4th International Symposium on Higher Order Structure of Protein Therapeutics (HOS 2015)

Symposium Co-chairs:
Jamie Moore, Genentech, a Member of the Roche Group
Linda Narhi, Amgen Inc.

April 13-15, 2015
Courtyard Downtown Boston
Boston, MA USA

Organized by
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The Organizing Committee gratefully acknowledges the Program Partners and Exhibitors for their generous support of the 4th Symposium on Higher Order Structure of Protein Therapeutics:

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- **Biogen**
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Welcome Reception

Waters Corporation
The Organizing Committee gratefully acknowledges the following media for their promotional consideration of the 4th Symposium on Higher Order Structure of Protein Therapeutics:

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Scientific Program Committee:
Wasfi Al-Azzam, GlaxoSmithKline Pharmaceuticals
David Bain, University of Colorado Anschutz Medical Campus
Katherine Bowers, FUJIFILM Diosynth Biotechnologies
Michael Brenowitz, Albert Einstein College of Medicine
Guodong Chen, Bristol-Myers Squibb Company
Steven Cohen, Northeastern University
Otmar Hainzl, Sandoz Biopharmaceuticals
Damian Houde, Biogen
David Keire, CDER, FDA
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Renee Olson, Senior Program Manager
Anna Lingel, Project Coordinator
Catherine Stewart, Finance Manager
CASSS Higher Order Structure Student Travel Grants
CASSS is pleased to provide a limited number of student travel grants for PhD students and post-docs who present applicable posters at the 4th International Symposium on Higher Order Structure of Protein Therapeutics (HOS 2015). PhD students or post-doctoral fellows conducting research at academia throughout the world are eligible.

This year’s grant winners include:

**Ion Mobility - Mass Spectrometry for Structural Analysis of Monoclonal Antibody Drug**
Carly Ferguson, *CDER, FDA*

**Hydrogen Exchange Mass Spectrometry Reveals Protein Interfaces and Distant Dynamic**
Arora Jayant, *University of Kansas*

**Epitope Mapping of Human Interleukin 23 Interacting with Antibody by Fast Photochemical Oxidation of Proteins (FPOP)**
Jing Li, *Washington University in St. Louis*

**Investigation of Protein-particle Interactions in Vaccine Formulations by Fluorescence Spectroscopy**
Annamaria Vilinska, *Columbia University*

**Characterization of Therapeutical Protein Stability and Aggregation at High Concentration via Concomitant DLS and Raman Spectroscopy**
Chen Zhou, *University of Colorado*
Social Program

Welcome Reception
Monday, April 13
17:30 – 20:00
The Engen Laboratory, a Waters Center of Innovation
Sponsored by Waters Corporation

CASSS and Waters Corporation invite you to the HOS 2015 Conference welcome reception to be held at Northeastern University. Professor John Engen, a leading expert on hydrogen-deuterium exchange mass spectrometry (HDX MS), will deliver a keynote presentation entitled “How Hydrogen Exchange Mass Spectrometry Can Help Investigate Protein Higher Order Structure”. Please join us and enjoy the evening with Professor Engen and his group at their lab. You will have the opportunity to:

• Network with fellow attendees at HOS 2015 and members of the Engen Laboratory
• Exchange your thoughts and learn from a leading expert on HDX MS
• Visit posters from the Engen Laboratory
• Visit the Engen Laboratory to see the technologies behind the modern HDX MS studies

Please meet the CASSS Staff in the lobby after the Late Breaking Session on Monday, April 13 to take transportation over to The Engen Laboratory.

Exhibitor Reception
Tuesday, April 14
17:40 – 19:00
Courtyard Boston Downtown in the Washington Ballroom
# 4th International Symposium on Higher Order Structure of Protein Therapeutics Scientific Program Summary

**Monday, April 13**

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<tr>
<th>Time</th>
<th>Event</th>
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<tr>
<td>07:30 – 17:00</td>
<td>Registration in the 5th Floor Foyer</td>
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<tr>
<td>07:30 – 08:30</td>
<td>Continental Breakfast in the Washington Ballroom</td>
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<tr>
<td>08:30 – 08:45</td>
<td>Welcome and Introductory Comments in the Theatre Ballroom</td>
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<tr>
<td></td>
<td>Linda Narhi, Amgen Inc., Thousand Oaks, CA USA</td>
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<tr>
<td></td>
<td><strong>Keynote Session</strong> in the Theatre Ballroom</td>
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<td><strong>Session Chair:</strong> Linda Narhi, Amgen Inc., Thousand Oaks, CA USA</td>
</tr>
<tr>
<td>08:45 – 09:45</td>
<td>Progress in Understanding Relationships between Higher Order Structure and Clinical Performance - <em>Is De Novo Protein Design Feasible?</em></td>
</tr>
<tr>
<td></td>
<td>Mark Schenerman, MedImmune, A member of the AstraZeneca Group, Gaithersburg, MD USA</td>
</tr>
<tr>
<td>09:45 – 10:15</td>
<td>Break – Visit the Exhibits and Posters in the Washington Ballroom</td>
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<tr>
<td>10:15 – 10:35</td>
<td>Higher Order Structure in Development Session in the Theatre Ballroom</td>
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<tr>
<td></td>
<td><strong>Session Chair:</strong> Katherine Bowers, FUJIFILM Diosynth Biotechnologies, Cary, NC USA</td>
</tr>
<tr>
<td>10:15 – 10:35</td>
<td>Biophysical Analyses of HOS Stability and Comparability for ADCs</td>
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<td></td>
<td>Yin Luo, Pfizer, Inc., Andover, MA USA</td>
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<tr>
<td>10:35 – 10:55</td>
<td>Microstructure of Amorphous Dense Phases of Proteins</td>
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<td>Abraham Lenhoff, University of Delaware, Newark, DE USA</td>
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<tr>
<td>10:55 – 11:15</td>
<td>Predicting and Understanding Aggregation of Antibody Therapeutics at Air-water Interfaces</td>
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<td>Ian Shieh, Genentech, a Member of the Roche Group, South San Francisco, CA USA</td>
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<tr>
<td>11:15 – 11:40</td>
<td>Discussion – Questions and Answers</td>
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<tr>
<td>11:40 – 13:10</td>
<td>Hosted Lunch in the Washington Ballroom</td>
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Monday, April 13

**Biological Consequences Session** in the Theatre Ballroom

**Session Chairs:** Aston Liu, GlaxoSmithKline, King of Prussia, PA USA
and Jamie Moore, Genentech, a Member of the Roche Group, South San Francisco, CA USA

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<th>Time</th>
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<tr>
<td>13:10</td>
<td>Higher Order Structures in Protein Therapeutics: Friend or Foe for Eliciting Immunogenic Responses</td>
<td>Binodh Desilva, Bristol-Myers Squibb Company, Princeton, NJ USA</td>
</tr>
<tr>
<td>13:30</td>
<td>Engineering Antibody Fab Fragments for Treatment of Ocular Disease</td>
<td>Bob Kelley, Genentech, a Member of the Roche Group, South San Francisco, CA USA</td>
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<tr>
<td>13:50</td>
<td>Functional Relevance of Mutations on the Higher Order Structure and Activity of a Therapeutic Enzyme</td>
<td>Flaviu Gruia, MedImmune, A member of the AstraZeneca Group, Gaithersburg, MD USA</td>
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<tr>
<td>14:10</td>
<td>Discussion – Questions and Answers</td>
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<tr>
<td>14:35</td>
<td>Poster Session in the Washington Ballroom</td>
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**Late Breaking Session** in the Theatre Ballroom

**Session Chairs:** Linda Narhi, Amgen Inc., Thousand Oaks, CA USA
and Jamie Moore, a Member of the Roche Group, South San Francisco, CA USA

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<td>15:35</td>
<td>Structural Mapping on the NIST Standard Monoclonal Antibody by 2D NMR Fingerprinting Methods</td>
<td>Robert Brinson, NIST, Rockville, MD USA</td>
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<tr>
<td>15:55</td>
<td>Hydrogen Exchange Mass Spectrometry Reveals Protein Interfaces and Distant Dynamic Coupling Effects during the Reversible Self-Association of an IgG1 Monoclonal</td>
<td>Jayant Arora, University of Kansas, Lawrence, KS USA</td>
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<tr>
<td>16:15</td>
<td>Including Protein Separation Methods in a Higher Order Structure Characterization Strategy</td>
<td>Yung-Hsiang Kao, Genentech, a Member of the Roche Group, South San Francisco, CA USA</td>
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<td>16:35</td>
<td>Thermal Unfolding of Developable Therapeutic mAbs: Implications for Candidate Selection and Formulation Design</td>
<td>Mark Brader, Biogen, Cambridge, MA USA</td>
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<tr>
<td>16:55</td>
<td>Discussion – Questions and Answers</td>
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Monday, April 13

17:30 – 20:00  Welcome Reception  
The Engen Laboratory, a Waters Center of Innovation  
Please meet the CASSS Staff in the hotel lobby for transportation over to The  
Engen Laboratory.
## Tuesday, April 14

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<tr>
<td>07:30 – 08:30</td>
<td><strong>Technical Seminar</strong></td>
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<td><strong>Beyond FT-IR &amp; Raman: Predictability and Sensitivity of ROA</strong></td>
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<td>Sponsored by BioTools, Inc.</td>
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<td>Theatre Ballroom</td>
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<tr>
<td>08:30 – 09:30</td>
<td><strong>Rational Approaches for the Development of Biotherapeutics</strong></td>
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<td>Bernhardt Trout, <em>Massachusetts Institute of Technology, Cambridge, MA USA</em></td>
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<tr>
<td>09:30 – 10:00</td>
<td><strong>Break</strong> – Visit the Exhibits and Posters in the Washington Ballroom</td>
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<td>10:00 – 10:20</td>
<td><strong>What Controls the Size and Structure of Protein Aggregates?</strong></td>
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<td>Christopher Roberts, <em>University of Delaware, Newark, DE USA</em></td>
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<td>10:20 – 10:40</td>
<td><strong>Quantifying Secondary and Tertiary Structure Interactions that Lead to Cooperative Protein Folding and Assembly</strong></td>
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<td>Doug Barrick, <em>The Johns Hopkins University, Baltimore, MD USA</em></td>
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<td>10:40 – 11:00</td>
<td><strong>New Approaches to Precision Biologics</strong></td>
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<td>Steven Almo, <em>Albert Einstein College of Medicine, Bronx, NY USA</em></td>
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<tr>
<td>11:00 – 11:25</td>
<td><strong>Discussion – Questions and Answers</strong></td>
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<tr>
<td>11:25 – 12:55</td>
<td><strong>Hosted Lunch</strong> in the Washington Ballroom</td>
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Emerging and Novel Technologies Session in the Theatre Ballroom
Session Chairs: David Bain, University of Colorado Anschutz Medical Campus, Aurora, CO USA
and Guodong Chen, Bristol-Myers Squibb Company, Princeton, NJ USA

12:55 – 13:15  A Novel Technology for Mapping Native Protein-protein Interfaces
Alessandra Luchini, George Mason University, Fairfax, VA USA

13:15 – 13:35  Complex Interaction in Serum Analyzed by FDS AUC
John J. Correia, University of Mississippi Medical Center, Jackson, MS USA

Maofu Liao, Harvard Medical School, Cambridge, MA USA

13:55 – 14:20  Discussion – Questions and Answers

Young Scientist Session in the Theatre Ballroom
Session Chairs: Wasfi Al-Azzam, GlaxoSmithKline, King of Prussia, PA USA
and Steven Cohen, SAC Analytical Consultants, Hopkinton, MA USA

