5th International Symposium on Higher Order Structure of Protein Therapeutics (HOS 2016)

Symposium Co-Chairs:
Jamie Moore, Genentech, a Member of the Roche Group
William Weiss, Eli Lilly and Company

April 11-13, 2016
Renaissance Long Beach
Long Beach, CA USA

Organized by

CASSS
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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 5th International Symposium on Higher Order Structure of Protein Therapeutics (HOS 2016). PhD students or post-doctoral fellows conducting research at academia throughout the world are eligible.

This year’s grant winners include:

Quinary Interactions and Protein Stability in Living Cells
Rachel Cohen, University of North Carolina at Chapel Hill, USA

Correlating the Impact of Different Well-defined Oligosaccharide Structures on Physical Stability Profiles of IgG1-Fc Glycoforms
Apurva More, University of Kansas, USA

A Hybrid Orbitrap/NEMS Instrument for Native Single Molecule Analysis
Adam Neumann, California Institute of Technology, USA

Higher Order Structural View of Polysorbate Interactions with Interferon Alpha-2a
Dinen Shah, University of Colorado Anschutz Medical Campus, USA
5th International Symposium on
Higher Order Structure of Protein Therapeutics
Scientific Program Summary

Monday, April 11

07:30 – 17:30  Registration in the 2nd Floor Foyer

07:30 – 08:30  Continental Breakfast in the Bixby Ballroom

08:30 – 08:45  Welcome and Introductory Comments in the Bixby Ballroom
Jamie Moore, Genentech, a Member of the Roche Group, South San Francisco, CA USA

Keynote Session in the Bixby Ballroom
Session Chair: Jamie Moore, Genentech, a Member of the Roche Group, South San Francisco, CA USA

08:45 – 09:45  A Lower Order Look at Higher Order Structure
Reed Harris, Genentech, a Member of the Roche Group, South San Francisco, CA USA

09:45 – 10:15  Break – Visit the Exhibits and Posters in the Bixby Ballroom

Higher Order Structure in Development Session in the Bixby Ballroom
Session Chair: Damian Houde, Biogen, Cambridge, MA USA

10:15 – 10:35  All the Time and Sample That We Can Save: Developability and Predictive Stability Techniques During Biologics Formulation
Deniz Temel, Amgen Inc., Thousand Oaks, CA USA

10:35 – 10:55  Practical Applications and Performance of Analytical Ultracentrifugation Sedimentation Velocity in Biopharmaceutical Development
Jared Bee, MedImmune, A member of the AstraZeneca Group, Gaithersburg, MD USA

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

11:15 – 11:35  Regulatory Considerations on Higher Order Structure Determination and Evaluation – An EU Perspective
Veronika Jekerle, European Medicines Agency, London, United Kingdom


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<td>Discussion – Questions and Answers</td>
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**Biological Consequences Session** in the Bixby Ballroom  
**Session Chairs:** David Keire, *CDER, FDA, St. Louis, MO USA*  
and Linda Narhi, *Amgen Inc., Thousand Oaks, CA USA*

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| 13:00 – 13:20 | The Type and Extent of Chemical Modification of Biotherapeutics May Impact the Potential for Biological Consequences  
Marisa Joubert, *Amgen Inc., Thousand Oaks, CA USA* |
| 13:20 – 13:40 | High Concentrations, Weak Interactions and Protein Stability  
Gary Pielak, *University of North Carolina at Chapel Hill, Chapel Hill, NC USA* |
| 13:40 – 14:00 | Regulatory Expectations for the Characterization of Higher Order Structure  
Maria Gutierrez-Lugo, *CDER, FDA, Silver Spring, MD USA* |
| 14:00 – 14:25 | Discussion – Questions and Answers                                                                  |
| 14:25 – 15:25 | Poster Session in the Bixby Ballroom                                                               |

**Late Breaking Session** in the Bixby Ballroom  
**Session Chairs:** Jamie Moore, *Genentech, a Member of the Roche Group, South San Francisco, CA USA*  
and William Weiss, *Eli Lilly and Company, Indianapolis, IN USA*

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| 15:25 – 15:55 | Reversible Self Association of a HSA Fusion Protein – Insights into Molecular Interactions through AUC, FFF and Molecular Modeling  
Haihong Fan, *GlaxoSmithKline, King of Prussia, PA USA* |
| 15:55 – 16:15 | Individual-Particle Electron Tomography (IPET): An Approach to Study Flexible Protein Structure, Dynamics, Mechanism and Aggregation  
Gang (Gary) Ren, *Lawrence Berkeley National Laboratories, Berkeley, CA USA* |
Mark Manning, *Legacy BioDesign LLC, Johnstown, CO USA* |
| 16:35 – 17:00 | Discussion – Questions and Answers                                                                  |
| 17:00 – 18:00 | Exhibitor Reception in the Bixby Ballroom                                                                |
Tuesday, April 12

07:30 – 08:30  Continental Breakfast in the Bixby Ballroom

08:00 – 17:00  Registration in the 2nd Floor Foyer

**Keynote Session** in the Bixby Ballroom  
**Session Chair:** Guodong Chen, *Bristol-Myers Squibb Company, Princeton, NJ USA*

08:30 – 09:30  **Mass Spectrometry-Based Biophysical Approaches for Affinity, Aggregation, and Structure of Therapeutic Proteins**  
               Michael Gross, *Washington University in St. Louis, St. Louis, MO USA*

09:30 – 10:00  **Break** – Visit the Exhibits and Posters in the Bixby Ballroom

**Fundamentals Session** in the Bixby Ballroom  
**Session Chairs:** David Bain, *University of Colorado Anschutz Medical Campus, Aurora, CO USA*  
and Jason Rouse, *Pfizer, Inc., Andover, MA USA*

10:00 – 10:20  **Islet Amyloid Formation, Beta Cell Death and Diabetes**  
               Daniel Raleigh, *Stony Brook University, Stony Brook, NY USA*

10:20 – 10:40  **Native Top-Down Mass Spectrometry Approaches for Characterizing Proteins**  
               Joe Loo, *University of California, Los Angeles, Los Angeles, CA USA*

10:40 – 11:00  **AUC as a Complementary Approach to Structural Techniques: Analysis of Protein Assembly, Linked Equilibria and Complicated Mixtures in Solution**  
               Andrew Herr, *Cincinnati Children’s Hospital Medical Center, Cincinnati, OH USA*

11:00 – 11:25  **Discussion – Questions and Answers**

11:25 – 12:25  **Hosted Lunch** in the Farrell’s Lounge

**Emerging and Novel Technologies Session** in the Bixby Ballroom  
**Session Chair:** Otmar Hainzl, *Sandoz GmbH, Oberhaching, Germany*

12:25 – 12:45  **Serial Crystallography of Membrane Proteins with X-Ray Lasers**  
               Vadim Cherezov, *University of Southern California, Los Angeles, CA USA*

12:45 – 13:05  **Deep Ultraviolet Resonance Raman (DUVRR) Spectroscopy of Therapeutic Proteins**  
               Sergey Arzhantsev, *CDER, FDA, St. Louis, MO USA*
Tuesday, April 12

13:05 – 13:25  Profiling Formulated Protein Therapeutics Using NMR Spectroscopy
Brad Jordan, Amgen Inc., Thousand Oaks, CA USA

13:25 – 13:50  Discussion – Questions and Answers

13:50 – 14:20  Break – Visit the Exhibits and Posters in the Bixby Ballroom

**Young Scientist Session** in the Bixby Ballroom
*Session Chair: Steven Cohen, Northeastern University, Boston, MA USA*

14:20 – 14:35  Quinary Interactions and Protein Stability in Living Cells
Rachel Cohen, University of North Carolina at Chapel Hill, Chapel Hill, NC USA

14:35 – 14:50  Mass Spectrometry of Nanoparticles with Nanomechanical Systems
Eric Sage, California Institute of Technology, Pasadena, CA USA

14:50 – 15:05  Correlating the Impact of Different Well-defined Oligosaccharide Structures on Physical Stability Profiles of IgG1-Fc Glycoforms
Apurva More, University of Kansas, Lawrence, KS USA

**Merging Experimental and Computational Approaches to HOS Characterization Session** in the Bixby Ballroom
*Session Chair: Michael Brenowitz, Albert Einstein College of Medicine, Bronx, NY USA*

15:05 – 15:25  Molecular Modeling and Machine Learning to Predict Chemical Stability
Lydia Beasley, Genentech, a Member of the Roche Group, South San Francisco, CA USA

Andrej Sali, University of California, San Francisco, San Francisco, CA USA

15:45 – 16:05  The Potential of the riboSNitch in Personalized Medicine
Alain Laederach, University of North Carolina at Chapel Hill, Chapel Hill, NC USA

16:05 – 16:30  Discussion – Questions and Answers

16:30 – 17:30  Regulatory Panel Discussion in the Bixby Ballroom
*Facilitators: Jamie Moore, a Member of the Roche Group, South San Francisco, CA USA and William Weiss, Eli Lilly and Company, Indianapolis, IN USA*
Wednesday, April 13

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

08:00 – 12:00  **Registration** in the 2nd Floor Foyer

**Beyond mAbs: New Challenges for Novel Frameworks Session** in Bixby Ballroom

**Session Chairs:** Katherine Bowers, *FUJIFILM Diosynth Biotechnologies, Cary, NC USA* and Guodong Chen, *Bristol-Myers Squibb Company, Princeton, NJ USA*

