
Symposium Co-Chairs:

Alain Balland, Amgen Inc.
Steven Cohen, SAC Analytical Consultants

September 9-12, 2014
Silverado Hotel
Napa, CA USA

Organized by
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Welcome to the 11th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry

We are pleased to welcome you to the 11th Symposium on the Practical Application of Mass Spectrometry in the Biotechnology Industry. The focus of this Symposium is the application of mass spectrometry for product characterization, process monitoring, formulation development and release testing in the pharmaceutical industry. Since mass spectrometry is a critical technology for a wide array of applications, the meeting will provide scientists in the industry an opportunity to share their data and learn from their colleagues. The symposium will feature a diversified selection of speakers from academia, industry and regulatory agencies, who will discuss applications of state-of-the-art mass spectrometry to proteins, glycans, nucleic acids, viral vectors and other molecules of therapeutic interest.

The success of this symposium will depend not only on our experienced and knowledgeable speakers and workshop leaders, but also on the interactions and open discussion that take place among the attendees. We encourage you to participate wholeheartedly in the discussion sections that have been designed to stimulate exchange of ideas and information.

We would like to thank the speakers who are giving generously of their time and resources, and you for your attendance, which will make this a successful endeavor.

We gratefully acknowledge the generosity of our sponsors and exhibitors. Thank you to AbbVie, Inc., AB SCIEX, Agilent Technologies, Biogen Idec Inc., BioProcess International, Bruker Daltonics, Inc., Eurofins Lancaster Laboratories, Inc., Genetic Engineering & Biotechnology News, Genedata AG, Genovis AB, IPQ Publications, New England Biolabs Inc., Pfizer, Inc., Promega Corporation, Protein Metrics, ProZyme, Inc., Roche Diagnostics GmbH & Genentech, a Member of the Roche Group, Royal Society of Chemistry, separationsNOW.com, Technology Networks Limited, The Analytical Scientist, Thermo Scientific and Waters Corporation. We are also thankful for the expert assistance and support of Renee Olson and CASSS, as well as the audiovisual expertise of Michael Johnstone from MJ Audio-Visual Productions.

We hope you enjoy the conference, build new contacts and return for new information in 2015!

THE ORGANIZING COMMITTEE:
Greg Adams, FUJIFILM Diosynth Biotechnologies
Alain Balland, Amgen Inc. (Co-chair)
Michael Boyne, CDER, FDA
Patrick Bulau, Roche Diagnostics GmbH
James Carroll, Pfizer, Inc.
Steven Cohen, SAC Analytical Consultants (Co-chair)
Eef Dirksen, Merck, Sharp & Dohme

Vivian Lindo, MedImmune
Anders Lund, Genzyme, a Sanofi company
Yelena Lyubarskaya, Biogen Idec Inc.
Jun Park, CDER, FDA
David Passmore, Bristol-Myers Squibb
Jason Rouse, Pfizer, Inc.
Hansjörg Toll, Sandoz GmbH
Christopher Yu, Genetech, a Member of the Roche Group

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CASMS Mass Spec Student Travel Grants

CASMS is pleased to provide a limited number of student travel grants for PhD students and post-docs who present applicable posters at the 11th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry (Mass Spec 2014). PhD students or post-doctoral fellows conducting research at academic institutions throughout the world are eligible.

This symposium offers insight to current topics and issues under discussion within the biotech and biopharmaceutical industries, and as such, provides an opportunity to bridge between industry, academia and regulatory agencies. The presentations and workshops are focused on the application of mass spectrometry to advance drug discovery and development in the biotechnology industry. Applications will highlight uses of MS in various areas of product development including lead selection & optimization, high throughput screening, identification of PTMs, process development and in-process testing, drug product characterization, higher-order structure, and adoption of innovative MS technologies. Participants will have an excellent opportunity to meet, network and participate in exchanging knowledge for mutual education with other MS practitioners.

Requirements are:
- Present a poster on a MS topic
- Proof of studentship/post-doc status
- Recommendation from the supervisor/advisor

This year’s grant winners include:
Host-Cell Protein Analysis of Therapeutic Monoclonal Antibodies Following Protein A Chromatography using Data Independent 2D LC-MS
Amy Farrell, National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland

Monoclonal Antibodies Complete Primary Structure and Biosimilarity Assessment in a Single Analysis using Transient Isotachophoresis Sheathless Capillary Electrophoresis-tandem Mass Spectrometry
Rabah Gahoual, University of Strasbourg, Strasbourg, France

Intact Cell MALDI-TOF Mass Spectrometry: Monitoring of Cell Stress in CHO Cell Cultures
Sebastian Schwamb, Center of Applied Biomedical Mass Spectrometry (ABIMAS), Mannheim, Germany

Characterization of Various IgG Platforms by Native Mass Spectrometry
Yang Yang, Utrecht University, Utrecht, The Netherlands

Development of Fast Photochemical Oxidation of Protein (FPOP) Platform for Protein Therapeutics: Validation and Application
Ying Zhang, Washington University in St. Louis, St. Louis, MO USA
The Organizing Committee gratefully acknowledges the following program partners for their generous support of this Symposium:

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Mass Spec 2014
Scientific Program Summary

Tuesday, September 9, 2014

07:30 – 08:30  Breakfast (Full Day and Morning Short Course Attendees ONLY)

08:00 – 13:30  Short Course Registration ONLY in the Louis M. Martini and Trefethen Foyer

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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<tr>
<td>08:30 – 12:00</td>
<td>Fundamentals of Mass Spectrometry in the Analysis of Protein Therapeutics&lt;br&gt;Anders Lund, Genzyme, a Sanofi company, Framingham, MA USA</td>
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<tr>
<td>12:00 – 13:00</td>
<td>Lunch (Full Day Short Course Attendees ONLY)</td>
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<tr>
<td>13:00 – 17:00</td>
<td>Applications of Mass Spectrometry to Characterize Protein Therapeutics&lt;br&gt;Anders Lund, Genzyme, A Sanofi company, Framingham, MA USA</td>
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<tr>
<td>14:00 – 19:00</td>
<td>Registration in the Ballroom Foyer</td>
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<tr>
<td>18:00 – 20:00</td>
<td>Welcome Reception – All Conference Attendees – Outdoor Arbor</td>
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Short Course in the Louis M. Martini and Trefethen Rooms
Session Chair: Anders Lund, Genzyme, a Sanofi company, Framingham, MA USA
Wednesday, September 10, 2014

07:30 – 17:30 Registration in the Ballroom Foyer

07:30 – 08:30 Breakfast in the Fairway Deck and Silverado East Ballroom

08:30 – 08:45 Welcome and Introductory Comments in the Silverado West Ballroom

08:45 – 09:45 Plenary I Session in the Silverado West Ballroom

Session Chair: Michael Boyne, CDER, FDA, Silver Spring, MD USA

08:45 – 09:45 The Role of Mass Spectrometry in Facilitating Process Understanding and Control

Patrick Swann, Biogen Idec Inc., Cambridge, MA USA

09:45 – 10:15 Break – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom

10:15 – 10:45 Process and Product Characterization Session in the Silverado West Ballroom

Session Chair: Eef Dirksen, Merck, Sharp & Dohme, Oss, The Netherlands

10:15 – 10:45 The Need of High Throughput Glycan Profiling of IgGs for Process Development

Markus Haberger, Roche Diagnostics GmbH, Penzberg, Germany

10:45 – 11:15 What We Can Learn from MS-based HCP Analysis

Qingchun Zhang, Amgen Inc., Thousand Oaks, CA USA


Li Zang, Biogen Idec Inc., Cambridge, MA USA

11:45 – 12:15 Rapid Level-3 Characterization of Therapeutic Antibodies by CESI-MS

Andras Guttman, University of Debrecen, Debrecen, Hungary

12:15 – 12:30 Break – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom
**Wednesday, September 10, 2014 (continued)**

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<th>Time</th>
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<tr>
<td><strong>12:30 – 13:30</strong></td>
<td><strong>Technical Seminar: Lunch and Learn</strong></td>
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<tr>
<td><strong>Characterization of Biotherapeutics with Mass Spectrometry: Addressing the Data Analysis Bottleneck</strong></td>
<td>Steven Pomerantz, <em>Janssen Pharmaceutical R&amp;D, LLC, Spring House, PA USA</em></td>
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<td>Sponsored by Genedata AG</td>
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<tr>
<td><strong>13:30 – 13:45</strong></td>
<td><strong>Break</strong> – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom</td>
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<tr>
<td><strong>Non-Traditional Antibody Molecules or Non-Antibody Molecules Session</strong> in the Silverado West Ballroom</td>
<td>Session Chair: Steven Cohen, <em>SAC Analytical Consultants, Hopkinton, MA USA</em></td>
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<td><strong>13:45 – 14:15</strong></td>
<td><strong>Structural Characterization of Lysine-linked Maytansinoid ADCs by Mass Spectrometry</strong></td>
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<td>Lintao Wang, <em>ImmunoGen, Inc., Waltham, MA USA</em></td>
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<td><strong>14:15 – 14:45</strong></td>
<td><strong>Emerging Mass Spectrometry Based Methods for Therapeutical Monoclonal Antibodies, Bispecifics and ADCs Deep Structural Characterization</strong></td>
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<td></td>
<td>Elsa Wagner-Rousset, <em>Centre d’Immunologie Pierre Fabre, St. Julien en Genevois, France</em></td>
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<tr>
<td><strong>14:45 – 15:15</strong></td>
<td><strong>Protein Chemical Characterization and Comparison of Recombinant Factor VIII Products</strong></td>
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<td>Per Franklin Nielsen, <em>Novo Nordisk A/S, Måløv, Denmark</em></td>
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<td><strong>15:15 – 16:15</strong></td>
<td><strong>Poster Session I</strong> – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom</td>
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<td><strong>Young Scientist Session</strong> in the Silverado West Ballroom</td>
<td>Session Chair: Michael Boyne, <em>CDER, FDA, Silver Spring, MD USA</em></td>
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<tr>
<td><strong>16:15 – 16:30</strong></td>
<td><strong>Host-Cell Protein Analysis of Therapeutic Monoclonal Antibodies Following Protein A Chromatography using Data Independent 2D LC-MS²</strong></td>
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<td></td>
<td>Amy Farrell, <em>National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland</em></td>
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Wednesday, September 10, 2014 (continued)

16:30 – 16:45  Intact Cell MALDI-TOF Mass Spectrometry: Monitoring of Cell Stress in CHO Cell Cultures
Sebastian Schwamb, Center of Applied Biomedical Mass Spectrometry (ABIMAS), Mannheim, Germany

16:45 – 17:00  Development of Fast Photochemical Oxidation of Protein (FPOP) Platform for Protein Therapeutics: Validation and Application
Ying Zhang, Washington University in St. Louis, St. Louis, MO USA

**Enology Session** in the Silverado West Ballroom
Session Chair: Jason Rouse, Pfizer, Inc., Andover, MA USA

17:00 – 17:30  Wine Pigment Structures Characterized by Mass Spectrometric Techniques
Jonathan Cave, University of California, Davis, Davis, CA USA

17:30 – 18:30  Exhibitor Reception – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom
Thursday, September 11, 2014

07:30 – 17:00  **Registration** in the Ballroom Foyer

07:30 – 08:30  **Breakfast** in the Fairway Deck and Silverado East Ballroom

07:30 – 08:30  **Technical Seminar**

**Effective Workflows for ADC Analysis: A Lively Breakfast Featuring ADC’s (Antibody-Drug Conjugates) and Mimosas (Champagne-Orange Conjugates)**
Scott Berger, *Waters Corporation, Milford, MA USA*

Sponsored by Waters Corporation

Silverado West Ballroom

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07:30 – 08:30  **Plenary II Session** in the Silverado West Ballroom
Session Chair: Alain Balland, *Amgen Inc., Seattle, WA USA*

08:30 – 09:30  **Increasing the Sensitivity, Coverage and Throughput of Proteomics Measurements Using Mobility-based Ion Manipulations**
Richard Smith, *Pacific Northwest National Laboratory, Richland, WA USA*

09:30 – 10:00  **Break** – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom

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**Innovative Approaches and New Technologies Session** in the Silverado West Ballroom
Session Chair: David Passmore, *Bristol-Myers Squibb Company, Redwood City, CA USA*

10:00 – 10:30  **Finding the Needle in the Haystack: Detecting Low Abundant Protein Species in Biopharmaceutical Development**
Florian Wolschin, *Sandoz GmbH, Kundl, Austria*

10:30 – 11:00  **Massively Multiplexed Single Cell Analysis to Reveal Mechanisms of Therapeutic Action and Resistance**
Sean Bendall, *Stanford University, Stanford, CA USA*

11:00 – 11:30  **Real-time Product Attribute Control (PAC) to Produce Recombinant Proteins with Homogeneous Critical Attribute Profiles**
Lowell Brady, *Amgen Inc., Seattle, WA USA*
### Thursday, September 11, 2014 (continued)

**11:30 – 11:45**  
**Break** - Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom

**11:45 – 12:45**  
**Technical Seminar: Lunch and Learn**

*Application of Label-free Discovery Proteomics Followed by MRM in Evaluating Sample Quality: From Blood Specimen Collection to Host Cell Proteins in Biologics*

Sushmita (Mimi) Roy, *Caprion Proteomics US LLC, Menlo Park, CA USA*

Sponsored by Agilent Technologies  
*Silverado West Ballroom*

**12:45 – 13:00**  
**Break** - Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom

#### Mass Spec Approaches for Pre-Clinical Quantitative Studies Session

in the Silverado West Ballroom  
Session Chair: Yelena Lyubarskaya, *Biogen Idec Inc., Cambridge, MA USA*

**13:00 – 13:30**  
**Mass Spectrometric Immunoassay (MSIA) Applications in Biopharma**  
Angela Goodenough, *Bristol-Myers Squibb, Princeton, NJ USA*

**13:30 – 14:00**  
**Approaches to Automated Sample Handling and LC-MS/MS Based Quantitation for Biologics in Pre-Clinical Pharmacokinetic Studies**  
Daniel Spellman, *Merck & Co., Inc., West Point, PA USA*

**14:00 – 14:30**  
**Mass Spec Based Proteomics for Preclinical Studies**  
Ru Wei, *Biogen Idec Inc., Cambridge, MA USA*

**14:30 – 15:45**  
**Poster Session II** - Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom

#### Regulatory Session

in the Silverado West Ballroom  
Session Chair: Vivian Lindo, *MedImmune, Cambridge, United Kingdom*

**15:45 – 16:15**  
**Mass Spectrometry in a Quality Control Laboratory: A Review and Perspective**  
Patrick Bulau, *Roche Diagnostics GmbH, Penzberg, Germany*

**16:15 – 16:45**  
**The Role of Mass Spectrometric Methods for Developing Biotechnology Products: FDA Perspectives**  
Jun T. Park, *CDER, FDA, Silver Spring, MD USA*
Thursday, September 11, 2014 (continued)

16:45 – 17:15  Applications of Mass Spectrometry in a Biologics Regulatory Organization
                Terry Cyr, Health Canada, Ottawa, ON Canada

17:15 – 18:30  Panel Discussion

Moderator:
Jun Park, CDER, FDA, Silver Spring, MD USA

Panelist:
Alain Balland, Amgen Inc., Seattle, WA USA
Patrick Bulau, Roche Diagnostics GmbH, Penzberg, Germany
Terry Cyr, Health Canada, Ottawa, ON Canada
Patrick Swann, Biogen Idec Inc., Cambridge, MA USA
Friday, September 12, 2014

07:30 – 13:00  **Registration** in the Ballroom Foyer

07:30 – 08:30  **Breakfast** in the Fairway Deck and Silverado East Ballroom

07:30 – 08:30  **Technical Seminar**

**Reliability of a Semi-automated, High Throughput MS Based Glycomics System for Discovery of Glycan Biomarkers and QbD studies of Glycoprotein Therapeutics**
Archana Shubhakar, *Ludger Ltd, Abingdon, United Kingdom*

Sponsored by Bruker Daltonics  
**Silverado West Ballroom**

08:30 – 08:45  **Poster Award Announcement** in the Silverado West Ballroom

08:45 – 09:45  **Plenary III Session** in the Silverado West Ballroom  
Session Chair: James Carroll, *Pfizer, Inc., St. Louis, MO USA*

08:45 – 09:45  **Rapid and Effective Methods for Determining Site-specific Glycosylation in Biotherapeutics and Biosimilars**
Carlito Lebrilla, *University of California, Davis, Davis, CA USA*

09:45 – 10:15  **Break** – Visit the Exhibits and Posters in the Fairway Deck and Silverado East Ballroom

10:15 – 10:45  **Relating Structures of Biopharmaceuticals to Function Session**  
in the Silverado West Ballroom  
Session Chair: Christopher Yu, *Genentech, a Member of the Roche Group, South San Francisco, CA USA*

10:15 – 10:45  **Hydrogen/deuterium-exchange Mass Spectrometry for Biopharmaceutical Developability**
Jonathan Phillips, *MedImmune, Cambridge, United Kingdom*

10:45 – 11:15  **Use of Mass Spectrometry to Explore the Structure-Function Relationships of Biotherapeutics**
Keith Johnson, *Pfizer, Inc., Andover, MA USA*

11:15 – 11:45  **Approaching Higher Order Structure Issues with Molecular Dynamics & Hydrogen Exchange Mass Spectrometry**
Benjamin Walters, *Genentech, a Member of the Roche Group, South San Francisco, CA USA*
**Plenary IV Session** in the Silverado West Ballroom  
Session Chair: Anders Lund, *Genzyme, A Sanofi company, Framingham, MA USA*

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| 11:45 – 12:45 | **Elucidating Drug Binding and 3D Protein Structure using Native Top-Down MS**  
Joseph Loo, *University of California, Los Angeles, Los Angeles, CA USA* |
| 12:45 – 13:00 | **Closing Comments** in the Silverado West Ballroom  
Steven Cohen, *SAC Analytical Consultants, Hopkinton, MA USA* |
The Role of Mass Spectrometry in Facilitating Process Understanding and Control

Patrick Swann

Biogen Idec Inc., Cambridge, MA USA

A major objective of a pharmaceutical quality system is to develop and use effective monitoring and control systems for process performance and product quality, thereby providing assurance of continued suitability and capability of processes (ICH Q10). The goal of Process Analytical Technology (PAT; “…a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality.”) is to enhance understanding and control the manufacturing process. Both ICH Q10 and PAT guidance stress the need for a science and risk-based approach for developing control strategies. However, many biotechnology control strategies (for both legacy and new products) have not applied more forward-thinking approaches.