14:20 – 14:35  Characterization of Therapeutical Protein Stability Aggregation at High Concentration via Concomitant DLS and Raman Spectroscopy
Chen Zhou, University of Colorado, Aurora, CO USA

14:35 – 14:50  Investigation of Protein-particle Interactions in Vaccine Formulations by Fluorescence Spectroscopy
Annamaria Vilinska, Columbia University, New York City, NY USA

14:50 – 15:05  Epitope Mapping of Human Interleukin 23 Interacting with Antibody by Fast Photochemical Oxidation of Proteins (FPOP)
Jing Li, Washington University in St. Louis, MO USA

15:05 – 15:20  Break – Visit the Exhibits and Posters in the Washington Ballroom

Imaging Approaches to Assess HOS Session in the Theatre Ballroom
Session Chairs: Damian Houde, Biogen, Cambridge, MA USA
and Yu Zhou, BioMarin Pharmaceutical Inc., Novato, CA USA

15:35 – 15:55  Structural-functional Analysis of a Recombinant Neurotrophic Factor After low-pH Viral Inactivation
Joe Arndt, Biogen, Cambridge, MA USA

15:55 – 16:15  X-ray Solution Scattering as a Probe of Higher-Order Structure in Proteins
Lee Makowski, Northeastern University, Boston, MA USA
Tuesday, April 14

16:15 – 16:40  Discussion – Questions and Answers

16:40 – 17:40

Regulatory Panel Discussion in the Theatre Ballroom
Facilitator: David Keire, CDER, FDA, St. Louis, MO USA

Panelists:
Yves Aubin, Health Canada, Ottawa, ON Canada
Barry Cherney, Amgen Inc., Rockville, MD USA
Ashley Gucinski, CDER, FDA, St. Louis, MO USA

17:40 – 19:00  Exhibitor Reception in the Theatre Ballroom
Wednesday, April 15

08:00 – 12:00  **Registration** in the 5th Floor Foyer

07:30 – 08:30  **Continental Breakfast** in the Washington Ballroom

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<tr>
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<td><strong>Biosimilars and Comparability Session</strong> in Theatre Ballroom</td>
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<td><strong>Session Chairs:</strong> Otmar Hainzl, <em>Sandoz GmbH, Oberhaching, Germany</em> and David Keire, <em>CDER, FDA, St. Louis, MO USA</em></td>
</tr>
<tr>
<td>08:30 – 08:50</td>
<td><strong>New Analytical Approaches and Data Visualization Tools to Assess Protein Higher-Order Structure and Pharmaceutical Stability as Applied to Comparability Assessments</strong></td>
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<td>David Volkin, <em>University of Kansas, Lawrence, KS USA</em></td>
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<tr>
<td>08:50 – 09:10</td>
<td><strong>Assessing Comparability and Biosimilarity from a Biophysical Perspective</strong></td>
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<td>Steven Berkowitz, <em>Consultant, Boston, MA USA</em></td>
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<tr>
<td>09:10 – 09:30</td>
<td><strong>Higher Order Structure Characterization: Which Methods are Best Suited for Evaluation of Product Comparability?</strong></td>
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<td>John Gabrielson, <em>Amgen Inc., Thousand Oaks, CA USA</em></td>
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<td>09:30 – 09:55</td>
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<td>10:25 – 10:45</td>
<td><strong>Protein Therapeutics Discovery and Candidate Selection Session</strong> in the Theatre Ballroom</td>
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<td><strong>Session Chairs:</strong> Linda Narhi, <em>Amgen Inc., Thousand Oaks, CA USA</em> and Satish Singh, <em>Pfizer, Inc., Chesterfield, MO USA</em></td>
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<tr>
<td>10:25 – 10:45</td>
<td><strong>Engineering Developability of Human Antibody Therapeutics</strong></td>
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<td>Karl Dane Wittrup, <em>Massachusetts Institute of Technology, Cambridge, MA USA</em></td>
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<tr>
<td>10:45 – 11:05</td>
<td><strong>Higher Order Structural Characterization of Engineered Bispecific Antibody</strong></td>
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<td>Jirong Lu, <em>Eli Lilly and Company, Indianapolis, IN USA</em></td>
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<tr>
<td>11:05 – 11:25</td>
<td><strong>Preclinical Candidate Selection using Higher Order Structure</strong></td>
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<td>Nicolas Angell, <em>Amgen Inc., Thousand Oaks, CA USA</em></td>
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<td>11:25 – 11:50</td>
<td><strong>Discussion - Questions and Answers</strong></td>
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<tr>
<td>11:50 – 12:00</td>
<td><strong>Closing Remarks</strong></td>
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<td>Jamie Moore, <em>Genentech, a Member of the Roche Group, South San Francisco, CA USA</em></td>
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Progress in Understanding Relationships between Higher Order Structure and Clinical Performance - Is De Novo Protein Design Feasible?

Mark Schenerman

MedImmune, A member of the AstraZeneca Group, Gaithersburg, MD USA

Understanding the relationships between changes in higher order structure for a therapeutic protein and the subsequent impact on clinical performance is key to successful biologics development. As technologies for measurement of HOS evolve, they need to be assessed against clinical performance attributes (safety and efficacy). Changes in HOS can sometimes be linked to changes in potency, PK/PD, toxicity, immunogenicity, and/or stability. However, the ability to measure the relevant structural changes and evaluate their significance continues to be a challenge. Case studies will be presented to illustrate how knowledge of HOS has led to a deeper understanding of the product and subsequent benefits to patients. The question of whether this enhanced knowledge can enable de novo protein design will also be explored.

NOTES:
Biophysical Analyses of HOS Stability and Comparability for ADCs

Yin Luo, Sharon Polleck, Lucy Liu

_Pfizer, Inc., Andover, MA USA_

Antibody drug conjugates (ADCs) are designed to be more effective biotherapeutic reagents, combining the benefits of potent cytotoxic drug molecules with the binding specificity of antibodies. However, conjugating drug molecules to antibodies requires covalent modifications, which necessarily impact the antibody’s primary structure, and may also alter their higher order structure. These modifications may or may not affect the antibody’s short-term and long-term stabilities, and/or the antigen-binding activities. Therefore, it is important to have methods that are sensitive to changes in protein higher order structure (HOS) to facilitate the studies of structure-function relationship and comparability for ADCs. In this presentation, the utility and limitations of several biophysical methods for providing information on the secondary, tertiary and quaternary structures of ADCs are shown. The caution required in data interpretation, and the value of cross-examining different methods to reach integrated conclusions, are demonstrated through case studies. These studies also indicated that the antibody structure may be influenced by the drug payload, including the hydrophobicity, the number and locations, as well as the conjugation chemistry. Potential implications of the antibody structure on the quality and safety of ADCs as biotherapeutics are considered.

NOTES:
Microstructure of Amorphous Dense Phases of Proteins

Abraham Lenhoff, Daniel G. Greene, Norman J. Wagner, Stanley I. Sandler

University of Delaware, Newark, DE USA

Protein dense phases, such as precipitates, crystals, gels and aggregates, appear in many guises in downstream processing, formulation and delivery; in some cases their appearance is desirable and in others not. The molecular structure and microstructure of the dense phase is known in exquisite detail in the case of crystals but is poorly understood for most amorphous dense phases. Such dense phases have been explored as candidate drug delivery vehicles, especially for monoclonal antibodies. An improved understanding of the structure and of structure–function relations can facilitate methods for designing new formulations, ameliorating the effects of high molecular weight aggregates, and designing novel separation schemes.

In this work we use scattering techniques and real-space imaging to explore a model protein system that has not been extensively investigated previously in order to understand better how protein interactions give rise to the observed phase behavior and to the microstructure of the resulting protein dense phases. The main system studied is a model one in which ovalbumin precipitates are formed in concentrated ammonium sulfate solutions. Small-angle neutron scattering (SANS) measurements show that macroscopically non-crystalline ovalbumin precipitate particles formed under high-salt conditions exhibit a microstructure at small length scales that can be described by a nanocrystalline cluster made up of a relatively small number of unit cells of ovalbumin. Additional structural features appear at longer length scales in the SANS data. The model fits of the SANS data can be correlated with direct real-space imaging using electron microscopy and electron tomography. Additional insights can be obtained from time-dependent observations that show the emergence of different structural features on a variety of time scales.

NOTES:
Predicting and Understanding Aggregation of Antibody Therapeutics at Air-water Interfaces

Ian Shieh, Danielle Leiske, Ankit Patel

Genentech, a Member of the Roche Group, South San Francisco, CA USA

Interactions at an air-water interface can accelerate aggregation of antibody therapeutics, particularly under agitation stress immediately prior to administration when both the product and the protective surfactants are diluted. A better understanding of interfacially mediated aggregation could assist during molecule candidate selection and in the design of more robust formulations to limit the formation of potentially immunogenic aggregates. A panel of monoclonal antibody therapeutics, exhibiting a range of aggregation propensities at an air-water interface, was characterized by multiple surface-sensitive techniques to enable the time-sensitive measurement of the intermolecular interactions, concentration, hydrophobicity, and mechanical properties of the interfacially adsorbed antibodies. The antibodies with the highest interfacial aggregation propensity exhibited more than a five-fold faster initial rate of surface pressure increase upon adsorption to the air-water interface compared to antibodies with a low aggregation propensity. The highest-risk antibodies also rapidly formed hydrophobic films upon adsorption, which indicated these molecules were susceptible to interfacially induced protein unfolding. Strong, essentially irreversible protein-protein interactions, likely dominated by hydrophobic interactions, resulted in high levels of interfacial aggregation for these antibodies. In contrast, the low-risk antibodies required many minutes of interfacial exposure to form hydrophobic films. These low-risk antibodies did not develop strong, irreversible interactions and as a result exhibited negligible aggregation. Overall, differences in the evolution of antibody film properties support a multistep aggregation mechanism at the air-water interface, where adsorption is a necessary but not sufficient condition for aggregation.

NOTES:
Higher Order Structures in Protein Therapeutics: Friend or Foe for Eliciting Immunogenic Responses

Binodh Desilva

Bristol-Myers Squibb Company, Princeton, NJ USA

The formation of aggregates and other higher order species in protein formulations is a major issue with the protein therapeutics. Most pharmaceutical companies make every effort to reduce these attributes in the final drug product to a minimum. This seminar will concentrate on the effect of higher order structures in the immunogenicity assessment. It will also focus on some attributes of immune-complexes which also can be of higher order structures.

NOTES:
Engineering Antibody Fab Fragments for Treatment of Ocular Disease

Robert Kelley

Genentech, a Member of the Roche Group, South San Francisco, CA USA

Less frequent dosing through use of long acting delivery (LAD) technologies is desirable for treatment of ocular diseases with therapeutic antibodies. Poor solubility, aggregation at high concentration, and chemical instability that affects potency can limit application of LAD to antibodies. Here we have increased the suitability of a therapeutic antibody Fab for LAD by using protein engineering to enhance the chemical and physical stability of the molecule. Structure-guided amino acid substitutions in a negatively charged CDR (CDR-L1) increase the chemical stability and solubility of the molecule. Variants show improved stability under both formulation and physiological conditions, and retain target binding and inhibitor potency comparable to the parental Fab. These variants have acceptable pharmacokinetic properties upon intravitreal injection in rabbits, and are suitable for $\geq 2$ month sustained release using triacetin-PLGA depot-forming formulation.
Functional Relevance of Mutations on the Higher Order Structure and Activity of a Therapeutic Enzyme

Flaviu Gruia, Arun Parupudi, Jared S. Bee

MedImmune, A member of the AstraZeneca Group, Gaithersburg, MD USA

The active enzyme is a non-covalent multi-subunit structure, with the active site located at the interface of the subunits. Biophysical investigations of recombinant mutants generated during early stage development showed that the enzymatic activity was modulated by subtle, tertiary structure changes. These conformational rearrangements were associated with a reversible low temperature melting transition in calorimetric measurements. Additional Higher Order Structure studies were employed to further understand the biological consequences of the conformational changes. These studies demonstrate the value of applying early biophysical characterization techniques for preclinical lead selection and identification.

