JANUARY 2022

IN THIS ISSUE

OVERVIEW

We start the new year with the January 2022 issue of *Staying Current: Formulation of Biopharmaceuticals*. This month, the main focus is on chemical stability, but you will also find articles on interfacial stability, polysorbate degradation, and particle formation, among others. As always, we hope these various articles 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 your support year after year. Together, we will continue to explore the many and varied aspects of the stabilization and formulation of biotherapeutics.

Staying Current
Formulation of Biopharmaceuticals

IRON-DEPENDENT PHOTODEGRADATION

Subelzu and Schöneich, Pharmaceutical Excipients Enhance Iron-Dependent Photo-Degradation in Pharmaceutical Buffers by Near UV and Visible Light: Tyrosine Modification by Reactions of the Antioxidant Methionine in Citrate Buffer. *Pharm. Res.* **2021**, 38: 915-930.

Abstract: The photostability of model peptides in acetate and citrate buffers in the presence of Fe^{3+} was investigated. Sugars (sucrose, trehalose), mannitol, and basic amino acids (Arg, His, Lys) promoted oxidation of met residues upon exposure to visible or near UV light. Addition of free Met to the solution reduced sulfoxide formation in these peptides. In citrate buffered solutions, the addition of met oxidation products to Tyr residues was observed.

Analysis: This study from the University of Kansas and Merck illustrates how commonly used excipients can promote the iron-mediated photooxidation of Met residues. This includes polyols, sugars, and amino acids. In addition, some novel Tyr degradants were observed in the presence of citrate buffer, which is also worth noting.

PHOTOOXIDATION OF Met RESIDUES UNDER ANOXIC CONDITIONS

Pędzinski et al., Radiation- and Photo-Induced Oxidation Pathways of Methionine in Model Peptide Backbone under Anoxic Conditions. *Int. J. Mol. Sci.* **2021**, 22: 4773.

Abstract: The reaction of •OH, generated by ionizing radiation, with Ac-Met-OMe was studied in aqueous solution under anoxic conditions. The result is a detailed mechanistic description of Met oxidation by •OH.

Analysis: Here is a second article on photo-induced oxidation of Met. This study from groups in Poland and Italy examined the impact of ionizing radiation on the generation of •OH and its subsequent oxidation of Met residues.

OXIDATION OF LYSOZYME BY PEROXYL RADICALS

Fuentes-Lemus et al., Oxidation of Lysozyme Induced by Peroxyl Radicals Involves Amino Acid Modifications, Loss of Activity, and Formation of Specific Crosslinks. *Free Radical Biol. Med.* **2021**, 167: 258-270.

Abstract: Lysozyme was incubated with AAPH, which is a source of peroxyl radicals (ROO•). Exposure to AAPH at 37º C led to decreased enzymatic activity. Side chain modifications were observed, including Met oxidation, dityrosine formation, and production of multiple Trp oxidation products. Specific crosslinks between Tyr20-Tyr23 (intramolecular) and Trp62-Tyr23 (intermolecular) were detected as well.

Analysis: Oxidative damage of lysozyme by peroxyl radicals was the focus on this article from the University of Copenhagen and Pontificia Universidad Católica de Chile. Exposure to AAPH at elevated temperature produced a variety of oxidation products, as well as some specific crosslinks, which appear to be related to chain flexibility and spatial proximity.

PREDICTION OF METHIONINE AND TRYPTOPHAN PHOTOOXIDATION

Delmar et al., Machine Learning Prediction of Methionine and Tryptophan Photooxidation Susceptibility. *Mol. Ther.: Meth. Clin. Dev.* **2021**, 21: 466-477.

Abstract: Until now, there has been no good predictive model for the photooxidation susceptibility for Met or Trp residues. Machine learning was used on a library of tryptic peptides analyzed by LC-MS/MS. The resulting models predict the total extent of Met and Trp photooxidation with a likelihood of 0.926 and 0.860, respectively. Prediction of Met oxidation rates had a rms error of 10.9%.