“Timely” measurement of quality attributes of biotechnology products can be a challenge so many PAT proposals to date have focused solely on performance attributes. However, it can also be challenging to use performance attributes to eliminate the need for testing of DS or DP (i.e. parametric release) as it is not clear what would be adequate information to ensure ongoing product quality. Often models can show a strong correlation between performance attributes and a quality attribute as measured on release but a detailed functional linkage is lacking. The use of mass spectrometry to analyze quality attributes from in-process materials has the capability to provide more detailed process understanding and increase the confidence in manufacturing models. Examples of the use of mass spectrometry to support a traditional control strategy as well as more exploratory analyses consistent with more forward thinking approaches will be discussed.

NOTES:
The Need of High Throughput Glycan Profiling of IgGs for Process Development

Markus Haberger

*Roche Diagnostics GmbH, Penzberg, Germany*

Fc glycosylation of IgGs is crucial for antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity. To monitor IgG Fc glycosylation, high-throughput techniques for glycosylation analysis are needed in the biotechnology industry. Here we describe the development of a fully automated high-throughput method based on glycopeptide analysis. Glycan species were identified by MS/MS characterization and isobaric structures analyzed by ion mobility separation. The IgG’s are purified directly from fermentation broths by means of immobilized protein A followed by trypsin digestion. Glycopeptides are purified by hydrophilic interaction solid-phase extraction and analyzed by electrospray mass spectrometry in the positive-ion mode. Data are automatically processed and relative intensities of the various IgG glycopeptides are obtained. The newly developed method is suitable to support process development studies for glycoengineered IgG’s with enhanced ADCC.

NOTES:
What We Can Learn from MS-based HCP Analysis

Qingchun Zhang, Gregory C. Flynn

*Amgen Inc., Thousand Oaks, CA USA*

Identification and unbiased quantification of individual host cell proteins (HCPs) by mass spectrometry can facilitate assessment of potential HCP-related safety risks to patients as well as forming the basis for a mechanistic understanding of HCP-mAb interactions with a view to eventual mitigation of these interactions. We have undertaken a systematic study to investigate how specific HCPs are retained or cleared at each step of a platform mAb purification process. The bulk of the HCP content remaining after Protein A affinity chromatography consists of some of the most abundant HCPs present in cell culture fluid, suggesting a roughly similar clearance factor for different HCPs at this step. HCPs remaining after the Protein A step exist bound to mAb, and a challenge for subsequent purification steps is to separate these complexes from free mAb. We will illustrate, with example, insights gained from these studies and how they may lead to refinements of mAb process development strategies.

NOTES:
Demonstration of Clearance of Individual Host-Cell Proteins (HCPs) in Biopharmaceutical Purification Process Using Proteomics Approach

Li Zang, Chong-Feng Xu, Suli Liu

*Biogen Idec Inc., Cambridge, MA USA*

Host-Cell Proteins (HCPs) are important process-related impurities for biopharmaceuticals. The common method used for monitoring HCP clearance in biopharmaceutical purification process is a multi-analyte ELISA assay, which potentially suffers from poor coverage, sensitivity and linearity due to the total reliance on a polyclonal antibody reagent to detect the HCPs. The use of null cell lysate to establish calibration curve in the ELISA assay is also likely to introduce bias towards some HCPs when the relative HCP levels change over the purification process. In this work, we developed a proteomics workflow to monitor the clearance of individual HCPs using heavy-isotope labeled whole cell lysate as internal standard. The workflow includes first identification of the HCPs in the process intermediates followed by target quantitation of abundant HCPs in process intermediates and drug substance. This method was successfully applied to monitoring the HCP clearance in the purification process of an Fc fusion protein. The clearance of multiple HCPs was demonstrated by comparing the process intermediates prior to and after the last purification step in the process.

**NOTES:**
Rapid Level-3 Characterization of Therapeutic Antibodies by CESI-MS

Andras Guttman$^{1,3}$, Bryan Fonslow$^{2,3}$

$^1$Horvath Laboratory of Bioseparation Sciences, University of Debrecen, Debrecen, Hungary,  
$^2$The Scripps Research Institute, La Jolla, CA USA, $^3$SCIEX Separations, a part of AB SCIEX, Brea, CA USA

With the increase of the number of approved protein therapeutics in the market, comprehensive and reproducible characterization of these new generation drugs is crucial for the biopharmaceutical industry and regulatory agencies. One of the largest groups of biotherapeutics is monoclonal antibodies (mABs) possessing various post-translational modifications (PTMs) and potential degradations during the manufacturing process that may affect efficacy and immunogenicity. The exceptionally high separation power of capillary electrophoresis (CE) in conjunction with mass spectrometry fulfills Level-3 Characterization requirements necessary to reveal such modifications and degradations. In this presentation a comprehensive characterization example will be given for a representative monoclonal antibody Trastuzumab (Herceptin), illustrating the benefits of the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) in a unified bioanalytical process (CESI) coupled with high resolution mass spectrometry. Both small and large peptides (3 – 65 amino acids) have been separated, identified with 100% sequence coverage and quantified, including degradative hotspots such as aspargine-deamidation, methionine-oxidation, glutamic-acid-cyclization, and C-terminal lysine heterogeneity using only 100 fmol of a single protease digest sample. The low flow rate of the system (>20 nL/min) ensured maximized ionization efficiency and dramatically reduced ion suppression. Special emphasis will be given to glycosylation microheterogeneity analysis to reveal potential in-source fragmentation mediated glycopeptide artifacts in view of the ADCC and CDC function assessment during the manufacturing process.

NOTES:
Structural Characterization of Lysine-linked Maytansinoid ADCs by Mass Spectrometry

Lintao Wang, Alexandru C. Lazar

ImmunoGen, Inc., Waltham, MA USA

Antibody-drug conjugates (ADC) have been increasingly used in the development of target-specific therapies for various diseases. ImmunoGen’s technology utilizes the reactivity between the lysine residues at the surface of the antibody and the N-hydroxysuccinimide group of the linker molecules to conjugate antibody with the maytansinoid payload, which are potent anti-microtubular cytotoxic agents. To date a number of antibody-maytansinoid conjugates (AMCs) have been developed using this technology for various anticancer therapies; one of them, Kadcyla or T-DM1, has been commercially available for patients with HER2-positive breast cancer. However, due to the large number of lysine residues in a typical antibody (approximately 90), the conjugation process produces a heterogeneous mixture of antibody molecules linked with varying number of payload molecules that are conjugated at different lysine residues. For a conjugate to be appropriate for clinical use the maytansinoid distribution profile (MDP) has to be controlled. In addition the unconjugated antibody (uMAb) and maytansinoid-antibody ratio (MAR) are also monitored as part of process optimization and product characterization. Although a number of analytical approaches (i.e., CE, HIC, UV, etc.) are potentially useful for such measurements, intact protein mass spectrometry has proven to be a versatile, reliable and informative assay to measure the above attributes and confirm the results of other assays as an orthogonal analytical method. Middle-down and subunit analysis of AMCs with mass spectrometry can provide further structural information. As a bottom-up approach with relatively longer turnaround, peptide mapping combined with LC/UV, LC/MS and LC/MS/MS analysis provides the most detailed structural information about AMCs regarding determination of the exact conjugation sites in both light and heavy chains of the antibody.

NOTES:
Emerging Mass Spectrometry Based Methods for Therapeutical Monoclonal Antibodies, Bispecifics and ADCs Deep Structural Characterization

Elsa Wagner-Rousset¹, Alain Beck¹, Marie-Claire Janin-Bussat¹, Olivier Colas¹, Nathalie Corvaïa¹, Sarah Cianferani², François Dabaene², Alain Van Dorsselaer²

Centre d’Immunologie Pierre Fabre St Julien en Genevois, France¹, Laboratoire de Spectrométrie de Masse Bio-organique, Strasbourg, France²

The early use in the R&D process of Mass Spectrometry (MS) methods helps to optimize the structure and the function of next generation mAbs (OptimAbs) as well as more sophisticated derivatives such as bispecific antibodies and Antibody Drug Conjugates (OptimADCs). In addition, these MS techniques afford comparability studies and biosimilarity evaluation. Case studies on deep structural characterization of antibodies and derivatives based on emerging methods such as middle-up/ down MS, native MS and Ion-Mobility MS will be showcased and discussed.

• Wagner-Rousset et al., Antibody Drug Conjugate model fast characterization by LC-MS following IdeS proteolytic digestion. MAbs, 2014, 1;6(1):173-84.


• Ayoub et al., Cetuximab correct primary structure assessment and extensive glyco-profiling by a combination of intact, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. MAbs, 2013, 5(5):699-710.


NOTES:
Protein Chemical Characterization and Comparison of Recombinant Factor VIII Products

Per Franklin Nielsen

Novo Nordisk A/S, Måløv, Denmark

Blood clotting factor VIII (FVIII) is a complex 2332 amino acid protein with the domain structure A1-A2-B-A3-C1-C2. The mature FVIII molecule circulates as a heterodimer consisting of a heavy and a light chain held together by a metal ion. The B-domain is not needed for hemostatic function and different forms of recombinant FVIII have been produced including full length and B-domain truncated/deleted versions. Turoctocog alfa (B-domain truncated form) and three commercial recombinant FVIII products (full length and B-domain deleted forms) have been protein chemically characterized and compared. The employed methods include LC-MS/MS based peptide mapping using Q-Tof Premier and Orbitrap mass spectrometers as well as non-mass spectrometric methods such as SEC-MALS and Edman degradation. The characterization has focused on the nature and abundance of degradation products and on selected post-translational modifications. These include the extend of sulfation at the important Tyrosine-1680 position and potential deamidation/IsoAsp formation/Methionine oxidation at labile residues. The results demonstrate, that full length recombinant FVIII products display a significantly higher heterogeneity with fragmentations in the B-domain region and free light chain accounting for most of this. One recombinant FVIII product showed a significant amount of non-sulfation at Tyrosine-1680 and minor deamidation, whereas IsoAsp formation and Methionine oxidation at suspected labile residues was not found at significant levels in any of the products.

NOTES:
Host-Cell Protein Analysis of Therapeutic Monoclonal Antibodies Following Protein A Chromatography Using Data Independent 2D LC-MS\textsuperscript{E}

Amy Farrell, Brian Morrissey, Jonathan Bones

*The National Institute for Bioprocessing Research & Training (NIBRT), Dublin, Ireland*

Host cell proteins (HCPs) are a major class of bioprocess-related impurities that pose a considerable risk to patient safety and as such their removal from therapeutic protein products is of critical importance. Protein A purification is the primary procedure for removal of HCPs from culture media in downstream processing. Typically HCP levels in drug substances are measured using multi-product assays such as enzyme-linked immunosorbent assay (ELISA). In the current study a quantitative high pH low pH reversed-phase data independent 2D-LC-MSE based proteomic method has been used, which enables the identification and quantitation of HCPs in various therapeutic protein samples.

Here, we have applied the proteomic platform to evaluate the suitability of four different elution buffers for the removal of HCPs from monoclonal antibody (mAb) samples following Protein A purification. The effect of elution buffer choice on the quality of the therapeutic protein was also evaluated. Of the different buffers assessed, an arginine-based buffer was found to have the most favourable impact on product quality and HCP profile.

Additionally, the developed proteomic platform was used to determine the impact of cell culture day of harvest on the HCP profile of a therapeutic protein following Protein A purification. mAb, from culture harvested at the beginning of the stationary phase of cell growth, was found to contain lower levels of HCPs when compared to mAb harvested at the end of the stationary phase of growth, following Protein A purification.

Finally, the proteomic platform was applied to gain quantitative and qualitative information of the HCP profile present in five commercially available drug products, revealing low levels of proteins involved in transcription, intracellular membrane trafficking and prevention of protein aggregation. The information obtained using MS was subsequently compared to ELISA values for total HCP concentration estimation.

**NOTES:**
Intact Cell MALDI-TOF Mass Spectrometry: Monitoring of Cell Stress in CHO Cell Cultures

Sebastian Schwamb¹, Mathias Hafner¹,²,³, Philipp Wiedemann¹,²

¹Center for Applied Biomedical Mass Spectrometry (ABIMAS), Mannheim, Germany,
²Mannheim University of Applied Sciences, Mannheim, Germany,
³Heidelberg University, Institute Medical Technology, Mannheim, Germany

Over the last 25 years, the cultivation of mammalian cells, especially Chinese Hamster Ovary (CHO), has found widespread acceptance as the method of choice for the production of biopharmaceuticals. Particularly the area of process monitoring and development - enforced by the PAT (Process Analytical Technology) initiative of the FDA - underwent changes from examining the bioreactor/culture as a “black-box” towards integrated methods, helping to gain deeper process understanding by assessing the cells themselves.

Here, we present the application of Intact Cell MALDI-TOF Mass Spectrometry (ICM MS) biotyping, a method originally used in clinical and environmental microbiology to identify microorganisms based on specific MS-fingerprints, to mammalian cell culture in order to detect early signs of cellular stress.

We report the identification of reproducible stress related changes in m/z signal intensities that allowed prediction of upcoming cell viability changes up to 24 h earlier than standard monitoring methods. Moreover, the identification of apoptosis onset was comparable to that using a sensitive, albeit off-line, detection method. Up to now, this approach was successfully applied to in total four CHO cell lines; three suspension adapted CHOK1 and one DUXB11 cell line. Many of the detected changes in m/z signal intensities were identified as apoptosis-specific by validation with apoptosis induced cultures.

Based on a condensed subset of m/z values, we built classification models allowing a reliable discrimination of unknown samples regarding their cell physiological state (viable, early and late apoptotic).

The fast, robust and automated acquisition of cell state specific MS signatures together with simple sample preparation could become a promising tool for at-line CHO culture monitoring. With this work, we want to highlight mass spectrometry as an alternative to conventional apoptosis detection assays and furthermore as an innovative addition to routine process monitoring.

NOTES:
Development of Fast Photochemical Oxidation of Protein (FPOP) Platform for Protein Therapeutics: Validation and Application

Ying Zhang, Michael L. Gross

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

Unlike small molecule drugs (< 600 Da), protein therapeutics (> kDa) undergo complicated folding and form higher order structures (HOS), posing a challenge for traditional analytical methods. Mass spectrometry-based protein footprinting can provide structural information and bring high sensitivity, fast turnaround, and small sample consumption. We report here the development of FPOP platform for HOS characterization of protein therapeutics.

Advantages of FPOP include fast labeling, targeting side chains, and making irreversible modifications, making FPOP complementary to hydrogen deuterium exchange. For characterization of protein therapeutics, FPOP requires monitoring protein conformations in native environments with high reproducibility. An asymmetrical mixing was integrated into the FPOP setup to allow proteins to undergo minimal exposure to H2O2. Each step is now more accurately controlled by time to increase precision. We used cyt c to evaluate these improvements. Changes in FPOP modification patterns were consistently observed. Only laser-plus-hydrogen peroxide gave high level modifications (+16, +32… ) than the two control experiments (laser only and H2O2 only). The FPOP modification pattern was quantitatively analyzed by custom-built software. The variation in modification extent is dramatically smaller for the modified platform (69.9 ± 0.8%) compared to the original platform (75.0 ± 3%). The modification level is lower for the improved platform, which we attribute to a reduction in non-FPOP-induced oxidation. The newly developed FPOP platform can now be used to evaluate the results from experiments at different times and laboratories. Different scavenger and buffer can be tested using the modified platform. We observed a similar FPOP modification pattern to that of the typical FPOP condition (Gln as scavenger or PBS as buffer). We envision that this modified platform will be useful for characterization of the HOS of protein therapeutics.

NOTES:
Wine Pigment Structures Characterized by Mass Spectrometric Techniques

Jonathan Cave¹, Evan Parker², Carlito B. Lebrilla², James Kennedy³, Andrew Waterhouse¹

University of California, Departments of¹Viticulture and Enology and ²Chemistry, Davis, Davis, CA USA, ³California State University, Department of Viticulture and Enology, Fresno, Fresno, CA USA

Wine pigment have been shown to provide persistent red wine color on aging, but also alter flavor perception, astringency, and cause softening of texture and mouthfeel. Wine pigments comprise a significant portion of the red wine matrix, and while some components have been identified, an inventory of these compounds remains fragmentary, despite its importance. To begin the identification, we are using Fourier Transform ion cyclotron resonance (FTICR) mass spectrometry which provides high accuracy molecular weights for mixtures of numerous compounds. These precise mass values lead to almost unique molecular formulas. These formulae are then paired with literature structures where known, and to date approximately 150 have been matched. To address the unknowns, we will be looking at fragmentation products in wither the FTICR or in a tandem quadrupole time of flight (QTOF) mass spectrometer. This will allow analysis to create information on molecular connectivity necessary to properly determine structure from the FTICR molecular formula. To date we have found over one hundred unknown compound candidate ions, and the determination of these structures may confirm proposed structures as well as bring to light new structures for which the mechanisms of production are unknown. Knowledge of wine pigment structure provides the basis for understanding their production mechanisms, which in turn will lead to cellar control for winemakers looking to enhance or suppress the formation of specific wine pigments.

NOTES:
Increasing the Sensitivity, Coverage and Throughput of Proteomics Measurements Using Mobility-based Ion Manipulations

Richard Smith

Pacific Northwest National Laboratory, Richland, WA USA

Mass spectrometry (MS)-based proteomics measurements are having profound impacts on broad areas of biological research. Advances in the quality, resolution, and the speed of e.g. polypeptide and protein separations have arguably been as important as mass spectrometric developments in improving the sensitivity and coverage of proteomics measurements. Both liquid phase, e.g., using liquid chromatography, and increasingly gas phase ion mobility separations, respectively, provide a basis for increasing the extent of proteome coverage. While these capabilities are challenged by very small sample sizes, the recent development of more efficient nanoelectrospray ion sources and MS interfaces has helped enable ultra-sensitive measurements. Increasingly MS platform advances involve gas phase ion manipulations that are conducted between the ion source and m/z analyzer. These manipulations include: ion transport through regions of elevated pressure, trapping, reactions (both ion-molecule and ion-ion), and mobility-based separations. This presentation will discuss the utility of ion mobility separations for proteomics applications, and also describe new gas phase ion manipulation approaches having broad utility for facilitating MS analysis capabilities. These various developments will be discussed with regard to their sensitivity, measurement throughput, and their utility for both broad (proteome-wide) and targeted quantitative measurements. The presentation will conclude with consideration of projected future developments.

NOTES:
Finding the Needle in the Haystack: Detecting Low Abundant Protein Species in Biopharmaceutical Development

Florian Wolschin, Veronika Reisinger, Johann Holzmann, Hansjoerg Toll

Sandoz GmbH, Kundl, Austria

Samples of purified biologics typically contain extremely low levels of sequence variants of the active pharmaceutical ingredient as well as host cell proteins. The reliable detection and identification of such molecules is greatly hindered by the presence of the active pharmaceutical ingredient. Nevertheless, the tremendous advances in mass spectrometry enable the identification and quantification of even these very low abundant proteins.

We present mass spectrometry-based approaches that enable a sensitive identification and quantification of low abundant protein species directly in samples containing large quantities of purified biologics. Potential pitfalls of such proteomic approaches and remedies to avoid misinterpretation of the results are discussed.

