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IN THIS ISSUE

OVERVIEW

Welcome to the first issue of *Staying Current: Formulation of Biopharmaceuticals* for 2019 with a Focus Area on analytical methods. There are eight articles from eight different journals on techniques that we think you may find helpful. In addition, there are articles on HOS determination and the relationship between formulation and patient safety. There are also two reviews on drug delivery and articles on chemical instability, including a novel pathway for glycan shedding. As always, there are a multitude of valuable articles being published, many of which are highlighted here. We hope that you find these summaries useful.

Staying Current
Formulation of Biopharmaceuticals

GLYCAN SHEDDING

Qian et al., Non-Enzymatic and Site-Specific Glycan Shedding; A Novel Degradation Pathway Observed in a Stabilized Form of RSV Prefusion F Protein. *Analytical Chem.* **2018**, 90: 10897-10902.

Abstract: A novel nonenzymatic gradation process was observed under stress conditions for an engineered form of RSV prefusion F protein. It involved the shedding of a glycan. The process is site-specific, is dependent on structural features and requires a glycine residue immediately after the glycosylation site. The loss of glycan does not appear to affect binding to the neutralizing antibody, D25. The mechanism of glycan shedding is similar to, but distinct from deamidation.

Analysis: This new degradation pathway was described by researchers at GSK Vaccines in Maryland USA. The mechanism has similarities to conventional deamidation, but some differences as well. It requires a Gly residue immediately after the glycosylation site, so structural and flexibility properties appear to be important for controlling relative rates.

REMOVAL OF AGGREGATION PRECURSORS

Senga and Honda, Suppression of Aggregation of Therapeutic Monoclonal Antibodies during Storage by Removal of Aggregation Precursors Using a Specific Adsorbent of Non-Native IgG Conformers. *Bioconjugate Chem.* **2018**, 29: 3250-3261.

Abstract: A strategy was devised for removing non-native IgG molecules that could serve as precursors to aggregation. This was accomplished using the artificial protein, AF.2A1, which binds to non-native conformers of antibodies. Removal of these species resulted in a suppression of aggregate formation.

Analysis: In this study from the National Institute of Advanced Industrial Science and Technology in Japan, an affinity chromatography method is described using AF.2A1, a protein that specifically binds conformationally altered IgG. Reduction in these species results in suppressed aggregation during storage.

HIGHER ORDER STRUCTURE BY CD SPECTROSCOPY

Barnett et al., Enhanced Precision of Circular Dichroism Spectral Measurements Permits Detection of Subtle Higher Order Structural Changes in Therapeutic Proteins. *J. Pharm. Sci.* **2018**, 107: 2559-2569.

Abstract: Higher order structure (HOS) can be an essential quality attribute of a therapeutic protein. In this study, circular dichroism (CD) spectroscopy was used to monitor changes in secondary and tertiary structure. A method was developed to enhance the precision through normalization by the protein concentration determined directly from the CD measurement. This approach allows one to detect subtle changes in HOS in numerous forced degradation studies.

Analysis: Assessing comparability of HOS has received a great deal of attention. In this work from Bristol-Myers Squibb (BMS) and Zymogenetics (a BMS company), the precision of using CD has been improved by avoiding the use of a separate method to determine protein concentration.

EFFECT OF OXIDATION ON A mAb

Shah et al., Effect of Peroxide- Versus Alkoxyl-Induced Chemical Oxidation on the Structure, Stability, Aggregation, and Function of a Therapeutic Monoclonal Antibody. *J. Pharm. Sci.* **2018**, 107: 2789-2803.

Abstract: Oxidation of an IgG monoclonal antibody (mAb) was performed using AAPH, t-butyl hydroperoxide and hydrogen peroxide. The conformational stability of the mAb was reduced for all three oxidized forms. In addition, the AAPH-oxidized formed aggregated to a greater extent. Biological activity was significantly reduced as well. The differences in the activity and stability of these three oxidized samples appear to be related to different chemical modification on various residues in the Fc and Fab regions of the mAb.