Analysis: This work from Yale University, AstraZeneca, and Northeastern University provides a framework for predicting the relative propensities of Met or Trp residues to undergo photooxidation. In addition, the authors discuss what chemical, physical, and formulation factors will influence photooxidation.

CHARACTERIZATION OF PROTEINACEOUS PARTICLES

Xu et al., Characterization of Proteinaceous Particles in Monoclonal Antibody Drug Products Using Mass Spectrometry. *J. Pharm. Sci.* **2021**, 110: 3403-3409.

Abstract: Characterization of visible and subvisible particles is important for understanding product quality. In particular, it is beneficial to be able to distinguish particles arising from the active protein compared to those from air bubbles, oil droplets and other exogenous sources. This study demonstrates the use of mass spectrometry to characterize proteinaceous particles and shows how this level of characterization can be integrated in a workflow aimed at product composition and quality.

Analysis: This article from Regeneron describes the use of LC-MS to characterize proteinaceous particles, allowing one to monitor the composition of particles upon storage or during product development.

ISOPEPTIDE BONDS IN AGGREGATES

Powell et al., Detection of Isopeptide Bonds in Monoclonal Antibody Aggregates. *Pharm. Res.* **2021**, 38: 1519-1530.

Abstract: In this study, the presence of intramolecular isopeptide bonds in aggregates of mAbs was detected and characterized for the first time. It was found that clipping at a Asp-Pro sequence in the C_H2 domain occurred during storage at elevated temperature. Peptide mapping revealed 14 isopeptide bonds between Asp at this site and Lys residues in adjacent antibody molecules.

Analysis: This study from UCB in the UK found chemical crosslinks in mAbs aggregates arising from chain scission at a Asp-Pro residue and subsequent reaction with Lys residues. This provides us with another type of crosslinking that can occur in mAbs, in this case, beginning with proteolysis of an Asp-Pro linkage.

MIMICKING LOW pH VIRUS INACTIVATION FOR mAbs

Kim et al., Mimicking Low pH Virus Inactivation Used in Antibody Manufacturing Processes: Effect of Processing Conditions and Biophysical Properties on Antibody Aggregation and Particle Formation. *J. Pharm. Sci.* **2021**, 110: 3188-3199.

Abstract: The low pH virus inactivation step commonly used for mAbs was examined using a lab-scale reactor. Adjustment of the pH with NaOH increased the number of particles, as measured using flow imaging. It was found that PS 80 was effective at reducing particles formed during stirring. In contrast, trehalose was found to increase particle levels. The conformational and colloidal properties of the mAb were measured in an effort to understand their role in particle formation under these conditions. Overall, the value of using a scaled down system to study aggregation during this unit operation is shown, even though the results were not fully predictive.

Analysis: The damage that can occur during a low pH hold step, common in mAb processing, is the focus of this article from BMS, the University of Colorado, and Dongguk University in Korea. The study provides insights about the impact of various physicochemical properties and certain excipients on particle formation during this widely used unit operation.

CLIPPING IN CDR OF mAbs

Atsumi et al., Identification and Characterization of a Monoclonal Antibody Variant Species with a Clipping in the Complementarity Determining Region Isolated by Size Exclusion Chromatography Under Native Conditions. *J. Pharm. Sci.* **2021**, 110: 3367-3374.

Abstract: Hydrolysis or clipping is usually associated with the hinge region of mAbs. However, clipping in the CDRs has been reported. In this study, fragments were separated from the parent compound using SEC under native conditions. This allows one to determine the relative potency of these chemically modified species. In particular, clipping of bevacizumab at Ser105-Ser106 was observed at 50º C using this methodology. This species exhibited lower antigen binding and poorer thermal stability.

Analysis: This study from Kyowa Kirin and Gunma University in Japan uses SEC under native conditions to separate hydrolytic degradation products that occur in the CDR. This then allows one to characterize these clipped species even further.

EFFECT OF Trp ON LIGHT-INDUCED AGGREGATION OF LACTALBUMIN

Zhao et al., Effect of Addition of Tryptophan on Aggregation of Apo-α-Lactalbumin Induced by UV-Light. *Foods* **2021**, 10: 1577.