NOTES:
Massively Multiplexed Single Cell Analysis to Reveal Mechanisms of Therapeutic Action and Resistance

Sean Bendall

Stanford University, Stanford, CA USA

The emergence of drug resistance leading to a failure in cancer therapy is commonly explained by the selection of cancer cells with resistance mutations. However, metastable non-genetic mechanisms have also been shown to play a role in resistance. The non-genetic variants are thought to produce diverse regulatory signaling states, some of which can confer resistance. Whether these resistant cells that persist in the face of therapeutic intervention possess a uniform or a more diverse set of signaling states could reveal essential functions involved in maintaining non-genetic resistant states in cancer.

We have previously used single cell (CyTOF) mass cytometry - a technology which leverages non-biological, heavy element isotopes chelated to epitope specific probes in atomic mass spectrometric analysis of single cells - to create a detailed response profile of the healthy primary human hematopoietic system with > 30 simultaneously measured cellular features on millions of individual cells per analysis. Now using mass cytometry to measure functional changes in regulatory proteins and epitopes in conjunction with reporters of cellular survival in the even of TRAIL-induced apoptosis we have found the array of signaling states that confers resistance. We termed these cells the Essential Persisters (EP). By constricting the signaling states available to EP cells, it was possible to significantly decrease resistance. Moreover, this profile in rare resistant cells can be further exploited to predict the effects of combinatorial kinase inhibitor effects on reducing resistant cellular behavior. These findings provide a new level of understanding in non-genetic resistance at the population-level and explain the failure of previous ‘bulk’ (non-single cell) methods to identify a mechanism of therapeutic resistance in rare cells.

NOTES:
Real-time Product Attribute Control (PAC) to Produce Recombinant Proteins with Homogeneous Critical Attribute Profiles

Lowell Brady, Phil Clark, Brittney Livingston, Richard Wu, Craig Zupke, Peter Slade, Arvia Morris, Bob Bailey

Amgen Inc. Seattle, WA USA

Understanding the impact of product quality attributes (PQAs) is an important aspect of biologic drug process development. Control of these attributes in real-time allows a shift to a true quality by design (QbD) approach for the development of biomolecules and ensures their safety and efficacy. This product attribute control (PAC) strategy has the potential to be of importance to both innovator product development and for the development of biosimilars, where matching PQAs is essential. In our work, we controlled PQAs on monoclonal antibodies produced in bioreactors by using real-time product quality testing by mass spectrometry (MS) combined with process control loops. These process control loops use real-time testing with a control element previously identified to produce a change in a PQA on the product. This approach has allowed us to generate more homogeneous final product and control attributes of interest within a narrow set-point during production. In one example, high-mannose glycans on a monoclonal antibody were controlled within 1% of a set-point across many days of bioreactor production at large scale. By decreasing the variability of this attribute, we produced a final product with a narrower distribution of high-mannose levels. The ability to control both product variability and target a set-point for specific PQAs enables the production of more homogenous and higher quality biopharmaceutical products. Beyond this, real-time analysis of bioreactor samples using powerful mass spectrometry approaches can be used for both attribute control (using feedback loops) and real-time disposition of drug substance when these MS techniques are used on both bioreactor and purification process samples. These efforts comprise a new strategy for production of biopharmaceuticals where biologically relevant PQA levels can be controlled within a defined range of a target value and used to generate a homogenous final product.

NOTES:
Mass Spectrometric Immunoassay (MSIA) Applications in Biopharma

Angela Goodenough, Richard Huang, John Mehl, Silvi Chacko, Ashok Dongre, Ramaswamy Iyer, Guodong Chen, Adrienne Tymiak

Bristol-Myers Squibb, Princeton, NJ USA

In recent years, there has been a dramatic increase in the need for robust, specific, and sensitive quantitative protein and antibody assays. This is primarily due to two factors: (1) an increase in the number of biologics on the market and in drug development and (2) an emphasis on protein biomarker measurements for use in patient stratification, monitoring PD effects, and understanding mechanism of action. Historically, ELISA has been the primary analytical workhorse supporting these efforts, but may suffer from poor specificity as well as suppression effects due to matrix (endogenous and/or antibody drug) interferences. LC/MS/MS assays provide this analytical specificity, but typically require some sample pre-treatment, such as immunoaffinity capture, prior to MS analysis for sufficient sensitivity. There are a variety of formats used for this immunocapture including beads and cartridges. However, there is not a standardized workflow for such applications that provides the desired assay characteristics that are afforded by ELISA. A new approach based on D.A.R.T. technologies, called Mass Spectrometric Immunoassay (MSIA), provides a potential solution to this issue. This presentation will discuss MSIA applications for qualitative and quantitative measurements of biologics and protein biomarkers, ranging in size from small proteins (~6-7 kDa) up to monoclonal Abs (~150 kDa). Various capture approaches, MS analyses (intact or post-digestion), and comparisons to magnetic beads will also be presented.

NOTES:
Quantitation of therapeutic monoclonal antibodies (mAb) using LC-MS/MS for pharmacokinetic (PK) studies is becoming an essential complement to traditional antibody-based ligand binding assays (LBA). Here we describe varied automated approaches to sample handling and multiple approaches to LC-MS/MS based quantitation. Among them, we describe a generic approach to mAb quantitation employing IgG1 conserved peptides, a heavy isotope labeled mAb internal standard, and anti-human Fc enrichment. All reagents in the method are commercially available with no requirement to develop novel assay-specific reagents. The method met traditional quantitative LC-MS/MS assay analytical characteristics in terms of precision, accuracy and specificity. The method was applied to the pharmacokinetic study of a mAb dosed in cynomolgus monkey and the results were compared with the immunoassay data. This methodology has the potential to benefit and accelerate the early biopharmaceutical development process, particularly by enabling PK analysis across species and candidate molecules with minimal method development.
Mass Spec Based Proteomics for Preclinical Studies

Ru Wei

Biogen Idec Inc., Cambridge, MA USA

Drug discovery has stepped into a new era embracing much more advanced technologies such as omics in various stages of drug discovery. Like genetics/genomics, proteomics is making its sounding impacts. Here we describe our work in applying proteomics in several areas in drug discovery; including small molecule-protein interactions and chemical proteomics, drug MOA, and biomarker discovery of disease progress or treatment responses.

NOTES:
Mass Spectrometry in a Quality Control Laboratory: A Review and Perspective

Patrick Bulau

Roche Diagnostics GmbH, Penzberg, Germany

The use of mass spectrometry in a quality control environment is presently being controversially discussed. Mass spectrometry based methods are typically applied to conduct QbD studies, host cell protein profiling, and sequence variant analysis. This raises the question of whether mass spectrometric test procedures should be added to the conventional electrophoretic and chromatographic methods currently used to release biologics. At Roche Pharma Technical Development Penzberg mass spectrometry is routinely used for the release testing of biologic marketed products. This presentation will summarize the strategy and challenges that were encountered during method development, validation, and maintenance of these assays.

NOTES:
Therapeutic biotechnology products are generally complex, heterogeneous, and subject to a variety of modifications during expression, cell culture, purification, and long-term storage. Today, mass spectrometry (MS) is an essential tool for monitoring protein products during manufacturing process development; and it is used in combination with other analytical techniques to comprehensively characterize therapeutic proteins. This presentation will focus on chemistry, manufacturing, and control (CMC)-related regulatory perspectives on the use of MS for the development of therapeutic proteins. Several examples will also be discussed.

NOTES:
Applications of Mass Spectrometry in a Biologics Regulatory Organization

Terry Cyr, Daryl Smith, Marybeth Creskey, Lisa Walrond, Michael Johnston, Michel Girard, Sean Li

Health Canada, Ottawa, ON Canada

The capability of mass spectrometry to provide information rich data on complex samples has led to its increased use in the characterization and control of biological products. This is well recognised in research environments and is being adopted in quality control labs and perhaps most recently in product submissions. Examples of studies from the mass spectrometry group of the Biologics and Genetic Therapies Directorate within Health Canada will provide our perspective of where mass spectrometry can constructively contribute to enhancing our understanding of biologics. Our main emphasis has been the strain identification of influenza in multivalent vaccines and the simultaneous quantitation of all major antigens. Other MS topics touched on will include relative quantitation using stable isotope labeling in proteomics studies, variable glycation patterns from differing expression systems, accelerated protein degradation and the identification of unknowns.

NOTES:
Rapid and Effective Methods for Determining Site-specific Glycosylation in Biotherapeutics and Biosimilars

Carlito Lebrilla

University of California, Davis, Davis, CA USA

The developments of analytical methods for proteins have produced highly effective tools for identification that are sensitive and comprehensive. The vast majority of proteins are post-translationally modified, however proteomics methods have not advanced as rapidly in this area. The most complicated post-translational modification is also one of the most common. Glycans are composed of up to 20 monosaccharide units as glyconjugates in proteins and lipids. Glycosylation is a modification where the primary structures are more complicated than that of the proteins. While sequences define the polypeptides, glycans are complicated by having many linkages, branching, and stereomeric residues. Understanding the roles of glycans depends on the ability to identify and quantitate hundreds of structures simultaneously. Research in our laboratory has focused on the development of rapid methods employing mass spectrometry and advanced liquid chromatography for the analysis of protein and lipid glycosylation. These methods are employed in determining biomarkers for diseases including cancer and autoimmunity. They are also used for monitoring health by determining the biologically active components in mammalian milk. In this presentation, the characterization of glycosylation in biologics and biosimilars are discussed as well as the role of glycosylation in the production and efficacy of these drugs.

NOTES:
Hydrogen/deuterium-exchange Mass Spectrometry for Biopharmaceutical Developability

Jonathan Phillips, Daniel Higazi, David Lowe

MedImmune, Cambridge, United Kingdom

Proteins are highly dynamic and it is this conformational flexibility that underpins their key physicochemical characteristics, notably folding, binding entropy (contributing to affinity), solubility, self-association, adsorption, viscosity and aggregation. However, it is experimentally challenging to determine the structural dynamics of the (often large) proteins that are prevalent in biotechnology, such as monoclonal antibodies and their derivatives. These molecules have proven themselves relatively intractable to study by high-resolution structural techniques, such as NMR and X-ray crystallography, in part due to their large size and dynamic freedom. In order to comment on structure and dynamics attributes of the drug or target molecules with sufficient spatial and temporal resolution to guide molecular engineering, recent advances in hydrogen/deuterium-exchange mass spectrometry (HDX-MS) have been made.

Here we present an analysis of structure and conformational dynamics in therapeutic human IgG1 antibodies by HDX-MS. This is correlated to biophysical attributes: thermodynamic stability, self-association/aggregation and chemical stability. This builds toward an approach in which HDX-MS can be integrated into lead optimisation and platform engineering of therapeutic proteins to enhance ‘developability’ criteria.

NOTES:
Use of Mass Spectrometry to Explore the Structure-Function Relationships of Biotherapeutics

Keith Johnson, Matt Thompson, David Cirelli, Lisa Marzilli, Jason Rouse

Pfizer, Inc., Andover, MA USA

An important component of biotherapeutics development is the elucidation of structural isoforms via mass spectrometry. Common protein isoforms resulting from N-glycosylation, deamidation, N- or C-terminal truncation, glycation, and oxidation may have the potential to modulate target interactions in antibodies and fusion proteins. Mass spectrometry structural endpoints combined with bioactivity assessments help define the relationship between structure and function, providing increased product understanding throughout the development cycle. In this presentation, we will review our structure-function approaches, and explore several examples of specific isoforms and their impact on antibody or fusion protein activity. Additionally, once the structure-function correlation for a particular isoform is known, we will describe the process of controlling these isoforms for success of the project, as well as monitoring for the long term. Taken together, a thorough understanding of the structure-function connections in each biotherapeutic leads to optimal product quality and consistency for sustained clinical safety and efficacy.

NOTES:
Approaching Higher Order Structure Issues with Molecular Dynamics & Hydrogen Exchange Mass Spectrometry

Benjamin Walters, Thomas Patapoff, Jennifer Zhang

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

Environmentally sensitive chemical degradation in therapeutic antibodies, such as Asp isomerization, creates potential safety concerns in addition to their impact on bioactivity. In an early stage therapeutic IgG1 antibody, we discovered through a four week thermal stress study that potency was almost completely abolished when formulated in a mildly acidic buffer. Peptide mapping uncovered isomerization at an Asp hotspot (A DD sequence) that reacted nearly to completion at pH 5 (much faster than isomerization rates in unstructured peptides) while remaining inert at pH 7. To investigate the cause for this extreme pH dependence, we incorporated both molecular dynamics (MD) and hydrogen exchange mass spectrometry (HX MS) as complementary techniques to study mAb-̃ structure under each condition. This effort has proven particularly fruitful.

We find by MD that the protonation of two histidine residues (pH 5) in the Fab region causes a conformational opening of heavy and light chains in vicinity of the Asp hotspot. We hypothesized this would produce measurable local differences in HX rates in the vicinity; however, HX MS measurements taken at different pHs cannot be compared directly. We developed a novel HX time correction to circumvent this problem. We set the pH 5 HX condition as our reference and compare data sets on the basis of effective exchange time (E[t]=t*10 ΔpH) vs. deuterium uptake. In doing so, we discover increases of exchange rates at pH 5 of >50 fold relative to pH 7 in the same structural regions identified by MD. These HX experiments corroborate conformational changes observed by MD and provide increasing confidence in the accuracy of our simulations. Through the combination of these technologies, we conclude that increased solvent accessibility, conformational flexibility, and likely pKa elevation of the labile Asp carboxylate occur as a direct consequence of the perturbed local structural environment at pH 5.

NOTES:
Elucidating Drug Binding and 3D Protein Structure using Native Top-Down MS

Joseph Loo

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

Advanced mass spectrometry (MS) has capabilities to offer structural biologists layers of insight into the details of protein complexes. 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. With electrospray ionization (ESI), MS can measure proteins and complexes from aqueous solution at near neutral pH, i.e., “native” MS. ESI’s gift for transforming solution-phase macromolecules into gas-phase ionized counterparts without disrupting covalent bonds and weak noncovalent interactions is key for applying MS to study protein complexes. Further, “top-down” mass spectrometry of protein complexes can be applied to probe ligand-binding sites and yield topological information of large proteins and complexes.

Recent data using high resolution Fourier transform ion cyclotron resonance (FT-ICR) MS will be shown to provide examples of the capabilities of native MS and top-down MS. We are using electron capture dissociation (ECD)/FT-ICR MS to investigate the molecular action of compounds that prevent amyloid fibril formation in neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease. Native top-down MS generates information on the surface topology, ligand binding sites, and post-translational modifications of protein complexes to greater than 800 kDa. Laser photodissociation of disulfide bonds shows promise as a tool to augment sequence coverage by top-down MS of large 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. Native top-down MS should be one of several approaches to provide important data for an integrated approach to structural biology.

Acknowledgments: Support from the US National Institutes of Health (R01GM103479, S10RR023045, S10RR028893) and the US Department of Energy is acknowledged.

NOTES:
NOTES:
Characterization of Biotherapeutics with Mass Spectrometry: Addressing the Data Analysis Bottleneck

Steven Pomerantz

Janssen Research & Development, LLC, Spring House, PA

Increasingly, the biopharmaceutical industry is turning to mass spectrometry for the characterization of both therapeutic candidates as well as reagent proteins throughout the entire discovery and development process lifecycle. Organizations can quickly become overwhelmed with data and often struggle to find new ways to automate data processing, analysis, and reporting. While most major vendors have provided some semblance of an informatics solution to address the need, the reality is that most biopharmaceutical mass spectrometry laboratories are a heterogeneous mélange from multiple manufacturers, each with its own software for processing data. This contributes significantly to the difficulty when trying to streamline and automate analysis and reporting, not to mention the additional costs associated with purchasing essentially the same software solution for each data generating platform. Thus, there is a clear need for a vendor-agnostic, platform neutral software system that is capable of processing mass spectrometry data from any source to address the analysis and reporting bottleneck through standardization and automation. Additional benefits accrue with respect to data integrity and traceability when automated analyses are employed, a special concern of GxP laboratories. This presentation will demonstrate recent advancements in automated characterization of biotherapeutics using the Expressionist software. This will include workflows that illustrate intact protein analysis, peptide mapping, glycopeptide analysis, disulfide bond network mapping as well as identification and quantification of other post-translational modifications, using data generated from multiple instrument vendors.

NOTES:
Effective Workflows for ADC Analysis: A Lively Breakfast Featuring ADC's (Antibody-Drug Conjugates) and Mimosas (Champagne-Orange Conjugates)

Scott J. Berger

Waters Corporation, Milford, MA USA

ADC's (Antibody-Drug Conjugates) are viewed as a leading biotherapeutic class, evidenced by the recent approval of the first potential-blockbuster drug of this type, and a rapidly expanding clinical pipeline against a variety of cancer indications. The characterization of ADC's presents the traditional challenges of defining structural variation for a mAb based biotherapeutic, and further expands the list of critical quality attributes to those describing the additional variation of that mAb conjugated with a population of drug-linker moieties. Foremost of these characteristics are the calculation of the average Drug to Antibody Ratio (DAR) for a given preparations, measurement of the distribution of various ADC forms, and determination of drug-linker site occupancy on the mAb.

In this session, we will discuss workflows for automated DAR determination, and describe 1D- and 2D-LCMS methodologies for the efficient investigation of sites of drug-linker attachment.

NOTES:
Agilent Technologies Lunch and Learn  
Thursday, September 11  
11:45 – 12:45  
Silverado West Ballroom  

Application of Label-free Discovery Proteomics Followed by MRM in Evaluating Sample Quality: From Blood Specimen Collection to Host Cell Proteins in Biologics  

Sushmita (Mimi) Roy  

*Caprion Proteomics US LLC, Menlo Park, CA USA*  

Label-free proteomics has matured into a quality-controlled analytical technology for discovery of clinical biomarkers. However, preclinical variables affect biomarker discovery results. Blood and cerebral spinal fluid sample collection, processing, handling and storage protocols are based on accepted practices rather than careful testing. We examined variables intrinsic to each step in the process of obtaining and storing clinical samples, beginning with electronically monitored collection of samples in controlled studies. Various blood collection tubes, times on bench, incubation temperatures, freeze-thaw cycles and freezer storage effects over 18 months were compared. Sample analysis was performed by high resolution mass spectrometry, leading to the identification of specific proteins affected by the parameters tested. A multiplexed MRM assay was assembled in order to determine sample integrity and establish utility for use of stored samples in clinical research.  

The versatility of this platform has also been utilized to assess sample quality in biologic pharmaceuticals by enumerating and quantifying trace levels of host-cell proteins in highly pure protein preparations.  