Analysis: This study on mAb oxidation was conducted by groups at Eli Lilly and the University of Colorado. It shows that different oxidizing species result in different profiles of modified residues, which, in turn, leads to differential effects on potency, aggregation and conformational stability.

INJECTION SITE REACTION CAUSED BY POLYSORBATE?

Singh et al., Are Injection Site Reactions in Monoclonal Antibody Therapies Caused by Polysorbate Excipient Degradants? *J. Pharm. Sci.* **2018**, 107: 2735-2741.

Abstract: Injection site reactions (ISRs) are common for injectable biotherapeutics. A case is reported where a patient developed ISRs to a commercial PCSK9i product to the point of having to discontinue therapy. The potential role of polysorbate and its degradation products in causing these ISRs is discussed. Thus, further research is needed to understand the underlying causes of ISRs, especially as they relate to excipients.

Analysis: There continues to be greater scrutiny of polysorbates and their ability to cause adverse biological responses as well as contributing to diminished storage stability of biopharmaceuticals. This work from Lonza in Basel and UnityPoint Cardiology in the US suggests that polysorbate degradation products are implicated in the ISRs observed in this case study. More importantly, it is a call to develop alternatives to polysorbates and understand how formulations may contribute to ISRs.

EXPOSURE TO LEACHABLES IN LYOPHILIZED PRODUCTS

Zdravkovic, Assessment of Patient Exposure to Leachables from Lyophilized Drug Formulations Following Reconstitution, Storage, and Administration via Polymeric Packaging/Delivery Systems. *J. Pharm. Sci.* **2018**, 107: 2837-2846.

Abstract: Rubber stoppers used in lyophilized products have greater potential to leach compounds compared to liquid formulations. The extent of patient exposure to leachables from two commercial lyophilized products was evaluated after administration from i.v. bags. The materials used in the bags did not impact the mass of leachables administered to the patient. The amount of leached material was related to the duration of administration and the storage temperature.

Analysis: This leachable study was conducted by Pharmaceutical Product Development located in Wisconsin. It shows how the storage and direction of administration play an important role in patient exposure to leachables from polymeric materials, along with the exact composition of the packaging materials employed.

IN-LINE FILTERS

Werner and Winter, Expanding Bedside Filtration- A Powerful; Tool to Protect Patients from Protein Aggregates. *J. Pharm. Sci.* **2018**, 107: 2775-2788.

Abstract: The efficiency of 'bedside filtration' to reduce the levels of protein particles was demonstrated with 19 stressed and non-stressed biopharmaceutical products. In addition, issues such as particle shedding from filters, losses due to adsorption, and hold-up volume were also evaluated.

Analysis: This study from Ludwig-Maximilians Universität in Germany shows that there is little, if any, negative impact of using in-line filters to reduce the levels of protein particles prior to administration to the patient. The work examined compatibility for a number of different filters and protein products.

VISCOSITY MODIFIERS FOR mAbs

Ke et al., Novel Salts of Dipicolinic Acid as Viscosity Modifiers for High Concentration Antibody Solutions. *Int. J. Pharm.* **2018**, 548: 682-688.

Abstract: Two specific salts, ethanolamine and diethanolamine, of dipicolinic acid (DPA) were selected based on solubility and crystallinity screens. These salts were found to reduce the viscosity of highly concentrated solutions of five different monoclonal antibodies (mAbs). These compounds did not reduce Tm values and they did cause loss of monomer upon extended storage at 5° C, although some increased degradation of three of the mAbs was seen at 40° C.

Analysis: Groups at AstraZeneca/ MedImmune and the University of Cambridge collaborated on the use of DPA salts as novel excipients to reduce viscosity in high concentration mAb formulations.

LOW pH STABILITY OF A mAb

Fukuda et al., Long-Term Stability and Reversible Thermal Unfolding of Antibody Structure at Low pH: Case Study. *J. Pharm. Sci.* **2018**, 107: 2965-2967.

Abstract: A monoclonal antibody (mAb) was stored for ten years at 4° C, where the T_m values, measured by DSC, were unchanged, even at pH values as low as 2.7. The unfolding at pH 2.7 was found to be highly reversible. At higher pH, thermal denaturation resulted in aggregation.

