

## PARTICULATE ANALYSIS

Sharma et al., Micro-Flow Imaging: Flow Microscopy Applied to Sub-visible Particulate Analysis in Protein Formulations. *AAPS J.* **2010**, 12: 455-464.

**Abstract:** The need to control and characterize proteinaceous sub-visible particles has been the focus of recent publications. For much of this size range no other has been available with sufficient sensitivity. This review covers the technique of micro-flow imaging (MFI). It discusses its ability to distinguish protein particulates from other particles, such as air bubbles and silicone oil droplets. It appears that MFI can accurately measure sub-visible protein particles even when other particle types are present.

**Analysis:** This is the first of a number of articles on the topic we will cover this month. This is a review article from Brightwell, makers of the MFI system. It covers many of the topics of interest to formulation scientists, such as what kinds of studies have been done with proteins as well as the effects of operational parameters. For anyone interested in this methodology, this is a good place to start to learn about the equipment and its various applications.

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### OVERVIEW

Flow microscopy/flow imaging as tools for measuring particulates in protein solutions takes center stage this month with five related articles. These studies are sure to add to the discussions about subvisible particles in protein therapeutics. In addition, we have a number of articles on antibody stability and structure.

## MICRO-FLOW IMAGING OF OPALESCENT MAb FORMULATIONS

Sharma et al., Quantification and Characterization of Subvisible Proteinaceous Particles in Opalescent mAb Formulations Using Micro-Flow Imaging. *J. Pharm. Sci.* **2010**, *99*: 2628-2642.

**Abstract:** Micro-flow imaging (MFI) appears to be more sensitive than light obscuration (LO) methods for measuring subvisible protein particulates in protein products. Given the potential difficulties in detecting particles in opalescent solutions, studies were performed to measure low concentrations of glass and polystyrene particles in an opalescent monoclonal antibody (MAb) solution. MFI was able to detect and quantify particles, even at levels of 10/ml. In addition, MFI showed excellent linearity in quantifying protein particles from 20 to 160,000 particles/ml. By comparison, the LO method significantly underestimates the number of protein particles, especially in the 2-10  $\mu\text{m}$  size range.

**Analysis:** Here we have a study from Brightwell, manufacturers of the MFI system. The primary focus is on the ability of MFI to detect low levels of foreign particles in the presence of high concentrations of proteins. The study also demonstrates linearity of the MFI technique for protein particles.

## MICRO-FLOW IMAGING OF A HIGH CONCENTRATION IgG1 FORMULATION

Wuchner et al., Development of a Microflow Digital Imaging Assay to Characterize Protein Particulates During Storage of a High Concentration IgG1 Monoclonal Antibody Formulation. *J. Pharm. Sci.* **2010**, *99*: 3343-3361.

**Abstract:** A micro-flow imaging (MFI) method was used to detect and quantify particulates in a high concentration IgG1 monoclonal antibody (MAb) formulation. The MFI assay was optimized and qualified. The stability profile for a 90 mg/ml formulation at 2-8° and -70° C for up to 18 months was evaluated. Accumulation of protein particles in certain size ranges was observed, along with a concomitant increase in the overall particle size distribution over time. The weight amount of protein particulates was ~0.022% after storage at 2-8° C for 18 months. It was found that the same formulation in different containers or even different lots of the same container yielded different particle stability profiles.

**Analysis:** This is an extensive study from Centocor using the MFI method to examine the stability of a high concentration MAb formulation. It includes information on qualification of the MFI method, but really speaks to the ability of MFI to follow stability of the protein over time. The mass associated with the subvisible particles was determined and found to be less than 0.1%. Much of this was associated with larger particles (> 25  $\mu\text{m}$  in size). This is a great case study to consider when looking to implement flow imaging as part of the characterization and stability package for a new formulation or product.

## HIGH MOLECULAR AGGREGATES IN REPACKAGED BEVACIZUMAB

Kahook et al., High-Molecular-Weight Aggregates in Repackaged Bevacizumab. *Retina* **2010**, 30: 887-892.