NOTES:
Structural Mapping of the NIST Standard Monoclonal Antibody by 2D NMR Fingerprinting Methods

Robert Brinson, Luke Arbogast, John Marino

National Institute of Standards and Technology, Rockville, MD USA

The development of advanced techniques for the characterization of the higher order structure (HOS) of protein therapeutics, including monoclonal antibodies (mAbs), is emerging as a major priority in the pharmaceutical industry. While nuclear magnetic resonance (NMR) spectroscopy provides information on HOS in proteins at atomic resolution, limits on sensitivity, molecular size, and sample requirements, as well as the perceived need for stable-isotope enrichment, have limited its widespread use in the biopharmaceutical industry. However, recent hardware advances in ultra-high field NMR field strengths and cryogenically cooled probes have enabled the development and application of high resolution 2D heteronuclear NMR 'fingerprinting' methodologies of protein therapeutics at natural isotopic abundance. The individual peak positions from NH and CH correlation spectra are sensitive to HOS and can serve as spectral indicators for establishing consistency in drug manufacturing, assessing stability of drug formulations and for establishing biosimilarity to an innovator reference product. To demonstrate the applicability of NMR fingerprinting techniques to the monoclonal antibody class of biopharmaceuticals, we will present the NMR amide and methyl fingerprint characterization of the intact IgG-based NIST standard monoclonal antibody and 50 kDa Fab and Fc fragments, derived from facile enzymatic digestion. We demonstrate that the utilization of rapid acquisition techniques affords a CH-methyl spectral fingerprint in approximately 30 minutes. Finally, we describe robust statistical approaches to establish structural comparability between mAb drug samples.

NOTES:
Hydrogen Exchange Mass Spectrometry Reveals Protein Interfaces and Distant Dynamic Coupling Effects during the Reversible Self-Association of an IgG1 Monoclonal Antibody

Jayant Arora¹, John M. Hickey¹, Ranahoy Majumdar¹, Reza Esfandiary², Hardeep S. Samra², Steven M. Bishop², C. Russell Middaugh¹, David D. Weis¹, David B. Volkin¹

¹The University of Kansas, Lawrence, KS USA, ²MedImmune, A member of the AstraZeneca Group, Gaithersburg, MD USA

Concentration-dependent, reversible self-association (RSA) is a phenomenon in which native protein molecules form non-covalent and reversible oligomers in a concentration-dependent manner. The RSA of monoclonal antibodies present both process development and formulation challenges such as abnormally high solution viscosity at high protein concentrations. To mitigate RSA through protein engineering or formulation strategies, it is essential to gain information about the site and nature of such non-covalent interactions.

In the present study, we developed a novel hydrogen exchange mass spectrometry (HX-MS) method to map the interface of RSA on an IgG1 mAb (mAb-C). First, a combination of biophysical methods was used to characterize RSA of mAb-C and to identify solution conditions resulting in either predominantly monomeric or predominantly self-associated species of mAb-C in solution. Second, we developed a stabilizing formulation and lyophilization cycle to freeze-dry mAb-C at low and high protein concentrations. Third, the lyophilized cakes were then reconstituted under RSA promoting solution conditions in a D₂O buffer, and HX-MS experiments were conducted. Results were then compared to identify regions in mAb-C that showed significant protection against deuterium uptake under conditions that promote RSA.

HX-MS experiments revealed two specific regions, mainly populated around two of the six complementarity determining regions (CDR2H and CDR2L) of mAb-C, which showed significant protection against deuterium uptake at high vs. low protein concentrations. These results define the major protein-protein interface associated with RSA of mAb-C. Surprisingly, certain segments in V₃H, C₃H1-C₃H2 interface (hinge), and C₃H2 domains of mAb-C showed significant increases in local flexibility at high vs. low protein concentrations. These results indicate the concomitant presence of long-range, distant dynamic coupling effects within the antibody molecule upon RSA of mAb-C.

NOTES:
Including Protein Separation Methods in a Higher Order Structure Characterization Strategy

Yung-Hsiang Kao

*Genentech, a Member of Roche Group, South San Francisco, CA USA*

Biophysical techniques, particularly the spectroscopic methods (e.g. CD, FT-IR, NMR etc.) are traditionally considered as the tools for characterizing higher order structures (HOS) of proteins. In recent years, the HOS tool box has expanded to include many more different techniques, such as deuterium-hydrogen exchange mass spectrometry and various “molecular painting” techniques, such as hydroxyl radical footprinting. Some other biophysical methods, whose outputs do not directly provide any HOS information but can be influenced by protein conformation change, are also included in the expanded tool box. For example, differential scanning calorimetry (DSC), a thermoanalytical technique for studying protein stability, has also been used as an indirect HOS characterization method. In other cases, biological and functional assays have been used to assess protein HOS as well. In this presentation, we will use several examples to demonstrate the capabilities of different chromatographic and electrophoretic techniques to detect and differentiate protein structural variants. In fact, while protein separation methods, similar to many other biophysical methods, cannot offer detailed protein structure information, these methods are usually much more sensitive and often the first methods to reveal structure variants. Therefore, these protein analytical methods should also be considered effective HOS tools. We propose that a balanced HOS characterization strategy should include not only employing a tool box of various biophysical techniques but also using various protein separation techniques to differentiate protein structural variants.

NOTES:
Thermal Unfolding of Developable Therapeutic mAbs: Implications for Candidate Selection and Formulation Design

Mark Brader

Biogen, Cambridge, MA USA

Screening protein formulations and candidate molecules using thermal-ramp-based techniques such as differential scanning calorimetry (DSC) and differential scanning fluorimetry (DSF) are widely used to support early development in the biotechnology industry. The popularity of these techniques derives from the principle that integrity and stability of higher order structure will be best enhanced under conditions more resistant to thermal unfolding. However, the interrelationships between intrinsic protein conformational stability, thermal denaturation, and pharmaceutical stability are complex - especially for multi-domain proteins such as mAbs. This presentation will examine the thermal unfolding profiles of a series of authentically developable mAbs and describe formulation development and candidate selection learning points exemplified by this series.

NOTES:
Rational Approaches for the Developability of Biotherapeutics

Bernhardt Trout

*Massachusetts Institute of Technology, Cambridge, MA USA*

We describe a new strategic approach for developability based on applying both molecular and macroscopic modeling tools in order to gain an understanding of degradation processes with unprecedented detail and accuracy. Taking into account higher order structure is key to the success of these tools. Target areas include aggregation, oxidation, and hydrolysis. Also, tools for viscosity and crystallizability are described.

NOTES:
What Controls the Size and Structure of Protein Aggregates?

Christopher Roberts

University of Delaware, Newark, DE USA

Protein aggregates range in “size” from dimers (~ 5-10 nm) to visible particles (~ hundreds of microns), and are a long-standing concern for pharmaceutical products. The size of aggregates, and their spatial arrangement (i.e., structure and/or morphology) is important from the perspective of product quality, as it influences the choice of analytical methodologies, pharmaceutical elegance of the product, and potentially affects immunogenicity when administered to patients. This presentation focuses on the question of what physical factors determine the size of aggregates that form in protein solutions, as well as their structure. Example systems include monoclonal antibodies and globular proteins, as a function of typical formulation variables. The results highlight strengths and weaknesses of current analytical technologies for assessing the higher order structure of protein aggregates in solution, and also illustrate that different aggregation mechanisms can yield greatly different results.

NOTES:
Cooperativity is fundamental to folding, though it is poorly understood. The interactions that give rise to cooperatively folded systems serve to suppress intermediate, partly folded states, in turn, suppressing misfolding and aggregation. Misfolding and aggregation are significant problems both in human diseases and in limiting the effectiveness of protein therapeutics. We have used linear repeat proteins to quantify cooperativity, both to better understand how proteins fold, and to understand what can go wrong with they misfold and associate. We have created a variety of consensus repeat protein families that can be terminated by minimally substituted capping repeats on either (or both) end(s). This strategy facilitates nearest-neighbor 1D Ising analysis that accounts for energetic variation from caps. Consensus ankyrin repeats reveal strong coupling energy between repeats [see Wetzel et al, JMB 376, 241 (2008), Aksel et al., Structure 19, 249 (2011)]. Coupling is entropically driven at low temperatures, consistent with hydrophobic desolvation. This coupling is considerably stronger than for TPR repeats [Kajander et al., JACS 127, 10188 (2005)].

To understand these differences, we built a series of capped consensus repeats of different architectures, and subjected these to 1D-Ising analysis. One series, based on an array of 42 residue TPR-like proteins, shows increased cooperativity but low unfolding midpoints, largely from intrinsic instability. Another helical family, the 34 residue TALE repeats, shows enhanced cooperativity compared to TPRs, due to increased interfacial stabilization and lower intrinsic stability. Recently, we have succeeded in capped LRR arrays, which show higher cooperativity than any of the helical repeat proteins. This stability results from a combination of very strong intrinsic stabilities and very unstable LRR units. Although the structural basis for these differences is unclear, there is some correlation between buried polar interactions and high cooperativity.

NOTES:
New Approaches to Precision Biologics

Steve Almo

*Albert Einstein College of Medicine, Bronx, NY USA*

Not available at the time of print.

**NOTES:**
A Novel Technology for Mapping Native Protein-protein Interfaces

Alessandra Luchini

George Mason University, Fairfax, VA USA

Protein-protein interaction interfaces are important drug targets. Unfortunately, intimate, functionally important contact regions are extremely difficult to characterize because they are hidden inside the binding interface. We recently developed a novel technology, protein painting, for the rapid direct isolation and sequencing of hidden native protein-protein interfaces. Protein painting employs small molecules (molecular paints) to tightly coat the surface of protein-protein complexes. Paint chemistries have extremely high affinities for proteins: rapid on-rates, and very slow off-rates that are ten to 100 times higher than most protein-protein interactions. Each paint molecule spans only 3 amino acids or less. The molecular paints, which block trypsin cleavage sites, are excluded from the binding interface. Following painting, the unbound paints are washed away and the associated proteins are dissociated, linearized, digested with proteolytic enzymes, and sequenced by mass spectrometry (MS). The paint molecules remain non-covalently bound even after the proteins are denatured, reduced and alkylated. Therefore, following MS, only peptides hidden in the interface emerge as positive hits, revealing the functional contact regions that are drug targets.

We use protein painting to discover contact regions between the three-way interaction of IL1β ligand, the receptor IL1RI and the accessory protein IL1RAcP. A very high correlation (p<0.0003) was found between the sequences found by protein painting and the protein-protein contact points revealed by crystal structure, with a 97% specificity for true positive hot spot sequences. Side-by-side experimental comparison with chemical crosslinking and hydrogen deuterium exchange documented the superiority of protein painting in terms of number of positive hits, specificity of hits and ease of use. Protein painting revealed new highly conserved 3 way interaction point between IL1RAcP and the receptor-ligand complex that we used to create peptides and monoclonal antibodies to block the interaction and abolish IL1β cell signaling. The technology is broadly applicable to discover protein interaction drug targets.