08:30 – 08:50  **Advanced Characterization of ADC for Defining CQAs**  
                HT Song, *Bristol-Myers Squibb Company, Princeton, NJ USA*

08:50 – 09:10  **Characterization of Protein Structure and Dynamics - New Insight into Structure-function/degradation for mAbs and Novel Molecular Formats**  
                Nick Bond, *MedImmune, A member of the AstraZeneca Group, Cambridge, United Kingdom*

09:10 – 09:35  **Discussion – Questions and Answers**

09:35 – 10:05  **Break** – Visit the Exhibits and Posters in the Bixby Ballroom

**Protein Therapeutics Discovery and Candidate Selection Session** in the Bixby Ballroom

**Session Chair:** Wasfi Al-Azzam, *GlaxoSmithKline, King of Prussia, PA USA*

10:05 – 10:25  **Formulation as a Layer Cake: Combining Materials and Study Types for Maximum Predictability and Efficiency**  
                Julie Wei, *Biogen, Cambridge, MA USA*

10:25 – 10:45  **Deamidation Potential in Therapeutic Antibodies: Bridging the Gap between Discovery and Development**  
                Shrikant Deshpande, *Bristol-Myers Squibb Company, Redwood City, CA USA*

10:45 – 11:05  **The Development of Biophysical Screening Methods to Identify Better Candidates**  
                Bryan Jones, *Eli Lilly and Company, San Diego, CA USA*

11:05 – 11:30  **Discussion - Questions and Answers**

11:30 – 11:40  **Closing Remarks**  
                William Weiss, *Eli Lilly and Company, Indianapolis, IN USA*
A Lower Order Look at Higher Order Structure

Reed Harris

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

Product quality decisions rely on the identification of: a) what to measure, b) how to measure it, and c) what test results are required to ensure safety and efficacy. Our capabilities for detecting aberrant structures decrease as the order increases. Currently, we can use higher order structure tools to understand chromatographic differences or potency differences for materials isolated using lower order structure methods including IEC, HIC and RP-HPLC, but examples where higher order methods revealed unknown protein structure variation remain elusive.

Additionally, we have been interested in higher order protein structure because of the potential for aberrant forms to aggregate, which could lead to immunogenicity. Recent studies have shown that non-covalent aggregates may not be as immunogenic as previously believed, and so the focus for immunogenic structures should shift to covalent modifications or cross-linked forms, which can readily be identified using lower order techniques.

Some higher order structure mysteries remain. We have found that IEC separates variants based on non-charge differences, which suggests that this technique remains useful for indirectly assessing structural changes that affect charge presentation.

NOTES:
All the Time and Sample That We Can Save: Developability and Predictive Stability Techniques During Biologics Formulation

Deniz Temel

Amgen Inc., Thousand Oaks, CA USA

A biomolecule must meet certain criteria to progress from the discovery to the development stage. It should possess favorable storage stability and solubility profiles. The expression of the molecule should be good and it must be able to be formulated at high concentrations with low aggregation and viscosity. Finally, low immunogenicity is required. According to these criteria, it is crucial to identify the physicochemical risks associated with the biomolecular candidates before they leave discovery and transition to development. Therefore, developability assessment may play an extremely important role in evaluating the various 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 stages. 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 over a wide range of concentrations.

NOTES:
Analytical Ultracentrifugation Sedimentation Velocity (AUC-SV) is a powerful tool that can be used to characterize the molecular weight (MW) and hydrodynamic properties of proteins and their complexes. In this presentation several different applications of AUC-SV for research and development of biopharmaceuticals are discussed. Quantitation of trace levels of aggregates and fragments is one of the most common applications of AUC-SV in biopharmaceutical development. The performance of AUC-SV for trace impurity analysis of highly purified monoclonal antibodies (mAbs), fusion proteins, and bispecific antibodies was assessed using multiple years of historical data. AUC-SV and high performance liquid chromatography (HPSEC) results are compared and discussed. We show how characterization of isolated protein aggregates by AUC-SV can provide insight into their structure that is complementary to other characterization methods. Multi-signal AUC-SV (MSSV) is a method that can be used to determine the stoichiometry of multi-protein complexes. Use of MSSV has provided unique insight into protein complex formation in support of protein engineering and design. More recently, new developments in analysis of variable field AUC-SV data has created the capability for characterization of species spanning an expanded sedimentation coefficient range in a single experiment. Examples where this new capability is being applied for characterization during biopharmaceutical development are discussed. Together, these case studies highlight the unique value that AUC-SV can provide in support of research and development of biopharmaceuticals.
Hydroxyl Radical Footprinting-Mass Spectrometry (HRF-MS): Probing Changes in the Higher Order Structure (HOS) of Biotherapeutics

Aaron Wecksler, Galahad Deperalta

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

Demonstrating structural consistency is important for ensuring product quality of biotherapeutics. Structural characterization of full-length monoclonal antibodies (mAbs) has been challenging due to the size, heterogeneity and formulation of the therapeutic proteins. One potential tool for evaluating the higher order structure (HOS) of therapeutic mAbs is hydroxyl radical footprinting-mass spectrometry (HRF-MS). At Genentech, we are developing HRF-MS technology which utilizes a high power laser to photo-lyse hydrogen peroxide into hydroxyl radicals, initiating the sub-microsecond surface oxidation of solvent exposed amino acid residues. Traditional peptide mapping using LC-MS/MS analysis then enables the identification and quantification of the oxidized residues corresponding to the different solvent accessible regions of a studied mAb. This technology is orthogonal to hydrogen-deuterium exchange (HDX), but has advantages that include: (1) ultra-fast labeling time scales, (2) irreversible protein modification (providing flexibility in the downstream analysis) and (3) information on the structural positioning of the protein side-chain residues (rather than amide backbone). HRF-MS thus has the potential to support critical studies in drug discovery and development, especially for formulation development, comparability assessments, and epitope mapping. In this presentation, we show multiple case studies demonstrating the utility of this emerging technology for the structural characterization of therapeutic mAbs.

NOTES:
Regulatory Considerations on Higher Order Structure Determination and Evaluation – An EU Perspective

Veronika Jekerle

European Medicines Agency, London, United Kingdom

The higher order structure of a therapeutic protein plays an important role in its stability, physicochemical attributes and biological potency and may have an impact on the safety and efficacy of the protein (e.g. PK/PD, immunogenicity). Changes in the higher order structure are frequently observed during marketing authorisation assessment following manufacturing changes pre- and post-marketing and in biosimilarity assessments versus the reference product. Likewise, changes in biological activity and/or clinical performance may be detected during development, which necessitate an in-depth investigation into the higher order structure of the protein.

For the above challenges, the appropriate set of analytical and biological methods needs to be determined in order to achieve a thorough product understanding that will help to recognise the structural/functional relationship of the therapeutic protein and its consequences (e.g. misfolding, aggregation, receptor binding).

This presentation will highlight European regulatory experience for the approach and methods used to characterise higher order structure, relevant scientific guidance and ways to interact with EU regulators on such questions. In addition, case studies will bring concrete examples covering monoclonal antibodies, biosimilars and antibody-drug conjugates.

NOTES:
The Type And Extent of Chemical Modification Of Biotherapeutics May Impact the Potential for Biological Consequences

Marisa Joubert

Amgen Inc., Thousand Oaks, CA USA

There are a number of factors that may impact the immunogenicity of a biotherapeutic drug product. We have investigated the potential biological impact of different product quality attributes, including chemical modification of amino acid residues and protein conformation, in an in vitro comparative immunogenicity assessment (IVCIA) assay. Human peripheral blood mononuclear cells (PBMC) from a population of donors, including up to 50 healthy and disease state individuals, was evaluated for cytokine secretion and T-cell proliferation for up to eight days. The results highlight potential differences in the relative risk of immunogenicity of specific product quality attributes and how they relate to the safety profile of a biotherapeutic.

NOTES:
High Concentrations, Weak Interactions and Protein Stability

Gary Pielak

University of North Carolina at Chapel Hill, Chapel Hill, NC USA

Quinary structure, which is responsible for organizing the cellular interior, is brought about by weak protein-protein interactions that occur under crowded conditions. These interactions also appear at the high protein concentrations used to deliver biologicals. Unfortunately, we know almost nothing about quinary interactions, because they are completely absent in the dilute buffered solutions used for most experiments.

The fact that proteins are almost always studied in dilute solutions rather than in cells means there is much more information about interiors than there is about exteriors. It is well known that interior side chains must be hydrophobic, the buried atoms must be exquisitely well packed and nearly all hydrogen bond donors and acceptors must be satisfied. This knowledge has yielded tremendous assets, including the ability to predict, manipulate and design protein structure and stability. The same knowledge-to-benefits claim cannot yet be made for exteriors. Our recent work sheds new light on protein surfaces.

We find that exteriors harbor as much information as interiors, but this information is only available when proteins are studied in cells or under crowded conditions in vitro. Specifically, changing the protein exterior affects the equilibrium thermodynamics of globular protein unfolding in cells in a manner that emphasizes a key role of surface charge-charge interactions. Our findings are important both for understanding fundamental aspects of biology and formulating biologics.


NOTES:
Regulatory Expectations for the Characterization of Higher Order Structure

Maria Gutierrez-Lugo

CDER, FDA, Silver Spring, MD USA

This presentation will provide an overview of the expectations for HOS characterization of biotechnology products. An overview of the methods used in regulatory applications will be also presented.