**Abstract:** Both ranibizumab (Lucentis) and bevacizumab (Avastin) are being used to treat ocular diseases. There have been recent reports of sustained elevation of intraocular pressure (IOP) after use of these agents. It is hypothesized that this is due to the presence of high molecular weight aggregates. Examination of repackaged bevacizumab from three compounding pharmacies found that the IgG concentrations varied significantly, with a trend for an increase in micron-sized particles (measured by micro-flow imaging) with a decrease in IgG concentration. It may be that large particulates block aqueous outflow and raise IOP.

**Analysis:** Repackaging of biopharmaceuticals presents its own risks. Here, MFI is used to measure the extent of aggregation in repackaged Lucentis for intraocular use. The amount of active ingredient and aggregated protein varies widely among the pharmacies evaluated. This raises serious toxicity issues. At the same time, it emphasizes for us that aggregates could cause toxicity beyond immunogenicity effects.

## SUBVISIBLE PARTICLES IN PROTEIN PHARMACEUTICALS

Singh et al., An Industry Perspective on the Monitoring of Subvisible Particles as a Quality Attribute for Protein Therapeutics. *J. Pharm. Sci.* **2010**, 99: 3302-3321.

**Abstract:** Recently, concerns have been raised about subvisible particles in protein products, as these are typically not measured. This article, representing the opinions of a number of industrial scientists, seeks to further the discussion with a review of the methodology surrounding particle characterization and the link to increased risk of immunogenicity. It is concluded that limitations in the measurement technologies, variability in protein concentration, packaging, viscosity, and sample handling, as well as inherent batch variability, makes these methods unsuitable for lot release, stability assessment, or for comparability testing, at the present time.

**Analysis:** This article was written in response to the now well-publicized article from Carpenter et al. (*J. Pharm. Sci.* **2009**, 98: 1201-1205) calling for monitoring of subvisible particles in protein products. Certainly, our understanding of the relationship between aggregated protein and safety, especially immunogenicity, is limited, with few systems (human growth hormone, interferon- $\alpha$ ) studied in detail. This article continues the dialogue that has been and is occurring at scientific meetings. A significant amount of data from marketed products is included and this forms the basis for arguments about the limitations of particle counting technologies. I expect that we will see many more studies in the future that should help clarify some of the issues that are currently under debate.

## PREDICTION OF AMYLOIDOGENIC REGIONS

Garbuzynskiy et al., FoldAmyloid: A Method of Prediction of Amyloidogenic Regions from Protein Sequence. *Bioinformatics* **2010**, 26: 326-332.

**Abstract:** Two characteristics appear to be able to identify amyloidogenic regions in protein sequences. These are the probability of forming hydrogen bonds and the expected packing density of the side chains. An algorithm, called FoldAmyloid, has been developed focusing on these parameters. It has been tested on a database of 407 peptides (including 144 amyloidogenic peptides) and shown to have good performance in predicting amyloidogenic sequences. This method is correct for 80% of the amyloidogenic peptides and 72% for the non-amyloidogenic peptides. For proteins, the method correctly found 10 or 11 amyloidogenic regions.

**Analysis:** The methodology continues to evolve for predicting aggregation propensity, and we have covered many such studies in *Staying Current: Formulation of Biopharmaceuticals*. This group has discussed efficient packing of side chains before, in terms of its importance in identifying amyloidogenic regions. Now, coupled with an assessment of hydrogen bond capabilities, a new algorithm is presented that displays fairly good accuracy.

## AGGREGATION-PRONE REGIONS IN ANTIBODY CDRs

Wang et al., Potential Aggregation-Prone Regions in Complementarity-Determining Regions of Antibodies and Their Contribution Towards Antigen Recognition: A Computational Analysis. *Pharm. Res.* **2010**, 27: 1512-1529.