**NOTES:**
Reliability of a Semi-automated, High Throughput (HT) MS Based Glycomics System for Discovery of Glycan Biomarkers and Quality by Design (QbD) Studies of Glycoprotein Therapeutics

Archana Shubhakar¹,², Claire Morgan¹, Richard A. Gardner¹, Manfred Wuhrer², Daniel I.R.Spencer¹, Daryl L.Fernandes¹

¹Ludger Ltd, Culham Science Centre, Abingdon, United Kingdom, ²VU University Amsterdam, The Netherlands

For most therapeutic glycoproteins the glycosylation patterns correlate strongly with the clinical safety and efficacy profiles. In biological tissues these patterns can also correlate with the state of health or disease of the individual. Given this, there is an increasing interest in accurately characterising changes in glycosylation — for example in QbD studies throughout biopharmaceutical development, in Genome Wide Association Studies (GWAS) as well as in glycan biomarker discovery for medical diagnostics.

Changes in glycosylation patterns can be complex and subtle and the numbers of samples needed to be analysed can be large, ranging from hundreds to thousands. To perform these studies, reliable systems for HT glycomics are needed. However, despite many advances in glycosylation analysis there are still problems with current technologies, including poor reliability, high cost per sample, low sample throughput, long turnaround times and high labour intensity.

This talk concerns “LongBow” — a system developed at Ludger for reliable HT glycomics. The “LongBow” system is made up of flexible, modular technologies for semi-automated processing of glycans from a variety of clinical and bio-therapeutic samples with analysis by MS, UHPLC or CE. The focus here will be on permethylated N- and O-glycans analysed by MALDI-MS. This analytical technique delivers the most cost effective, fast and HT method for our QbD and biomarker studies. However, the reliability of glycan quantitation using this method has been challenging.

Here we will give an overview of our approach to improving the reliability of the system. The topics include robotisation, HT permethylation, sample clean-up, sample preparation using AnchorChip™ plate, MS acquisition parameters and analysis with Bruker GlycoQuest™ software. Applications include the analysis of the impact of bioreactor conditions on glycosylation patterns of a therapeutic IgG4 monoclonal antibody (mAb), N- and O-glycosylation profiling of a biosimilar erythropoietin (rhEPO) and serum N-glycan profiling in inflammatory bowel disease.

NOTES:
Automating Characterization of mAb Charge Variants Isolated by Cation Exchange Chromatography Using Integrated LCMS Peptide Mapping and Informatics Workflow

Stephane Houel, Robert Birdsall, Ying Qing Yu, Scott J. Berger, Weibin Chen

Waters Corporation, Milford, MA USA

Therapeutic monoclonal antibodies often contain low-level charge variants that alter protein surface charge, and can significantly impact mAb function and efficacy. These charge variants can result from modifications including: C-terminal lysine variants, deamidation, oxidation, and glycan sialylation. Characterization of these modifications presents an analytical challenge due to the sample heterogeneity and low abundance of the charge variants. We applied Ion Exchange Chromatography (IEX) in the isolation and enrichment of mAb charge variants of Infliximab, followed by LC/MSE peptide mapping for characterization of isolated acidic charge variant peaks.

On-line enrichment was performed for 5 acidic charge variants of infliximab separated using IEX as the 1st dimension on a multidimensional chromatographic system. The 2nd dimension column acted as a trapping/desalting column to retain and enrich multiple 1st dimension heart-cuts. The data was processed automatically using the UNIFI Scientific Informatics System. Modifications that contribute to form acidic components including deamidation, glycation, gamma-carboxyglutamic acid E, as well as acidic glycoforms with NeuAc and NeuGc sialylated species were considered in peptide mapping analysis. Total acidic residues amount relative to the non-acidic fraction was 22% based on integrated chromatographic areas. High levels of deamidation (33% as oppose to 1%) in the T7 peptide from the heavy chain of infliximab was prominent in the most acidic fraction, while the rest of the deamidated peptides remained the same across all injections. It was shown that the profile of % modification of various acidic glycoforms changed dramatically from the most acidic to non-acidic fractions. This study shows an efficient automated workflow that successfully pin-pointed differences in charge variant profiles across samples and also localized and identified modifications giving rise to individual charge variant peaks.

NOTES:
Advancement in the characterization of biopharmaceutical molecules has been driven by improvements in mass spectrometry technologies. As applications become mainstream in biopharmaceutical research and development, organizations seek to deploy solutions in GxP environments. Biotherapeutics require extensive release testing to confirm that all critical quality attributes fall within acceptable tolerances. There is ongoing discussion and debate about how to develop best practices and standard protocols to qualify and validate processes. Ideally, characterization methods developed during the research and development phases can be leveraged for release testing in production. Software solutions capable of supporting both R&D and the QA lab will have to process data from multiple instruments while providing automation, reporting, and compliance. Here we discuss these topics and provide analysis results using data generated from reference standards such as the NIST IgG1 monoclonal antibody reference material. While data was generated on a variety of mass spec instruments, a single software solution is used for data processing and analysis. Analyses include intact mass, peptide mapping, glycopeptide analysis, disulphide bond mapping as well as analysis of released glycans.

NOTES:
A Rapid and High-throughput Method for Quantifying Trisulfide Bond Content in mAb Molecules

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Trisulfide bonds are a posttranslational modification formed by the insertion of a free sulfur atom into disulfide bonds, resulting in a 32 Da mass increase. Trisulfides have been observed in natural and recombinant monoclonal antibodies (mAbs), but in-vivo they are limited to low levels due to the elimination of the extra sulfur atom by disulfide exchange reactions. With several disulfide bonds, mAb molecules are potential targets for trisulfide formation, and peptide map experiments showed that the primary site of this modification is at the disulfide bonds linking the antibody heavy and light chains.

In recombinant processes, trisulfide bond content may be indicative of variability in the culture redox environment and elevated trisulfide content can reduce the efficiency of antibody-drug conjugation. Additionally, changes in the integrity of the disulfide bonds in mAb molecules are of concern due to the potential impact on protein stability. Thus, it is useful and sometimes necessary to monitor trisulfide bond content during mAb production.

Trisulfide bonds can be detected by intact mass analysis of non-reduced molecules, where they manifest as additional peaks adjacent to the main peaks. Furthermore, detection of trisulfide bonds is facilitated by digesting the mAb with an enzyme, such as Fabricator, which yields Fab’2 and Fc fragments with a reduced size that improves the quality of the intact mass spectra. Here we describe a new method for measuring trisulfide content that is based on intact mass analysis of mAb preparations digested with Fabricator, with and without the inclusion of L-Cysteine during the reaction, which facilitates thiol-disulfide exchange reactions. Intact mass analysis of the pairwise samples provides one mass spectrum that shows the extra peaks from the trisulfide bonds and another from which these peaks have been eliminated. The trisulfide content is then measured by comparing the peak area distributions of the two samples.

**NOTES:**
Improving Identification of Sequence Variants by an Integrated Mass Spectrometric and Informatics Workflow

Weibin Chen, Stephane Houel

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An analytical approach aims to identify sequence variant peptides by comparing all the ion features identified for multiple therapeutic proteins is presented. A detection software (Progenesis QI) was used to identify and quantify all of features in each sample (replicate injection) after performing systematic retention time alignment and signal normalization for the features detected in all samples. Features were compared among samples, and unique features for each samples was then acquired. An inclusion list containing the unique ions was automatically generated and exported to acquire MS/MS in data-dependant acquisition (DDA) mode. The data was searched with UNIFI to identify the sequence variant peptide. In the searching method, the number of point mutation as well as the likelihood of the mutation and the number of missed cleavage can be defined.

The performance of the workflow is demonstrated by analyzing samples containing spiking synthetic peptides corresponding to amino acid substitutions in a monoclonal antibody. Three synthetic mutant peptides corresponding to amino acid substitutions in a monoclonal antibody were spiked in a tryptic digest of Trastuzumab (10 pmol/ul) at a concentration of 0.1% of the native peptide (10 fmol/ul) to create Sample A. In the same time, two additional Trastuzumab digests without spiked-in peptides (Sample B and C) were created in parallel digestion. Triplicate runs of these samples were performed. HDMSE data was acquired, and Progenesis QI software was used to identify and quantify ion-clusters. Totally we identified 7830 common features among all three samples but only 9 unique features including the spiked peptides were identified sample A. An inclusion containing 9 unique ions was automatically exported to acquire MS/MS in data-dependant acquisition (DDA) mode. Submission of the DDA data to UNIFI, the spiked-in mutant peptides containing one or two point mutation were successfully identified.

NOTES:
Advantages of Ion Mobility Mass Spectrometry for Identification and Quantification of Host Cell Proteins in Protein Therapeutics

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A major portion of biopharmaceuticals today are produced by recombinant DNA-technology using a well-selected host cell system. Even after sophisticated purifications steps, low levels (1-100 ppm) of host-cell proteins (HCPs) may still remain in the final purified biopharmaceutical. Mass spectrometry-based assay have been recently developed for identification and quantification of HCPs in biopharmaceuticals by using data-independent analysis and 2D chromatography[1-2]. Ion mobility gas phase separations, which can be performed in the timescale of milliseconds, can be used as orthogonal separations, between chromatographic separations and mass spectral analysis. In this study, 2D chromatography was combined with ion mobility to identify and quantify HCPs in biotherapeutic samples.

A highly-purified murine monoclonal antibody (mAb) was obtained from National Institute of Standards and Technology (USA). The sample was denatured, reduced, alkylated and digested with trypsin. The mAb digest was spiked with known concentrations of four protein digests used as internal calibration standards for quantification purposes. The peptide mixture was fractionated by RP chromatography at high pH (pH 10) in the first dimension, followed by an orthogonal separation at low pH (pH 2.5) in the second dimension, in a ten-step fractionation experiment [1-2]. Peptides were detected by a QTOF instrument in MSE mode. 2D LC/MS separations were performed with and without ion mobility and results were compared.

In the absence of ion mobility separation, four HCPs were identified in the mAb sample with concentrations in the range of 300 - 20 ppm. The same 4 proteins were identified when using ion mobility, and one more HCP was detected, extending the quantification range down to 5 ppm.


NOTES:
Characterization of Low Level Protein Impurity Peaks Observed in HPLC Assays Using LC-MS and JMP® Pairwise Statistical Analysis

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Protein products are often analyzed by RP-HPLC and SEC to identify product related impurities resulting from post-translational modifications (PTM) or degradation. However, impurity peaks can often be at levels too low (~0.3% of total peak area in this study) to identify with standard LC-MS methods. To overcome these challenges, multiple rounds of fraction collection can be used to isolate and enrich impurities for intact protein or peptide mapping with LC-MS. In this study, fraction collection of RP-HPLC peaks was shown to be infeasible due to artifacts occurring during sample collection and preparation. Fraction collection of the SEC peaks did not introduce interfering modification artifacts. Therefore, to identify the RP-HPLC minor peaks required an alternative approach with minimal sample manipulation.

A statistical analysis approach was taken to minimize the interference of method related artifacts. Concurrent analyses of 6 samples containing varying levels of RP-HPLC and SEC impurity peaks were performed. The methods include intact protein and peptide mapping with LC-MS analysis as well as RP-HPLC and SEC with UV detection. Results were compiled in JMP® software for the statistical analysis. Using the Pairwise statistical analysis tool in JMP® correlations between the varying levels of impurity peaks and observed PTM levels become apparent. To demonstrate the value of the statistical analysis procedure, a Cys dioxidation (CysO2) was found to be significantly correlated (p=0.0005) to an SEC impurity peak. Using peptide mapping with LC-MS analysis of the fraction collected SEC impurity peak, the same association with CysO2 was identified; therefore, establishing the effectiveness of the method. This statistical analysis thus reveals associations between multiple PTM’s and the RP-HPLC and SEC impurity peaks. Correlations were also observed between impurity peaks of the two HPLC methods.

Sequence Variant Analysis of Therapeutic Protein using LC/MS/MS

Ning Tang

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Sequence variants in therapeutic proteins may have an unpredictable impact on clinical safety and efficacy. Therefore, sequence variant analysis is an important component of biological product characterization during clone selection and bioprocess development. Amino acid mis-incorporation in protein products occurs due to either DNA mutations or RNA transcriptional or
translational errors. Sequence variants can be identified during peptide mapping analysis via LC/MS/MS experiments. In this work, peptide mapping of an IgG (1) therapeutic monoclonal antibody (mAb) was performed using LC/Q-TOF. The gradient was 60 minutes and MS/MS were collected throughout the run. 100% sequence coverage of both light chain and heavy chain were achieved. Multiple post translational modifications, such as methionine oxidation, deamidation and pyroglutamate were identified. In addition, the data was searched by allowing a +/- 130 Da mass gap between the observed mass and the theoretical peptide masses. Four potential sequence variants have been identified by Spectrum Mill. The suggested amino acid substitution is labeled in red. The specific amino acid changes are shown in the third column with the observed delta mass of the sequence variant compared to the regular sequence. Manual validation included examining other potential explanation of the spectra. Number 1 sequence variant S7C has an alternative explanation as methionine oxidation as the delta mass is 15.99 Da. MS/MS spectra did not show evidence of fragments to exclude the possibility of Met oxidation. Number 2 variant G16N could be attributed to Cys alkylation. Number 4 variant Y109W has a delta mass of 22.9730 Da and potentially be the sodium adduct of the regular peptide. This peptide also has the same retention time as the regular peptide, which further support the sodium adduct. MS/MS could not provide evidence for the mutation. Number 3 variant I34M is a true variant and validated by MS/MS.

NOTES:
P-109-W

Novel Workflows for Identification and Profiling of Disulfide Bonds in Biopharmaceuticals

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The correct location of disulfide bonds (DSB) in biologics such as antibodies is important for their safety and efficacy. Hence DSB analysis is an indispensable quality-control step that is often done by a time-consuming approach that involves comparing peptide maps of reduced and non-reduced samples. In order to avoid this differential approach, mass-spec based workflows using only non-reduced protein digests were evaluated. DSBs are cleaved in a first step either by electron-transfer dissociation (ETD) on an ion-trap (IT) instrument or by in-source decay (ISD) on a MALDI mass spectrometer. In a second step the separated peptides are analyzed by collision induced dissociation (CID) for identification and mapping to the protein sequence under investigation. In the case of the ion trap this was done using a second LC-MS3 CID run based on a precursor list found in the prior LC-MS2 ETD run. For improved ETD efficiency the CaptiveSpray technology was used to move the charge state distribution to higher charges for disulfide-linked peptides. The MALDI workflow relied on acquiring CID spectra from the respective spot using parent masses found in the ISD data acquired during the first step. The position of the disulfide bridge in these cysteine-containing peptides could then be unambiguously assigned from the peptides identified in the second step: This allows analyzing DSB scrambling without requiring a differential approach and without having to calculate all possible combinations of peptides. We applied this novel workflow to model proteins, e.g. a-lactalbumin and to adalimumab, an important antibody candidate for biosimilar development.

P-110-T

Comprehensive Sequence and Post-translational Modifications Analysis of Monoclonal Antibody by Flash Digest and LC-High Resolution Orbitrap MS

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As well established and fast growing biotherapeutics, monoclonal antibodies (mAbs) have been approved for the treatment of many diseases. To ensure product efficacy and safety, the quality of biotherapeutics needs to be closely monitored. Here, we report a fast and sensitive approach by combing fast enzymatic digestion, high resolution mass spectrometry and user friendly new data processing software for sequence and post translational modifications (PTMs) analysis. This approach provides an effective way to characterize protein therapeutics in bioprocess development.
Using the Flash Digest kit, digestion time was optimized by incubating native, non-reduced IgG mAb at 70°C at various duration times. Without reduction of disulfide linkages, a 30-min digestion time is adequate to achieve good sequence coverage of >83% for light chain and >79% for heavy chain, indicating an excellent digestion efficiency of Flash Digest kit. When the same IgG is further reduced and alkylated, sequence coverages of both IgG light and heavy chains are near 100%. A comparison between overnight digestion and the Flash Digestion kit will be generated.

PTMs are identified with high confidence levels as shown in the summary table from software. The relative abundance of each modification in different samples is calculated. The oxidative study of IgG shows that oxidation of methionine 49, 304 and 393 in the heavy chain is dose-dependent as the oxidation reaction time increases from 30 to 120 min. Relative percentages of each glycoform do not change over the reaction time as expected. Double and triple oxidation of cysteine were monitored but not observed. This result combined with other toxicology studies could be used to find critical quality attributes of biotherapeutics and guide the setting of corresponding thresholds for QC. This workflow could greatly shorten the sample preparation, data analysis time while providing great sensitivity to detect low level PTMs.

NOTES:
P-111-W

Data Independent SWATH-MS for the Quantitative Analysis of Host Cell Proteins in Biotherapeutic Purification

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Analysis of residual Host Cell Protein (HCP) present in biotherapeutics by mass spectrometry provides complementary information to ELISA HCP assay and enhances understanding of purification process. We present in this poster a routine methodology for unbiased and comprehensive HCP analysis using SWATH-MS, a data-independent acquisition (DIA) mass spectrometry technique. SWATH MS2 spectra are used to detect and quantify a number of HCPs in the presence of highly abundant product protein with MRM-like quality and sensitivity equal to triple quadrupole instruments yet devoid of heavy upfront method development and validation time.

A CHO IgG1 was subjected to 2-step purification, Protein A affinity chromatography and anion-exchange chromatography. Spike-in samples were prepared by mixing Protein A eluate (% w/w) into anion-exchange eluate at various amounts. The spike-in samples were digested with trypsin, and then analyzed by microflow LC-MS using a 40-minute gradient. SWATHTM data-independent acquisitions were performed using a 25 Da Q1 window width to produce high-resolution MSMS spectra of every precursor ion window between m/z 400 and 1200. Results Quantitative analysis was performed via peptide fragment ion chromatogram extraction using the PeakView® Software. For the top detected HCPs and the IgG product, three to five peptides were extracted for SWATH quantitation. In all cases, protein-level quantitation reflects summation of the individual peptide XIC signals. We observed a range of 2-log linearity of the HCPs against the IgG product and limits of detection ranging from 2 - 16 ppm in 6 of the 10 major proteins consistent in the purified IgG product sample. These results indicate extremely high quantitative reproducibility, independent of protein identity, suggesting that this approach should be broadly applicable for the unbiased quantitation of HCPs down to low ppm levels - using a fast single 1D 45 min LC method.

NOTES:
Comparability and Biosimilar Analysis

P-112-T

Analytical Characterization of Biosimilars by Mass Spectrometry

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Biosimilar medicines are the follow-on versions of the original biological medicines. Unlike small-molecule drugs, biologics generally are more complex and sensitive to changes in manufacturing processes. Therefore, Reference to the innovator product is an integral component of the approval. Analytical tools play important roles in physical and chemical characterization of the biosimilars. In this work, we focus on characterization of biosimilar and comparison with the originator using LC/MS. First, the originator mAb and its biosimilar were analyzed using LC/Q-TOF to compare intact mass and subunit masses. The masses matched very well. At this level, difference in major glycoforms relative abundances can be clearly seen. The biosimilar product and the originator were also digested and peptide mapping was performed using LC/MS/MS. Post-translational modifications were identified and semi-quantified for both samples. The glycan profiles were analyzed using mAb-Glyco chip and clear difference in glycan patterns were verified. Finally, two mAbs will be analyzed using ion mobility MS for structure comparison between the two molecules.