Abstract: The photo-induced aggregation of apo-α-lactalbumin (LA, calcium-depleted form of the protein) was studied in the presence of Trp, which can act as a photosensitizer. Under aerobic conditions, the lowest levels of disulfide-mediated aggregation were observed. At the same time, light caused oxidation of Trp under these conditions. Under anaerobic conditions, one observes higher levels of disulfide-crosslinked aggregates when compared to the absence of Trp.

Analysis: This work from Arla Foods and the University of Copenhagen examines the impact of free Trp on UV light-induced aggregation of LA. It illustrates how Trp can be a photosensitizer, but also shows that the Trp itself can undergo photooxidation.

PRO ISOMERIZATION IN β_2-MICRO-GLOBULIN

Maschio et al., Proline Isomerization Effects in the Amyloidogenic Protein β_2-Microglobulin. *Phys. Chem. Chem. Phys.* **2021**, 23: 356-367.

Abstract: The potential of amyloid formation in β_2-microglobulin being triggered by cis-trans Pro32 isomerization was studied using molecular dynamics (MD) simulations. The simulations are in good agreement with experimental data and are consistent with Pro isomerization generating a destabilized isomer that is more aggregation-prone.

Analysis: Here we have a theoretical study that provides molecular insight into the fibrillation behavior of β_2-microglobulin. In doing so, we understand more about the subtleties that control conversion from the 'native' state to an aggregation-competent conformation.

EXCIPIENT EFFECTS ON IL-2 UNDER HEAT STRESS

Moradi et al., Computational Investigation on the Effects of Pharmaceutical Polymers on the Structure and Dynamics of Interleukin2 in Heat Stress. *J. Biomol. Struct. Dyn.* **2021**, 39: 4536-4546.

Abstract: MD simulations were employed to study the impact of various carbohydrate excipients on IL-2 subjected to elevated temperatures. It was found that chitosan and cyclodextrin were more effective than pectin, alginate, or hyaluronic acid at inhibiting thermal denaturation of the protein.

Analysis: Researchers at Kermanshah University in Iran used MD simulations to probe the effects of polymeric carbohydrates to stabilize IL-2 at elevated temperatures. It is another example of using MD simulations to gain molecular level information that would be quite difficult to obtain experimentally. Moreover, they show that chitosan and cyclodextrin might be effective stabilizers for this protein.

PROTEIN ADSORPTION TO PUMP SURFACES

Roffi et al., Adsorbed Protein Film on Pump Surfaces Leads to Particle Formation during Fill-Finish Manufacturing. *Biotechnol. Bioeng.* **2021**, 118: 2947-2957.

Abstract: Aggregation of proteins can occur during various steps in manufacturing. In this study, the absorption of protein onto pump surfaces of rotary piston pumps was found to lead to increased particle formation. As these types of pumps are used in fill-finish operations, this can be of great concern. A scaled-down version was employed to quantify the extent of adsorption and particle formation. Ceramic debris from the pump head was not found to influence aggregation to any appreciable extent.

Analysis: Here is a study from Pfizer on the role of surface adsorption and film formation/disruption in the subsequent formation of subvisible particles when using a rotatory piston pump.

BUFFER SELECTION FOR FROZEN rAAV2 PRODUCTS

Pandharipande et al., Considerations for Buffering Agent Selection for Frozen rAAV2 Mediated Gene Therapy Products. *J. Pharm. Sci.* **2021**, 110: 3535-3539.

Abstract: The impact of freezing rates and buffer selection on rAAAV2 stability was investigated. Aggregation of rAAV2 is maximal at pH 5.5 to 6.5. Using slow freezing rates, phosphate was found to cause sizable effect on concentration loss. These effects were mitigated by using Tris buffer instead of phosphate.

Analysis: Development of gene therapy products is ever increasing. In this case study from Voyager Therapeutics, the impact of buffer choice, pH, and freezing rate is examined for a recombinant adeno-associated virus being stored in the frozen state.

POLYSORBATE DEGRADATION AND PARTICLE FORMATION

Roy et al., Polysorbate Degradation and Particle Formation in a High Concentration mAb: Formulation Strategies to Minimize Effect of Enzymatic Polysorbate Degradation. *J. Pharm. Sci.* **2021**, 110: 3313-3323.