**Abstract:** A database of 29 antibody-antigen complexes having high-resolution structures was examined. All Fabs contained at least one aggregation-prone region (APR). Aggregation and antigen recognition may be coupled via aromatic residues, like Tyr and Trp. It was found that APRs were infrequent in the heavy chain CDR3 (H3), but common in H2 loops, occurring 45% of the time. Coincidence of APRs and antigen recognition sites suggests that aggregation will lead to loss of function.

**Analysis:** Interestingly, the groups that allow antibodies to be specific in antigen recognition also lead to greater propensity to aggregate. This group from Pfizer continues earlier work of theirs looking at APRs in MABs. Previously, they evaluated a number of marketed MABs for the presence and distribution of APRs. This concept of aromatic residues playing important roles in aggregation, as well as in antigen recognition, is seen in the paper by Wu et al. discussed below.

## PHASE SEPARATION OF A IgG1

Nishi et al., Phase Separation of an IgG1 Antibody Solution under a Low Ionic Strength Condition. *Pharm. Res.* **2010**, 27: 1348-1360.

**Abstract:** Liquid-liquid phase separation (LLPS) was observed for a IgG1 monoclonal antibody (MAB). A phase diagram was constructed using data from dynamic light scattering, analytical ultracentrifugation, and viscosity measurements. The MAB has a clear appearance under isotonic conditions, but undergoes LLPS at low ionic strength. The two phases become miscible as the ionic strength and temperature are raised. It appears that the MAB self-associates at low ionic strength at a concentration above the critical concentration of 16.5 mg/ml. There appears to

be an attractive protein-protein interaction in the protein-rich phase, inducing LLPS.

**Analysis:** Here is a study from Osaka University and Daiichi Sankyo on a MAb that undergoes LLPS at low ionic strength. It is a detailed study, with detailed characterization of the subsequent phases. We have too few studies like this on LLPS, especially with proteins of pharmaceutical relevance. I would consider this a 'must-read' article for anyone working on MAb formulation development.

### ANALYSIS OF THERMAL UNFOLDING PROFILES

Saini et al., Determination of Heat Capacity of Unfolding for Marginally Stable Proteins from a Single Temperature Induced Protein Unfolding Profile. *Thermochim. Acta* **2010**, *506*: 28-33.

**Abstract:** For marginally stable proteins, the pre-transition region of the unfolding curve may not be fully populated, making estimation of the heat capacity change ( $\Delta C_p$ ) for denaturation difficult. Analysis of denaturation profiles for cytochrome c and myoglobin at low pH shows that an accurate value of  $\Delta C_p$  can be obtained from a single unfolding profile obtained spectroscopically by including low temperature data.

**Analysis:** I think it is always helpful to highlight papers that discuss the best methods for data analysis. This study provides guidance on how to evaluate unfolding curves, even when portions are incomplete. What they demonstrate is that small differences in the estimated  $\Delta C_p$  lead to large variations in the fit of the experimental data. Therefore, one can obtain a relatively accurate estimation of  $\Delta C_p$ , even when using spectroscopic methods to monitor protein denaturation.

### ANALYSIS OF AGGREGATION KINETICS AND LAG TIME

Auer and Kashchiev, Insight into the Correlation Between Lag Time and Aggregation Rate in the Kinetics of Protein Aggregation. *Proteins* **2010**, *78*: 2412-2416.

**Abstract:** In this study, the correlation between lag time and the maximal rate of aggregation was analyzed. It was found that the product of these two quantities depends on a single numerical parameter, the kinetic index of the curve describing the time evolution of the fraction of protein aggregated. This index seems to be independent of the conditions or the protein being studied. Finally, it is shown that this kinetic index is a basic parameter related to a recently proposed theory of protein aggregation.

**Analysis:** While it is appreciated that aggregation processes proceed during the lag phase, here we have a study that connects the lag time with the rate of maximal aggregation, albeit within the context of specific aggregation kinetic scheme. Even so, every study like this provides us with a greater understanding of how aggregation rates are related to specific mechanisms. Given the importance and complexity of this issue, new insights are always appreciated.