NOTES:
Glycan and Glycopeptide Analysis

P-113-W

Procainamide Labeling Enables Sensitive and Efficient Glycoprofiling by HILIC-FLR-MSn

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The identification and relative quantification of glycans is fundamental to the characterization of glycoproteins, and is thus central to burgeoning focuses ranging from therapeutic biomolecule production control to glycomics-driven biomarker discovery. The definitive identification of glycans specifies their composition, branching, linkage, and stereochemical structure; owing to this glycans do not lend themselves to simple analysis. Quantification also requires coupling to a chromophore as glycans are UV-Vis transparent. As a result, methods for glycan analysis are still being developed after more than 30 years, encompassing different chemical derivatization, chromatography, and MS methods. Recently, procainamide has been introduced as a labeling agent because of its dual properties as a sensitive fluorophore and as an easily ionizable moiety for enhance positive mode ESI. Despite these advantages, procainamide labeling is not yet widely used, in part because the chromatographic behavior of other fluorophore-labeled glycans has been well cataloged. We report on a robust procainamide labeling and UPLC-FLR-MSn method for glycan identification and quantification. Hierarchical workflows are discussed, highlighting MS/MS and MSn methods on an LC-timescale utilizing hydrophilic interaction chromatography (HILIC), coupled in line to a fluorescence detector and LTQ mass spectrometer. These workflows capitalize on the simplification of MS/MS fragmentation owing to y-type ion proclivity, minimizing and enabling judicious usage of exoglycosidases for definitive identification. Demonstrating the utility of the procainamide UPLC-FLR-MSn workflow, the chromatographic- and mass spectrometric-resolved glycans at sub 1% abundance for transferrin, human IgG, and cetuximab are presented. Finally, the optimization of cleanup, chromatography, ionization, and data analysis are discussed, with a focus on balancing maximal information gain with minimal starting material and time.

NOTES:
Flow-Through Electron Capture Dissociation Mass Spectrometry for High Throughput Glycoproteomics

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ECD and ETD using ion trap devices are often implemented in high throughput mass spectrometers with a particular advantage for post-translational-modification analysis. The objective of this research is to develop a more efficient ECD method using a novel ion trap device: “Chimera” with ~100% sample consumption duty cycle. The novel “Chimera” trap consists of eight L-shaped electrodes comprising four branches, where each branch has a linear quadrupole structure. One aligned pair of the branches is used for precursor introduction and product extraction, and another pair, exposed to a magnetic field, is used for electron injection. This Chimera ECD device was installed between the first quadrupole for precursor selection and the second quadrupole for CID in a Q-TOF instrument. This approach demonstrates a clear pathway to providing previously challenging techniques into a routine environment, while maintaining the fidelity of mature technology.

To maintain fragmentation efficiencies, control of the product extraction lens in millisecond time ranges was important. Fragment signals were enhanced dramatically with an ECD efficiency >60% during simultaneous injection of the electron beam and the precursor ions. This adapted “quasi” flow-through mode also produced more fragments than a conventional trapping ECD mode. To prove coverage and information depth, BSA digested by Lys C was injected onto a reversed phase C18 UPLC-ESI, with acetonitrile concentration between 2% to 40% for 10min, and five ECD spectra were obtained per second. This ECD technique provided sequence coverage of 85% (Lys C) and 75% (trypsin). The technique was applied on tryptic digests of glycosylated proteins on an LC- time scale, showing numerous O-glycosylated and N-glycosylated peptides with sialic acids being successfully identified using information dependent LC-ECD MS measurement.

Improved Glycopeptide Analysis using Acetonitrile Enriched Sheath Gas and Oxonium Ion Dependent ETD

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Mass spectrometric analysis of protein glycosylation is a major topic. In the instance of protein drugs, the glycan profile needs to be carefully selected and controlled to ensure safety and efficacy.
A great variety of different approaches is available to analyze intact glycoproteins, glycopeptides and released glycans. Glycopeptide analysis is of particular interest as detailed information about glycans, peptide and glycosylation site is needed in order to build a comprehensive understanding of the molecule. This study uses solvent-enriched sheath gas to enhance glycopeptides signal intensities for improved detection. An efficient combination of collision induced dissociation (CID) and oxonium ion triggered electron transfer dissociation (ETD) allows for extensive protein sequence coverage and detailed glycopeptide profiling in a single run. A standard protein mixture was reduced, carbamidomethylated and digested with trypsin. The tryptic peptides were separated on a nano HPLC system. A quadrupole ion trap MS (amaZon speed ETD, Bruker Daltonics) equipped with a CaptiveSpray nanoBooster ionization source was used for data dependent MS/MS experiments. Spectra were acquired in “Fragment Triggered ETD” mode to selectively acquire ETD spectra upon detection of glycan fragments in the CID MS/MS experiment. Acetonitrile-enriched nitrogen was used as sheath gas to enhance the intensities of glycopeptides and increase charge states for higher ETD efficiency. Data processing was performed using ProteinScape 3.1 software. First, glycopeptide CID spectra were automatically detected and searched against a database using the GlycoQuest engine. ETD spectra were then used for glycopeptide sequencing to associate the knowledge about the peptide primary sequence and its glycan profile.

NOTES:
P-116-T

High Sensitivity Analysis of N-glycans on Novel UHPLC HILIC Columns by FLD-MS

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Analysis of N-linked glycans has become a critical capability for biopharmaceutical analysis due to their effects on product efficacy and immunogenicity. A widely used approach relies on 2-AB labelling of enzymatically released glycans with separation by hydrophilic interaction chromatography (HILIC) and detection using fluorescence (FLD). While HILIC-FLD of labelled glycans is sufficiently sensitive for most applications, peak assignment can be challenging, especially when potential N glycan structures share the same retention time. Efforts to assist peak assignment by mass spectrometry are hampered by the low sensitivity of ESI-MS for glycans. Here, three different approaches to increase MS sensitivity in HILIC-FLD-MS of N-glycans were investigated: (1) Use of an ionization promoting tag: procainamide (2) Use of Jet Stream and dual ion funnel-enabled MS instruments (3) Use of MRM on a QQQ. These investigations were carried out using two novel HILIC phases available on sub-2 µm porous particles or 2.7 µm superficially porous particles.

As expected, QTOF-based analysis of 2-AB labelled glycans was less sensitive than FLD, even for ion funnel enabled instruments. However, the sensitivity of ion funnel QQQ MRM of 2-AB labelled glycans was similar to that of FLD. When using procainamide, we show that the QTOF can achieve similar sensitivity to the FLD measurements, making this workflow much more powerful than using fluorescence detection alone. More pronounced improvements are afforded by combining procainamide tagging with ion funnel QQQ MRM, where sensitivity often exceeded that of FLD detection. The sensitivity enhancement afforded by procainamide was most pronounced for high mannose glycans, which exhibit the poorest ionization in positive mode. Overall, ion funnel QTOF or QQQ measurement of procainamide labelled glycans was very useful for comprehensive analysis of N-glycans, where mass information permitted assignment of low abundance compositions.

P-117-W

The Biopharmaceutical System Solution with UNIFI: A Holistic Workflow for LC/MS Acquisition, Processing, and Reporting of Fluorescent Labeled Glycans

Ying Qing Yu, Weibin Chen

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The majority of the therapeutic proteins are glycosylated, and the attached glycans have significant impact on the efficacy and safety of the biotherapeutic. The International Conference on Harmonization Guideline Q6B requires the analysis of carbohydrate content, structural
profiles, and characterization of the glycosylation site(s) within the polypeptide chain(s). The most widely adopted analytical workflow for routine N-linked glycan characterization involves labeling the enzymatically released glycans with a fluorescent molecule (such as 2-aminobenamide, or 2-AB), resolving the labeled glycans by hydrophilic interaction liquid chromatography (HILIC), and detecting with the labeled glycan peaks with a fluorescence detector. The assignment of glycan peaks during routine analysis is fundamentally based on matching their retention time to established values. For non-routine analysis, glycosidase arrays or MS analysis are employed to verify tentative assignments or resolve ambiguous peak assignments. In order to best control method variation (between runs, days, instruments, scientists, labs) glycan profiles from the HILIC separation are often calibrated and normalized against a 2AB-labeled dextran ladder (glucose homopolymer). Glycan peaks in an unknown sample can be assigned glucose unit values from the GU vs. RT dextran curve.

The UNIFI 1.7 informatics tool enabled automated data acquisition, processing, and reporting. The major workflow utilized for this analysis employed collection of dextran ladder calibrated UPLC HILIC retention (Glucose Units or GU) for 2-AB labeled N-glycans. Assignments were produced using an experimentally derived Glycan GU library search, based on GU value and accurate mass search criteria. We characterized and compared the innovator and a biosimilar Etanercept N-glycans profile using the glycan workflow in UNIFI system, and the results from qualitative and quantitative analysis will be discussed in details.

NOTES:
Higher Order of Structure Analysis

P-118-T


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Therapeutic applications with proteins and especially with monoclonal antibodies (mAb) have been rapidly growing in recent years. The function, efficacy, and safety of therapeutic proteins are tied to their three-dimensional structure. The analysis and verification of this higher order structure is critical in demonstrating manufacturing consistency and in analyzing the absence or presence of structural changes in response due to changes in production or stability. It is, therefore, essential to have reliable and high sensitive biophysical tools capable of interrogating protein structure and conformation. The analytical tools available to study and evaluate this critical attributes, which are in general the classical spectroscopic methods like FT-IR, fluorescence or circular dichroism, are lacking sensitivity and spatial resolution. Hydrogen/deuterium exchange (HDX-MS) offers significant hope to improve this. The use of HDX-MS for protein biotherapeutics can include epitope mapping, binding, protein-drug interaction studies, aggregation studies, effect of mutation on conformation and localization of conformational changes as a result of post-translational modifications. Due to recent developments in robotic automation of sample handling and improvements in chromatographic separation and mass spec detection along with the development of appropriate computer software, HDX-MS has now become a technology that is capable of making significant contributions in biopharmaceutical characterization by providing more spacial resolved information compared to the classical spectroscopic methods. It has also the potential to be used as a tool to support biopharmaceutical comparability studies. This poster will present several examples of HDX analysis applied to mAb samples to elucidate possible structural changes caused by aggregation and storage conditions and induced by ligand binding.

NOTES:
Overcoming the Challenges of Disulphide Bonds in Proteins under HDX Quench Conditions

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The presence of disulphide bonds in proteins is an obstacle for anyone engaging in HDX-MS. Particularly if the disulphide bonds are numerous either throughout the sequence or within a single domain. The conditions necessary for quenching the HDX reaction (low pH, cold temperature, short time for incubation) are unfavourable for reducing disulphide bonds. Here we present how optimization of the HDX quench conditions as well as pepsin digestion enables us to get near complete sequence coverage for the blood coagulation factor XIII B-chain (FXIII-B), which is a 75 kDa protein containing 20 disulphide bonds. We also present how an alternative to chemical reduction, namely an electrochemical reactor, efficiently reduces the disulphide bonds of FXIII-B online the HPLC system.

NOTES:
Intact Protein Mass Spectrometry

P-120-T

Integrated Intact/Topdown Analysis of A Standard Protein Mix using RP-LC-MS On Q Exactive HF

Jenny Chen¹, Benjamin Cutak², Jim Blasberg², Kevin Ray², Keith Waddell¹

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In recently years, biopharmaceutical proteins have been increasingly developed. These molecules are more complex to analyze than conventional low molecular weight drugs, and thus need powerful analytical approaches. Analytical groups are often tasked with performing LC-MS analysis of intact proteins with a wide array of characteristics in a variety of sample matrices and a range of available sample amount. A general work flow is developed for Intact/topdown analysis of a standard protein mix using RP-LC-MS on Q Exactive HF. With the advances in the high resolution Orbitrap, intact molecule weight measurements and topdown sequencing are integrated in one single LC run. A Sigma protein standard mix is used to cover a wide MW range from 5000 Da to 80000 Da with different hydrophobicity. The proteins include insulin, Ribonuclease B, Lysozyme C, Transferrin, BSA, b?-Lactoglobulin A, Carbonic Anhydrase, Lectate Dehydrogenase. The protein solutions were directly injected and analyzed by using capillary LC coupled with a Q Exactive HF mass spectrometer. Parameters of mass spectrometer were optimized, including in-source activation energy, s-lens values, AGC target and HCD collision energy. Protein Deconvolution was used for intact MS spectra deconvolution and ProSightPC 2.0 was used for topdown spectra analysis. The intact mass of standard proteins was accurately measured with low ppm mass error. Isotopic resolution was achieved for proteins with MW <30kDa. Topdown analysis was conducted in Intact Protein Mode using High Mass Resolution (240k) for fragments detection. High sequence coverage was obtained for the standard proteins with N-termini and C-termini identification. Using a protein mixture with known masses helps to establish processing parameters for proteins of a given size. The LC MS-based intact/topdown method can serve as system suitability check. Furthermore, parameters used to produce accurate results for well-defined standards can be applied as starting point to analyze variant proteins.

NOTES:
Mass Spec Approaches for Pre-Clinical Quantitative Studies

P-121-W

LC/MS Protein Quantitation and Characterization Enabled by a Sample Preparation Platform that Automates Purification, Digestion, and Cleanup

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There are an ever growing number of candidate biotherapeutics that need to be quickly and accurately quantified and characterized so timely decisions can be made that move these candidates efficiently through the discovery and development process. Great strides have been made in the performance of LC/MS instruments but actionable data requires that the precision of these instruments be matched throughout the entire workflow. Currently, sample preparation upstream of LC/MS is often performed manually by highly skilled scientists. There is an urgent need to replace the tedious but critical manual sample preparation workflows with automation that is simple to use, highly precise, scalable, transferable between operators/sites, and frees skilled scientist to do more value added work. We report results from two workflows that are routinely used to quantify and characterize biotherapeutic proteins. Peptide quantification was performed using the AssayMAP Bravo to purify 96 antibody samples in parallel with PG-W (immobilized Protein G) cartridges, digest the antibodies, and clean up the peptides with C18 cartridges. LC/MS analysis indicated that the workflow was highly reproducible with CVs < 10 \%. Sequence coverage characterization was performed using the AssayMAP Bravo to purify antibody samples, digest the antibodies with three different proteases in parallel, and clean up the peptides. LC/MS analysis indicated that the combined sequence coverage from all three enzymes was 99 \% and 100 \% for the heavy and light chains, respectively. Finally, we report analytical figures of merit for antibody purification using PG-W and PA-W (immobilized Protein A) cartridges and the AssayMAP Affinity Purification application. Antibodies were purified with CVs less than 5 \% and elution volumes of less than 10 \mu L.

NOTES:
NOTES:
Quantification of Chinese Hamster Ovary Homologs to a Human Protein Target in an Antibody Therapeutic by LC-MS Analysis with Selected Ion Monitoring

John Briggs, Adam D Catherman, Matt Kalo

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In the course of developing a recombinant monoclonal antibody therapeutic, it was discovered that a Chinese hamster ovary (CHO) homolog of the human antibody target (CHO Target) was co-purifying with the antibody. Upon further investigation it was determined that up to 10 clipped forms of the CHO Target were found in the purified materials. Rather than attempting to quantify 10 different species, a common proteolytic fragment was selected for quantification. In preparation for quantification, purified materials were spiked with serial dilutions of 15N-labeled CHO Target and then digested with trypsin. The resultant peptides were then separated by reverse-phase chromatography and analyzed using a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer. As part of the mass spectrometric analysis, ions associated with the native and 15N-labeled common proteolytic fragment were monitored by selected ion monitoring (SIM) and the native fragment was quantified relative to a standard curve generated using the 15N-labeled fragment.

Applications of Mass Spectrometry for Studying the Effect of Raw Materials on N-linked Glycosylation of Biotherapeutic Protein

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Detailed characterization of glycans present on recombinant glycoproteins remains an important challenge in the development and production of biotherapeutics. An analytical platform consisting of reversed phase chromatography combined with a high-resolution quadrupole time of flight mass spectrometer has been developed for monitoring protein glycosylation for bioreactor samples. To perform the analysis, the pre-concentrated and buffer-exchanged cell culture samples were desalted by reversed phase chromatography prior to elution into the mass spectrometer. The post-column infusion of 25% acetic acid in acetonitrile was used to increase the ionization efficiency of bioreactor samples.

Six glycans were monitored and classified into two groups: (1) non-sialylated and sialylated biantennary oligosaccharides (BiNA0, BiNA1, BiNA2, BiNA3) and (2) sialylated triantennary oligosaccharide structures (TriNA3, TriLacNA3). Repeatability and intermediate precision values for % antennary distribution of the most abundant glycoforms (i.e. BiNA2, TriLAcNA3 and TriNA3) were determined to be in the range of 0.4-1.6% and 1.0-12.7 %RSD, respectively.
The samples were shown to be stable in autosampler over a period of 4 hours, which allows analysis of up to 10 samples at a time.

The method has been applied for systematic screening of protein glycosylation in bioreactor and in-process samples. We found that atypical levels of the tri-antennary and bi-antennary glycoforms have been observed as a result of a change in concentration of an impurity in one of the raw materials. Dosing this impurity back into the medium to historical concentrations was sufficient to restore the glycan profile. The finding confirmed that the glycosylation profile of therapeutic antibodies needs to be routinely analyzed throughout development to monitor the impact of process parameters and to ensure consistency, efficacy, and safety for clinical and commercial batches of therapeutic products.

NOTES:
Utility of the NIST Monoclonal Antibody Reference Material: A Case Study for Method Pre-Qualification and Pre-Validation

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Biopharmaceutical products are carefully engineered to provide maximum therapeutic results while minimizing off-target effects. But because protein therapeutics are produced in living cellular systems, the resultant drug products contain a certain level of inherent variability that must be well-characterized prior to approval and release. This heterogeneity is a product quality attribute that must remain constant throughout production and storage to ensure safety and efficacy for the patient. In-house product-specific reference standard programs are required for stringent qualification and validation of the analytical characterization methods and quality control platforms to be employed. However, the need for a class-specific reference material to supplement these activities is highlighted by the fast pace of technological development, particularly in the field of mass spectrometry. The availability of a reliable and well-characterized reference material would facilitate the evaluation of new analytical methods and instrumentation as well as their implementation into a regulatory-compliant setting. An IgG1κ monoclonal antibody reference material is currently under development by the National Institute of Standards and Technology (NIST) and soon to be released along with relevant analytical data. This material will be of value for a number of uses in the biopharmaceutical industry including system suitability testing, method pre-qualification and pre-validation, and method transfer. As an example, utility of the NIST mAb will be demonstrated as a relevant external control to monitor forced degradation conditions as well as evaluate analytical methods for characterization of product heterogeneity and glycan composition. We also anticipate the NIST mAb to act as a non-proprietary tool that will foster open discussion between members of the industry or between industry and regulatory agencies.