Abstract: The stability of a high concentration formulation of a mAb was investigated when subvisible particles (SVPs) appeared abruptly during storage at 5º C. These particles appear to be associated with the production of free fatty acids from enzymatic polysorbate degradation. Various formulation interventions were evaluated, including the use of solubilizers, lipase inhibitors, and alternative surfactants to reduce SVP formation.

Analysis: Scientists at Janssen R&D investigated different approaches for slowing SVP formation in a high concentration mAb formulation. In addition to efforts to control host cell protein levels, they found various formulation strategies that seem to be effective to reducing particles arising from free fatty acids.

MODIFIED PS 20 TO REDUCE FFAs

Doshi et al., Evaluating a Modified High Purity Polysorbate 20 Designed to Reduce the Risk of Free Fatty Acid Particle Formation. *Pharm. Res.* **2021**, 38: 1563-1583.

Abstract: A new modified, high purity polysorbate 20 (PS 20) was evaluated that contained lower levels of stearate, palmitate, and myristate esters. Formation of free fatty acids (FFAs) was delayed under stress conditions compared to PS 20 itself. The interfacial protection and oxidation propensities were comparable. This study shows that control over long chain fatty acids can reduce the occurrence of FFAs in pharmaceutical products.

Analysis: This study from Croda, Roche, and Genentech examines a new version of PS 20, one where the long chain fatty acid content has been modified. The modified PS 20 appears to protect against interfacial stress in a similar manner to PS 20, but results in lower levels of FFAs under stress conditions.

mAb AGGREGATION ASSOCIATED WITH OXIDATION

Zheng et al., Monoclonal Antibody Aggregation Associated with Free Radical Induced Oxidation. *Int. J. Mol. Sci.* **2021**, 22: 3952.

Abstract: Exposure of a mAb to AAPH causes Met and Trp oxidation. In addition, there is an increase in aggregation observed by SEC. Analysis by SDS-PAGE indicates that intermolecular disulfide bonds are involved in formation of these aggregates. Furthermore, evidence of crosslinking through di-Tyr linkages was also detected. Excipient screening indicated that addition of Tyr, Trp or pyridoxine could reduce aggregation caused by oxidation stress.

Analysis: In this work from Genentech and the University of Michigan, the ability of free radicals generated from AAPH to cause covalent crosslinking and aggregation was evaluated. Both reducible and non-reducible crosslinking was detected. The addition of sacrificial additives, such as Tyr or Trp, can be affective at slowing or inhibiting these processes.

IMPROVED PREDICTION OF HIGH CONCENTRATION mAb VISCOSITY

Roche et al., Towards an Improved Prediction of Concentrated Antibody Solution Viscosity using the Huggins Coefficient. *J. Colloid Interf. Sci.* **2022**, 607: 1813-1824.

Abstract: A viscometry technique was developed to measure the intrinsic viscosity ([η]) of high concentration formulations of mAbs, along with the Huggins coefficient, k_h. The viscometric parameters were compared to k_D and B_{22} values for two different mAbs. A strong correlation was observed for the viscosity with kh, but not with k_D or B_{22} values. Trends of [h] and k_h were tabulated as a function of pH and ionic strength and are discussed in terms of protein-protein interactions.

Analysis: Groups in Germany and the UK collaborated on improving the ability to predict the viscosity behavior of mAbs at high concentrations. Interestingly, the Huggins coefficient was found to be a better metric for prediction than well established measures of protein-protein interactions, like k_D and B_{22} values.

EFFECT OF SHEAR RATE ON FIBRILLATION

Adam et al., Effects of Shear Rate and Protein Concentration on Amyloidogenesis via Interfacial Shear. *J. Phys. Chem. B* **2021**, 125: 10355-10363.