NOTES:
Complex Interaction in Serum Analyzed by FDS AUC

John J. Correia

University of Mississippi Medical Center, Jackson, MS USA

The development of the fluorescence detection system (Aviv-FDS) for the AUC allows a single fluorescently labeled species to be quantitatively characterized against a highly concentrated and heterogeneous background. During our use of the FDS to characterize ELP, a novel drug delivery vector (see Lyons, et. al., Biophys. J. 104, 2009-2021, 2013), in serum, we encountered the Johnston-Ogston (J-O) effect. The J-O effect is a classical anomaly in sedimentation theory describing the non-ideal sedimentation properties of a component as a function of high concentrations of other components. We examined the J-O effect using recent advances in AUC hardware, the AU-FDS (AVIV Biomedical), and data-analysis methods, primarily Sedanal global direct boundary fitting. We empirically quantified the self and cross-sedimentation non-ideality properties of ELP and the two most ubiquitous serum proteins, Albumin (~35 mg/ml) and γ-Globulins (~10-15 mg/ml). We have verified and measured the presence of cross-term hydrodynamic and thermodynamic non-ideality by running SV studies on a fluorescently labeled component (~100 nM) in a titration experiment with high concentrations of unlabeled components. This has been accounted for through the introduction of a 3x3 non-ideality matrix of Ks and BM1 values into Sedanal. ELP experiments with mixtures of Albumin and γ-Globulins were also performed in an attempt to recapitulate the J-O behavior of a serum solution. Clearly other components or effects contribute to the J-O effect. Additional experiments with lipids, lipidated serum albumin and PEG solutions are planned. These studies lay the groundwork for bringing quantitative hydrodynamic analyses into crowded environments, and will allow measurement of hydrodynamic and equilibrium macromolecular properties in a physiological state.

NOTES:
Single-particle Cryo-electron Microscopy: A Transformative Method for Structural Analysis

Maofu Liao

Harvard Medical School, Cambridge, MA USA

High-resolution structure of biological macromolecules in action has been very difficult to study, due to the size, complexity, and dynamics of target molecular machines. The structure determination through crystallography is often hampered by the necessity of forming well-ordered crystals from highly homogeneous protein preparations, a major roadblock particularly for solving the structure of membrane proteins, whose flexibility and lipid environment required for function further confound protein crystallization. The very recent advancement of single-particle cryo-electron microscopy (cryo-EM) has revolutionized our ability to reveal the structure details of macromolecules in multiple function states. This method circumvents the requirement of crystal contact. Proteins in their native conformations are embedded in vitreous ice at liquid nitrogen temperature and imaged directly using EM. We have determined the structure of TRPV1 ion channel at 3.4 Å resolution, which enabled de novo atomic model building of the complete tetrameric channel. Three high-resolution cryo-EM structures of TRPV1 in distinct conformations have further revealed the mechanisms of channel activation via ligand binding. Representing a landmark in the evolution of cryo-EM, our work has demonstrated the transformative power of single-particle cryo-EM to solve novel macromolecule structures, dissect mixed conformations, and aid rational drug design.

NOTES:
Characterization of Therapeutical Protein Stability and Aggregation at High Concentration via Concomitant DLS and Raman Spectroscopy

Chen Zhou¹, Wei Qi², E. Neil Lewis², John F. Carpenter¹

¹University of Colorado, Aurora, CO USA, ²Malvern Instruments, Inc., Columbia, MD USA

Raman spectroscopy and dynamic light scattering (DLS) were combined to concomitantly characterize the structure and size of therapeutic proteins. We first evaluated the capability of the combined system by investigating human serum albumin (HSA) during heating and as a function of pH. Then we studied the thermal stability/aggregation propensity of therapeutic antibodies: intravenous IgG (IVIG), Bevacizumab, and Rituximab.

For HSA heating studies, HSA was dialyzed against 20mM phosphate-citrate buffer with NaCl (pH 3, 5, and 8) and protein concentration was adjusted to 37mg/mL. IVIG was prepared in commercial formulation at 51mg/mL. Bevacizumab was analyzed in its commercial formulation at 25mg/ml. Rituximab was studied in its commercial formulation and was concentrated to 20mg/mL before analysis.

Heating of HSA at different pHs showed that for both secondary structure and size the values for Tonset (pH 8)>T onset (pH5)>T onset (pH3). IVIG started to aggregate at ~56°C according to DLS size measurement. Tertiary structure alterations were reflected in changes in the Raman aromatic side chains Trp1550, Tyr850 and 830. And with difference spectra the change in secondary structure could also be observed during heating. Bevacizumab had aggregation onset of 63.8°C and grossly precipitated at 68°C, where there were still no apparent structural alternation. Rituximab had two size transitions: 54.2 and 67.4°C, and grossly precipitated at >68°C. Raman aromatic side chains had noticeable changes before grossly precipitation.

The new approach combining Raman spectroscopy with DLS is capable of capturing structural change and protein aggregation simultaneously under a variety of experimental conditions. This new instrument will provide valuable insights into protein structure and stability for formulation development and comparability assessments. And it will be particularly useful for studying samples with high protein concentrations.

NOTES:
Investigation of Protein-particle Interactions in Vaccine Formulations by Fluorescence Spectroscopy

Annamaria Vilinska¹, Michael Chin¹, Parag Purohit¹, Min Huang², Satish Singh², Ponisseril Somasundaran¹

¹Columbia University, New York, NY USA, ²Pfizer, Inc., Chesterfield, MO USA

The effect of adsorption of CRM-197, a non-toxic mutant protein of diphtheria toxin, onto aluminum phosphate (AlPO₄) adjuvants was studied using adsorption, zetapotential and intrinsic fluorescence based techniques. Adsorption of CRM-197 on AlPO₄ adjuvants at pH 5.8 causes surface charge changes which could disrupt the interparticle force balance leading to particle aggregation and agglomeration. The adsorption of CRM-197 to AlPO₄ adjuvants is primarily electrostatically driven, reducing the overall net charge of the particle with protein adsorbed. Such adsorption also induces protein structure changes, partial folding and unfolding as well as exposing certain regions of the CRM-197 structure. Intrinsic, time-resolved and fluorescence anisotropy was employed to take advantage of the 5 tryptophan (Trp) residues located throughout the CRM structure. Upon adsorption of CRM onto AlPO₄, mobility of only a portion of Tryptophan residues are reduced. This can be attributed to tryptophan 50 and 153 which are located near the surface. This also agrees with FTIR data indicating a loss of α-helical structure, as well as the charge density map – which indicates that portion of the protein near Trp 50 and 153 is more positively charged and would preferentially absorb onto the negatively charged AlPO₄ particle adjuvant. Fluorescence decay studies indicate changes in decay time (T1) of outer Tryptophan groups of CRM-197 with increase in adsorption. T1 contribution decreases with the increase in CRM-197 adsorption indicating possible multilayer or dimer formation. Since the IEP of CRM-197 is near the formulation pH, inter protein dimer and aggregate formation can further influence the stability of interparticle forces. These results, combined with FTIR and synchronous fluorescence define the particular orientation of CRM-197 attached to the AlPO₄ particle, enhancing our understanding of the physical system and what forces may be responsible for particle aggregation.

NOTES:
Epitope Mapping of Human Interleukin 23 Interacting with Antibody by Fast Photochemical Oxidation of Proteins (FPOP)

Jing Li¹, Guodong Chen², Richard Y.-C. Huang², Hui Wei², Adrienne A. Tymiak², Michael L. Gross¹

¹Washington University in St. Louis, St. Louis, MO USA, ²Bristol-Myers Squibb Company, Princeton, NJ USA

Human interleukin (IL)-23 is a pro-inflammatory heterodimeric cytokine that plays a key role in pathogenesis of several autoimmune and inflammatory diseases. Targeting IL-23 is a validated therapeutic approach to immune-mediated diseases. IL-23 is comprised of a distinct p19 subunit and a p40 subunit; the binding site of a Fab is known to be on the p19 subunit. Here we demonstrate the use of fast photochemical oxidation of proteins (FPOP) to probe the binding interface at the residue level by locating regions with reduced solvent accessibility and show that FPOP is a sensitive method for epitope mapping.

FPOP labeling experiments of IL-23 and IL-23/Fab complex were performed. Owing to the lack of R and K residues (6%), typical trypsin digestion yielded six peptides covering 54% of IL-23 p19 sequence, and three are long peptides (>20 AA) that gave poor MS/MS. Because these long peptides and the corresponding missing regions are important, it is essential to digest and form many short peptides that afford good MS/MS to map the interface at the residue level. To this end, we developed a multi-enzyme digestion approach that significantly improves the sequence coverage of IL-23 p19. Specifically, we used trypsin/Asp-N, chymotrypsin, and pepsin separately to digest IL-23 under various digestion conditions prior to LC-MS/MS analysis. The final protocol yielded 18 overlapping peptides spanning 98% of the IL-23 p19 sequence. FPOP labeling of IL-23/Fab complex showed a high degree of protection for six peptides that are located at N-terminus, the ‘tip’ of Helix A, C, D, and the loops surrounding the ‘tip’ of IL-23p19. Residue-level analysis resolved important residues that exhibit significantly reduced level of oxidation in IL-23/Fab complex. The FPOP results are consistent with those from HDX that identified similar regions as potential epitopes, demonstrating that FPOP and HDX are sensitive and complementary protein footprinting methods for epitope mapping.

NOTES:
Structure-function Analysis of a Recombinant Neurotrophic Factor After low-pH Viral Inactivation

Joseph Arndt, Chao Quan, Blake Pepinsky, Bang-Jian Gong, Damian Houde

Biogen, Cambridge, MA USA

A detailed knowledge of interrelationships between structure and function is a requirement for the development of biologics. Environmental perturbations from the manufacturing process such as temperature, pH, and solute additives can impact higher order structure and stability in solution. In this talk, a case study will be presented in which a protein was rendered inactive, with significantly reduced viscosity, as a result of a commonly used viral inactivation step using low-pH incubation. The structure and dynamics of the native and viral inactivated protein were examined using orthogonal structural techniques, including crystallography, SAXS, LS, and HDX-MS, to understand the underlying origin of the activity loss and change in rheological behavior. These studies highlight the structure-function complexity of protein biologics that must be maintained at all stages of the drug development.

NOTES:
X-ray Solution Scattering as a Probe of Higher-Order Structure in Proteins

Lee Makowski

Northeastern University, Boston, MA USA

High concentration protein solutions have significant therapeutic potential, but detailed characterization of their properties remains a significant challenge. Small angle x-ray solution scattering (SAXS) has the capability of characterizing protein solutions over a very large concentration range. At low concentrations, it can be used to determine protein size, oligomerization state and shape. At higher concentrations, detailed analysis of protein-protein interactions can be made. In this talk the methods used will be described and their application to several human IgGs will be demonstrated.

NOTES:
New Analytical Approaches and Data Visualization Tools to Assess Protein Higher-Order Structure and Pharmaceutical Stability as Applied to Comparability Assessments

David Volkin

University of Kansas, Lawrence, KS USA

This presentation will examine the challenges and opportunities of using new analytical approaches and data visualization tools to assess protein physical stability profiles as part of comparability assessments. Illustrative case studies will include (1) high-throughput biophysical analysis examining the structural integrity and conformational stability of different IgG1-Fc glycoforms, (2) implementing new data visualization tools to assess protein aggregation and particle formation profiles between mAb samples, (3) using a novel chaperone protein-based biosensor (GroEL-BLI) format to probe for presence of early, preaggregate species in antibody solutions.

NOTES:
Assessing Comparability and Biosimilarity from a Biophysical Perspective

Steven Berkowitz

Consultant, Boston, MA USA

In the biopharmaceutical industry, the process of assessing the comparability or biosimilarity of two or more different samples of the same biopharmaceutical in principle rests on the same idea of proving how identical these two or more biopharmaceutical samples are when characterized biologically, biochemically, biophysically and clinically. In this presentation we will focus our attention on just the biophysical part of this “Totality of the Evident” data package.