NOTES:
P-125-W

Application of High Resolution MS for a Quantitative Multi-attribute Method for Quality Control and Release Testing of Bio Therapeutic Molecules

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Release testing in a Quality Control (QC) environment is performed with well-established chromatographic and electrophoretic methods designed to ensure the process consistency and safety of bio therapeutic molecules. Applying high resolution mass spectrometry (MS) techniques during release testing can increase product understanding particularly around molecule critical quality attributes (CQAs) that may impact safety and efficacy. The current use of multiple HPLC and CE based release assays can be a burden in the QC environment due to cost, required instrumentation, and importantly analyst time. Using the principles of high resolution MS, a method has been developed that is able to monitor a large number of modifications in a single assay. In this communication, a high resolution MS method capable of quantifying multiple CQAs including glycoforms and other amino acid modifications are presented. CQA values reported by this method were compared to values reported by traditional methods (CEX, HILIC, and rCE-SDS) and showed comparable trends across techniques. These data demonstrate that the MS based technique was well suited to accurately monitor and measure CQAs that are deemed important for monoclonal antibodies. Looking to the future, the use of a MS based Multi-Attribute Method will allow us to monitor and quantify multiple CQAs with one encompassing assay resulting in a comprehensive understanding of our products, while also reducing the resource demand in a QC environment. The data presented supports the ability of this high resolution MS technique to be used for the quantitative analysis of critical quality attributes of monoclonal antibodies and release of biologics.

P-126-T

MS in QC: A Fully Compliant Multi-attribute Quantitative Method for Quality Control and Release Testing of Biologics

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Application of highly resolving mass spectrometry (MS) techniques has resulted in better understanding at the molecular level of the attributes that are crucial for safety and efficacy of complex biotherapeutic molecules. Application of a Quality by Design (QbD) approach would require leveraging the knowledge of these critical quality attributes (CQAs) to guide manufacturing processes and drug disposition. We have developed a method that integrates a
MS-based multi-attribute method into a fully compliant package required for quality control and release testing of biologics in a regulated environment. At the first phase of development, we used the stand-alone software program, Pinpoint, to simultaneously quantify multiple CQAs such as glycoform species and amino acid modifications. In the second phase, we addressed the question of detecting unexpected events during manufacturing by relatively comparing peaks detected by the mass spectrometer, using the stand-alone program Sieve. In the third phase, we aim to integrate the software elements in a compliant package to be able to apply the method in a regulated environment. We chose to modify a pre-existing compliant chromatography data system, Chromeleon 7.2, which is used in quality control for UV-based release methods. The system controls acquisition from the mass spectrometer and insures integrity of LC-MS workflows and data processing. In addition, the LC-MS software fulfils 21 CFR Part 11 requirements for electronic records and electronic signatures. Integration of Pinpoint and Sieve into Chromeleon would create a fully compliant package that can be validated to operate in a regulated environment. Development of such a tool for quantitative analysis of CQAs of monoclonal antibodies in a quality control environment is a step forward in the implementation of QbD principles in manufacturing and release of biologics.

NOTES:
A Novel 6x5 LCMSMS Peptide Reference Mixture for Instrument Performance Monitoring

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For the Characterization of Biologics, consistent and optimal monitoring of instrument performance continues to be challenging and has yet to be standardized across all MS laboratories. Towards this end, we have prepared a novel peptide mixture. In combination with an accompanying software tool, this mixture reports on LC-MS instrument performance. The novelty of the mixture is based on the following: The mixture contains 6 peptide sequences in which each sequence is a mixture of 5 isotopologues. Each isotopologues is mixed at different molar ratios so that a linear curve, based on mass and intensities can be produced to assess instrument sensitivity and dynamic range. In addition to reporting on all critical instrument parameters, the software can also report on parameter history, compare instruments, and display XIC traces for the peptides. This is the first example of a peptide mixture designed to give a reporting of all critical LC and MS parameters in a single run. All of the peptide sets resolve with identical chromatography and are distinguished only by molecular mass. In doing so, we have the advantage of verifying the sensitivity and dynamic range of MS detectors in a single run. In our studies, based on Orbitrap and Q-TOF type instruments, we have detected peptide abundances as low as 20 amole (200 fmole of the heaviest peptide loaded on column). The software tool is also able to detect the peptides spiked into a complex background.

Enhanced Performance on Peptide Quantitation using Integrated LC-MS Workflow

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Sample complexity and low concentration of some biomarkers are the main challenges in the multiple reaction monitoring (MRM)-based biomarker verification methodology. Consequently, the development of MRM-based methods in biological matrix has focused on improving the sensitivity and increasing the dynamic range, and improving the robustness in the high-throughput clinical research environments. This work demonstrates a novel targeted protein/peptide quantitation workflow which incorporates data independent All Ions MS/MS technique and fully integrated skyline software to achieve straightforward development of an MRM method. The sensitive and robust determination of human plasma peptides using a newly designed Triple-Quadrupole-Mass-Spectrometer is also achieved. Tryptic digest BSA was used as a model system to demonstrate the MRM method development workflow. The peptides were first analyzed by All Ions MS/MS technique on a QTOF mass spectrometer. Skyline software
was then used to select target peptides with transitions based on the data and create a QQQ targeted MRM method. Sensitivity and robustness were assessed using both a standard peptide in a simple matrix as well as a plasma protein assay using commercially available kits on a QQQ mass spectrometer. All ions MS/MS alternates between low and high energy scans without a MS1 filtering during acquisition. Once imported into Skyline, the top ranked peptides with transitions were selected based on the evaluation of the retention time, precursor ion and production abundance. The detection limit of the system for a standard peptide was achieved down to 3 amol on-column. After more than 600 injections of plasma digest (40 µg per injection), area response remained stable with area RSDs of QC peptides lower than 15%. The retention times were extremely stable (average of 0.46% RSD for all peptides across all runs) which allowed the weeks of analyses to be performed with no modification of retention time windows.

NOTES:
Non-Traditional Antibody Molecules and Non-Antibody Molecules

P-129-W

Characterization of Isoforms of Cysteine-Conjugated Antibody Drug Conjugates (ADCs) Using On-line 2D-LC/MS

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Hydrophobic interaction chromatography and mass spectrometry are leading techniques for the characterization of critical quality attributes (CQA) of ADCs including average drug-to-antibody ratio (DAR) and drug loading distribution. However, chromatographic peaks are not always readily interpreted and may be associated with conjugate isoforms, incomplete conjugations, or hydrolysis products. Due to non-volatile HIC mobile-phase components, elucidation of the peak identity/structures often involves a time-consuming process of offline fractionation and/or identification using alternative techniques. Here, an on-line 2D-LC/MS approach was applied to elucidate the structures of some cysteine-conjugated ADC isoforms in HIC fractions.

Hydrophobic interaction chromatography was used to determine CQAs including drug load distribution and DAR values for an ADC sample. Five main peaks from a HIC separation were observed and were associated with 0, 2, 4, 6, and 8 drug-loaded conjugates, respectively. Shoulder peaks distributed throughout the chromatogram could not be assigned unambiguously based on conventional LC/UV data due to the lack of corresponding ADC standards. These peaks required further analysis for identity and structural elucidation. Shoulder peaks were isolated automatically from the 1st dimension and the eluent flow was directed onto a reversed phase column for mass spectrometric analysis. QTOF-MS data were used for unequivocal peak identification and proper DAR value assignment. The DAR value was determined to be 5.97 for the ADC sample. Subunits of ADC isoforms (e.g. HC, LC, 1/2Fc, Fab, or Fd) were generated online, and analyzed using the 2D LC/MS platform for the identification of site conjugation via a middle-down approach. These results demonstrate the LC/LC/QTOF-MS method facilitates a comprehensive and unambiguous determination of DAR values for the ADC isoforms in HIC fractions, and provides information for conjugation sites on those isoforms.

NOTES:
Combining On-Bead Conjugation and MS Analysis Following IdeS Digestion Simplifies the Production and Characterization of ADCs

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Antibody-drug conjugates (ADCs) are a growing class of targeted therapies designed to deliver cytotoxic drugs to cancer cells. The process to make ADCs can be tedious, involving optimization of numerous variables including linker design, attachment chemistry and loading density. We have developed an improved method to produce ADCs utilizing antibodies immobilized on high-capacity magnetic protein A or G particles. We employed this method to label Rituximab with a fluorescent dye, and characterized the conjugate using mass spectrometry techniques including intact mass and subunit-level analyses after digestion with IdeS protease. Conjugation of AlexaFluor to Rituximab immobilized on high capacity magnetic protein A or G particles was accomplished after mild reduction of the hinge disulfides followed by covalent attachment using maleimide chemistry. Intact mass analysis of PNGase F-treated naked and conjugated Rituximab demonstrated characteristic mass shifts representative of different payload distributions of the dye. Full MS of the IdeS-digested ADC revealed mass shifts consistent with loading on both the Fd and light chain. The combination of on-bead conjugation followed by IdeS digestion and subsequent MS analysis simplifies the workflow for production and characterization of ADCs.

The Neisseria Meningitidis GNA1030 is a Ubiquinone 8 Binding Protein

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¹Novartis Vaccines and Diagnostics, Siena, Italy, ²Institut Pasteur Centre François Jacob, Paris, France

Bexsero® is the newly vaccine against Neisseria meningitidis serogroup B (MenB) approved in more than thirty countries. It is composed of three main protein antigens identified by reverse vaccinology: NHBA (Neisserial Heparin Binding Antigen, NadA (Neisserial adhesin A)) and fHbp (factor H binding protein) and the Outer Membrane Vesicles derived from the New Zealand strain, NZ 98/254. NHBA is present as fusion to GNA1030 antigen to further increase its immunogenicity. The gene encoding for the GNA1030 is present in all Neisseria strains and is highly conserved in sequence, but its biological function has not been to date elucidated. Native mass spectrometry was used to demonstrate that GNA1030 forms a homodimer associated with two molecules of Ubiquinone 8 (Ub8), a cofactor mainly involved in the electron transport chain.
and with antioxidant properties. To investigate the role of GNA1030 in binding to Ub8, we performed functional assays on MenB cultured under stress conditions and demonstrated that GNA1030 is not involved in oxidative stress and electron chain transport, but it is able to carry Ub8 from the cytoplasmic to the periplasmic compartment and contribute to constitutively refill the inner membrane with Ub8. We propose to name the protein Neisserial Ubiquinone binding protein (NUbp).

NOTES:
P-132-T

Characterization of the Fab Fragment of the NIST-Monoclonal Antibody by Hydrogen Deuterium Exchange-Mass Spectrometry

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Hydrogen deuterium exchange-mass spectrometry (HDX-MS) is a powerful tool for characterizing protein structure, dynamics, and binding interactions. Recently, the pharmaceutical industry has expressed interest in utilizing this method for analyzing biologics and biosimilars to verify product quality and evaluate changes in manufacturing protocols. HDX-MS monitors the mass increase of a protein over time associated with exchange at amides along the protein backbone. Though global analyses have been utilized to probe protein structure, it is more common to perform a proteolysis step prior to the liquid chromatography-MS analysis to achieve peptide-level resolution of the exchange events. Though many laboratories utilize bottom-up HDX-MS, a comprehensive examination of typical intermediate precision and inter-laboratory reproducibility of the method has not been reported. Here we describe the initial characterization of the Fab fragment of a monoclonal antibody that is being developed by NIST as a candidate standard reference material. This work illustrates the preliminary qualification of this material, which will be used as a standard protein for an inter-laboratory analysis of the reproducibility of the bottom-up HDX-MS method. Fab is prepared by enzymatic digestion (papain) of the monoclonal antibody, followed by purification on a Protein A spin column. During HDX-MS, the online digestion (pepsin) results in over 120 peptides that correspond to 94% and 65% coverage of the heavy and light chain, respectively. Preliminary HDX-MS studies illustrate that this protein exhibits a large dynamic range of exchange events at 25 °C and pH 7.4, with the detected peptides having 10 to 91% deuterium uptake after exposure to D₂O for 30s. Measurements at longer times reveal similar dynamics for Fab. This work provides the foundation for the multi-laboratory comparison, establishing the reproducibility of the bottom-up HDX-MS method.

P-134-T

Development of Integrated Informatics Workflows for the Automated Assessment of Comparability for Antibody Drug Conjugates (ADCs) Using LC/UV and LC/UV/MS

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Hydrophobic interaction chromatography (HIC) and mass spectrometry (MS) are among the most commonly used analytical techniques for characterizing antibody-drug conjugates (ADCs). Very often, different informatics tools and manual calculations are needed for data
Structural information from different analytical methodologies, especially for critical quality attributes (CQAs) such as average drug-to-antibody ratio (DAR) and drug load distribution (which measures the homogeneity of the ADC population), are not readily integrated to provide effective assessment for this important modality. An integrated informatics workflow was developed to streamline data acquisition, processing and reporting for ADC analysis by LC/UV or LC/MS so that information from multiple analytical techniques can be effectively synergized for rapid and quantitative assessment of comparability of ADCs. Analyses of ADC samples were performed using LC/UV and LC/UV/QTOF-MS. Samples were analyzed at the intact protein level by LC(HIC)/ UV and by LC/MS under either native (SEC-MS) or denatured conditions (reversed phase LC/MS) with the control of a common informatics platform. The total run time for both HIC-LC and LC/MS experiments was 20 min. Both cysteine and lysine conjugated ADC samples were used to test the workflow. Samples with different drug loading were synthesized and analyzed. The analyses focus on comparison of the experiment results for critical quality attributes such as average drug-to-antibody ratio (DAR) and drug loading distributions between the optical detection (HIC) and MS detection methodologies, and subsequent comparability assessment is made among the samples analyzed based on analytical results. Preliminary results show that the HIC method and the LC/MS method (native and denatured) achieved excellent agreements on the DAR values and on the drug loading distributions for all cysteine-conjugated ADC samples. The experimental results demonstrate that the integrated informatics workflow is well suited for robust method development and can be easily automated to increase the productivity for ADC drug development.

NOTES:
Analysis of Antibodies and Antibody Drug Conjugates Using a High-resolution Quadrupole Time-of-flight Mass Spectrometer

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Intact protein characterization by mass spectrometry is often used to provide rapid assessment to the heterogeneity of biomolecules. Isotopic resolution in combination with exact mass measurement allows direct detection of modifications such as sequence variants and reduction of disulfides at the intact protein or subunit level. In this presentation, a sensitive, high-resolution quadrupole/time-of-flight mass spectrometer equipped with a novel segmented quadrupole collision cell was used to analyze intact mAbs and ADCs or their subunits.

Monoclonal antibodies (Trastuzumab, Rituximab, PTP-1) from multiple lots were studied. Their subunits were generated either by DTT reduction or Limited Lys-C digestion plus DTT reduction. Antibody from each specific lot, its reduced form, and a mixture of intact protein and subunits were separated on a UPLC SEC column and analyzed. Where native SEC-MS analysis was performed, ammonium acetate at neutral pH was used as mobile phases. Acetonitrile with acidic modifiers were used for denaturing SEC separation. The use of native and denaturing SEC directly coupled to mass spectrometer for the molecular weight measurements provided greater details on sample heterogeneity and improved the productivity for MS analysis.

Intact proteins or mAb subunits were also separated by high resolution RP chromatography to resolve isoforms in the samples. High-resolution MS detection was able to isotopically resolve proteins up to 30 kDa. Identities of either intact protein, or the subunits of mabs (LC, Fd and 1/2Fc) were confirmed by deconvoluting the mass spectra using both maximum entropy and BayesSpray deconvolution algorithms. The utility of the approach to characterize protein modification with small mass changes such as disulfide bond breakage will be illustrated. The further optimization in experimental parameters for released glycan and middle-down fragmentation analysis on the new mass spectrometer will also be presented.

NOTES:
Rapid UHPLC-HRMS Peptide Mapping: Monitoring MAb Degradation

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Therapeutic monoclonal antibodies (MAbs) are susceptible to chemical degradation during production, purification, formulation, transportation, storage and use. These chemical modifications have been shown to generate products with different biological and immunological activity compared to their native MAbs, raising product quality and safety concerns. The ability to monitor chemical degradations during pharmaceutical development is of utmost importance. Peptide mapping using reversed-phase chromatography is commonly employed in the characterization and quantification of degradation products. In order to achieve sufficient resolution, peak shape, and signal, conventional HPLC separations require long gradient times, limiting the number of samples that can be analyzed. By utilizing Ultra High Performance Liquid Chromatography (UHPLC), gradient times can be shortened with increased resolution and throughput. When used in combination with high resolution, accurate mass spectrometry, coeluting peptides can be further separated and quantified based upon their masses.

Here, we developed a rapid UHPLC-High Resolution MS (HRMS) method for relative quantitation of chemical modifications. The reproducibility and linearity of the method was tested through the separation of tryptic peptides by a fast, 17 minute linear gradient, while detection of native and modified peptides was performed using a high resolution, accurate mass spectrometer. This novel method allows for in-process monitoring and relative quantitation of MAb chemical degradation products during pharmaceutical development.

Toward a Comprehensive Bottom-up and Top-down Analysis of the NIST Reference Monoclonal Antibody

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Careful characterization of therapeutic proteins is required due to their inherent variability. A reference protein standard can represent a major analytical tool for the industry, but it must be
well-characterized in order to serve as a reference. In this study, high resolution mass spectrometric data of the NIST mAb interim reference material (RM 8670, lot 3F1b) was analyzed by new bioinformatics tools to efficiently identify and quantify modifications, including oxidation, deamidation, glycation, glycosylation, and sequence variants.

Heavy and light chain components were measured by both bottom-up and top-down (sometimes called middle-down) approaches by a high resolution Thermo Fisher Orbitrap Elite mass spectrometer employing CID, HCD and ETD fragmentation modes. The data was analyzed by a combination of the search engine Byonic™ and new inspection software Byologic® that combines and compares MS1 and MS2 data streams and performs label-free quantification by taking the ratio of extracted ion chromatograms (XICs) of the modified to unmodified peptide. In addition, new peptide mapping software, Byomap™, was employed to quantify and annotate the peptide map.

Bottom-up analysis with just a few data sets led to extensive site-specific identification and quantification of sequence variants, glycations, glycosylations, oxidations, and deamidations. Glycosylation (both bottom-up and top-down) was measured for the intact molecular forms. The top-down analysis by Byonic provided annotation of fragments spanning the entire sequences, with ETD, at least initially, providing the greatest degree of fragment coverage. Oxidation sites, glycosylation and truncations were observed in the top-down analysis. Overall, with a modest amount of high quality mass spectra, extensive quantitative analysis of the NIST interim reference standard was performed in a rapid fashion.