## DESTABILIZATION BY 2-HYDROXY- PROPYL- $\beta$ -CYCLODEXTRIN

Maloletkina et al., Effect of 2-hydroxypropyl- $\beta$ -cyclodextrin on Thermal Inactivation, Denaturation and Aggregation of Glyceraldehyde-3-phosphate Dehydrogenase from Rabbit Skeletal Muscle. *Int. J. Biol. Macromol.* **2010**, 46: 487-492.

**Abstract:** The effect of 2-hydroxypropyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD) on the thermal stability of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was investigated using dynamic light scattering. In the presence of HP- $\beta$ -CD, the rate of aggregation increased and larger protein particles were formed. The accelerated degradation of GAPDH was due to destabilization of the multimeric structure of the enzyme, as evidenced by differential scanning calorimetry (DSC).

**Analysis:** The number of studies on the effects of cyclodextrins on protein stability is still relatively small, but most have found there to be a benefit to using them as stabilizers. Here we have evidence that they disrupt multimeric enzymes, affecting the quaternary structure. In doing so, they lower the thermal stability, at least for GAPDH. I expect this may be a general finding for multimeric enzymes, but such studies are yet to be done. Still, it is helpful to know that certain excipients may not always improve stability.

## ADSORPTION OF A SURFACTANT AND A MAb TO FILTERS

Mahler et al., Adsorption Behavior of a Surfactant and a Monoclonal Antibody to Sterilizing-Grade Filters. *J. Pharm. Sci.* **2010**, 99: 2620-2627.

**Abstract:** Lab-scale testing of adsorption behavior of different filter materials from different suppliers was conducted for solutions containing a monoclonal antibody and polysorbate 80. Subsequently, these were tested at manufacturing scale. Nylon filters showed the highest level of protein adsorption. At the manufacturing scale, adsorption of polysorbate 80 was observed. Thus, adsorption of both protein and surfactant must be considered when designing filtration processes and assessed on a case-by-case basis.

**Analysis:** For anyone involved in aseptic processing, this information is quite helpful, as it shows the material and vendor dependence of protein adsorption. The differences are substantial. It also emphasizes that surfactants can also interact with filters, potentially altering polysorbate levels (and changing the degree of protein adsorption as well).

## HIGH-THROUGHPUT FORMULATION DEVELOPMENT OF ANTIBODIES

Zhao et al., Formulation Development of Antibodies Using Robotic System and High-Throughput Laboratory (HTL). *J. Pharm. Sci.* **2010**, 99: 2279-2294.

**Abstract:** An automated system, capable of performing sample preparation, testing and data management, is described. Up to 500 liquid samples can be prepared and distributed to microplates for forced degradation studies. The system can be used to analyze samples using UV spectroscopy, turbidity, DLS, and various kinds of HPLC (RP, CEX, and SEC). The data generated with this system were comparable to those obtained with classical approaches.

**Analysis:** Here is a detailed description of a high throughput robotic system put in place at Novartis. Unlike other articles on the topic, we are given an extensive comparison of data from the robotic system to those collected by standard methods for a number of antibody formulations. Anyone interested in the topic would benefit from careful study of this article.

## VCD OF PROTEINS

Ma et al., Near-Infrared and Mid-Infrared Fourier Transform Vibrational Circular Dichroism of Proteins in Aqueous Solution. *Appl. Spectroscopy* **2010**, 64: 615-626.

**Abstract:** Vibrational circular dichroism (VCD) of various proteins in water is reported, including, for the first time, in the near-infrared (NIR) region. The NIR VCD spectra show distinct spectral features for different secondary structure compositions. In particular, the A-II and B-II combination bands, consisting of NH stretching motions seem to be valuable in distinguishing proteins of different structure. The NIR VCD spectrum of an insulin fibril sample is reported, where the growth of the fibrils in solution can be monitored.