Abstract: Insulin solutions at different concentrations were subjected to shear flows using a knife-edge viscometer. Fibrillation rates and onset times were measured. The study indicates that increasing interfacial shear rate results in a monotonic increase in the rate of fibril formation and a monotonic decrease in the onset time. On the other hand, the protein concentration did not have a significant effect on either parameter. Gelation was observed in this system and there does appear to be a minimal insulin concentration needed for gelation to occur.

Analysis: This work on shear and fibril formation comes form Rensselaer Polytechnic University and the University of Colorado. These researchers found that interfacial shear accelerated the rate of insulin fibrillation and decreased onset times, but that insulin concentration had little effect, except on the subsequent gelation process.

EXCIPIENTS AND OXYTOCIN STABILITY

Ghasemisarabbadieh et al., The Effect of Trehalose, Antioxidants, and Acetate Buffer Concentration on Oxytocin Stability. *J. Pept. Sci.* **2021**, 27: e3324.

Abstract: The stability of liquid formulations of oxytocin were examined. Acetate buffer was found to provide better stability than the use of citrate/phosphate buffer. Lower concentrations (< 25 mM) were more effective than higher concentrations. The antioxidants, BHT and uric acid, had negligible impact on stability, while the use ascorbate actually resulted in faster degradation. Surprisingly, trehalose had little effect on the stability of oxytocin, even at concentrations up to 1 M.

Analysis: This work from the University of Iceland, Calor ehf, and University of Malawi examines the stability of oxytocin at pH 4.5. The buffer effects are interesting, especially the acetate concentration effects. Also, the use of trehalose had minimal effects, unlike the stabilization one often sees for globular proteins. The impact of antioxidants is also worth noting.

Met RESIDUES AND LLPS

Aledo, The Role of Methionine Residues in the Regulation of Liquid-Liquid Phase Separation. *Biomolecules* **2021**, 11: 1248.

Abstract: Reversible demixing into two distinct liquid phases, in what is termed liquid-liquid phase separation (LLPS), is reviewed. The focus is on structural elements that promote LLPS. In this context, the post-translational modification (PTM) of Met residues (i.e., formation of Met sulfoxide) can change protein-protein interactions (PPIs), thereby altering the dynamics of phase separation. The discussion covers proteins of low structural complexity whose composition is biased towards Met residues.

Analysis: This review from the Universidad de Malaga in Spain examines how modifications to Met residues can affect colloidal stability, which, in turn, will alter the propensity to phase separate. At the heart of this topic is the concept that PTMs can impact the colloidal stability of protein, which will then change the physical stability of a protein.

HYPOCHLORITE-INDUCED OXIDATION OF FIBRINOGEN

Rosenfeld et al., Hypochlorite-Induced Oxidation of Fibrinogen: Effects on Its Thermal Denaturation and Fibrin Structure. *Biochim. Biophys. Acta* **2021**, 1865: 129970.

Abstract: Using a wide range of hypochlorite concentration, the oxidation of fibrinogen was studied with respect to its impact on unfolding and aggregation. It was found that oxidation affects the thermal stability of region D directly and that of region E indirectly. The oxidized material tends to form abnormal fibrin structures.

Analysis: The interrelationship of chemical and physical stability is also seen in this article from groups in Moscow, Russia. Not only is the conformational stability adversely affected, the subsequent assembly to form fibrin fibers is impacted.

AGGREGATION OF EGG WHITE PROTEINS

Hong et al., Aggregation of Hen Egg White Proteins with Additives during Agitation. *LWT* **2021**, 146: 111378.

Abstract: The aggregation of hen egg white proteins (HEWPs) at the air-water interface was examined. This type of aggregation is significantly repressed by the addition of ionic surfactants. On the other hand, excipients like ArgHCl, NaCl, and Gly had a negligible effect on suppressing interfacial damage. Lysozyme and ovalbumin strongly formed co-aggregates upon agitation, while lysozyme and ovotransferrin did so to a lesser extent. Covalent crosslinking through disulfide plays a significant role in the formation of aggregates in HEWPs.

Analysis: This work from Kewpie Corporation and the University of Tsukuba in Japan examines the interfacial damage of HEWPs. Surfactants provide a sizable degree of protection, while many other excipients do not. Covalent co-aggregation is seen along with non-covalent interactions.