NOTES:
Monitoring Advanced Glycation End Products within an E. coli-Derived Monoclonal Antibody

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Advanced glycation end products (AGEs) are a heterogeneous set of protein modifications derived from the degradation of glycated amino acid residues or reactions with dicarbonyl compounds. Previous characterization of an E. coli-derived monoclonal antibody using both reduced mass analysis and tryptic peptide mapping revealed mass shifts consistent with AGE modifications. However, these methods alone were insufficient for characterization of AGEs due to the lack of site localization by reduced mass analysis and the inability to accurately quantify arginine and lysine modifications by tryptic peptide mapping. To provide a more thorough and quantitative investigation into the presence of AGEs, we developed an AspN peptide mapping procedure coupled with UHPLC-MS/MS. The utility of this approach was first demonstrated by monitoring AGEs in forced glycation samples, produced by reacting the mAb with either glyoxal or methylglyoxal. AspN peptide mapping allowed for the detection and quantification of AGEs on both lysine and arginine residues across many different peptides.

Based upon the observed modifications, mAb samples and acidic variant enriched fractions were also analyzed. While a minor amount of AGE species was detected in representative material, significant levels were detected in samples containing increased levels of acidic variants. Moreover, a heterogeneous set of highly modified peptides was detected within the acidic variant enriched fractions. The predominant AGE modification observed was methylglyoxal-derived hydroimidazolone, with the highest levels found in the Fc region. Due to the low level of glucose-adduction within the antibody, it is hypothesized that the AGE modifications are likely formed from reactive glycolytic intermediates produced during cell culture. Overall, this work provides a platform for detecting and quantifying AGEs which can be used for optimizing cell culture and purification conditions as well as monitoring product quality.

Identification and Characterization of Hydrophobic Interaction Chromatography Fractions of an IgG1 Monoclonal Antibody by LC-MS and MS/MS

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Hydrophobic interaction chromatography (HIC) separates populations of molecules in order of increasing hydrophobicity. Molecules bind to a weakly hydrophobic stationary phase in the
presence of high concentration of salt and desorb into the mobile phase as the concentration of salt decreases. HIC is widely used in a protein purification setting for isolating therapeutic antibodies and proteins. HIC is also used as an analytical tool to monitor various degradation products and isolate those isoforms for further characterization and assessment of their binding activities. Preparative HIC separation of the antibody was performed on a TSKgel® Ether-5PW HIC column. Collected fractions were concentrated and buffer exchanged to 1XPBS buffer pH7 using 10 kDa molecular weight cutoff (MWCO) spin filter for further analysis. Both non-reduced and reduced intact mass analysis was performed on a reversed phase column coupled with a quadrupole time-of-flight (QToF) mass spectrometer. The tryptic digest of the HIC fractions were analyzed by LC-MS and MS/MS on a LTQ XL ion trap mass spectrometer. We successfully characterized and identified seven HIC peaks from an IgG1 monoclonal antibody using intact mass measurement (non-reduced and reduced antibodies), tryptic peptide mapping analysis and tandem mass spectrometry sequencing. Non-reduced mass analysis required minimum sample preparation and gave a global protein characterization. Reduced mass analysis revealed that the modifications occurred in the heavy chain of antibody. Tryptic peptide mapping and tandem mass spectrometry sequencing enables localization of the modifications to specific amino acid sites. Asp54 isomerization, Met56 oxidation and Trp52 oxidation were found to cause the separation of different fractions in the hydrophobic interaction chromatography profile. All the three modification sites are located in the second complement-determining region of the heavy chain region of the antibody.

NOTES:
Upstream and Downstream Homogeneity Determination of Proteins Recombinantly Expressed in E. coli

Carl Hemond

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Various E. coli strains are used as preferred industrial-scale expression system for recombinant proteins and are routinely compared to maximize productivity. In all cases, overexpression represents a heavy burden for the host and can introduce modifications on the target protein.

Protein heterogeneity can also increase during downstream processing through chemical reactions, while peptide bond breakage is commonly observed. The spontaneous modification of amino acid residues is another phenomenon dependent on purification conditions that has to be tracked. Although the impact of these post translational modifications on the final immunogenicity of the product is not known, it can represent a source of challenge if the level of modified protein is not reproducible from batch to batch or scale to scale or if it evolves over time.

In most cases, it is possible to eliminate heterogeneities or fix stability issues at the upstream or downstream level, hence the need to develop analytical tools, such as LC-MS, that are capable of providing a feedback throughout the manufacturing process despite the crudeness of samples.

To obtain such results, it was identified that developing an appropriate sample preparation would provide the simplest and most rapid way to alleviate E. coli related matrix effect. Since host cell proteins (HCP) were released as well during the process, they also had to be taken into consideration when dealing with soluble recombinant proteins to avoid interference in LC-MS. In the case of inclusion bodies, the target protein was found in concentration and purity amenable to MS after solubilization.

In conclusion, sample treatments were successfully applied to decrease HCP interference and to evaluate the level of PTM of recombinant protein in various types of complex samples such as E.coli harvest. The impact of changing the strain on protein heterogeneity was obtained providing insightful information to better select the expression conditions.

**NOTES:**
A Streamlined Workflow for Characterizing Low-Abundance Glycans on Therapeutic Proteins

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A strategy is presented for characterizing low-abundance N-glycans using single exoglycosidase digestions with LC-MS and LC-MS/MS. N-glycans from Rituxan® (rituximab) and Enbrel® (etanercept) were released and labeled with Procainamide (PCA), treated with a single exoglycosidase, and the products analyzed. Using a threshold of 0.01%, forty-one (41) N-glycans were identified on Rituxan and seventy-one (71) on Enbrel. Minor species included N-Glycolylneuraminic acid (NGNA), hybrid glycoforms and triantennary N-glycans (Enbrel only).

Understanding Cysteine Modifications in Therapeutic Proteins

Adriana Kita, Charles Cheng, Hongcheng Liu, Gomes Ponniah, Bruce Andrien, Dino Miano

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Monoclonal antibodies are an important class of therapeutic proteins, and they are also notorious for their heterogeneity in nature. N-terminal pyroglutamine cyclization, C-terminal lysine variants, incomplete disulfide bond formation, deamidation, and oxidation are just a starting list of modifications commonly found in an antibody. High resolution mass spectrometry, with its specificity in nature and ability to handle complex mixture, has been playing a central role in understanding the chemical nature of the heterogeneity. The work presented here focuses on cysteine modifications observed during therapeutic antibody development. A mass spectrometric strategy of combining intact and middle down analysis as well as bottom up approach is employed to understanding the chemical nature of the low level impurities ultimately attributed to cysteine modifications.

Two examples will be demonstrated in this presentation. In the first case, a low level impurity (mass addition of about 32 Da) was observed in an antibody protein during process development. FabRICATOR digestion localized the addition to the F(ab’2) region. Protein digestion of the lot in question along with the reference lot using the optimized protocol revealed a low level peptide with MS data consistent with N-terminal peptide with free Glutamate and as trisulfide content.

The second example involves also a low level impurity in the antibody production where a higher mass addition of 240 Da species was observed. Despite multiple efforts to isolate the
impurity protein, we have yet to identify a chromatographic condition that can enrich or isolate the species in question. A similar approach was taken where the modification was localized to the F(ab′)2 region of the protein. Protein digestion revealed a peptide that is consistent with the addition of two cysteine residues (cysteinylation x2).

NOTES:
PeptideAnalyzer: An Integrated Platform for Efficient In-depth Characterization of Therapeutic Proteins

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Mass spectrometry is an extremely valuable tool for detailed characterization of protein samples capable of detecting even low levels of protein variants such as post translational modifications or sequence variants resulting from DNA mutations, mistranslation or misincorporation. Unfortunately, the data evaluation part of these analysis is tedious, error-prone and time consuming as software solutions tailored to these workflows of biopharmaceutical applications are rare. Based on these observation/limitation we describe the PeptideAnalyzer software platform that evolved from this unmet need of our labs. It provides an integrated application facilitating the data evaluation tasks from LC-MS/MS data acquisition to reporting including access to different in-house databases covering our labs collected experiences from previous protein samples.

Development of a Universal Sample Preparation Platform for Disulfide Linkage Analysis of IgG1 Antibodies by LC-MS

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Monoclonal antibody (mAb) is composed of two identical heavy chains and two identical light chains linked by disulfide bonds to form a "Y" shaped structure. The number and positions of disulfide linkages present play a crucial role in forming and stabilizing a correct mAb structure which is critical to its function. Thus, it is important to characterize all disulfide linkages to ensure mAb drug function and quality. Peptide mapping by LC-MS analysis of enzymatically digested mAb under non-reducing condition is a powerful method for disulfide linkage assignment. However, the development of a robust sample preparation method with high digestion efficiency and minimized disulfide scrambling for non-reducing disulfide linkage analysis is necessary but challenging.

In this study, we developed a universal sample preparation platform for IgG1 disulfide linkage analysis by LC-MS. We prepared IgG1 samples using the conventional trypsin digestion at pH 8 and 37˚ C. Due to the steric hindrance, the hinge region disulfide linkage cannot be observed in this digestion condition. Instead, Lys-C plus trypsin digestion was investigated to see if higher digestion efficiency can be achieved. In addition, it is known that higher digestion temperature and higher pH will introduce more disulfide bond scrambling. Then lower digestion temperature (25˚ C) and lower digestion pH (pH 6.5) were also examined. Our results showed that Lys-C plus
trypsin digestion at pH 8 and 25°C is a better sample preparation condition because of better digestion efficiency (all expected disulfide linkages can be confidently observed) and minimal disulfide scrambling.

NOTES:
Investigating Cysteine Desulfurization by TCEP (tris(2 carboxyethyl)phosphine) and Its Impact on the Tryptic Peptide Mapping/LC MS Analysis of a Therapeutic Monoclonal Antibody

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Tryptic peptide mapping is an important tool for the characterization of site-specific post-translational modifications in therapeutic proteins, allowing for the identification of intrinsic modifications that are potential critical quality attributes. However, for quality attribute assessments, it is important to differentiate between intrinsic modifications and modifications induced by sample preparation conditions. During routine tryptic peptide mapping of a therapeutic monoclonal antibody, previously unidentified peptide peaks were observed in the UV profile. LC MS characterization revealed that these peaks were attributed to primary sequence substitutions of cysteine by alanine (Cys to Ala) in many cysteine containing peptides throughout the molecule. This poster will describe the detailed evaluation undertaken to determine the cause of the Cys to Ala substitutions detected in the peptide map of this therapeutic antibody. From this analysis, it was determined that TCEP, which was used as the disulfide bond reducing agent in the tryptic peptide mapping procedure, was responsible for converting cysteine to alanine by desulfurization. The observation of TCEP-induced cysteine desulfurization re-emphasizes the importance of understanding sample preparation-induced modifications. In this case, the artificially induced modifications could have been incorrectly assigned as a mutation or potentially interfered with the detection of an actual post-translational modification of interest. This study demonstrates that TCEP conditions should be carefully assessed to ensure that desulfurization of cysteine is not induced during tryptic peptide map/LC MS characterization of protein therapeutics.

Analysis of Cystine Knots in a Protein Biopharmaceutical using LC-MS with Electron Transfer Dissociation and a Stabilizing Sample Pretreatment

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Cystine knots are known to often play critical roles in the structural stability and efficacy of protein biopharmaceuticals. The accurate characterization of cystine knots is therefore important to ensure consistent stability and efficacy profiles of a biopharmaceutical throughout its clinical development. Despite the advances in technology for complex disulfide characterization, e.g. peptide mapping using high-resolution LC-ETD/MS² (ref Wu, JASMS, 2013), the sample
preparation involved in the analysis, such as enzymatic digestion, has a potential to introduce disulfide scrambling particularly among the less stable disulfides in a cystine knot structure, which can result in significant method-induced artifacts and sometimes mistaken assignment of the disulfide linkages. The approach described here was therefore developed to minimize potential scrambling and other method-induced artifacts in the characterization of cystine knots in therapeutic proteins. An optimized partial reduction of the weakest disulfide bonds in the target molecule, followed by alkylation of the free cysteines, was developed to protect the disulfide bonds involved in cystine knots that are particularly susceptible to scrambling during enzyme digestion. Protection of the target molecule’s weakest disulfide bonds was found to prevent random scrambling during subsequent enzyme digestion, which ensures the reproducible and accurate assignment of the disulfides involved in the cystine knots. This method may provide an effective approach in biopharmaceutical development for the characterization of complex cystine knot structures containing less stable disulfides.

NOTES:
Relating Structures of Biopharmaceuticals to Function

P-148-T

Increasing Transfer Efficiency in Native Mass Spectrometry of Supermolecular Protein Complexes with a Modified Dual-Funnel QTOF

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Mass spectrometry has become a valuable tool for the analysis and the characterization of proteins. The majority of the MS-based proteomics approaches are identification, quantification and/or characterization of proteins differing in their primary sequence or in their PTM profile. Contrarily, the MS analysis of non-covalent complexes opens insights into the supermolecular chemistry, i.e. the study of higher orders of protein structure. Native-spray MS analysis of large molecules requires specific experimental setup and sometimes altered hardware that impacts performances of the mass spectrometer for other applications. Here we describe a modification of a QTOF-instrument for native MS of supermolecular structures with no influence on performance factors of other applications.

In this study ions of non-covalent protein complexes were introduced into a modified dual funnel QTOF. Native-spray of larger protein complexes result in supermolecular ions with relatively low numbers of charges. For high m/z protein complex ions (m/z range 8000 -14000), the transfer efficiency from the glass capillary into the first funnel was enhanced by increasing the back pressure in the first funnel region. Two model systems were selected to evaluate the improvement of transmission efficiency. For both systems the efficiency and S/N ratio was relatively low in standard conditions but could be improved 10-folds after pressure adjustments.

The increased pressure facilitates in-source CID, further improving the desolvation process. The supermolecular structure stays intact as the higher pressure limits the dissociation of the protein complex. Further improvement can be obtained by tuning the pressure and potential of the collision cell.

NOTES:
Identification of Light Chain Variants in Monoclonal Antibodies

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Non-reduced reversed phase high performance liquid chromatography (NR RP-HPLC) is a commonly used method to monitor the disulfide mediated structural isoforms of human IgG2 monoclonal antibodies (mAbs).

NR RP-HPLC analysis of a fully human IgG2 type recombinant mAb reveals two minor pre-peaks in addition to the canonical peaks. Isolation of these two minor pre-peaks followed by characterization using total mass analysis and peptide mapping indicates that the pre-peaks are cysteinylated light chain (LC) and covalently linked LC dimer, respectively. The LC dimer was determined to be linked through disulfide bonds at the C-terminal cysteines, as has previously been reported in literature for Bence-Jones protein.

Our data further suggests that the discovered two LC variants are non-covalently bound to the intact mAb molecule. Size-exclusion HPLC (SE-HPLC) analysis demonstrates the enrichment of the LC variants in the high molecule weight fraction, similar to previously reported association of general clipped species with aggregated species in antibodies.


NOTES:
Protein phosphorylation is one of the most common PTMs interrogated by bottom-up mass spectrometric techniques. Due to the low relative abundance of phosphopeptides, enrichment is often required and presents itself as one of the most challenging sample preparation techniques to perform reproducibly. While there is certainly no single source of variability in phosphopeptide enrichment strategies, we argue that a significant portion is from the manual nature of most phosphopeptide enrichment protocols limiting the potential for protocol standardization. To address the issue of reproducibility, we developed an automated phosphopeptide enrichment protocol on a liquid handler capable of mating to microchromatography cartridges packed with titanium dioxide (TiO2) resin and report the technique’s analytical figures of merit. The maximum capacity of the TiO2 cartridge was determined to be more than 110 µg for the synthetic phosphopeptide RRLIEDAEyAARG, yet recovery of 0.16 ng of this peptide from a BSA digest matrix exceeded 83% with a CV of 5.4% (n = 4). To assess phosphopeptide enrichment reproducibility of low mass amounts of phosphopeptides, 100 ng to 2.5 µg of digested α-casein was spiked into 150 µg of digested BSA. The median peak area CV for the 26 α-casein phosphopeptides confidently identified by MS/MS ranged from 9.1% at 100 ng to 4.1% at 2.5 µg spike levels. For an automated workflow featuring in-solution digestion, C18 cleanup, and enrichment of phosphopeptides from 150 µg of α-casein, the median peak area CV of α-casein phosphopeptides was 5.3% across 8 replicates. Greater than 76% of the total signal from peptides identified in the enriched α-casein samples was from phosphopeptides. We also describe the application of automated phosphopeptide enrichment to a more complex biological system demonstrating that comprehensive and reproducible phosphopeptide enrichment is feasible using standard flow ESI-LC/MS with a sensitive Q-TOF mass spectrometer.
Pressurized Online Pepsin Digestion of Proteins for Hydrogen/deuterium Exchange Mass Spectrometry

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Pepsin digestion is an integral part of hydrogen/deuterium exchange mass spectrometry (H/DX MS) for probing the location of the incorporated deuterium in order to obtain localized conformational information of a whole protein. Online digestion has been widely accepted and utilized because of its high digestion reproducibility; minimized introduction of pepsin autolysis fragments into the LC /MS system, etc. However, the typical online pepsin digestion may not generate sufficient digestion for some proteins under low temperature. In this study, we report an improvement in protein digestion efficiency using a high pressure approach.

High pressure promotes protein denaturation, which mechanically stretches the proteins to expose more cleavage sites. Pressurized digestion of highly soluble and easy-to-digest proteins (e.g. BSA and phosphorylase B) may not yield more peptides compared with conventional digestion method [1].The most significant enhancements are generally observed with hydrophobic proteins or other difficult-to-digest targets, such as native IgGs. In this study, a high-pressure sustainable BEH column with immobilized pepsin [2], was applied in the digestion of monoclonal antibodies (IgG1 and IgG2) under different temperatures, 15, 10, and 0˚ C, respectively. The digestion efficiency of this column under high pressure (up to ~15,000 psi) and under normal pressure (~ 1000 psi) was compared. A higher number of overlapping peptic peptides were observed 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.


Reducing Time-to-Characterization of Monoclonal Antibodies using Microfluidic LC/MS Approaches

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The goal of this work was to perform comprehensive analysis of a monoclonal antibody (mAb) in the most rapid manner possible. In order to achieve this goal, microfluidic technologies that
minimize sample handling were utilized. The approach included microfluidic LC/MS, microfluidic electrophoresis, and capillary isoelectric focusing. These experiments were conducted on a candidate mAb reference material provided by NIST (8670 mAb lot #3F1b). The combination of the approaches permitted analysis of size variants, charge variants, glycan profile, glycopeptide profile, and measurement of the untreated and PNGase-F deglycosylated masses of the intact molecule or its IdeS-generated fragments. The use of microfluidic approaches for this purpose was advantageous because of the minimization of sample handling as well as time to measurement.