Analysis: Most mAbs exhibit a marked decrease in T_m at acidic pH values. In this study from Alliance Protein Labs, the University of Tokyo, Osaka Prefecture University, and Sysmex, the low pH unfolding event was found to be highly reversible, resulting in no change in conformational stability over ten years of storage.

PPIs IN HIGHLY CONCENTRATED PROTEIN FORMULATIONS

Baek and Zydney, Intermolecular Interactions in Highly Concentrated Formulations of Recombinant Therapeutic Proteins. *Curr. Opin. Biotechnol.* **2018**, 53: 59-64.

Abstract: In order to allow for subcutaneous injection, high concentration (HC) formulations of therapeutic protein have been developed, but often resulting in very high viscosities. Recent work on the intermolecular interactions (i.e., protein-protein interactions or PPIs) governing viscosity behavior is reviewed. The impact of these interactions on UF/DF operations is discussed.

Analysis: Formulation development work on HC formulations continues. This review from Pennsylvania State University provides a valuable synopsis on PPIs and how they impact drug delivery, storage stability, and bioprocessing.

STABILIZERS OF EPO

Mortazavi et al., Physicochemical Screening for Chemical Stabilizer of Erythropoietin to Prevent Its Aggregation. *Prep. Biochem. Biotechnol.* **2018**, 48: 121-127.

Abstract: A number of compounds were screened for their ability to inhibit thermally induced aggregation of erythropoietin (EPO). Glycine betaine was found to stabilize EPO at concentrations as low as 1 mM. Stabilizers like mannitol also provided some degree of protection, but required much higher concentrations.

Analysis: Various groups in Iran contributed to this work. Using turbidity measurements on a microtiter plate platform, numerous compounds were evaluated for their ability to inhibit aggregation at 50° C. Of these, betaine proved to be the most effective, providing protection even at concentrations as low as 1 mM.

DILUTION APPROACH TO STUDYING ANTIBODY STABILITY

Svilenov et al., A New Approach to Study the Physical Stability of Monoclonal Antibody Formulations- Dilution from a Denaturant. *J. Pharm. Sci.* **2018**. 107: 3007-3013.

Abstract: A dynamic light scattering plate reader was used to measure aggregation of monoclonal antibody (mAb) samples that were subject to incubation and dilution from a denaturant (GnHCl). The method correlates with other measures of protein aggregation propensity. Thus, this method provides an isothermal, label-free approach towards screening the relative stability of a mAb in various formulations.

Analysis: A new DLS method is described in this work from groups at Ludwig-Maximilians Universität and Wyatt Europe in Germany. It is relatively rapid and requires fairly small amounts of material.

DEAMIDATION-RESISTANT MUTANTS OF mAbs

DiCara et al., High-Throughput Screening of Antibody Variants for Chemical Stability: Identification of Deamidation-Resistant Mutants. *MABS* **2018**, 10: 1073-1083.

Abstract: Developability assessment of monoclonal antibodies (mAbs) includes identification of potential chemical instabilities. A high throughput assay was developed to characterize the propensity for asparagine (Asn) deamidation. Ninety variants were screened using this method. Surprisingly, a mutation that is five residues removed from the Asn greatly reduced the extent of deamidation.

Analysis: Groups at Genentech developed this method to assess the degree of deamidation of various mAb mutants. In doing so, they found some surprising results in terms of remote effects on Asn reactivity. In doing so, one can rapidly evaluate the developability of mAb variants in terms of deamidation propensity.

INTRANASAL DELIVERY OF BIOLOGICS

Rohrer et al., Advanced Formulations for Intranasal Delivery of Biologics. *Int. J. Pharm.* **2018**, 553: 8-20.

Abstract: The potential of excipients to facilitate intranasal delivery of biologics is reviewed. This includes penetration enhancers, mucolytic agents, and mucoadhesive compounds. The goal is to highlight recent advancements in nasal delivery of macromolecules.

Analysis: This review article from the University of Innsbruck in Austria examines the role of formulation in facilitating intranasal delivery of proteins and other macromolecules. This route of administration could provide significant advances in the use of biotherapeutics.