**Analysis:** Potentially, VCD could provide additional structural information compared to infrared (IR) spectroscopy. However, for a long time, the limitation had been access to commercially available equipment. This study describes new VCD equipment that can capture IR spectra at the same time and monitor spectral changes in the NIR as well. This equipment also yields better signal-to-noise than earlier instruments. While VCD is still quite a weak phenomenon and requires lengthy data accumulation, it is interesting to see some of what VCD could provide in terms of structural characterization of proteins.

## IMPROVED SOLUBILITY OF MABs

Wu et al., Structure-Based Engineering of a Monoclonal Antibody for Improved Solubility. *Prot. Eng. Des. Selection* **2010**, 23: 643-651.

**Abstract:** An anti-IL-13 monoclonal antibody (MAB) was engineered for improved solubility. Three approaches were taken, including (i) modifying the pI, (ii) decreasing the overall surface hydrophobicity, and (iii) reintroducing an N-linked glycosylation site within a CDR. One mutant with a modified pI value showed a 2-fold increase in solubility. Several mutants with decreased overall surface hydrophobicity showed moderate increases in solubility. The glycosylated variant showed greatly increased solubility. Of the three approaches, adding an N-linked carbohydrate was the most effective at increasing solubility.

**Analysis:** Here we have a direct comparison of different ideas about how to improve the solubility of a MAB. Reintroducing a glycosylation site that was most occupied had the biggest effect. However, it did interfere with antigen binding, although alternative sites could, in theory, be used. In addition, this study found that a specific group of residues (Phe-His-Trp) in the heavy chain CDR3 was responsible for aggregation in this MAB. We have seen other studies indicating that groups important for antigen binding also being aggregation prone, so this appears to be an emerging theme in antibody stabilization.

## COLLOIDAL STABILITY OF LYSOZYME

Le Brun et al., Correlation of Protein-Protein Interactions as Assessed by Affinity Chromatography with Colloidal Protein Stability: A Case Study with Lysozyme. *Pharm. Dev. Technol.* **2010**, 15: 421-430.

**Abstract:** Lysozyme-lysozyme interactions were measured using self-interaction chromatography (SIC), for both the native state (25° C) and the denatured state (80° C). Addition of salt reduced the osmotic second virial coefficient ( $B_{22}$ ) for both states. The decrease was more pronounced for the denatured state. Lysozyme formulation presenting more positive  $B_{22}$  values showed better stability when subjected to thermal and agitation stress. Thus, colloidal stability, as measured using SIC, leads to formulations that are more stable.

**Analysis:** While information on the colloidal stability of proteins continues to grow, there are still too few that have demonstrated a correlation between  $B_{22}$  values and the physical stability of proteins. Rarer still are studies that look at the colloidal stability of the unfolded or denatured forms. This work speaks to both of these issues, making it definitely worth careful study.

## MONITORING MANUFACTURING USING RAMAN SPECTROSCOPY

Wen et al., Application of Raman Spectroscopy in Biopharmaceutical Manufacturing. *Am. Pharm. Rev.* **2010**, 13(4): 46-53.

**Abstract:** Raman spectroscopy and Raman microscopy are well suited for analysis of solid samples. The laser beam can be focused to allow characterization of very small particles, even through glass, removing any need for sample manipulation. These techniques were used to investigate incidents during biopharmaceutical manufacturing, including gelation in a syringe and particulate identification.

**Analysis:** This group from Amgen has previously reported on the value of Raman methods in particle characterization. Here are some new examples, including powders on the outside and inside of vials. There is also an interesting study on gelation within a syringe.

## RADICAL-INDUCED HINGE CLEAVAGE

Yates et al., Histidine Residue Mediates Radical-Induced Hinge Cleavage of Human IgG1. *J. Biol. Chem.* **2010**, 285: 18662-18671.

**Abstract:** Hydroxyl radicals were found to induce hinge cleavage in a human IgG1 via radical formation at Cys231. Direct involvement of His229 was implicated as mutation to remove it reduced hinge cleavage by 75 to 85 %. It is hypothesized that the imidazole ring is capable of forming a radical center than can extract a protein from neighboring residues. This work suggests that more stable antibody structures could be

engineered that are capable of resisting hinge cleavage.