NOTES:
Concentration dependent reversible self-association (RSA) has been observed in many therapeutic proteins. RSA may have impact on solubility and stability of these proteins and in some cases leads to abnormal behaviors in process, formulation and certain analytical assays. Understanding the nature of molecular interaction that leads to RSA, and how it can be controlled by altering solution conditions could be critical in biopharmaceutical product development.

Here we present a case study on reversible self-association of a HSA fusion protein. SV-AUC data suggest the protein exists mostly in monomer-dimer equilibrium in solution. Acidic pH appears to promote self-association, and ionic strength has opposite impact at near neutral vs. acidic pHs. High salt concentration promotes association at pH 7.5, but suppresses association at pH 5.5. In line with experimental observations, molecular modeling was performed to provide insights on nature of interactions at the potential dimerization site, and how pH and ionic strength could impact association states and strength of the interactions. Examples will be given to illustrate how this RSA behavior may have impact in different aspects of product development. Considering the low throughput, relatively labor intensive nature of AUC experiments, FFF-MALS was also explored as an alternative tool to map out the solution behavior of this protein.

NOTES:
Individual-Particle Electron Tomography (IPET): An Approach to Study Flexible Protein Structure, Dynamics, Mechanism and Aggregation

Gang (Gary) Ren

Lawrence Berkeley National Laboratory, Berkeley, CA USA

Proteins have the unique ability to function specifically and efficiently, which is attained through its three-dimensional (3D) structures and flexibility, as well as necessary conformational changes. However, structural study on proteins that have large-scale flexibility, dynamics, and heterogeneity is challenging by current techniques, including X-ray crystallography, nuclear magnetic resonance (NMR) spectrum, small angle scattering (SAXS) and electron microscopy (EM) single-particle reconstruction. The common modality of these techniques in determining the structure rely on the “signal” averaged from thousands to millions of different protein molecules under an assumption that the proteins remain in one or few identical conformations (no continuously changing conformation). Although this assumption works for some rigid-body proteins, for many macromolecules that are naturally soft and flexible, such as DNA, lipoproteins and antibodies, this assumption can have serious consequences.

A fundamental approach to study the structure of flexible proteins should be based on the signal from each individual protein molecule itself instead of averaging from different protein molecules. EM provide a novel tool to image each individual molecule at atomic resolution level, while electron tomography (ET) provide an approach to image a targeted molecule from a series of tilt angles. Although the signal obtained from an individual molecule has been believed for decades to be too weak to achieve any 3D structure with a meaningful resolution, we recently re-investigated this possibility carefully and proposed an individual-particle electron tomography (IPET) approach with a “focused electron tomography reconstruction” (FETR) algorithm to improve the 3D structure resolution via decreasing the reconstructing image size with an iterative refinement process. IPET does not require a pre-given initial model, class averaging of multiple molecules or an extended ordered lattice, but can provide near one nanometer resolution 3D structure from an individual protein molecule. Through the structure determination of each individual molecule, the comparison of these molecular structures provides a new opportunity to reveal the dynamic character, equilibrium fluctuation, mechanism, aggregation and even structural changes in proteins during a chemical reaction or biological event.

NOTES:
Comparing higher order structure (HOS) in therapeutic proteins is a significant challenge. Previously, we showed that changes in solution conditions produced detectable changes in the second-derivative amide I Fourier transform infrared (FTIR) spectra for a variety of model proteins. Those comparisons utilized vector-based approaches, such as spectral overlap and spectral correlation coefficients to quantify differences between spectra. In this study, chemometric analyses were performed to classify samples into different groups based on solution conditions. The solution conditions were composed of various combinations of temperature, pH, and salt types. At first, principal component analysis (PCA) was used to visually demonstrate that FTIR spectra respond to changes in solution conditions, which presumably indicates variations in HOS. This is observed when samples from the same solution condition form clusters within a PCA score plot. The second approach, called soft independent modeling of class analogy (SIMCA), was conducted to account for the within-class experimental error for the lysozyme spectra. The DModX values, indicative of the distance of each spectra to their respective class models, was found to be a more sensitive quantitative indicator of changes in HOS when compared with the modified area of overlap algorithm. The SIMCA approach provides a metric to determine whether new observations do, or do not belong to a particular class or group. Thus, SIMCA is the recommended approach when multiple samples from each condition are available.
Mass Spectrometry-Based Biophysical Approaches for Affinity, Aggregation and Structure of Therapeutic Proteins

Michael Gross

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

There is a strong need for methods to characterize protein therapeutics to meet the challenge that they, unlike small-molecule drugs, can form various higher order structures and can oligomerize. Most mass-spectrometry-based methods have high sensitivity, high information content, and fast turnaround, motivating their use for therapeutic protein studies. We will review HD exchange, specific amino-acid labeling, and fast photochemical oxidation of proteins (FPOP) that meet the challenge to assess the higher order structure of therapeutic proteins and to map epitopes. The outcomes establish that HDX and FPOP are complementary, orthogonal approaches. We will also describe other MS-biophysical methods that can be used to follow protein oligomerization and aggregation and play a role in drug development for amyloid proteins. In a third application, we will describe an HDX approach that measures affinity of a protein interacting with small-molecule drug candidate, providing both the affinity and the binding site.

NOTES:
Islet Amyloid Formation, Beta Cell Death and Diabetes

Daniel Raleigh, Andisheh Abedini, Martin Zanni

Stony Brook University, Stony Brook, NY USA

Amyloid formation plays a key role in a broad range of diseases including neurodegenerative disorders and type-2 diabetes. The mechanism of amyloid formation has yet to be derived in detail for any protein, the nature of the toxic species produced during amyloid formation is controversial and efforts at drug development have been disappointing. This talk outlines our new approaches to address these fundamental issues. Our work is focused on islet amyloidosis and its role in type-2 diabetes and beta-cell death. Key questions include: (1) What is the mechanism of amyloid formation by islet amyloid polypeptide (IAPP, Amylin) the causative agent of islet amyloidosis? (2) What are the properties of the toxic species produced during islet amyloidosis? (3) Can IAPP toxicity be inhibited by small molecules? (4) Can non-toxic soluble but bioactive analogs of IAPP be designed for use adjuncts to insulin therapy?

NOTES:
Native Top-Down Mass Spectrometry Approaches for Characterizing Proteins

Joseph Loo

University of California, Los Angeles, Los Angeles, CA USA

Mass spectrometry (MS) is a technique that weighs molecules, but this simple measurement can reveal much more than size. MS has capabilities to offer structural biologists layers of insight into the details of protein complexes. Using electrospray ionization (ESI), mass measurements deliver information on stoichiometry of binding partners directly, even for multi-ligand hetero-complexes and molecular machines with masses well beyond 1 MDa. Relative charging by ESI can give some information on protein folding. Top-down mass spectrometry is an effective tool for protein sequencing. We use high resolution Fourier transform mass spectrometry (FTMS) to probe ligand-binding sites and to generate topological information for large proteins and complexes, particularly with electron capture dissociation (ECD). Native top-down MS with ECD and photodissociation generates information on the surface topology, ligand binding sites, and post-translational modifications of protein complexes and membrane proteins. We aim to relate the 3D architecture of the gas phase protein to the solution phase state as a means to further develop MS for structural biology.

NOTES:
AUC as a Complementary Approach to Structural Techniques: Analysis of Protein Assembly, Linked Equilibria and Complicated Mixtures in Solution

Andrew Herr

*Cincinnati Children's Hospital Medical Center, Cincinnati, OH USA*

Analytical ultracentrifugation (AUC) is a powerful and flexible technique for characterizing the behavior of macromolecules in solution. AUC offers several advantages over other hydrodynamic techniques and allows both qualitative characterization of multi-component samples as well as quantitative analysis of reversible self-assembly. AUC is particularly well-suited as a complementary technique to high-resolution structural approaches. This presentation will focus on the analysis of single-species through multi-component systems by AUC, including examples of reversible self-assembly, ligand-induced oligomerization as linked equilibria, and aggregation phenomena. Finally, the deconvolution of sedimentation velocity data into 2D size-and-shape distributions will be discussed, with applications for complicated mixtures or certain poorly-resolved multi-species systems. These approaches represent powerful tools for both fundamental characterization of biopharmaceuticals and optimization of formulation conditions.

NOTES:
Serial Crystallography of Membrane Proteins with X-Ray Lasers

Vadim Cherezov

University of Southern California, Los Angeles, CA USA

Structural studies of many biomedically relevant membrane proteins and complexes are hampered by challenges related to growing sufficiently large crystals capable of withstanding radiation damage and yielding high-resolution data at X-ray synchrotron sources. We have developed a new approach of using a membrane mimetic gel-like matrix known as lipidic cubic phase (LCP) for growth and delivery of membrane protein microcrystals for data collection by serial femtosecond crystallography (SFX) at X-ray free electron lasers (XFEL). Microcrystals are delivered to the intersection point with an XFEL beam using a specially designed LCP injector, allowing to adjust LCP flow-rate and minimize crystal consumption. LCP-SFX uses highly intense and ultra-short XFEL pulses to minimize radiation damage and collect room temperature high-resolution data from sub-10 μm crystals. Protein consumption is reduced by 2-3 orders of magnitude compared to liquid injectors, making the LCP-SFX method attractive for structural studies of challenging proteins, and their complexes. Recent applications of this method resulted in solving difficult structures of the human δ-opioid receptor in complex with a bi-functional peptide ligand, a blood pressure regulator - angiotensin receptor, and a major GPCR signaling complex between rhodopsin and arrestin.