HOS ASSESSMENT OF PROTEINS

Orphanou and Gervais, Higher-Order Structure and Conformational Change in Biopharmaceuticals. *J. Chem. Technol. Biotechnol.* **2018**, 93: 2477-2485.

Abstract: Higher order structure (HOS) is critical for proper function of therapeutic proteins. Post-translational modification (PTMs) can impact HOS. A HOS assessment strategy is proposed with the goal of ensuring product safety and efficacy, as well as helping develop robust processes for biopharmaceuticals. When changes in HOS do occur, there is increased risk of aggregation and immunogenicity.

Analysis: This review from Porton Biopharma in the UK examines HOS assessment from the perspective of quality control, risk mitigation, and safety. As such, it is a helpful overview of the importance of HOS characterization in biopharmaceutical product development.

ANTIBODY DRUG DELIVERY

Awwad and Angkawinitong, Overview of Antibody Drug Delivery. *Pharmaceutics* **2018**, 10: art. 83.

Abstract: Monoclonal antibodies (mAbs) are the dominant class of protein among biotherapeutics. There are opportunities to develop mAb formulations for lifecycle management, improved patient compliance, and alternative routes of administration. This review examines technologies currently being developed to facilitate mAb delivery, including those for extended release.

Analysis: This drug delivery review from University College London and the National Institute for Health Research in the UJ examines a variety of antibody drug delivery technologies, including scaffolds, polymeric matrices, and PEGylation.

FOCUS AREA: ANALYSIS OF BIOTHERAPEUTICS

PROTEIN CHARACTERIZATION USING IEX-MALLS

Amartely et al., Coupling Multi Angle Light Scattering to Ion Exchange Chromatography (IEX-MALLS) for Protein Characterization. *Sci. Rep.* **2018**, 8: 6907.

Abstract: Multi-angle laser light scattering (MALLS) detection is often used with SEC. However, SEC can exhibit limited resolution, especially for complex systems. In this study, MALLS was used in conjunction with the higher resolution ion exchange (IEX) chromatography. This approach was able to resolve and characterize both higher and lower molecular weight species in various protein and peptide systems.

Analysis: Our focus on analytical methodology starts with this article from groups in Israel about the utility of using MALLS detection with IEX chromatography. The degree of characterization is striking, suggesting that this approach might be valuable for a wide range of biotherapeutics.

DETERMINATION OF PS 80

Koppolu et al., A Universal Method for the Determination of Polysorbate 80 in Monoclonal Antibodies and Novel Protein Therapeutic Formulations. *Analytical Meth.* **2018**, 10: 1296-1304.

Abstract: A mixed-mode chromatography method coupled with ELSD is described for quantitation of polysorbate 80 (PS 80) in various protein formulations. The method appears to be free of matrix interference from the protein species and can detect PS 80 in the range of 0.05 to 0.6 mg/ml. It was able to detect losses in PS 80 during long-term storage.

Analysis: This article from MedImmune is worth noting for two reasons. The first is the method itself and its ability to be adapted to other analytes, such as poloxamer 407. In addition, it shows the instability of PS 80 under stress and long-term storage, which is an important focus within protein formulation, with both technical, regulatory, and toxicology implications.

RAPID PROTEIN CONCENTRATION DETERMINATION

McKechnie et al., Accurate and Rapid Protein Concentration measurement of In-Process, High Concentration Protein Pools. *Biotechnol. Prog.* **2018**, 34: 1234-1241.

Abstract: Protein concentration measurements at high concentrations can be challenging. The use of UV slope spectroscopy for analysis of in-process samples is demonstrated here. It allows measurements over a range of 0.5 to 315 AU at 280 nm with < 7% error. This meets the internal acceptance criteria of 10% set for such measurements.

Analysis: The use of slope spectroscopy to measure high protein concentrations is described here in this work from Merck Research Labs in the US. It is a detailed method assessment study and will be helpful for anyone using this technique and for anyone developing high concentration protein formulations.