In the biophysical case, assessing the comparability or biosimilarity would ideally amount to assessing and providing direct information as to the level of how close the overall detailed higher order structure, 3-dimensional (3-D) structure or conformation (in terms of spatial and temporal attributes) of each biopharmaceutical sample is to each other. Unfortunately, this is typically not a practical or feasible approach. Rather, in the case of biophysical comparability and biosimilarity a more realistic approach is to use a collection of biophysical tools and techniques to assess an array of biophysical properties and behavior that proxies as a source of indirect orthogonal information about each biopharmaceutical sample’s 3-D structure (and potentially heterogeneity of this 3-D structure). By so doing, a much more practical biophysical comparison can be achieved. In this presentation we will take a critical and realistic look at the present state of affairs concerning our capability, limitations and the pitfalls in carrying out such types of comparisons.

NOTES:
Higher Order Structure Characterization: Which Methods are Best Suited for Evaluation of Product Comparability?

John P. Gabrielson¹, Kelly K. Arthur¹, Nikita N. Dinh¹, John B. Jordan², Leszek Poppe², Brent S. Kendrick¹, and Linda O. Narhi²

¹Amgen Inc., Longmont, CO USA, ²Amgen Inc., Thousand Oaks, CA USA

Demonstration of analytical comparability of biopharmaceutical products is necessary following changes to their manufacturing processes. Analytical comparability studies occur at multiple stages of the product development lifecycle from early development through post-approval. Ensuring that the higher order structure (HOS) of the protein is maintained in the post-change product is a critical component of a well-developed comparability study design, yet method selection is often not straightforward. This presentation provides important considerations for selecting HOS methods and establishing criteria by which to evaluate the results. We detail a case study demonstrating how multiple HOS methods may be directly compared to each other to aid in the method selection decision.

NOTES:
Engineering Developability of Human Antibody Therapeutics

Karl Dane Wittrup

Massachusetts Institute of Technology, Boston, MA USA

The field of antibody engineering has evolved from simply plucking binders out of libraries to engineering molecules with industrial-grade expression, stability, solubility, and specificity. For small molecules, the “Lipinski Rule of 5” provides a widely accepted rule of thumb for structural features consistent with successful drug development. Is there a similar pattern in the values for biophysical properties of developable antibody drugs? Analysis of a broad spectrum of features of a large sample of antibodies in commercial clinical development will be presented with an eye towards the emergence of such criteria for antibodies.

NOTES:
Higher Order Structural Characterization of Engineered Bispecific Antibody

Jirong Lu

Eli Lilly and Co., Indianapolis, IN USA

Many diseases are heterogeneous and multiple points of pathway blockade are needed for optimal efficacy. Bispecific antibody capable of engaging dual targets present a novel promising class of therapeutic agents. Various approaches have been explored to develop stable bispecific antibodies. Significant progress has been made to engineer tetravalent bispecific antibody with IgG-scFv format. In some cases, significant improved efficacy was shown over combination of two antibodies due to increase avidity. We have discovered an alternative fold called mAb-diabody structure in the context of IgG-scFv. This presentation will cover development of methods to identify and characterize the structure, stability, and biological activity of this alternative fold, and engineering strategies to minimize the structure heterogeneity.

NOTES:
Preclinical Candidate Selection using Higher Order Structure

Nicolas Angell

Amgen Inc., Thousand Oaks, CA USA

With advances in protein technology, the time allowed to identify a target, generate candidates and optimize those candidates through protein engineering, has decreased. Organizations can find themselves overwhelmed with multiple and diverse protein candidates towards a single target. And while biology is still the predominant factor in candidate progression, organizations are embracing the concept of a developability assessment where a candidate’s potential liabilities from an expression, purification and formulation perspective are identified. The goal of this developability assessment strategy is to produce a single lead molecule that lends itself towards a robust production process and phase appropriate formulation, delivering to the target product profile. We discuss the role that HOS techniques can play in providing high throughput information during candidate engineering and as part of a data package to identify a single lead molecule.

NOTES:
Technical Seminar Abstracts

BioTools, Inc. Technical Seminar
Tuesday, April 14
07:30 – 08:30
Theater Ballroom

Beyond FT-IR & Raman: Predictability and Sensitivity of ROA

Rina Dukor

BioTools, Inc., Jupiter, FL USA

The use of FT-IR spectroscopy as a probe of secondary structure is now widespread throughout the biopharmaceutical industry. And Raman spectroscopy, although a well-established technique in analytical sciences, is now gaining some popularity for structure elucidation of biologics. Its advantage is ability to detect the conformation of disulfide bonds and gain information from side-chains, in addition to secondary structure. More recently, several studies have shown an enhanced sensitivity of ROA (Raman Optical Activity) with differences observed when none are observed with any other spectroscopic techniques. In this presentation, we will discuss advances in four forms of vibrational spectroscopy as applied to structural studies of proteins, with emphasis on predictability (and thus sensitivity) nature of ROA.

NOTES:
Comparability: Biosimilars, Development and Post-Marketed Changes

P-101

Physical Stability Comparisons of IgG1-Fc Variants, Effects of N-glycosylation Site Occupancy and Asp/Gln Residues at Site Asn 297

Mohammad Alsenaidy\textsuperscript{1,2}, Solomon Z. Okbazghi\textsuperscript{2}, Jae Hyun Kim\textsuperscript{2}, Sangeeta B. Joshi\textsuperscript{2}, C. Russell Middaugh\textsuperscript{2}, Thomas J. Tolbert\textsuperscript{2}, David B. Volkin\textsuperscript{2}

\textsuperscript{1}King Saud University, Riyadh, Saudi Arabia, \textsuperscript{2}University of Kansas, Lawrence, KS USA

The structural integrity and conformational stability of various IgG1-Fc proteins produced from the yeast Pichia pastoris with different glycosylation site occupancy (di-, mono-, and nonglycosylated) were determined. In addition, the physical stability profiles of three different forms of nonglycosylated Fc molecules (varying amino-acid residues at site 297 in the CH2 domain due to the point mutations and enzymatic digestion of the Fc glycoforms) were also examined. The physical stability of these IgG1-Fc glycoproteins was examined as a function of pH and temperature by high-throughput biophysical analysis using multiple techniques combined with data visualization tools (three index empirical phase diagrams and radar charts). Across the pH range of 4.0-6.0, the di- and monoglycosylated forms of the IgG1-Fc showed the highest and lowest levels of physical stability, respectively, with the nonglycosylated forms showing intermediate stability depending on solution pH. In the aglycosylated Fc proteins, the introduction of Asp (D) residues at site 297 (QQ vs. DN vs. DD forms) resulted in more subtle changes in structural integrity and physical stability depending on solution pH. The utility of evaluating the conformational stability profile differences between the various IgG1-Fc glycoproteins is discussed in the context of analytical comparability studies.

NOTES:
P-102

Ion Mobility - Mass Spectrometry for Structural Analysis of Monoclonal Antibody Drug Products

Carly N. Ferguson, Michael T. Boyne II, Ashley C. Gucinski

CDER, FDA, St. Louis, MO USA

Patent exclusivity for several monoclonal antibody products is scheduled to end within the next two years, and several follow-on applications are expected in the US. These follow-on products may have different manufacturing processes than the original application that could cause alterations in structure and therefore purity, potency and safety. The complexity of monoclonal antibodies necessitates the use of advanced analytical methods to fully characterize these products and identify any changes in higher order structure (HOS). The suitability of ion mobility mass spectrometry (IM-MS) to rapidly distinguish monoclonal antibodies (mAbs) was evaluated. As proof of concept, mAbs from two different IgG classes, rituximab (IgG1) and panitumumab (IgG2), were compared by arrival time distribution plots from several charge states of each protein using IM-MS. Collision-induced unfolding (CIU), first reported by the Ruotolo laboratory, was also performed on these two samples over a range of trap collision energies from 0 V to 200 V to identify any potential structural differences that could be identified based on changes in unfolding patterns. A comparison of CIU IM-MS profiles allowed for rituximab and panitumumab to be distinguished from one another. Similar experiments were performed using multiple lots of rituximab obtained from two different manufacturers to evaluate the ability of IM-MS, with or without CIU, to detect changes between lots and/or manufacturers.

P-103

Quantitative Comparison of the Higher-Order Structures of Four Clinically Relevant Insulins by Automated Spectroscopy and Numerical Analysis

David Gregson, Doug Marshall, Tom Hampson, James Law, Lindsay Cole

Applied Photophysics Ltd., Surrey, United Kingdom

Automated CD spectroscopy was used in combination with a novel ‘comparison suite’ data analysis package to compare quantitatively the higher-order structures of four real-world biosimilars. Samples of human insulin, insulin glargine, insulin lispro and insulin aspart were presented in 96-well plate format and measurements made in the near- and far-UV. The inclusion of replicate samples allowed the comparison suite to determine the uncertainty of the measurements and, using approaches based on recent publications (1, 2), report on multiple numerical assessments of similarity for all the sample pairings, including comparison with self, in an interactive environment.

This study shows how data collected and analysed in less than 24 hours can provide a quantitative assessment of the similarity of higher-order structure of biotherapeutics. It directly addresses several issues that were ranked high amongst the barriers to an effective method of comparison in a recent
survey (3), specifically: the complexity of testing methods, a lack of automation (poor productivity), and a lack of quantitative comparison methods.

3. Survey of practising US-based biopharmaceutical laboratory managers and supervisors, by Coalesce Corporation, Mill Valley, California, on behalf of Applied Photophysics Ltd.

NOTES:
Enhanced Comparability Assessment for the Higher Order Structure of Biotherapeutics

Robert Kutlik, Thomas Lerch, Caitlin Wappelhorst, James Zobel, Qin Zou

Pfizer, Inc., St. Louis, MO USA

Higher order structure (HOS) characterization and comparability assessments are an integral part of regulatory filings for biotherapeutics, and typically involve biophysical analyses such as circular dichroism (CD) and differential scanning calorimetry (DSC). For comparability exercises, the near- and far-UV CD spectral profiles of representative test materials from the original manufacturing process and/or site typically are compared to the improved process and/or new site, together with the reference material, using a visual inspection process, following side-by-side analyses. The superimposition quality, or similarity, of spectral data between the two processes and/or sites helps ensure comparable HOS, structure/function characteristics, safety, and efficacy, complementing the results from QC release assays and other heightened characterization analyses targeting primary structure integrity. Effort has been made to enhance the spectral comparison process using orthogonal methods, statistical analysis, chemometrics, and perturbation studies, in addition to visual inspection. Using chemometrics and statistical analysis in combination with a perturbation study of proteins, it is possible to provide a similarity score for a spectrum. Using heating as the perturbation method, DSC analysis was used for added confidence in the spectral similarity score. This approach was used to analyze a number of drug substance batches representing the original and improved manufacturing processes to understand any potential impacts to HOS from the manufacturing changes. By applying this method to near-UV CD and DSC experimental data, the similarity scores generated aid in the interpretation and discrimination of batches of the drug substance material. The chemometric method described would be compatible with, and could potentially be applied to, the data from a number of different analytical techniques providing a wide range of potential applications.