**Analysis:** Here is another study from Amgen, this one a detailed biochemical study on hydroxyl radical-induced cleavage of the peptide backbone on the hinge region of a human IgG1. The work follows up on their initial report of this process (Yan et al., *J. Biol. Chem.* **2009**, 284: 35390-35402). Typically, we think of hydroxyl radicals causing oxidation reactions. This one actually could lead to His to Asp conversion, along with cleavage of the hinge region, all without affecting the surrounding amino acids. This is one more degradation pathway that needs to be considered for monoclonal antibodies.

## AGGREGATION OF CYSTATINS

Esposito et al., Aggregation Mechanisms of Cystatins: A Comparative Study of Monellin and Oryzacystatin. *Biochemistry* **2010**, 49: 2805-2810.

**Abstract:** The aggregation behavior of two members of the cystatin superfamily were investigated: monellin and oryzacystatin I (ORYZ), a plant cystatin. Despite their structural similarities, monellin forms amyloid fibrils while ORYZ only forms amorphous aggregates. Mutants of ORYZ, designed to make it more similar to monellin, still formed amorphous aggregates. It was observed that ORYZ undergoes some type of structural transition at 50° C, well below T<sub>m</sub>. Also, removal of the unstructured terminal tails of ORYZ does seem to increase the likelihood for fibril formation. Therefore, ORYZ may be a promising model protein for studying protein aggregation mechanisms.

**Analysis:** More examples are emerging of proteins following distinct pathways to either amyloid or amorphous aggregates. Studies like this one, where higher order structural

similarities still lead to different types of aggregates provides some insights into what controls these pathways. Systems like this also allow one to assess whether amyloid formation is a truly general property of proteins (given sufficient destabilization of the native structure) or not.

## FLEXIBILITY OF ANTIBODIES

Zimmermann et al., *Molecular Description of Flexibility in an Antibody Combining Site. J. Phys. Chem. B* **2010**, *114*: 7359-7370.

**Abstract:** The finite number of Germline antibodies suggests that they are polyspecific and specificity increases during maturation. The one biophysical property that should coincide with polyspecificity is flexibility. The present work extends a previous study (*Proc. Natl. Acad. Sci. USA* **2007**, *104*: 8821-8826) that found flexibility decreased during affinity maturation. The current work provides a concrete link between structural features and spectroscopic measures of flexibility. Along with molecular dynamics simulations, a molecular level description of how protein dynamics and biological function are interrelated is emerging.

**Analysis:** Flexibility continues to be an important theme in protein structure and stability. Our understanding of how it connects to stability is still far from complete, but here we are provided with some ideas of how flexibility is related to binding affinity in antibodies and what structural changes are made to modulate flexibility. It is definitely helpful to read the earlier paper along side of this one.

## STABILITY ENGINEERING OF scFvs

Miller et al., *Stability Engineering of scFvs for the Development of Bispecific and Multivalent Antibodies. Protein Eng. Des. Select.* **2010**, *23*: 549-557.

**Abstract:** Single chain Fvs can be used as therapeutic and diagnostic agents themselves or be used to form bispecific antibody structures. Focused libraries of scFvs were designed using statistical analyses and structure-based methods to improve stability. Thermally stable scFvs were obtained that retain full antigen binding. Single mutations increased the  $T_m$  by as much as 14° C relative to the wild type. Combinations of mutations increased  $T_m$  by an additional 12° C. Introduction of these mutations enable the scalable production of scFvs and bispecific antibodies containing these fragments.

**Analysis:** This study comes from Biogen Idec, as they looked to improve upon the conformational stability of scFvs, which are often quite unstable. The mutations were based on model building but also on the relative frequency of a residue at a certain position. In doing so, significant increases in structural stability (as indicated by  $T_m$  values) were obtained. As many groups are interested in scFvs themselves and in platforms incorporating these fragments, this study should be informative and helpful.