FREE THIOLS IN mAbs

Welch et al., Dacile Quantitation of Free Thiols in a Recombinant Monoclonal Antibody by Reversed-Phase High Performance Liquid Chromatography with Hydrophobicity-Tailored Thiol Derivatization. *J. Chrom. B* **2018**, 1092: 158-167.

Abstract: Free thiol content is an important quality attribute of monoclonal antibodies (mAbs). A RP HPLC method is described for determination of free thiols in a mAb following derivatization with N-tert-butylmaleimide. The method exhibits good specificity, linearity, accuracy and robustness. The results correlated to measurements by orthogonal methods, such as LC-MS.

Analysis: This method was developed by Genentech to monitor conveniently the free thiol content of mAbs. It works well in both an R & D environment as well as QC.

SV-AUC FOR HIGH CONCENTRATION PROTEIN SAMPLES

Chaturvedi et al., Measuring Macromolecular Size Distributions and Interactions at High Concentrations by Sedimentation Velocity. *Nature Commun.* **2018**, 9: 4415.

Abstract: A sedimentation velocity analytical ultracentrifugation (SV-AUC) technique is described that allows one to examine size distributions and thermodynamic interactions at concentrations up to 50 mg/ml.

Analysis: Methods directed at higher protein concentrations continues with this work from Dr. Schuck's group at NIH in the US. It allows one to look at size distributions as well as protein-protein interactions with high resolution.

CHARGE VARIANTS OF mAbs

Liu et al., Characterization of Recombinant Monoclonal Antibody Charge Variants Using WCX Chromatography, icIEF, and LC-MS/MS. *Analytical Biochem.* **2019**, 564-565: 1-12.

Abstract: Charge variants of a monoclonal antibody (mAb) were separated into four fractions using weak cation exchange chromatography (WCX). Peptide mapping allowed for further identification of the modified sites using LC-MS/MS and icIEF. Basic variant 1 was due to cysteinylation and dehydration of Asn residues. Basic fraction 2 was fully accounted for by variations in the N-terminal leader sequence of the heavy chain. About 18% of the acidic variants is due to Asn deamidation in the heavy chain, while 15% is due to N-terminal variants of the light chain. Approximately 54% of the acidic variants still could not be explained.

Analysis: Here we have a detailed examination of the basic and acidic charge variants in a mAb from groups in Jilin, China. This provides a clearer picture of the heterogeneity that can and does occur in modifications of the primary sequence of a mAb.

CROSS-INTERACTION CHROMATOGRAPHY

Hedberg et al., Cross-Interactions Chromatography as a Rapid Screening Technique to Identify the Stability of New Antibody Therapeutics. *Eur. J. Pharm. Biopharm.* **2018**, 133: 131-137.

Abstract: Cross-interaction chromatography (CIC) allows one to examine the colloidal stability of multiple proteins without the need to prepare individual columns for each protein. In this study, three monoclonal antibodies (mAbs) were examined for changes in B_{23} values as a function of NaCl concentration. The B_{22} values from self-interaction chromatography (SIC) correlated well with the B_{23} values from CIC. Use of pre-prepared columns of a known mAb allowed for one to evaluate an unknown protein while using up to fifty times less material than SIC might require.

Analysis: The use of CIC as a rapid screening tool is illustrated here in this work from Imperial College London and Fuji Diosynth. Using a single column SIC column, the rank ordering of colloidal stability can be determined rapidly for many unknown mAbs.

FLOW CYTOMETRY FOR PROTEIN AGGREGATES

Hu et al., Light-Scattering Detection within the Difficult Size Range of Protein Particle Measurement using Flow Cytometry. *Nanoscale* **2018**, 10: 19277-19285.

Abstract: Characterization of protein particles at or below 1 μm in size has remained a significant technical challenge. Using submicrometer spheres of polystyrene and silica, a flow cytometry method was developed using light scattering detection that is capable of analyzing particles down to 200 nm in size. The results were benchmarked against field-flow fractionation (FFF). Using this technique, the distribution of protein aggregates from 200 nm to 10 μm was monitored.

Analysis: This study from groups in Beijing China demonstrates that flow cytometry could be used to measure both subvisible and submicron protein particles when using light scattering detection.