Application of Bayesian Analyses and F-statistics for Accurate Quantitation

Lucas Wafer, Marek Kloczewiak, Yin Luo

Pfizer, Inc., Andover, MA USA

Analytical ultracentrifugation - sedimentation velocity (AUC-SV) is increasingly used to quantify high molecular mass species (HMMS) present in biopharmaceuticals. Although these species are often present in trace quantities, they have received significant attention due to their potential immunogenicity. Commonly, AUC-SV data is analyzed as a diffusion-corrected, sedimentation coefficient distribution, or c(s), using SEDFIT to numerically solve Lamm-type equations. SEDFIT also utilizes Maximum-Entropy or Tikhonov Phillips regularization to further allow the user to determine relevant sample information, such as the number of species present, their sedimentation coefficients, and their relative abundance. However, this methodology has several, often unstated, limitations, which may
impact the final analysis of protein therapeutics. These include regularization-specific effects, artificial “ripple peaks,” and manufactured shifts in the sedimentation coefficients. In this investigation, we have experimentally verified that an explicit Bayesian approach, as implemented in SEDFIT, can directly correct for these effects. Clear guidelines on how to implement this technique and interpret the resulting data, especially for samples containing micro-heterogeneity, are also provided. In addition, we have demonstrated how the Bayesian approach can be combined with F-statistics to draw more accurate conclusions and rigorously exclude artifactual HMMS from the analysis of biopharmaceuticals. Numerous examples, with IgG, IgA, and antibody-drug conjugates have been included to clearly illustrate the strengths and drawbacks of each technique.

NOTES:
Nanoparticle tracking analysis (NTA) is a powerful technique for analyzing nanoparticles. Its use in analyzing therapeutic proteins has not been thoroughly characterized, nor a standard protocol developed. Application of NTA in this area has been limited by several key factors, including effects of high concentrations of protein formulations. In this study, a systematic analysis of a high concentration therapeutic product, IVIg, was performed to develop a methodology for analyzing such formulations.

The impact of a high concentration protein background on particle detection and analysis was first investigated by adding particles to solutions with increasing protein concentration. Synthetic particles such as polystyrene were easily identified regardless of protein background concentration, whereas identification of protein particles was highly dependent on the solution protein concentration. Particle dilution studies determined NTA is feasible for background protein concentrations of 5 mg/mL and less. Visualization of particles in an agitated 100 mg/mL IVIg sample is possible through thorough camera setting optimization to produce a linear response to particles; however, the raw counts are significantly lower than should be expected. It is therefore recommended that NTA be used for comparative studies within the same protein background.

Taken together, the results of this study illustrate a standardized method for applying NTA to protein therapeutics. Obtaining correct, absolute counts in formulations with high protein concentrations is not feasible. However, the changes in particle concentration are easily quantified in samples with an identical high protein background. Also, relative changes in particle content in samples originally containing high protein concentration can be accurately assessed after sample dilution. Based on these results, a method to analyze high concentration protein therapeutics using optimized instrument settings is provided.

NOTES:
Advanced LC-MS Based Characterization and Higher Order Structure Analysis by HDX-MS for Orthogonal Determination of CQA in Biologic Drugs

Peter Li¹, Terry Zhang², Jonathan Josephs² Emma Zhang¹, Chen Li¹, Billy Wu¹

¹BioAnalytix Inc., Cambridge, MA USA, ²Thermo Fisher Scientific, San Jose, CA USA

Effective correlations between structural critical quality attributes (CQA) and impact on higher order structure are becoming increasingly important in biopharmaceutical development. In these studies we first conducted several advanced analytic methods including peptide mapping coupled with top-down LC-MS for precise sequence modification, and ETD with CID for accurate disulfide linkage and glycostructure analysis in biologic drugs. Several structural modifications initially defined by the advanced methods were then further evaluated by HDX-MS to assess any potential influence on the protein’s higher order structure as an orthogonal CQA. Specifically, product variants such as N149 deamidation, M125 oxidation, and C53 free cysteine in human growth hormone (hGH), as well as N28 deamidation at Glucagon-Like Peptide-1 (GLP-1) and free cysteine and disulfide scrambling at hinge region of an IgG4 mAb were initially characterized, and observed with proportionally increasing levels under stressed conditions. The modifications were then correspondingly evaluated by HDX-MS. In the HDX-MS analysis, no significant higher order changes were found with N149 deamidation and M125 oxidation, however, a significant change of ~30% deuterium uptake was observed in the region of C53 free cysteine at hGH. Using the same approach, no significant change was observed for free cysteine and scrambling at the hinge region of the IgG4 mAb, however a 50% increase of deuterium uptake was observed for N28 deamidation at GLP-1. For further confirmation of the structural changes, a crystal structure of hGH indeed reflected N149 and M125 at the protein surface, and C53 connected to a large loop of disulfide bonds, both consistent with the results seen by HDX. Such correlations in the observed modifications with or without induction of higher order structure changes may be increasingly useful in providing a more relevant understanding of the CQA’s in bioprocess development going forward.

Combining Raman Spectroscopy, Dynamic Light Scattering and Microrheology

Wei Qi, Stacy Kenyon, Samiul Amin, E. Neil Lewis

Malvern Instruments, Inc., Columbia, MD USA

The combination of dynamic light scattering (DLS), DLS-microrheology and Raman spectroscopy offers concomitant measurements of protein conformation viscosity and size. By linking the changes observed in the molecular structure as measured by Raman with the nanoscale physical properties derived from DLS and DLS-microrheology, unique insights into the optimum solvent (formulation) conditions that stabilize the native state of the protein can be obtained. In particular the Raman measurements provide insight into secondary and tertiary structural changes as well as the
conformations around the disulfide linkages and changes in intermolecular (protein-protein) interactions as measured by changes in the extent of hydrogen bonding. In addition a number of kinetic and thermodynamic parameters can also be obtained through temperature studies and/or the use of deuterium exchange experiments. Specifically we have studied a number of mAbs, modified mAbs and simple smaller proteins under a variety of solvent conditions to demonstrate the utility of the approach. We will present data that demonstrates the tool can be used to gain insight into the mechanism and pathways of protein aggregation as well as some of the driving forces associated with changes in viscosity.

NOTES:

Inigo Rodriguez-Mendieta

SGS Life Science Services, West Chester, PA USA

A collective group of analytical methods, loosely defined as biophysical techniques, provides a convenient experimental approach to characterise Higher Order Structure of proteins (HOS). However, the selection and use of these types of prevailing methods in characterization remain elusive when applied to formulation development of Biologics. This would mainly be due to limitations imposed by the throughput and high sample requirements. Although HOS is a key quality attribute of the molecule that should be thoroughly characterised during formulation development, it is usually compromised when the value of data generation is weighed against time and sample economy. This presentation discusses the use of traditional biophysical techniques for the elucidation of protein conformation and protein dynamics. In addition, the importance of using an appropriate selection criteria for such orthogonal biophysical techniques will be put in perspective, particularly with regards to avoiding redundancy of data generation and to maximizing outcome value on experimental strategies.

The NIST mAb: A Reference Material to Advance Biopharmaceutical Characterization Technologies

John Schiel, Trina Formolo, Karen Phinney

NIST, Gaithersburg, MD USA

Monoclonal antibodies (mAbs) are the fasted growing class of therapeutics, the speed of which is reflected in rapidly advancing technologies for their characterization. Perhaps the greatest advances in recent years have been targeted at higher-order structure. Characterization platforms applied to both new and legacy products must co-evolve with these changing/evolving analytical test methods. Qualification of new technologies would be greatly supplemented by a widely available representative test material. This presentation will discuss the establishment of an IgG1k Reference Material expected to more firmly underpin technology implementation decisions and facilitate the development of originator and follow-on biologics. The RM is intended for a variety of uses including, but not necessarily limited to: establishing method or instrument performance and variability, comparing changing analytical methods, assisting in method qualification, etc. The NIST mAb characterization and certification will be discussed in association with potential utility during an originator or follow on manufacturer’s process/product development process. Global conclusions drawn from the unprecedented industry-wide book collaboration “State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization” will also be presented.

NOTES:
P-111

Studies on Developability and Predictive Stability Techniques

Deniz B. Temel, Mark Brader

*Biogen, Cambridge, MA USA*

Developability assessment may play an extremely important role in assessment of biomolecule candidates behavior and prevention of their possible failure during preclinical and clinical development. Developability studies are integrated with and would bridge between the discovery/design and development/delivery. My research focuses on various measured and calculated properties of monoclonal antibodies that may then serve as predictors and indicators of suitability of these important biomolecules in wide range of concentrations.

P-112

Lessons Learned from a Comparison Between Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC) and Size Exclusion Chromatography (SEC) to Quantify Protein Aggregates

Aditya V. Gandhi, David L. Bain, John F. Carpenter

*University of Colorado, Aurora, CO USA*

This study compared the ability of SEC and SV-AUC to size and quantify the monomer and aggregates in intravenous immunoglobulin (IVIg) formulations. Additionally, the authors would like to share some lessons learned from the process of comparing SEC and SV-AUC analyses.

IVIg solution (0.5 mg/mL in 0.2M glycine, pH 4.2, with 25mM NaCl) was agitated at 20 rpm, and samples were withdrawn at 0, 90, 180 and 270 minutes. To test the effects of mobile phase composition of SEC analysis, the IVIg samples were analyzed using mobile phases composed of 0.2M sodium phosphate (pH 7.0) plus NaCl at 0, 50, 100, 200 or 400 mM. The agitated IVIg samples were also analyzed using SV-AUC, and the effects of protein concentration and formulation composition were assessed.

The NaCl concentration in the SEC mobile phase affected the mass recovery of protein; e.g., ranging from 89.4 ± 0.5 % to 99.1 ± 0.6 % for 0 mM to 400 mM NaCl for 0 min agitated IVIg samples. The c(s) distributions obtained with SV-AUC and sedfit analysis had better resolution of high molecular weight species (HMWS) than did the chromatograms obtained with SEC, wherein the HMWS species eluted as a single peak. Solution properties (protein concentration and formulation buffer) affected the quality of c(s) fitting obtained with sedfit analysis. The data fit was significantly improved with decreasing protein concentration (rmsd value for 0.75 mg/mL: 0.00723 ± 0.00016 and 0.5 mg/mL: 0.00452 ± 0.00014). Similarly, the presence of 25 mM NaCl (rmsd: 0.00548 ± 0.00015) in the formulation buffer improved the c(s) fit when compared to that obtained in absence of NaCl (rmsd: 0.00723 ± 0.00016).
This research highlights the importance of quantifying aggregates using orthogonal techniques. Furthermore, with both SEC and SV-AUC the methods must be optimized to obtain reliable and accurate results.

NOTES:
Emerging and Novel Technologies

New Features in Bioinformatics Software for Automated Processing of HDX-MS Data

Asish Chakraborty, Jing Fang, Ying Qing Yu, Keith Fadgen, Michael Eggertson, Rose Lawler

Waters Corporation, Milford, MA USA

Hydrogen/deuterium exchange mass spectrometry (HDX-MS) has emerged as a powerful technology for studying many aspects of proteins, especially probing protein conformation and dynamics. HDX-MS offers important advantages including small sample amounts, automation, and is not limited by the sizes of the target proteins. In HDX-MS studies, replicate data are collected across multiple time points and varied species (native vs. mutant, innovator vs. biosimilar, bound vs. unbound). Manually curating data is not time-efficient and requires expert interpretation. Here we report an improved HDX-MS Data Analysis Software tool, DynamX 3.0 that allows researchers to assess possible conformational changes in proteins quickly in an automated fashion. It automates processing of intact protein, peptide digest, and electron transfer dissociation (ETD) fragment level HDX-MS data. It is designed to systematically select spectra and measure the mass change of the deuterated form. Taking advantage of the high resolution separation from micro-scale UPLC and high-resolution MS data, along with comprehensive data-independent MSE detection, DynamX software is able to automate data sorting and display. Analysis time is significantly reduced with the automated capabilities. Proteins, peptides and fragments are tracked across replicates, ensuring consistent measuring of deuterium uptake. The software has display tool to illustrate the results in comparative views: uptake curves, butterfly charts, coverage maps, and difference charts.

