in NaCl solutions) and then used to make measurements on two new protein samples.

DRIED POWDERS OF A mAb

Pan et al., Spray-Dried and Spray-Freeze-Dried Powder Formulations of an Anti-Interleukin-4Rα Antibody for Pulmonary Delivery. *Pharm. Res.* **2022**, 39: 2291-2304.

Abstract: A monoclonal antibody against the receptor for IL-4 is being developed for the treatment of asthma. The mAb was prepared as dried powder by spray-drying or spray-freeze-drying (SFD) with the intention of pulmonary administration. Hydroxypropyl-β-cyclodextrin was incorporated into the powder as both a stabilizer and an aerosol performance enhancer. The SFD material displayed satisfactory performance with an emitted fraction of 80% and a fine particle fraction of ~50%. The spray-dried material was hindered by having a high residual moisture content, but both preparations were stable for one year of storage at ambient temperatures.

Analysis: Groups in Hong Kong and Shanghai worked to develop inhalable powders of this particular mAb for treatment of asthma. The critical excipient was HP-β-CD, with SFD producing a powder with acceptable performance.

NMR STUDIES OF G-CSF

Kellerman et al., NMR Reveals Functionally Relevant Thermally Induced Structural Changes within the Native Ensemble of G-CSF. *Mol. Pharm.* **2022**, 19: 3242-3255.

Abstract: NMR was employed to identify residues within G-CSF that are critical for global stability of the native structure. Four particular residues (G4, A6, T133, Q134) were found to be significant in terms of environmental and dynamic changes upon increased temperature. In addition, four structural clusters were identified that appear to cluster around loop AB, which has been implicated in forming an aggregation prone state upon conformational rearrangement.

Analysis: This article shows how NMR can be employed to identify residues critical for function and stability, at least in smaller proteins. Researchers at University College London and NIBSC used NMR methods to study G-CSF as a function of temperature to identify residues that appear to be critical for activity and stability.

PHOTO-OXIDATION BY SINGLET OXYGEN

Brunell et al., Photo-Oxidation Mechanisms in Liquid Pharmaceutical Formulations: The Overlooked Role of Singlet Oxygen Presented as a Case Study. *Pharm. Res.* **2022**, 39: 2529-2540.

Abstract: Several factors can affect the oxidative sensitivity of drug products. For photo-oxidation, this can be particularly challenging, as many possible mechanisms could occur. The degradation of MK-1454 upon exposure to light was examined, where losses were mostly likely due to singlet oxygen causing chemical modification.

Analysis: This work from Merck emphasizes the role that singlet oxygen plays in photo-oxidation of APIs in liquid formulations. Using this case study, the authors raise awareness for the potential for singlet oxygen to participate in oxidative processes that could damage pharmaceutical products, including proteins.

HCPs IN mAb AGGREGATES

Hu et al., Host Cell Protein Identification in Monoclonal Antibody High Molecular Weight Species. *J. Chrom. B* **2022**, 1210: 123448.

Abstract: High molecular weight (HMW) species are product-related variants that can impact product quality. In this study, analysis of host cell proteins (HCPs) within aggregates from five different mAbs was performed. In mAb2, a chemokine was found to be enriched 46 times more than in HMW species than in the drug substance.

Analysis: This work from Regeneron illustrates how aggregates can retain certain HCPs at levels well in excess of what is found in the overall drug substance. Presumably this is due to preferential binding to aggregated material. This finding indicates that aggregate removal can also be an effective strategy to lower HCP levels.

STABILIZATION BY ALGINATE BINDING

Chang et al., Protein Stabilization by Alginate Binding and Suppression of Thermal Aggregation. *Biomacromolecules* **2022**, 23: 4063-4073.

Abstract: Addition of alginate was found to increase the T_m values of the enzyme, PGK (by 14.5º C) and hPin1 WW domain (by 3.5º C). This effect appears to be due to direct binding to alginate and not from a crowding or excluded volume effect. The effect of ionic strength on this behavior suggests that the binding is primarily electrostatic in nature. Alginate binding also suppresses aggregation at elevated temperatures.

Analysis: Scientists at the University of Illinois found that alginate bound directly to these two different proteins, resulting in increased conformational stability and reduced aggregation upon heating. This strategy may also provide stabilization of therapeutic proteins as well.

SURFACE-INDUCED AGGREGATION OF FVIII

Chae et al., Recombinant Factor VIII Protein Aggregation and Adsorption at the Liquid-Solid Interface. *J. Colloid Interf. Sci.* **2022**, 628: 820-828.

Abstract: The adsorption and aggregation of recombinant Factor VIII (rFVIII) was studied using a variety of biophysical techniques. The effect of PEGylation, temperature, pH and ionic strength were investigated. It was found that PEGylation provided increased stability at the air-water interface over a wide range of solution conditions. This work illustrates how proteins can adsorb and aggregate on a solid surface.

Analysis: The stability of rFVIII and its PEGylated version were studied by groups at Bayer, Cal-Berkeley, and Lawrence Berkeley National Laboratory. This work provides us with a case study in the relationship between interfacial behavior and resulting aggregation.

ADSORPTION OF SURFACTANTS

Kanthe et al., Differential Surface Adsorption Phenomena for Conventional and Novel Surfactants Correlates with Changes in Interfacial mAb Stabilization. *Mol. Pharm.* **2022**, 19: 3100-3113.

Abstract: Competitive adsorption studies were performed for a mAb, polysorbate 80, and the novel surfactant, FM1000. It was determined that FM1000 saturates the interface faster than PS 80 and occupies a greater percentage of the interfacial area, suggesting that FM1000 could be effective at lower bulk concentrations that PS 80. In the presence of a mAb, more PS 80 is required to prevent protein adsorption, whereas a fixed amount of FM1000 is effective. Agitation studies found that FM1000 was more protective against particle formation then PS 80, which is consistent with the measurements made on adsorption of the surfactants and mAb at the air-water interface.

Analysis: In an effort to find alternatives to polysorbates, this study from Bristol-Myers Squibb (BMS), University of Chicago, and City University of New York compared the interfacial behavior of FM1000 and PS 80 and found that adsorption behavior was indicative of the degree of protection afforded to a mAb upon agitation.