NOTES:
Deep Ultraviolet Resonance Raman (DUVRR) Spectroscopy of Therapeutic Proteins

Sergey Arzhantsev, Chen Qiu, John Kauffman, Justin Bueno

CDER, FDA, St. Louis, MO USA

Proteins are complex macromolecules with secondary and tertiary structures that may easily be disturbed, potentially resulting in loss of therapeutic activity. Deep ultraviolet Resonance Raman (DUVRR) spectroscopy is distinctively advantageous for quality assessment of formulated proteins because it is sensitive to changes in protein higher order (secondary and tertiary) structure, and is capable of analyzing proteins at concentrations of approximately 1 mg/mL, lower than the typical concentrations of formulated products. The deep ultraviolet resonance Raman enhancement from proteins (approximately $10^4$) allowed for analysis in the presence of excipients in aqueous media, which exhibit relatively weaker Raman signals. These attributes have allowed us to analyze the secondary structure of formulated monoclonal antibodies (mAbs) with minimal sample preparation. DUVRR spectroscopy was applied for the quality assessment of formulated Rituxan® and Avastin®. The formulated products were degraded with chemical and thermal stress and the degradation products were subjected to DUVRR spectroscopic analysis. Chemical stresses included acidification and addition of surfactant to the formulated product. Changes were observed in the DUVRR spectra with each level of stress, and multivariate statistical models were applied to identify trends within the data. The results indicate that DUVRR spectroscopy can be used as a rapid, sensitive and specific tool to detect degradation of formulated mAb pharmaceuticals.

NOTES:
Profiling Formulated Protein Therapeutics Using NMR Spectroscopy

Brad Jordan

Amgen Inc, Thousand Oaks, CA USA

Nuclear magnetic resonance (NMR) is arguably the most direct methodology for characterizing proteins in solution. Modern structural characterization of proteins by NMR typically utilizes multi-dimensional heteronuclear experiments. However, for formulated monoclonal antibody (mAb) therapeutics, the use of these approaches is difficult due to the large size of the proteins, restraints imposed by various formulations, and the time requirements for the use of non-isotopically labeled samples. PROFILE-NMR is a one-dimensional NMR method that allows differences in protein structure to be detected with high sensitivity. The use of this method will be elaborated on and the potential for further use of NMR in HOS assessment of proteins will be discussed.

NOTES:
Quinary Interactions and Protein Stability in Living Cells

Rachel Cohen

University of North Carolina at Chapel Hill, Chapel Hill, NC USA

Until recently there were just four accepted levels of protein structure: primary (amino acid sequence), secondary (helices, sheets and turn), tertiary (the three dimensional structure of globular proteins) and quaternary (specific protein-protein interactions). Quinary interactions, weak protein-protein interactions that occur only in cells, are essential because they organize the cellular interior, but remain a mystery because they are both weak and difficult to study. We have overcome these challenges and shown, both qualitatively and quantitatively, how the intracellular pH modulates quinary interactions in living Escherichia coli cells. To accomplish this goal we buffered the cellular interior and used nuclear magnetic resonance spectroscopy (NMR) to probe properties of a test protein, the B domain of protein G (GB1, 6.2 kDa) inside living cells. From a qualitative viewpoint, decreasing the intracellular pH degrades the resolution of NMR spectra because the pH-induced increase attractive charge-charge interactions between E. coli proteins and GB1 increases the effective molecular weight of the test protein. To quantify this finding, we used NMR-detected amide proton exchange to determine the free energy of unfolding in cells. Near neutral pH, the unfolding free energy in cells is comparable to that in buffered solution, but at lower intracellular pH values, the increased number of attractive interactions destabilizes GB1 relative to buffer. Our quantification of the strength of quinary interactions supports the hypothesis that these interactions have an electrostatic component. These results not only enhance the understanding of protein chemistry in living cells but also have important implications for formulating biologics.

NOTES:
Mass Spectrometry of Nanoparticles with Nanomechanical Systems

Eric Sage

California Institute of Technology, Pasadena, CA USA

Nano Electro Mechanical Systems (NEMS)-based Mass Spectrometry is an emerging analytic technique able to weigh individual molecules as they adsorb onto the surface of a nanoscale resonant mechanical system and change its resonance frequency. Unlike well-established MS technique, NEMS-MS directly measures the mass of an analyte of interest instead of deducing it from a m/z distribution. In this work, we illustrate this unique capability by demonstrating the first mass spectra of neutral particles obtained with NEMS-MS in a dedicated setup.

The NEMS device is inserted in the deposition chamber of a sputtering-gas aggregation setup able to produce nanometric metallic clusters with tunable deposition rate and diameter. The mass deposition rate can be measured with a Quartz Crystal Microbalance (QCM) while the mass distribution is acquired by a TOF Mass Spectrometer. After mass sensitivity calibration of the NEMS and TOF analysis of the cluster flux, individual particles are weighed individually as they land on the NEMS and incrementally build a mass spectrum. We show that NEMS-MS analysis is insensitive to charge state: the spectrum consists of a single peak whatever the species’ charge state, making it significantly clearer than the TOF spectrum. In subsequent tests, all charged particles are electrostatically removed from the beam, and unlike TOF-MS, NEMS-MS can still measure masses. This demonstrates the possibility to measure mass spectra for neutral molecules.

These results show that with NEMS-MS, challenges arising from the difficulty to deduce a mass spectra from a m/z distribution are circumvented, like removing complex charge state identification procedure, simplifying mass spectra interpretation and avoiding peaks overlap in heterogeneous samples. Neutral particle MS opens up the possibility to develop novel techniques to introduce particles in the gas phase regardless of ionization yield and study analytes that are incompatible with current ionization techniques.

NOTES:
Correlating the Impact of Different Well-defined Oligosaccharide Structures on Physical Stability Profiles of IgG1-Fc Glycoforms

Apurva More, Vishal Toprani, Solomon Okbazghi, Jae Kim, Sangeeta Joshi, C. Russell Middaugh, Thomas Tolbert, David Volkin

University of Kansas, Lawrence, KS USA

Therapeutic efficacy of IgG antibodies depend on their conformational stability, local flexibility and biological functionality. IgG-Fc glycosylation is important for the structural integrity and effector function activities. Better correlation between glycosylation and pharmaceutical stability of well defined IgG1-Fc glycoforms serves as a model system to enable rational design and optimization of stable IgG formulation conditions to avoid conformational destabilization and aggregation issues during their manufacture, long-term storage and administration. This study also contributes towards ongoing development of data visualization and mathematical modeling tools for biosimilarity assessments.

In this study, four highly purified, well-defined recombinant IgG1-Fc glycoform variants were prepared: 1) High-mannose-Fc (HM-Fc), 2) Man5-Fc (truncated glycoform), 3) GlcNAc-Fc (truncated glycoform), and 4) Non-glycosylated-Fc (N297Q-Fc) by yeast expression, Protein G Hydrophobic Interaction chromatography followed by in vitro enzymatic digestion with α-1,2 mannosidase1A and endoglycosidase-H, respectively, for Man5-Fc and GlcNAc-Fc. The four proteins were characterized by SDS-PAGE and mass spectrometry (for purity), capillary isoelectric focusing (for charge heterogeneity) and size exclusion chromatography (for aggregates). Initial comparisons of relative apparent solubility by polyethylene glycol (PEG) precipitation assay and conformational stability by differential scanning calorimetry (DSC) were carried out at pH 4.5 and pH 6.0. A trend of decreasing apparent solubility (thermodynamic activity) by PEG precipitation (pH 4.5, 6.0) and lower conformational stability by DSC (pH 4.5) was observed with reducing size of the N297-linked oligosaccharides. The physical stability of these four proteins was evaluated by combination of high-throughput biophysical techniques like intrinsic tryptophan, extrinsic fluorescence spectroscopy and solution turbidity in two formulations (with NaCl or sucrose) across different pH (4.0-7.5) and temperature conditions (10° C-90° C). Data visualization tools (empirical phase diagrams and radar charts) were utilized to summarize and compare the physical stability profile results. The overall physical stability profiles showed a correlation with oligosaccharide structure (HM-Fc, Man5-Fc>GlcNAc-Fc>N297Q-Fc), solution pH (higher>lower) and formulation composition (sucrose>NaCl).

NOTES:
Molecular Modelling and Machine Learning to Predict Chemical Stability

Lydia Beasley

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

Early assessment of the stability and manufacturability of multiple potential molecule candidates can be crucial to the selection of lead molecules that (1) have a high probability of technical success later in development, and (2) enable the use of platform processes that accelerate development. Molecular dynamics (MD) simulations provide the opportunity to predict the chemical stability of molecule candidates even before the processes to generate a sample of the molecule have begun.

MD simulations offer the ability to perform in-depth analyses of physiochemical properties by generating estimates of thousands of conformations of each molecule and tracking the position of each atom over time. This atomic-resolution simulation data can be used to make detailed chemical and structural calculations of each molecule. We demonstrate that careful analysis of MD trajectories can predict the risk of methionine (MET) and tryptophan (TRP) oxidation events that have the potential to impact efficacy and shelf life of antibody therapeutics.

Experimental oxidation data was used as a training set to establish the relationship between MD analyses and MET/TRP oxidation. For each molecule, fully atomistic molecular dynamics simulations of the antibody Fv-region were executed. Strong predictive relationships were found between certain MD outputs and MET/TRP oxidation risk. Prediction of additional physiochemical properties using MD outputs will be discussed.