To highlight improvements made in the DynamX 3.0, the structural stability of human IgG2 was studied under denaturing condition using HDX-MS. The IgG2 confirmations were disturbed by the addition of a small amount of GdnHCl for the HDX-MS study that utilized DynamX data processing. The samples were then labeled with D2O buffer at 25 °C at various time points. The labeling reactions were then quenched by reducing the temperature to zero and pH to 2.5. This study demonstrates the susceptibility of IgG2 to denatureation and ranks its structural stability.

NOTES:
Pressurized Online Pepsin Digestion of Proteins for Hydrogen/deuterium Exchange Mass Spectrometry

Jing Fang, Ying Qing Yu

Waters Corporation, Milford, MA USA

To probe the location of the incorporated deuterium and obtained localized structure conformation of a whole protein using hydrogen/deuterium exchange mass spectrometry (H/DX MS), pepsin digestion is typically carried out after the labeling reaction is quenched. Online digestion has been widely accepted and utilized by benefiting from high reproducibility; minimized introduction of high concentrated pepsin fragments from self-digestion into the LC and MS steps, etc.. However, for some globular proteins that are resistant to digest or that contain hydrophobic segments, online digestion may not provide a completed digestion. Here we report a quick and facile pepsin digestion method utilizing enhanced pressure to solve the problems.

High pressure promotes protein denaturation, which mechanically stretches the proteins and can expose new cleavage sites to enzymatic hydrolysis making it vulnerable to proteolysis. Pressurized digestion of highly soluble and easy-to-digest proteins (e.g. BSA and phosphorylase B) may not be substantially different from standard digestion [1]. The most significant enhancements are generally observed with hydrophobic proteins or other difficult-to-digest targets (eg. ubiquitin or native IgG). In this study, a high-pressure sustainable BEH column with immobilized pepsin [2], called Enzymate column, was applied in the digestion of monoclonal antibodies (IgG1 and IgG2) under different temperature 15, 10, and 0 °C. The digestion efficiency of the Enzymate column under enhanced pressure (up to ~15,000 psi) and normal pressure (~ 1000 psi) was compared. A higher number of overlapping peptic peptides were identified for both IgG1 and IgG2 under enhanced pressure, which substantially increases the protein sequence coverage and redundancy score. Furthermore, the peptides generated under high pressure are shorter in length, which also improves the spatial resolution in order to locate the deuterium.

Characterization of Factors Affecting Nanoparticle Tracking Analysis Results

Aaron B. Krueger, John F. Carpenter

University of Colorado Anschutz Medical Campus, Aurora, CO USA

Submicron particles in therapeutic protein products have been suggested to contribute to immunogenicity and nanoparticles can cause aggregation of protein solutions during production and in the final formulation. Therefore, characterizing nanoparticles is important during product development. Current regulatory guidelines exist for reporting visible and microparticles because robust methods are available, whereas methods to monitor nanoparticles are currently being explored. A recent technique, nanoparticle tracking analysis (NTA), has proven useful for the characterization of submicron particles. However, several key factors may affect results, including a limited analysis volume and individual sample characteristics such as unknown polydispersity and presence of microparticles in a protein sample. This research explores how these factors affect the NTA results.

A flow pump was used to increase sample volume analyzed. Although increasing sampling volume, the addition and increase in flow rate caused a reduction in total particle counts for both polystyrene and protein particles. In addition, data for polydisperse samples tended to lose peak resolution at higher flow rates, masking distinct particle populations. Furthermore, in a bimodal particle population, a bias was seen towards the larger species within the sample. The impacts of sample preparation methods and the operating parameters including “MINexps” and “blur” were investigated to optimize the method and make recommendations. For example, because the presence of large particles affect NTA results, the effect of filtration on samples was explored and found not to have a major impact on sample characterization. Taken together, this study provides an approach to applying NTA to thoroughly characterize a sample, provides instrument setting recommendations, and aims to understand how sample characteristics affects the measured results.

NOTES:
Mapping Molecular Surfaces by Pyrite Shrink-Wrap Laminate - Mediated Benchtop Oxidation

Micheal Leser¹, Dibyendu Samanta¹, Steve Almo¹, Michael Brenowitz¹, Mohammed El Makkaoui², Jon Pegan², Michelle Khine², Matt Law²

¹Albert Einstein College of Medicine, Bronx, NY USA, ²University of California, Irvine, Irvine, CA USA

While folding and packing dictate the structure and stability of proteins, the biology that they regulate typically occurs on their surfaces. The development of effective biologics relies on understanding the stability, structure and physical and chemical nature of the therapeutic agent and its target as well as the interface that mediates their interaction. In the absence of or in complement with atomic resolution structural models, approaches referred to as ‘molecular mapping’ or ‘footprinting’ can identify the solvent accessible surface of proteins and their complexes with as fine as single residue resolution. The hydroxyl radical (•OH) has proven to be an effective probe of protein structure due to its small size and high reactivity toward amino acid side chains. Hydroxyl radical can be generated for footprinting by radiolytic, photolytic, electrochemical, and chemical means. We have developed the novel material, Pyrite-shrink wrap laminate, which catalyzes the production of •OH by the Fenton reaction for molecular mapping in an easy to implement protocol.¹ Pyrite-shrink wrap laminate is fabricated from synthetic pyrite (FeS2) nanocrystals deposited onto a thermo-labile plastic (Shrinky Dink) that when heat shrunk forms a physically and chemically stable surface. Thermoformed dimples in the pyrite-coated surface provide a receptacle for small volumes containing sample to be oxidized. The only sample handling equipment required is a single or multi-channel pipet. Generation of the dose-response curves that define the oxidation rates needed for protein footprinting is conveniently achieved by adjusting either sample incubation time or H₂O₂ concentration. Validation of the technology is being conducted using the protein Programed Death 1 (PD-1). PD-1 is a cell surface receptor belonging to the immunoglobulin superfamily that is expressed on T cells and pro-B cells and binds two ligands, PD-L1 and PD-L2.

New Approaches to Investigating the Self-Association and Colloidal Stability of Protein Pharmaceuticals at High Concentrations

John Philo , N. Karl Maluf

Alliance Protein Laboratories, San Diego, CA USA

It is well known that self-association or non-specific molecular interactions in protein products at high concentrations can significantly impact solution viscosity, which in turn affects drug delivery and product manufacturability. Long term colloidal stability (minimizing aggregation and particle formation) is often highly correlated with the solution second virial coefficient, B₂₂, which quantifies the non-specific attractive or repulsive interactions between protein molecules. Unfortunately experimental
methods to probe the solution molar mass of samples at ~100 mg/mL and for measuring virial coefficients are limited and can require prohibitive amounts of sample.

Here we show results obtained for protein samples at up to ~120 mg/mL via sedimentation equilibrium (SE-AUC) using extremely small volumes (only 15 uL). The small volume allows equilibration to occur in only a few hours, increasing throughput, and the use of refractive index (RI) detection and low rotor speeds keeps the concentration gradients across the SE cell within a workable range despite the high protein concentrations. The low gradients also mean the concentration at the center of the cell remains essentially at the loading concentration, which simplifies calculation of the weight average molar mass of the sample.

NOTES:
Electron Microscopy as an Analytical Tool for Characterizing Antibodies, Antibody Complexes, and Aggregates

Anette Schneemann, Joyce Sung, Sean K. Mulligan, Joel Quispe, Bridget Carragher, Clinton S. Potter

NanoImaging Services, Inc., San Diego, CA USA

Transmission electron microscopy (TEM) is an emerging analytical tool for the structural characterization of antibodies, antibody complexes and submicron aggregates. The power of TEM is presented in three examples that address a range of issues in structure-function relationships of antibodies and antibody-antigen complexes. Different imaging and analysis techniques were employed to probe specific aspects in each case: (i) Negative staining and 2D class averaging were used to visualize and characterize chicken IgY, a type of antibody that structurally remains poorly characterized. In contrast to current hypotheses, which predict IgY to be a relatively rigid molecule, we find that it is highly flexible and comparable to IgG in this regard. As expected, its Fc region is longer than that of IgG given the presence of an extra constant domain in this part of the molecule. (ii) Alignment and 2D class averaging of mammalian IgM, combined with selective masking and sub-classification, revealed that the five antibody monomers are arranged asymmetrically around a central hub. This, too, is in contrast to prevailing models which show the monomers to be symmetrically arranged. (iii) We will also present analysis of a dual-specific, tetravalent IgG-like molecule, termed dual variable domain immunoglobulin (DVD-Ig), which is engineered to bind two targets. The architecture and dynamics of the DVD-Ig molecule and its parental mAbs were examined in detail including three dimensional (3D) reconstruction. Hinge angles measured for the DVD-Ig molecule were similar to the inner antigen parental mAb. The outer binding domain of the DVD-Ig molecule was highly mobile and 3D analysis showed binding of inner antigen caused the outer domain to fold out of the plane with a major morphological change. Docking high-resolution X-ray structures into the 3D TEM map supports the extraordinary domain flexibility observed in the DVD-Ig molecule allowing antigen binding with minimal steric hindrance.

Development of Isotope-coded Carboxyl Group Footprinting for Determining Protein Higher Order Structure (HOS)

Hao Zhang, Haijun Liu, Robert E. Blankenship, Michael L. Gross

Washington University in St. Louis, St. Louis, MO USA

Mass spectrometry (MS)-based protein footprinting is an emerging approach for HOS studies of protein therapeutics (FEBS Lett(588)308). In this approach, residues on the protein surface are labeled by solution reagents. Residues inside the protein HOS are protected by hydrophobic or hydrophilic interactions and are less-labeled. Protein HOS and its variations determine the extent of labeling and can be monitored by MS. Advantages include high speed and sensitivity, making protein footprinting a
potentially effective tool in HOS studies of protein therapeutics (J Am Soc Mass Spectrom(24)835,Anal Bioanal Chem(406)6541). Here, we report a method to monitor protein HOS by using isotope-coded carboxyl-group footprinting. These side chains of aspartic and glutamic acids are major contributors in protein hydrophilic interactions that often determine protein HOS. The basis of the approach is a glycine ethyl ester (GEE) labeling strategy for protein HOS studies (Int J Mass Spectrom(312)78). We and others applied this strategy in HOS studies including those of mAbs (MAbs(6)1486). The rate limiting step in implementation of protein footprinting is the quantitative analysis of labeling extents. We are developing an isotope-coded approach to improve the speed and reproducibility of GEE labeling of protein therapeutics. In the new development, isotope-coded “heavy” and “light” GEE are used to label separately the two states of model protein, OCP. Two digested samples are 1:1 mixed and analyzed by a single LC-MS experiment. The difference in labeling extent between the two states is determined by the ratio of the abundances of the “heavy” and “light” peptides. Changes in HOS are measured by comparing signal ratios. Combining isotope-coded MS quantitative analysis and carboxyl-group footprinting dramatically reduces the time of MS analysis and improves the precision of footprinting. Other improvement and implementation of this new development are ongoing for protein therapeutics.

NOTES:
Evaluation of Resonant Mass Measurement (RMM) in Characterization of Sub-visible Particles in Therapeutic Proteins Formulations

Chen Zhou¹, Wei Qi², John F. Carpenter¹

¹University of Colorado Anschutz Medical Campus, Aurora, CO USA, ²Malvern Instruments, Inc., Columbia, MD USA

Resonant Mass Measurement (RMM) is capable of characterizing not only proteinaceous particles but also air bubbles and oil droplets, based on particle buoyant mass. We evaluated the effects of particle concentration and high protein concentrations on measuring particles in therapeutic protein formulations. In addition, we investigated the capability of RMM in to differentiate bubbles from protein particles in lyophilized samples after reconstitution.