NOTES:
The networks and spatial structures of biomolecular interactions provide insights into their function and thus help us to understand the workings of living cells. Detailed structural characterization of large and often dynamic assemblies and their networks is generally impossible by any single existing experimental or computational method. This challenge can be overcome by hybrid approaches that integrate data from diverse biophysical experiments (e.g., X-ray crystallography, NMR spectroscopy, electron microscopy, chemical cross-linking, yeast-two hybrid system, and various chemical genetics and proteomics approaches). We formulate the hybrid approach to structure and/or network determination as an optimization problem, the solution of which requires three main components: the representation of the assembly or network, the scoring function, and the optimization method. The ensemble of solutions to the optimization problem embodies the most accurate characterization given the available information. The key challenges remain translating experimental data into restraints on the structure and/or network, combining these spatial and/or network restraints into a single scoring function, optimizing the scoring function, and analyzing the resulting ensemble of solutions. The approach will be illustrated by several applications to specific biological systems, including the structure determination of the nuclear pore complex and the mapping of the gulonate pathway in Haemophilus influenzae.

NOTES:
RNA higher order structure plays a significant role in the cellular function of a majority of messages (mRNAs). Single nucleotide variants (SNVs) have the potential to disrupt functional elements because RNA folds in a sequence-specific manner, much like proteins. A riboSNitch is an element of RNA structure with a specific function that is disrupted by an SNV or a single nucleotide polymorphism (SNP; or polymorphism; SNVs occur with low frequency in the population, <1%). RiboSNitches are particularly relevant to interpreting the results of genome-wide association studies (GWAS). Often GWAS identify SNPs associated with a phenotype mapping to noncoding regions of the genome. Because a majority of the human genome is transcribed, significant subsets of GWAS SNPs are putative riboSNitches. The extent to which the transcriptome is tolerant of SNP-induced structure change is still poorly understood. Recent advances in ultra-high-throughput structure probing begin to reveal the structural complexities of mutation-induced structure change. In this talk I will review our current understanding and ability to predict SNV and SNP-induced structure change in the human transcriptome and discuss the importance of riboSNitch discovery in interpreting GWAS results and massive sequencing projects.

NOTES:
Advanced Characterization of ADC for Defining CQAs

Hangtian Song, Tapan Das

Bristol-Myers Squibb Company, Princeton, NJ USA

Development of antibody-drug conjugate as a treatment option for several unmet medical need areas gained significant traction with launch of multiple ADCs in recent years. Currently in the US, there are around 30 ADCs being tested in clinical trials. As a hybrid of a large biological molecule and multiple small molecule components, ADCs inherit physicochemical properties as well as quality attributes from both sides. Additionally, there are unique properties of ADCs that differentiate them from proteins and small molecules – such as drug-to-antibody ratio (DAR). Understanding these unique properties is crucial to gain comprehensive knowledge about the safety and efficacy of an ADC. This talk will include discussions on advanced characterization methods for ADCs and a case study to demonstrate available analytical tools for understanding ADC quality attributes.

NOTES:
Distinct molecular attributes can critically modulate the physicochemical and pharmacological properties of protein therapeutics. A detailed understanding of key structural features and degradation pathways enables protein design and development to proceed in a rational manner, whereby critical attributes are understood and controlled.

Case studies will be presented where insight into protein structure and dynamics of antibody and novel therapeutic protein formats has been gained using a combination of established and emerging analytical tools for biopharmaceutical product characterization.

NOTES:
Formulation as a Layer Cake: Combining Materials and Study Types for Maximum Predictability and Efficiency

Julie Wei

*Biogen, Cambridge, MA USA*

Abstract not available at the time of print.

NOTES:
Deamidation Potential in Therapeutic Antibodies: Bridging the Gap between the Discovery and Development

Shrikant Deshpande

_Bristol-Myers Squibb, Redwood City, CA USA_

Asparagine deamidation is one of the key quality attributes of many therapeutic antibodies. Though NG containing sequences are considered to be most susceptible for deamidation and iso-aspartic acid formation, a number of other asparagine containing motifs have shown deamidation potential as well. Recognizing these motifs and understanding their deamidation patterns in the early stages of antibody discovery is important in the lead selection process. Deamidation can potentially affect structural integrity of the antibodies leading to aggregation, poor target binding and CMC as well as clinical consequences. This presentation deals with exploring viable solutions to decrease or even eliminate deamidation risks of therapeutic antibodies in discovery.

NOTES:
The Development of Biophysical Screening Methods to Identify Better Candidates

Bryan Jones

*Eli Lilly and Company, San Diego, CA USA*

The successful development of therapeutic antibodies is complex, requiring both the correct biological activity, and suitable biophysical properties to enable the delivery of safe and effective products. Given the multitude of sources of therapeutic antibodies, and the availability of a variety of protein engineering approaches to alter desired properties of interest, the ability to assess biophysical properties becomes overwhelming, as typically these assessments are both material- and time-intensive. Therefore, we have sought to develop much more efficient methodologies that can provide predictive information about a molecule’s biophysical properties. This presentation will cover the development and integration of these methods with respect to providing a more thorough biophysical assessment of potential candidate molecules, and their application to both engineering activities and selection of potential candidate molecules.

NOTES:
NOTES:
**Regulatory Panel Discussion**

**Panelists:**
Yves Aubin, *Health Canada*
Maria Gutierrez-Lugo, *CDER, FDA*
Kate Hutterer, *Amgen Inc.*
Veronika Jekerle, *European Medicines Agency*
Pin Yee Wong, *Genentech, a Member of the Roche Group*

**Topic 1: Leveraging Links Between HOS and (Bio)potency to Accelerate Development**

- What would it take to reduce bio(potency) testing by leveraging one or more HOS methods? What would a suitable data package look like?
- What are the considerations at different phases of development? How about development vs. QC environment?
- Would HOS methods be better/worse than a non-cell-based assay? Why?
- What about cases where a relevant cell-based bioassay is not technically feasible? What is/could be the role for HOS methods in this case?
- Can functional assays be used in place of traditional HOS methods?
- What would be the scope and considerations for bridging studies to ensure that the HOS assay accurately selects the active form of the molecule (especially for proteins that demonstrate subtle structural changes, oligomerization, etc. that have direct effects on bio-potency)?
- What are the challenges and feasibility of qualifying and validating HOS assays to be used in a QC environment?
- How can a HOS characterization method(s) facilitate the development of a cell-based or bio-potency assay?

**Topic 2: Playing for Both Teams: HOS Characterization as Both Innovator and Biosimilar Developer**

- For the development of a biosimilar, what level and extent of testing of the innovator molecule is sufficient to understand the magnitude of variability in critical quality attributes for a marketed product? How is that information leveraged for the approval process of the biosimilar?
- What differences and similarities exist in HOS strategy for: (1) elucidation of structure (2) process/product characterization and enabling changes?
- How is the scope/depth of HOS characterization different or similar? Are different tools used? What about at different phases?
- Are all HOS characterization data packages created equal, or does the experience of the sponsor play a role? How/why?
- What is the importance of comparative forced degradation studies in assessing bio-similarity? What stress conditions and analytical assays are considered sufficient to convince a regulator that bio-similarity exists?
Biological Consequences of HOS

P-101

An Efficient Workflow for Mapping Disulfide Bonds of Therapeutic Proteins

Asish Chakraborty, Henry Shion, Liuxi Chen, Ying Qing Yu, Weibin Chen

Waters Corporation, Milford, MA USA

Disulfide bond formation is critical for establishing the 3-D folded structure, and maintaining the stability, and biological functions of therapeutic proteins. Disulfide bond linkage assignment, localization and monitoring are therefore of great importance to ensure process consistency and product integrity during biotherapeutic drug development. Regulatory agencies have specific requirements for mapping the disulfide bonds in biotherapeutics, such as monoclonal antibodies.

In this study, we describe a streamlined disulfide bond mapping workflow that includes on-line reversed-phase separation of tryptic digests using UPLC with on-line UV and QTof MS detections with automated data acquisition, processing, and reporting under compliant ready architecture. Experimental results from disulfide bond mapping analysis of mAbs will be used to demonstrate the streamlined common analytical workflow.

P-102

Higher Order Structural View of Antimicrobial Preservative-induced Aggregation of Interferon Alpha-2a

Krishna Mallela, Regina Bis, Surinder Singh, Javier Cabello-Villegas

University of Colorado Anschutz Medical Campus, Aurora, CO USA

Antimicrobial preservatives (APs) are included in liquid multi-dose protein formulations to combat the growth of microbes and bacteria. These compounds have been shown to cause protein aggregation, which leads to serious immunogenic and toxic side-effects in patients. We examined the biophysical and structural mechanisms by which APs induce protein aggregation using a pharmaceutical protein interferon alpha-2a (IFNA2). IFNA2 is used in treating various disorders including leukemia and hepatitis C, and APs have been used in its multi-dose formulation. APs induced IFNA2 aggregation, demonstrated by the loss of soluble monomer and increase in solution turbidity. The extent of IFNA2 aggregation increased with the increase in AP concentration. IFNA2 aggregation depended on the nature of AP, and followed
the order m-cresol > phenol > benzyl alcohol > phenoxyethanol. We examined the structural mechanisms by which benzyl alcohol (BA), the most widely used AP in protein formulations, cause IFNA2 aggregation. Denaturant melts measured using protein intrinsic fluorescence and that of the 1-anilinonaphthalene-8-sulfonic acid (ANS) dye indicated that increased BA concentration populated a partially unfolded intermediate of IFNA2. Changes in 2D nuclear magnetic resonance (NMR) chemical shifts and hydrogen exchange (HX) rates identified the structural nature of this intermediate, which correlated with an aggregation “hot-spot” predicted by computational methods. These results indicate that APs induce IFNA2 aggregation by partially unfolding the protein. Using protein NMR and HX helped us in pinpointing the local structural regions that unfold with the addition of APs.

*This work was funded by the PhRMA Foundation and the National Institutes of Health (NIH).*

NOTES:
Sensitivity of Circular Dichroism and Raman Spectroscopy to Local Environment of Individual Tryptophan in mAbs

Lucas Wafer, Sharon Polleck, Lucy Liu, Peter Richard, Jaime Lee, Yin Luo

Pfizer, Inc., Andover, MA USA

Antibody drug conjugates (ADCs) are covalent conjugates of a cytotoxic drug (payload) to a monoclonal antibody (mAb) that selectively target an antigen on the surface of target cells. ADCs offer substantial advantages over traditional therapies. This is due not only to the enhanced specificity that results from antigen-antibody interactions, but also to the superior mechanism of action (MoA)—the intracellular delivery of a lethal drug payload following receptor-mediated endocytosis. In order to fully realize the potential of therapeutic ADCs, it is critical to understand the impact of payload conjugation on the mAb. To address this issue, the aggregation propensity, higher-order structure, safety and efficacy of three IgG mAbs were compared before and after conjugation.

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 was observed in the region of C53 free cysteine at hGH. The crystal structure of hGH 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. Using the same approach, no significant change was observed for free cysteine and scrambling at the hinge region of the IgG4 mAb, however drastic change was observed for N28 deamidation at GLP-1. Additionally, different glycoforms
at Fc of IgG4 were found to affect the deuterium uptake. 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.

NOTES:
Application of Ion Mobility-Mass Spectrometry and HDX-MS for Investigating Monoclonal Antibody Conformations during Process Characterization

Tasneem Bahrainwala, Ratnesh Pandey, Yanhong Yang, Min Zhu, Mandy Xie, Michael Washabaugh

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

Monoclonal antibodies may undergo complicated folding and exist in a number of low-energy structures that can pose challenges for classical biophysical techniques such as circular dichroism (CD). Recently development in Mass spectrometric (MS) methods such as ion-mobility MS (IMS-MS) and hydrogen-deuterium exchange-MS (HDX-MS) can provide high resolution structural information with high sensitivity and less sample consumption. These techniques add complimentary information to other traditional methods on higher order structure (HOS).

An in process IgG4 protein which was held at different temperatures up to 35 days was used as a case study. After evaluating by multiple analytical methods including gel electrophoresis, intact mass analysis and peptide mapping, unexpected modifications and fragmentations were observed on the stressed samples. Data suggest different degradation pathways could present under this hold condition than that predicted from the force degradation studies using drug substance. The HOS of the stressed samples are further evaluated by IMS-MS and HDX-MS to assist the identification of these pathways. The IMS-MS experiments are carried out on the denatured and native sampling conditions to probe the global conformational changes, and HDX-MS is being performed to locate the regions where the conformational changes occurred.

This work demonstrated the capability of combining IMS-MS with HDX-MS to provide conformational information both on the global and residue level that would not be readily obtained through traditional HOS methods. This approach can be implemented as part of the structural characterization and ad-hoc investigations tools for biopharmaceutical development.

NOTES:
NMR Profile for Higher Order Structure Analysis in Comparability and Similarity Assessment of Biopharmaceuticals

Mats Wikstrom, Brad Jordan, Tsang-Lin Hwang, Shawn Cao

Amgen Inc., Thousand Oaks, CA USA

NMR spectroscopy represents a direct method for characterizing higher order structure (HOS) of proteins in solution since chemical shifts and lineshapes are intrinsically sensitive to the spatial arrangement of the amino acids. The 1D PROFILE method provides a fingerprint of a formulated protein therapeutics by performing line shape analysis, and has therefore the ability to detect and quantify subtle structural differences between samples. NMR has the potential advantages over methods currently used for HOS characterization (CD, FT-IR, and DSC) because NMR gives HOS attributes a priori at atomic resolution with high sensitivity. In addition, experiments can be performed on intact protein in formulation buffers, and at protein concentrations used in drug product formulations. 2D NMR methods have, in addition to provide complementary information on HOS similarity/comparability, the potential to offer further specific molecular detail on the site(s) if difference(s). In this presentation, we present experimental data on the use of NMR methods to access HOS attributes in biopharmaceutical process development and potential applications for assessing comparability and similarity.

NOTES:
Emerging and Novel Technologies

P-108

Increasing Throughput of Antigen-antibody Interaction Epitope Mapping by Oxidative Footprinting

Yining Huang¹, Ke Li¹, Manolo Plasencia¹, Henry Rohrs¹, Michael Gross¹, Yong Kil², Marshall Bern², Eric Carlson², Ilker Sen², Christopher Becker²

¹Washington University in St. Louis, St. Louis, MO USA, ²Protein Metrics, San Carlos, CA USA

Epitope mapping of antibody-antigen interactions is important for both therapeutic protein development and biological research. Mass spectrometry offers a critical alternative to X-ray and NMR analyses, which may be impossible or impractical for the biochemical system under study. Footprinting by irreversible chemical labeling (e.g., fast photochemical oxidation of proteins) is complementary, and can even be an alternative, to hydrogen-deuterium exchange (HDX). These mass spectrometry methods probe solvent accessibility.

Here we report on oxidative footprinting using the experimental technique known as fast photochemical oxidation of proteins (FPOP), with sub-microsecond exposure of OH radicals by pulsed laser photolysis of H₂O₂. We address a past limitation of this approach, namely the speed and ease of data analysis. The antigen-antibody pairs under study are Interleukin-6 receptor (IL6R) bound to adnectins, and Domain III of West Nile Virus envelope glycoprotein (WNV E-DIII) bound to a neutralizing antibody (mAb E16).

High resolution liquid chromatography – mass spectrometry (LC-MS) data of trypsin digested specimens were acquired on the bound pairs and separately on the individual proteins. Peptides were identified by the Byonic™ search engine and the relative degree of oxidation was quantified, tabulated, and plotted for each tracked peptide by Byologic™ software. The FPOP results agree with those from prior HDX and X-ray crystallography studies. The results from the automated software were compared with manual data processing showing good consistency. Thus the identification, validation, and quantitation of FPOP modified peptides was completed within a single pipeline, significantly improving efficiency.

NOTES:
NOTES:
P-109

Practical NMR Applications for Structural Assessment of Protein Therapeutics

Robert Brinson¹, Luke Arbogast¹, Yves Aubin², Frank Delaglio¹, John Marino¹

¹NIST/IBBR, Rockville, MD USA, ²Health Canada, Ottawa, ON Canada

Techniques for the facile characterization of higher order structure (HOS) in biotherapeutics are in great demand for establishing consistency in drug manufacturing, detecting process-related drug product variations and comparing a biosimilar to an innovator reference product. While nuclear magnetic resonance (NMR) spectroscopy provides structural information in proteins at atomic resolution, limits on sensitivity, molecular size and sample volumes, as well as the perceived need for stable-isotope enrichment, have limited its widespread use in the biopharmaceutical industry. Recent advances in NMR hardware and techniques have greatly ameliorated these limitations, and 2D ¹H-¹³C and ¹H-¹⁵N correlated spectra can now be collected on biologics at natural isotopic abundance. The individual peak positions in ‘fingerprint’ amide or methyl spectra are sensitive to structure and can serve as spectral indicators for establishing consistency in drug manufacturing, assessing stability of drug formulations, and determining biosimilarity to an innovator reference product. To demonstrate the applicability and practicality of NMR fingerprinting techniques to biopharmaceutical applications, we will present approaches for the analysis of 2D NMR spectra that are representative of drug-like proteins at natural isotopic abundance. We will further report on the robustness of NMR methods for mapping the structure of these biologics and the evaluation of statistical measures for comparability assessment.

P-110

HDX-MS for Assessing Comparability between Innovator and Biosimilar Biotherapeutics

Asish Chakraborty, Jing Fang, Ying Qing Yu

Waters Corporation, Milford, MA USA

Determining similarity between innovator and biosimilar biotherapeutics or between batches of a biotherapeutic are fundamental elements of biopharmaceutical development. Higher order structure can serve as a means for establishing comparability or lack of comparability between an innovator and a biosimilar, as well as between batches of an innovator biotherapeutic before and after process changes.