Silica beads (0.5μm and 1μm) and 1μm latex beads suspended in Millipore water were used to evaluate the particle concentration detection limit. To evaluate the effect of protein concentrations, four different types of particles (0.5μm/1μm silica beads, 1μm latex beads, silicone oil droplets and IVIG (intravenous immunoglobulin) aggregates) were spiked into Human Serum Albumin (HSA) solutions with protein concentrations of 0.1, 1, 5, 10, 25, 50 mg/mL. For the lyophilized samples, 4 different formulations were prepared: 2 mM citrate at pH 5, 2 mM citrate at pH 5 with 0.03% PS20, 2 mM citrate at pH 5 with 5% trehalose and 2 mM citrate at pH 5 with 5% trehalose and 0.03% PS20. Both placebo and samples with 1 mg/mL IVIG were prepared for each formulation and freeze-dried. Samples were reconstituted with distilled water and analyzed.

RMM was found to count and size particle concentration from tens of thousands to around twenty millions particles per milliliter, in the size range from about 0.2 to 5 micron. For all of the particle types spiked into HSA solutions, the protein concentration did not affect particle count or sizing. In the lyophilized samples with or without 1 mg/mL IVIG, trehalose formulation had the highest level of particles, and PS20 could effectively reduce the subvisible particles. Upon reconstitution, RMM showed that most of subvisible particles in lyophilized placebo samples had a density less than the bulk solution because they were air bubbles. In contrast, in samples with 1 mg/mL IVIG the majority of subvisible particles had a density greater than the bulk solution and were due to protein aggregates.

RMM is capable of measuring particles over a wide particle concentration range from tens of thousands to tens of millions particles per milliliter. RMM counting and sizing of particles is not affected by protein concentration. Subvisible particles including protein particles and air bubbles in lyophilized/reconstituted samples can be effectively differentiated and characterized by RMM.

NOTES:
Protein Therapeutics Discovery and Candidate Selection

P-121

Conformation Characterization of Therapeutic Antibody Oxidation Variants with Fully Automated Hydrogen/Deuterium Exchange Mass Spectrometry

Terry Zhang, David Horn, Shanhua Lin, Xiaodong Liu, Jonathan Josephs

Thermo Fisher Scientific, San Jose, CA USA

Monoclonal antibodies (mAbs) have been increasingly used for detection and treatment of diseases. Characterization of chemical degradation of mAb-based drugs is a primary concerns for biopharmaceutical development. It is thus important to have an analytical tool that can detect these minor conformational changes. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to investigate the conformation of intact proteins, including mAbs. In this study, an optimized HDX workflow was developed and used to probe the conformation of Herceptin and its oxidation variants.

Both non oxidized and oxidized mAb were diluted with labeling buffer and incubated for multiple time points. The samples were quenched and digested online with a pepsin column in a automated HDX system. The digested peptides were injected into a reverse phase column. MS analysis was performed with orbitrap mass spectrometer. Data was processed with Proteome Discoverer 1.4™ software for peptide identification. Peptide mapping and PTM analysis was performed with PepFinder 1.0™ software. HDX experiment data were analyzed by HDExaminer and Mass Analyzer HDX algorithm.

Nearly 100% sequence coverage was achieved for both herceptin and oxidized herceptin samples. More than 200 peptides were identified. These were subsequently used to probe the conformation of the two samples by HDX. The deuterium uptake data were analyzed by HDExaminer. Most of the peptides have similar deuterium uptake profile between the two samples indicating no significant conformational changes for most region of protein. However, local solvent exposure differences in the vicinity of the peptides containing methionine oxidation were observed. Single amino acid residue level protection factors were calculated by using Mass Analyzer HDX algorithm. Protection factor decreases were observed for nine amino acids around one of the methionine oxidation sites, which correlated well with HDExaminer results.

NOTES:
Late Breaking

LB-01

Rapid Conformational Analysis of Protein Drugs by Combining Solid Phase Extraction (SPE) and Hydrogen/Deuterium Exchange Mass Spectrometry

George M. Bou-Assaf\textsuperscript{1}, Damian Houde\textsuperscript{1}, Andy Weiskopf\textsuperscript{1}, Zeinab E. Nazari\textsuperscript{2}, Kasper. D. Rand\textsuperscript{2}

\textsuperscript{1}Biogen, Cambridge, MA USA, \textsuperscript{2}University of Copenhagen, Copenhagen, Denmark

SPE setup enables not only sample cleanup of protein drugs from formulation excipients prior to ESI-MS, but can be combined with HDX-MS for higher order structure characterization of biologics and biosimilars.

Hydrogen Deuterium Exchange coupled to Mass Spectrometry (HDX-MS) is gaining more and more popularity in investigating higher order structure and dynamics of proteins. The method is based on mapping the local hydrogen/deuterium exchange that occurs at backbone amide hydrogens of the protein, when the protein is diluted in deuterated buffer. The m/z shift in MS signal due to incorporation of heavy deuteriums will then give information on higher order structure of the protein \cite{1, 2}. In recent years, the application of HDX-MS in characterization of higher order structure of biologic drugs and their biosimilar candidates has gained much attention from both academia and biotech industry and has been referred to as the “ideal method” for conformational comparability studies of biosimilars \cite{3, 4}. However, there is still need for fast and reliable methods for pre-screening of biologic formulations containing excipients, in regards to quality control of biologics prior to the conventional and extensive HDX-MS studies. In this work, we present a novel system, that address such need by extraction of protein pharmaceuticals from their very ”final formulations” prior to MS, and could also be used in HDX-MS studies.

The system is a custom-made Solid Phase Extraction (SPE) setup that consists of a low back-pressure custom-built microbore column \cite{5}, and a Rheodyne six-port two-position injection valve coupled to a HPLC binary solvent manager. Upon manual injection of the samples to the column, the protein is separated from other formulation materials by use of one or multiple solvents of choice and the ”naked” protein is eluted directly to the mass spectrometer.

The system was optimized to ensure optimal separation of a protein from its excipients, while allowing minimum level of back exchange (deuterium loss) during HDX-MS. In order to do so, ten different set-ups were tested, where fully-deuterated and non-deuterated preparations of Angiotensin II and Insulin as model compounds were compared, and the level of back exchange was quantified for each setup. The lowest level of back exchange was obtained when SPE system parts were submerged in ice bath, and peptide/protein samples were eluted by isocratic elution.
Based on these optimizations, we are applying our setup for rapid conformation analysis to study Interferon-β-1a (IFN). We have evaluated the efficacy of the system in removing formulation excipients for this sample and have been successful in obtaining high quality MS spectra indicating efficient sample cleanup and removal of excipients from IFN’s formulation under conditions suitable for HDX-MS analysis. We have further evaluated a wide range of different compositions of organic and aqueous solvents and identified optimal “solvent cocktails” customized for different formulations.

Here, we report that SPE-MS system could be used as a fast, reliable and robust method for fast conformational analysis of proteins in formulation. Our setup offers the following unique advantages: a) The microtrap column used in SPE-MS setup, costs less than $8, and could be readily re-packed manually, b) The setup allows the use of multiple solvents of choice for excipient removal for individual protein sample formulations, prior to MS. While useful for primary structure analysis of proteins, The SPE-MS system is particularly promising for HDX-MS studies on higher order structure of proteins. Conformational analysis by HDX-MS can be performed in less a few minutes making the setup of use for higher-order structure characterization of proteins in formulation at increased through-put compared to existing workflows. HDX-MS can be performed with low back exchange value, using only small sample amounts. We are currently expanding the application of our integrated SPE-HDX-MS setup to analyze other biologics drugs and their biosimilar candidates.


NOTES:
Effect of Lipid Peroxidation Products on the Higher Order Structure and Function of GRP78/BiP

Dinen Shah, Surinder Singh, Swati Bandi, Krishna Mallela

University of Colorado Anschutz Medical Campus, Aurora, CO USA

Free radical generation is a common outcome of factors like alcoholism, smoking and chemotherapy. These free radicals attack the cell membranes and form reactive lipid peroxidation products such as 4-ONE. Earlier proteomic analysis indicated that Immunoglobulin Binding Protein (BiP) is a key protein that is modified by 4-ONE. BiP also known as GRP78, is a chaperone for the folding of numerous proteins, which include millions of antibodies in human body. Hence, any damage to this protein could result in a failure of protein folding machinery inside the cell and may cause devastating effects. In this study, we probed how 4-ONE modification affects the structure and function of GRP78.

To probe the protein structure and function, we have used techniques like circular dichroism, protein fluorescence, denaturant and thermal melts, mass spectrometry, ATPase and citrate synthase aggregation suppression assays.

Upon modification with 4-ONE, BiP loses significant stability as evident from CD denaturant and thermal melts. Thermal melts indicate that the modified protein populate partially unfolded states which may be non-functional. ATPase assay indicates that the modified protein substantially loses its ATPase activity. However, the modified protein suppresses the citrate synthase aggregation to the same extent as the unmodified protein. Mass spectrometry shows that 4-ONE treated protein is significantly modified.

The above results indicate that modified BiP loses its stability and ATP-dependent active role in folding upon modification, but retains its passive role as an aggregation suppressor. Molecular chaperones like BiP are critical for protein folding and the stress management of the ER. Understanding the protein at a mechanistic level will therefore give us a better understanding of the impact it has on human health.

High-Resolution Multinuclear NMR Studies of Higher-Order Molecular Interactions in Isoionic (Self-Buffering) Antibody Solutions

Feng Ni, Ping Xu, Ping Wang, Jamshid Tanha, Gregory Hussack, Denis L'Abbe, Louis Bisson, Yves Durocher, Melanie Arbour, Denis Bourque, and Luke Masson

National Research Council of Canada, Montreal, QC Canada

With the rise of antibody-based therapeutics, there is an increasing need for an adequate understanding of the physical stability of bulk protein solutions, especially that of high-concentration antibody formulations. We here focus on buffer-free (isoionic) and excipient-free antibody preparations whereby antibody proteins provide both the self-buffering capacity and the stabilization for high-concentration
solutions. We show, using high-resolution proton NMR spectroscopy, that some antibodies exhibit transient and reversible protein-protein interactions while at the same time preserve their higher-order structures (HOS) in high-concentration solutions. Multinuclear NMR spectroscopy, including natural-abundance 13C, 31P and quadrapolar 23Na and 35Cl/37Cl, is used to examine the molecular interactions of a number of antibody formats, including VH/VL single-domain antibodies, Fc-VH fusions and a full-length monoclonal antibody. Surprisingly, all antibody proteins were found to retain specific ions even after extensive dialysis, especially the chloride ion, phosphates and imidazole depending on the history of protein production and purification. These findings can have important implications for antibody epitope mapping and high-resolution ordered structure (HROS) analysis as well as for excipient-free antibody formulations and the design of efficient purification methods in scale-up antibody productions.

NOTES:
LB-04

Application of High Concentration SV-AUC

Julie Wei

Biogen, Cambridge, MA USA

SV-AUC is traditionally applicable to low concentrations of proteins. Recent development in this area has pushed the concentration to 100-150 mg/mL. Here we will present assessment of this technique and explore possible applications of this technique. B22 calculation is possible with this technique, and level of HMW can be calculated as well.

LB-05

Effect of Higher order structure on immunogenic potential of aggregates

Marisa K. Joubert, Vibha Jawa, Quanzhou Luo, Vivian Bi, Linda O. Narhi

Amgen, Inc. Thousand Oaks, CA USA

One of the safety concerns around protein therapeutics is the potential for inducing immunogenicity, ultimately resulting in anti-drug antibodies. Protein aggregates, especially in the micron size range, have been shown to induce cytokine release from immune cells in vitro, and weak and transient antibody generation in humanized mouse model systems. Protein aggregates are very heterogeneous mixtures, with different sizes, chemical modifications and conformations. In this poster we describe work done to begin to understand which of these characteristics contributes to the immunogenic potential of protein aggregates.

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