This study highlights the use of Hydrogen-deuterium exchange MS (HDX MS) for assessing comparability between an innovator therapeutic mAb Remicade® (Infliximab) and a commercial biosimilar product Inflectra™. Several batches of innovator products during manufacturing process were also analyzed at intact protein and peptide levels in order to gain insight into the conformational difference. The HDX MS experiments were conducted on an automated HDX platform. Details on the experimental conditions optimized exclusively for these antibodies are discussed. The conformational differences between Remicade and Inflectra are also discussed in details.
NOTES:
P-111

Complementary Particle and Biophysical Techniques for Evaluating Biosimilarity

Amber Fradkin¹, Aaron B. Krueger², Matt Baker¹, John Carpenter²

¹KBI Biopharma Inc., Boulder, CO USA, ²University of Colorado Denver, Aurora, CO USA

Significant advances have been made in analytical technology for the characterization and identification of protein therapeutics. With these advances comes a tremendous amount of new data with which to characterize particles in therapeutic protein products. Careful interpretation of data and in-depth understanding of the method limitations is of utmost importance for using complementary methods to establish ‘normal’ particle profiles. This study demonstrates how multiple particle and biophysical techniques can be used to characterize particle populations and stability of therapeutic protein products. Two commercially available brands of filgrastim, as well as vial and syringe configurations were analyzed by flow Imaging microscopy, resonant mass measurement (RMM), Nanosight, Viscosizer, Morphologi G3-ID, Helix, FTIR and DSC techniques. Image analysis, coupled with Morphologi G3-ID and RMM were applied to classify the particles as intrinsic, inherent and extrinsic. By applying a combination of analytical techniques, valuable information is gained about the product particle profiles, demonstrating that a single method to characterize particles may be insufficient for properly assessing therapeutic protein product particle profiles for comparability/biosimilarity.

P-113

Raman Spectroscopy for Higher Order Structure Elucidation

Thomas Lerch

Pfizer, Inc., Chesterfield, MO USA

Raman spectroscopy relies on vibrational and other low-frequency modes of light scattering to provide spectral features specific to chemical bond identity and geometry. Protein Raman spectra in the 1800 – 400cm⁻¹ region (“fingerprint region”) contain spectral bands unique to secondary structure and tertiary structure. In addition, Raman spectra offer a greater abundance of spectral features and improved data dispersion over more standard biophysical tools, including CD and fluorescence emission spectroscopies. These features give Raman spectroscopy the potential for improved higher order structure elucidation and product comparability capabilities over more traditional methods employed in the development of biotherapeutics. Ever-increasing product protein concentration in mAb product development further leverages the capabilities of this technology. This presentation will explore Raman spectroscopy for elucidation of higher order structure, comparability exercises, and as an alternative tool for more complex modalities that can be refractory to standard biophysical methods.
Development of a Microsecond X-Ray Hydroxyl-Radical Footprinting Facility at the Advanced Light Source to Study the Structure and Dynamics of Complex Biological Macromolecules

Corie Ralston, Sayan Gupta

Lawrence Berkeley National Laboratory, Berkeley, CA USA

Radiolytic footprinting is an increasingly popular method for structural elucidation of macromolecules in the solution state. X-ray hydroxyl radical footprinting in particular has developed in the past decade into a nearly routine technique and had been applied to a diverse range of biological systems, yielding unique structural insights impossible to obtain using the more standard structural methods of crystallography, NMR, electron microscopy and small angle scattering. The technique was pioneered at the National Synchrotron Light Source (NSLS) over the past decade; now with the user community continuing to grow and the closure of this facility, it is imperative that x-ray footprinting continue to be developed. Towards this end we are commissioning a new footprinting facility at the Advanced Light Source (ALS) synchrotron which has been supporting the NSLS users during the commissioning of the NSLS-II, and is also continuing to develop new methodologies. In particular, we show that flux densities produced by an ALS focused white-light bend magnet beamline are high enough to allow microsecond exposures yielding sufficient modification to conduct footprinting experiments. Examples of the application of microsecond footprinting to several important biological systems are given.

A Fully Automated Robotic Set-Up based on Ultrahigh-Resolution QTOF Mass Spectrometry for Hydrogen Exchange Experiments

Jan Wiesner¹, Nikolay Hentze¹, Eckhard Belau², Catherine Evans³, Guillaume Tremintin⁴, Detlev Suckau², Wolfgang Jabs², Matthias Mayer¹

¹Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), DKFZZMBH-Alliance, Heidelberg, Germany, ²Bruker Daltonik GmbH, Bremen, Germany, ³Bruker Daltonics Ltd, Coventry, United Kingdom, ⁴Bruker Daltonics, Inc., Fremont, CA USA

Most proteins require a defined secondary and tertiary structure for full activity and many proteins progress through different conformations in the course of their activity cycle. Thus, it is very important to analyze secondary and tertiary structure of proteins in solution in a time-resolved manner to determine their native state, their stability and activity, and their functional dynamics. Hydrogen exchange mass spectrometry (HX-MS) is a highly versatile method to detect solvent accessibility of the polypeptide backbone and therefore secondary structure and conformational changes of the protein of interest at the 10 s to several hours timescale. We have established a fully automated system, consisting of a Leap robot, an Agilent HPLC-system and a Bruker ultrahigh resolution (UHR) QTOF mass spectrometer that reliably performs HX-MS experiment in a highly flexible way. To benchmark the system we performed HX-MS
experiments with *E. coli* Hsp90, a 140 kDa dimer, which undergoes dramatic conformational changes in response to ATP. The obtained data from time course experiments from 10 to 1000 s are fully comparable with previously published data from a manual HX-MS system but of higher overall quality and reproducibility.

**NOTES:**
Luminex Beads-based Protein Conformational Array for mAb HOS Analysis

Xing Wang, Guofeng Fu, Michael Davies

Array Bridge Inc., St. Louis, MO USA

Biologics Higher Order Structure (HOS) is important to its safety and efficacy but difficult to define. A novel technology is developed using antibody arrays to analyze monoclonal antibody Higher Order Structure. This technology provides a sensitive, systematic and high-throughput approach for mAb Higher Order Structure comparability analysis, generating valuable information for cell line selection, process development and formulation development. Recently, the Protein Conformational Array has been adapted to the Luminex beads-based platform with similar profile readout but much improved automation and throughput. It has been used by many of the leading biotech companies and biosimilar developers in mAb development including regulatory filings. Case studies will be presented to demonstrate the application of the Luminex-based antibody array in biosimilar as well as novel mAb development and its complementary value to the bioassays and other analytical technologies.

Automated de novo Identification and Profiling of Disulfide Bonds in Monoclonal Antibodies Including Analysis of Low Level DSB Scrambling

Jason Wood

Bruker Daltonics, Inc., Billerica, MA USA

The location of disulfide bridges (DSBs) in biologics, such as antibodies, affects their spatial structures and can impact their safety and efficacy. DSB analysis is therefore required during the characterization of biologics. It is often performed by a time-intensive, data-processing approach, involving the comparison of peptide maps of reduced and non-reduced samples. In order to avoid this differential approach, a workflow analyzing non-reduced protein digests by LC-MALDI-MS/MS was investigated where the disulfide bonded peptides are reduced in the mass spectrometer. These innovations in LC-MALDI MS and new processing software make it possible to determine disulfide linkages much faster and without prior knowledge about their positions (de novo). This method was applied to adalimumab, a likely target for biosimilar development. Additionally, a disulfide scrambling experiment was performed on adalimumab, and the results presented.

NOTES:
Structural Characterization of the Therapeutic Antibody and Biosimilar Product with Optimized Hydrogen/Deuterium Exchange Mass Spectrometry

Terry Zhang, Michael Blank, Kai Scheffler, Shanhua Lin, Xiaodong Liu, Jonathan Josephs

1Thermo Fisher Scientific, San Jose, CA USA, 2Thermo Fisher Scientific, Sunnyvale, CA USA

Recombinant monoclonal antibodies (mAb) and derivatives have become the fastest growing class of human therapeutics since 1980s. The patent expiration of first generation mAb therapeutics provides opportunity for biosimilar mAbs to enter biotherapeutics market. FDA expects that extensive characterization of both biosimilar product and the innovator reference product as the foundation of biosimilarity. In addition, protein secondary, tertiary and quaternary structure should be considered. 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 characterize the conformation of trastuzumab and its biosimilar product.

Therapeutic antibodies were diluted 10 times with labeling buffer and incubated at room temperature for multiple time points. The samples were then quenched and digested online with a pepsin column in a fully automated manner using H/D-X PAL™ (LEAP Technology). The digested peptides were injected into a Pepmap C18 reverse phase column at 0°C with a short gradient. MS analysis was performed with Thermo Scientific™ Orbitrap Fusion™ mass spectrometer. Data was processed with Thermo Scientific™ Proteome Discoverer 1.4™ software for peptide identification. HDX experiment data were analyzed by HDExaminer (Sierra Analytics) and Mass Analyzer HDX algorithm.

Nearly 100% sequence coverage was achieved for both innovator and biosimilar samples. More than 200 peptides generated by online pepsin digestion from the optimized HDX workflow were identified. These were subsequently used to probe the conformation of the two samples by HDX. The deuterium uptake data were analyzed by HDExaminer. The two analyzed samples showed very similar deuterium uptake profiles for both light and heavy chain indicating that they have very similar three dimensional structures.

NOTES:
The Small Peptide Teduglutide Self-Associates to Form Stable Pentamers with a Defined Tertiary Structure

John Philo, Tsutomu Arakawa

Alliance Protein Laboratories, Inc., San Diego, CA USA

The peptide teduglutide (monomer mass 3.75 kDa) is a homolog of the hormone glucagon-like peptide 2 (GLP-2) in which glycine is substituted for alanine at position 2 to improve its half-life in serum. Teduglutide is used clinically to treat short bowel syndrome. Other members of the glucagon-like peptide family are well known to reversibly associate, and teduglutide had previously been reported to form dimers based on SEC-MALS. The general view has been that these peptides associate to form small micelle-like structures.

We have now studied the association of teduglutide using a combination of SV-AUC and circular dichroism at up to ~2 mg/mL, and by DLS and SE-AUC at concentrations up to ~15 mg/mL. Modeling of both the SE and SV data shows that below ~2 mg/mL teduglutide undergoes a concerted association to form a pentamer. Interestingly CD shows that this pentamer formation causes a significant increase in $\alpha$-helix content, and surprisingly near-UV CD shows that the pentamer has a distinct stable tertiary structure. The AUC and DLS data at concentrations above 2 mg/mL are dominated by very strong non-ideality due to electrostatic repulsion (despite an ionic strength over 40 mM), with a second virial coefficient ~25-fold higher than predicted from excluded volume alone. Nonetheless fitting the AUC data to models including non-ideality indicates that at higher concentrations larger oligomers are forming. The strong non-ideality makes it quite difficult to distinguish different assembly models, but we believe the larger oligomer is most likely a decamer (a dimer of the pentamer). Thus overall we picture this peptide as forming a specific pentamer where each monomer has a unique tertiary structure rather than a micelle-like state with flexible stoichiometry and structure, and then these pentamers in turn self-associate at higher concentrations.

This work was part of studies sponsored by NPS Pharmaceuticals/Shire.

NOTES:
Understanding or preventing degradation is essential when developing complex biotherapeutics. This study used a novel approach to HOS analysis – quantitative circular dichroism – to detect and determine the significance of changes in HOS of IgG1 caused by important degradation pathways.

IgG1 was subjected to conditions which promote degradation through deamidation of asparagine/glutamine residues and isomerization of aspartic acid residues, glycation, or oxidation. Degraded and control IgG1 samples were presented to a Chirascan-auto qCD spectrometer in 96-well plate-format and CD and absorbance spectra were recorded in the near and far UV regions.

The combination of automation and the high reproducibility of this system allowed large datasets to be accumulated which informed on minute HOS differences and, through application of appropriate statistical methods, their significance.

Working at the 2-sigma confidence interval we were able to objectively identify significant differences in the near UV CD spectra of IgG1 for all degradation treatments with the exception of one mild glycation treatment. No significant differences were found the far UV CD spectra. These results are indicative of changes occurring to the local environment of aromatic amino acid side chains with minimal changes to the peptide backbone occurring.

Subjective ‘visual comparisons’ of spectra were omitted and in place a robust, reproducible and audible statistical approach was used. The ability to generate truly quantitative data will substantially strengthen the role of CD analysis throughout biotherapeutic development programs and help fulfil regulatory demands.

NOTES:
P-121

Quinary Interactions and Protein Stability in Living Cells

Rachel Cohen

*University of North Carolina at Chapel Hill, Chapel Hill, NC USA*

Until recently there were just four accepted levels of protein structure: primary (amino acid sequence), secondary (helices, sheets and turn), tertiary (the three dimensional structure of globular proteins) and quaternary (specific protein-protein interactions). Quinary interactions, weak protein-protein interactions that occur only in cells, are essential because they organize the cellular interior, but remain a mystery because they are both weak and difficult to study. We have overcome these challenges and shown, both qualitatively and quantitatively, how the intracellular pH modulates quinary interactions in living *Escherichia coli* cells. To accomplish this goal we buffered the cellular interior and used nuclear magnetic resonance spectroscopy (NMR) to probe properties of a test protein, the B domain of protein G (GB1, 6.2 kDa) inside living cells. From a qualitative viewpoint, decreasing the intracellular pH degrades the resolution of NMR spectra because the pH-induced increase attractive charge-charge interactions between *E. coli* proteins and GB1 increases the effective molecular weight of the test protein. To quantify this finding, we used NMR-detected amide proton exchange to determine the free energy of unfolding in cells. Near neutral pH, the unfolding free energy in cells is comparable to that in buffered solution, but at lower intracellular pH values, the increased number of attractive interactions destabilizes GB1 relative to buffer. Our quantification of the strength of quinary interactions supports the hypothesis that these interactions have an electrostatic component. These results not only enhance the understanding of protein chemistry in living cells but also have important implications for formulating biologics.

P-122

Correlating the Impact of Different Well-defined Oligosaccharide Structures on Physical Stability Profiles of IgG1-Fc Glycoforms

*Apurva More, Vishal Toprani, Solomon Okbazghi, Jae Kim, Sangeeta Joshi, C. Russell Middaugh*

*University of Kansas, Lawrence, KS USA*

Therapeutic efficacy of IgG antibodies depend on their conformational stability, local flexibility and biological functionality. IgG-Fc glycosylation is important for the structural integrity and effector function activities. Better correlation between glycosylation and pharmaceutical stability of well defined IgG1-Fc glycoforms serves as a model system to enable rational design and optimization of stable IgG formulation conditions to avoid conformational destabilization and aggregation issues during their manufacture, long-term storage and administration.
This study also contributes towards ongoing development of data visualization and mathematical modeling tools for biosimilarity assessments. In this study, four highly purified, well-defined recombinant IgG1-Fc glycoform variants were prepared: 1) High-mannose-Fc (HM-Fc), 2) Man5-Fc (truncated glycoform), 3) GlcNAc-Fc (truncated glycoform), and 4) Non-glycosylated-Fc (N297Q-Fc) by yeast expression, Protein G Hydrophobic Interaction chromatography followed by in vitro enzymatic digestion with α-1,2 mannosidase and endoglycosidase-H, respectively, for Man5-Fc and GlcNAc-Fc. The four proteins were characterized by SDS-PAGE and mass spectrometry (for purity), capillary isoelectric focusing (for charge heterogeneity) and size exclusion chromatography (for aggregates). Initial comparisons of relative apparent solubility by polyethylene glycol (PEG) precipitation assay and conformational stability by differential scanning calorimetry (DSC) were carried out at pH 4.5 and pH 6.0. A trend of decreasing apparent solubility (thermodynamic activity) by PEG precipitation (pH 4.5, 6.0) and lower conformational stability by DSC (pH 4.5) was observed with reducing size of the N297-linked oligosaccharides. The physical stability of these four proteins was evaluated by combination of high-throughput biophysical techniques like intrinsic tryptophan, extrinsic fluorescence spectroscopy and solution turbidity in two formulations (with NaCl or sucrose) across different pH (4.0-7.5) and temperature conditions (10°C-90°C). Data visualization tools (empirical phase diagrams and radar charts) were utilized to summarize and compare the physical stability profile results. The overall physical stability profiles showed a correlation with oligosaccharide structure (HM-Fc, Man5-Fc>GlcnAc-Fc>N297Q-Fc), solution pH (higher>lower) and formulation composition (sucrose>NaCl).

NOTES:
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A Hybrid Orbitrap/NEMS Instrument for Native Single Molecule Analysis

Adam Neumann\textsuperscript{1}, Eric Sage\textsuperscript{1}, Warren Fon\textsuperscript{1}, Maria Reinhardt-Szyba\textsuperscript{2}, Dmitry Boll\textsuperscript{2}, Alexander Makarov\textsuperscript{2}, Michael Roukes\textsuperscript{1}

\textsuperscript{1}California Institute of Technology, Pasadena, CA USA, \textsuperscript{2}Thermo Fisher Scientific GmbH, Bremen, Germany

Nanoelectromechanical systems (NEMS) based mass spectrometry (MS) is a promising technique able to detect individual molecules as they adsorb onto a resonant nanostructure. Each adsorption event abruptly changes the device's resonance frequency, which is tracked in real time using a phase locked loop and custom FPGA electronics. Based on the frequency shifts of two resonant modes, we can calculate a mass spectrum of the delivered protein complexes, in contrast with an m/z spectrum obtained via traditional instrumentation. By tracking more resonant modes, we can also calculate the size and shape of individual molecules. This provides additional information compared to rotationally-averaged collisional cross-sections obtained via ion mobility measurements.

We have combined the unique abilities of NEMS-MS with the analytic power of an Orbitrap detector in a hybrid setup by modifying a Thermo Fisher Q Exactive Plus EMR with an additional transfer chamber and ion optics. We anticipate that this new instrumentation with single-molecule resolution will provide enhanced capabilities for native mass spectrometry and higher order structure research.

P-124

Higher Order Structural View of Polysorbate Interactions with Interferon Alpha-2a

Dinen Shah, Krishna Mallela

University of Colorado Anschutz Medical Campus, Aurora, CO USA

Polysorbates are commonly used aggregation suppressors in protein therapeutic formulations. Their interaction with proteins has long been the subject of interest to formulation scientists. We probed the interactions of polysorbate 20 (PS20) and polysorbate 80 (PS80) with a therapeutic protein interferon alpha-2a (IFNA2) using various biophysical tools that include circular dichroism, fluorescence, infrared and Raman spectroscopies. Denaturant melts using guanidinium chloride indicate that IFNA2 populates a partially unfolded intermediate in the presence of PS80. High-resolution NMR studies are in progress to characterize the local structural changes in the protein with the addition of polysorbates. Changes in chemical shifts and hydrogen exchange rates will be used to identify the partial protein unfolding. We also examined the effect of chemical degradation products of polysorbates, in particular peroxides, on the structure and stability of IFNA2. We have observed enhanced aggregation of IFNA2 in the presence of peroxides. Our goal is to use high-resolution NMR and HX to measure the chemical modification effects on protein structure and stability of local protein regions. We also plan to undertake accelerated stability
studies to understand the effect of the polysorbate degradation products on IFNA2. We have successfully done basic structural characterization of IFNA2. The effect of chemical modifications on the protein will be tested using the above mentioned spectroscopic techniques, as well as using mass spectrometry and cell activity based assays. From this study, we hope to gain a significant understanding of the interaction that polysorbates and their degradation products have with proteins.

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