NOTES:
Capillary Electrophoresis ESI MS Characterization of Therapeutic Monoclonal Antibody by Intact Mass Analysis, Reduced Intact Mass Analysis and Peptide Mapping

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In support of biotherapeutic development, comprehensive characterization of therapeutic monoclonal antibodies (mAbs) is essential, as impurities and unwanted heterogeneity can negatively impact both safety and efficacy. Intact mass analysis, reduced intact mass analysis, and peptide mapping of mAbs provides critical qualitative and quantitative information about impurities and heterogeneity. Capillary electrophoresis (CE) demonstrates exceptional separation efficiency and capabilities for analysis of mAbs by mass spectrometry. Previously reported, was the development of an electrospray ionization (ESI) emitter integrated with CE, combining CE separation and ESI into a single dynamic process (CESI). In this study we present data for intact mass, reduced intact mass and peptide mapping analyses of a representative mAb, Trastuzumab. A Beckman Coulter CESI 8000 system (sold through SCIEX Separations, a part of AB SCIEX), with a prototype neutral-coated capillary, coupled to an AB Sciex TripleTOF® Qq-TOF mass spectrometer were used to perform all analyses.

The results illustrate benefits of the integration of CE and ESI alongside fast high-resolution mass spectrometry. CESI separation and low-nanoflow electrospray conditions provide the sensitivity to achieve 100% peptide map sequence coverage and dramatic separation of the intact heavy and light chains of this mAb. At flow rates below 20 nL/min, ionization efficiency is maximized and ion suppression is dramatically reduced, allowing strong ionization of low-level modified peptides and glycopeptides. With comprehensive sequence coverage, identification and quantification of mAb purity, stability, and glycoform heterogeneity is possible from CESI-MS analysis. Intact and reduced mAb analysis is routine at low nanogram levels. In peptide map analysis, we can routinely identify low-level deamidation, oxidation, glutamic acid cyclization, and map all glycopeptides from injections in the fmol range.

Routinely Coping with Biomolecules of All Shapes and Sizes with Mobility

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Ion mobility is now accepted as a valid technique for orthogonal separation of biomolecules, but its routine use has generally been challenging in industry due to a variety of factors including physical implementation in specialist instruments, the need for software, specialist users, or
understanding of the parameters used. We present here a new implementation of differential ion mobility on accurate mass systems that can be routinely applied to a wide variety of biomolecules and therapeutic proteins. The tool is established in high throughput environments for small molecules and peptides, and here we demonstrate its further utility in facilitating the routine analysis of large proteins. Differential ion mobility mass spectrometry (DMS) separates ions based on their dipole moment, and this configuration provides an orthogonal separation prior to mass spectrometry analysis that avoids the need for dedicated software.

We optimized the voltages of the Selexion™ DMS device in front of a modified Qq-TOF mass spectrometer for a number of experiments. We differentially transmitted mAb or ADCs separately from a number of co-eluting contaminant molecules; we also show how a direct comparison can be made between reduced and non-reduced mAbs where DMS can rapidly help to distinguish differences; we were also able to clarify spectra for species with large heterogeneity, such as fusion proteins. Additionally, we demonstrate the ability to modify the mobility behavior of peptides with gas phase modifiers without recourse to any internal instrument modifications. This latter aspect demonstrates a distinct advantage of this configuration for investigative work without the need for specialist engineering. This technique can provide molecular weights accurately and quickly, even when other molecules confound analysis of the sample. Sample analysis time saved can be in days of method development time per protein, and have a better chance of providing clearer information content.

NOTES:
P-155-T

Nanodiscs as a Launch Pad for MS Studies of Membrane Embedded Therapeutic Target: A Case Study of Vitamin K Epoxide Reductase

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Many membrane-embedded proteins, like epidermal growth factor receptor (EGFR), are important therapeutic targets. Detergents or lipids are required to solubilize them for investigation. This raises both concerns as to relevance and challenges for appropriate MS-based analysis. Nanodiscs (FEBS Letters 584 (9): 1721 1727) open prospects for new approaches for MS-based biophysical studies of membrane-embedded therapeutic proteins. Protein targets are inserted into an assembly of phospholipids of the disc, the size of which is controlled by the length of a helical protein belt, termed the membrane scaffold protein (MSP). The resulting discs provide a native-like, soluble phospholipid bilayer environment that satisfies stability and functional requirements of the incorporated target. Two MS-based biophysical approaches, native MS coupled with top-down and protein footprinting coupled with bottom-up, could be used to generate information of protein targets (FEBS Letters 588 (2): 308-317). We report here an application of nanodisc-based MS study of Vitamin K epoxide reductase (VKOR). VKOR is a membrane-embedded enzyme and the target of warfarin, the most widely prescribed oral anticoagulant drug in North America. We assembled a VKOR bacterial homolog into a Nanodisc, and the intact VKOR-nanodisc was directly introduced by native MS and analyzed by top-down strategies, mainly collision-induced dissociation and electron-capture dissociation. The intact membrane embedded VKOR can be observed in native MS by using a Nanodisc as a launching pad. The conformational information of VKOR in the Nanodisc is ongoing by using the protein footprinting approach, Fast Photochemical Oxidation of Protein (FPOP).

NOTES:
Metabolic profiling of body fluids is crucial for monitoring and discovering markers of health and disease and for providing insights into human physiology. Urine and plasma each contain an enormous diversity of metabolites with a broad range of physicochemical properties such as size and hydrophobicity. As a consequence, single liquid chromatography systems coupled to mass spectrometry (MS) do not allow a full coverage of the metabolome. Hydrophilic interaction liquid chromatography (HILIC) offers complementary information to reverse-phase liquid chromatography (RPLC) by enabling the analysis of polar metabolites. To maximize the metabolome coverage in an untargeted fashion, we systematically investigated the performance of various HILIC and RPLC columns when coupled to MS using standards and biological samples. The performance of five HILIC columns operated at different pH (acidic, neutral, basic) and five C18 silica RPLC columns were compared. The zwitterionic column ZIC-HILIC operated at neutral pH gave the optimal results (HILIC-MS) while Hypersil GOLD and Zorbax SB aq performed the best for urine and plasma samples (RPLC-MS), respectively. Intra-batch reproducibility of the optimized HILIC-MS analytical procedure was excellent and similar to RPLC-MS in term of retention time (CV < 1%) and peak area (CV < 12%). Long-term inter-batch (40 days apart) reproducibility of the HILIC-MS procedure was good with a retention time CV < 1.25% and a peak area CV < 22%. Combining the optimal HILIC- and RPLC-MS approaches greatly expanded metabolome coverage with the detection of 44% and 99% new metabolic features on urine and plasma samples, respectively compared to RPLC alone. Overall, the use of the optimized combined approaches enables the monitoring of more than 25,000 unique metabolic features from urine and plasma and enables unprecedented coverage of the human metabolome.
Host-Cell Protein Analysis of Therapeutic Monoclonal Antibodies following Protein a Chromatography using Data Independent 2D LC-MS²

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Host cell proteins (HCPs) are a major class of bioprocess-related impurities that pose a considerable risk to patient safety and as such their removal from therapeutic protein products is of critical importance. Protein A purification is the primary procedure for removal of HCPs from culture media in downstream processing. Typically HCP levels in drug substances are measured using multi-product assays such as enzyme-linked immunosorbent assay (ELISA). In the current study a quantitative high pH low pH reversed-phase data independent 2D-LC-MSE based proteomic method has been used, which enables the identification and quantitation of HCPs in various therapeutic protein samples.

Here, we have applied the proteomic platform to evaluate the suitability of four different elution buffers for the removal of HCPs from monoclonal antibody (mAb) samples following Protein A purification. The effect of elution buffer choice on the quality of the therapeutic protein was also evaluated. Of the different buffers assessed, an arginine-based buffer was found to have the most favourable impact on product quality and HCP profile.

Additionally, the developed proteomic platform was used to determine the impact of cell culture day of harvest on the HCP profile of a therapeutic protein following Protein A purification. mAb, from culture harvested at the beginning of the stationary phase of cell growth, was found to contain lower levels of HCPs when compared to mAb harvested at the end of the stationary phase of growth, following Protein A purification.

Finally, the proteomic platform was applied to gain quantitative and qualitative information of the HCP profile present in five commercially available drug products, revealing low levels of proteins involved in transcription, intracellular membrane trafficking and prevention of protein aggregation. The information obtained using MS was subsequently compared to ELISA values for total HCP concentration estimation.

NOTES:
Monoclonal Antibodies Complete Primary Structure and Biosimilarity Assessment in a Single Analysis using Transient isotachophoresis Sheathless Capillary Electrophoresis-tandem Mass Spectrometry

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Patents on some approved monoclonal antibodies (mAbs) are going to end giving the opportunity for alternative companies to produce copies, referred as biosimilars. Regulation agencies requires for approval to demonstrate the chemical similarity between a biosimilar candidate and the original mAbs. mAbs are highly complex glycoproteins, displaying a wide range of microheterogeneities so adapted analytical techniques may be a huge asset for development/approval of biosimilars. A sheathless CE-MS platform was used to develop a CE-ESI-MS/MS method to perform fast and robust characterization of mAb primary structure and assess biosimilarity between two mabs samples.

mAbs samples, digested by trypsin, were analyzed using a CESI-MS platform coupled to 5600 TripleTOF mass spectrometer. Peptides, postranslational modifications (PTM) and glycosylations were simultaneously characterized. 2 approved mAbs and respective biosimilar candidates were considered. CESI-MS/MS allowed to obtain 100% sequence coverage systematically for studied mAbs plus up to 90% y/b ions could be identified. Amino acid (AA) sequence characterization could be performed only through digested peptides without miscleavages using a single injection. Same dataset also enabled to precisely characterize and estimate occurrence levels of all PTMs hotspots used to monitor product stability. Data were used to structurally characterize and estimate relative abundances of glycosylations. Indeed it was possible to characterize 15 N-glycans for trastuzumab while 2 different glycosylation sites could be distinguished with site dependent glycoforms semiquantification established for cetuximab.

Trastuzumab data comparison allowed to distinguish trastuzumab from its candidate biosimilar by a difference of one AA and dissimilarities in glycoforms distribution. For cetuximab, the candidate biosimilar proved complete similarity in AA sequence while expression of toxic glycoforms were pointed out excluding the candidate.

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NOTES:
Computer Assisted Design of CE-ESI-MS Interface for Biotechnology Applications

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One of the most widely used analytical techniques in biotechnology is online hyphenation capillary electrophoresis – mass spectrometer (CE-ESI-MS). In this study a novel CE-ESI-MS design is investigated by means of computational fluid dynamics (CFD) simulation. The developed device features two narrow channels for nebulizer gas and the system is simulated in 2D mirror symmetry layout in order to reduce computational time. The model applies the turbulent form of the Navier-Stokes equation assuming a compressible one phase flow with same physical characteristics as nitrogen at 293.15 K. Since the flowing media is assumed to be compressible gas, its density is corrected with the function of pressure. The velocity field distribution and the pressure drop are calculated by a commercially available CFD software package.

An ESI interfaces’ primary aim is transfer as much ionized analyte molecules as possible by physical positioning the CE capillary close to the MS orifice, closing the electrical circuit for both the CE and ESI sides and supporting the proper droplet formation required for creation of free, gas phase ions. To speed up their development, CFD modeling techniques will be introduced in this poster presentation. The demonstration will focus on the investigation of the pressure and velocity field distribution in order to discover unexpected low transfer rate problems inside the microchip.

As an example, an obtained velocity field is shown below (the warmer the color, the higher the velocity, unit is m/s). The full-length inflow pressure is 3 atms. The simulated velocity profile shows how the nebulizer gas could drag the flow from the separation channel suggesting avoidance or sharp edges.

Comparison of Glatiramer Acetate through Label Free Differential Mass Spectrometry

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Glatiramer acetate is an immunomodulator drug used in the treatment of multiple sclerosis. The product is currently marketed as Copaxone\textsuperscript{®} by Teva. The active pharmaceutical ingredient consists of a mixture of peptide chains synthesized from four amino acids (glutamic acid,
alanine, lysine, and tyrosine) with a range of molecular weight from 5,000 to 9,000 Da. Thus, modern analytical technologies which can characterize this complex mixture are desirable. Here, using a commercially available comparator (i.e. Copolymer-1, which is also a mixture of Ala, Glu, Lys, Tyr polymers) and multiple marketplace glatiramer acetate lots from the originator company, LC-MS approaches to detect significant analytical differences between the materials were explored. A label free differential mass spectrometry is presented for evaluation of detectable differences in comparator composition.

LC-MS of glatiramer acetate lots and Copolymer-1 was performed after digestion using either Lys-C or Glu-C. Samples were analyzed using HILIC separation with either a Thermo LTQ-Orbitrap XL or QExactive mass spectrometer. The resultant data were time aligned using Sieve 2.1. Alignment scores were all greater than 0.9 indicating that the chromatography of the different samples were similar. However, distinct variations between glatiramer acetate and Copolymer-1 chromatograms were detected. Differential analysis was conducted using Soft Independent Modeling of Class Analogy (SIMCA). SIMCA class projections were generated in Pirouette 4.0 and interclass distances were calculated. These analyses revealed minimal variation between glatiramer acetate lots, while significant differences were shown between glatiramer acetate lots and Copolymer-1.

NOTES:
Work Flow for At-line Glycosylation Analysis of Darbepoetin alfa during CHO Cell Cultivation

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Glycosylation has a huge influence on the biological function of proteins. In the case of erythropoietin (EPO) and its derivatives (like Darbepoetin alfa), increasing grade of sialylation leads to decreasing association with the corresponding receptor \cite{1} and reduced serum clearance \cite{2}. With better understanding of glycosylation effects on therapeutic proteins, the development of adequate process monitoring methods \cite{3} is required to assure the desired product quality. Analysis of glycosylation patterns is typically performed by Mass spectrometry (MS) in combination with separation methods like capillary electrophoresis (CE) or HPLC. There are three common analysis strategies using either the intact glycoprotein, glycopeptides generated by proteolytic cleavage, or N-glycans released by PNGase F treatment.

For relative quantification of glycans and monitoring variations at different time-points during cultivation, we used an established method of chemical derivatization (reductive amination) \cite{3}. This involves derivatization of the initial $t_n$-sample with $^{13}$C$_6$-anthranilic acid whereas all further $t_{n+x}$-samples were derivatized with $^{12}$C$_6$-anthranilic acid. 1:1 mixtures of $t_n$ and one further $t_{n+x}$ sample were analyzed and glycan-pairs were baseline detected with a mass difference of 6 Da. The major drawback of this approach is its time-consumption making it not capable for process concomitant analysis. In this work, we present a streamlined approach (based on \cite{3}) of quasi-quantitative MS glycan-analytic, for at-line monitoring of glycosylation pattern of Darbepoetin alfa, expressed in CHO cells. We successfully reduced the required overall time from sampling to MS result from exceeding 24h to only 8h (approx. one working day). This could be achieved by accelerating time-consuming glycan release, derivatization process and corresponding cleanup procedures.


\cite{3} Tep, S, Hincapie, M & Hancock, WS (2012) A MALDI-TOF MS method for the simultaneous and quantitative analysis of neutral and sialylated glycans of CHO-expressed glycoproteins. Carbohydr Res, 347, 121-129.

NOTES:
NOTES:
Characterization of Various IgG Platforms by Native Mass Spectrometry

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The successful use of native mass spectrometry (MS) studying Ab-based pharmaceuticals has been demonstrated for intact IgG, mAb mixtures and antibody drugs conjugates (ADCs).\textsuperscript{1-3} It allows fast, reproducible characterization of mAb composition including its molecular heterogeneity, in a qualitative and even quantitative manner. Here we highlight how this unique MS-based platform brings along new opportunities to gain insights into field of therapeutic mAbs analysis.

Using native MS we propose new strategies to validate glycan structure assignments on intact IgG using various exoglycosidases. Advantages of native MS in conjunction with the specificity of individual glycosidase enzyme facilitates the quantification of each glycoforms in IgG mAbs. This approach simplifies the analysis of the glycan fingerprinting to a great extent, as it excludes the laborious and possibly less reproducible steps of glycan release, derivatization, labeling and HPLC separation.\textsuperscript{3}

By taking advantages of a high resolution Orbitrap mass spectrometer with an extended mass range and equipped with a high-mass quadrupole, we investigated by native MS the drug load, drug-localization and the effect of drug binding on the antibody-drug conjugates (ADC) structural integrity. We show numerous advantages of this novel workflow.

Finally, we will demonstrate how native MS was used to optimize the propensity of IgGs to hexamerize in solution. Following this optimization stable IgG hexamers were found to bind with high efficiency to the C1q complement system, thereby activating the initial complement activation step tremendously.\textsuperscript{4}

References:


NOTES:
Development of Fast Photochemical Oxidation of Protein (FPOP) Platform for Protein Therapeutics: Validation and Application

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Unlike small molecule drugs (< 600 Da), protein therapeutics (> kDa) undergo complicated folding and form higher order structures (HOS), posing a challenge for traditional analytical methods. Mass spectrometry-based protein footprinting can provide structural information and bring high sensitivity, fast turnaround, and small sample consumption. We report here the development of FPOP platform for HOS characterization of protein therapeutics.

Advantages of FPOP include fast labeling, targeting side chains, and making irreversible modifications, making FPOP complementary to hydrogen deuterium exchange. For characterization of protein therapeutics, FPOP requires monitoring protein conformations in native environments with high reproducibility. An asymmetrical mixing was integrated into the FPOP setup to allow proteins to undergo minimal exposure to H2O2. Each step is now more accurately controlled by time to increase precision. We used cyt c to evaluate these improvements. Changes in FPOP modification patterns were consistently observed. Only laser-plus-hydrogen peroxide gave high level modifications (+16, +32...) than the two control experiments (laser only and H2O2 only). The FPOP modification pattern was quantitatively analyzed by custom-built software. The variation in modification extent is dramatically smaller for the modified platform (69.9 ± 0.8%) compared to the original platform (75.0 ± 3%). The modification level is lower for the improved platform, which we attribute to a reduction in non-FPOP-induced oxidation. The newly developed FPOP platform can now be used to evaluate the results from experiments at different times and laboratories. Different scavenger and buffer can be tested using the modified platform. We observed a similar FPOP modification pattern to that of the typical FPOP condition (Gln as scavenger or PBS as buffer). We envision that this modified platform will be useful for characterization of the HOS of protein therapeutics.

NOTES:
Late Breaking

LB-01

Initial Characterization of a Novel Cysteine Protease for Antibody Sub Unit Analysis

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The IdeS enzyme (FabRICATOR) has emerged as a powerful tool for rapid structural characterization of antibody based biotherapeutics. Due to the extremely high specificity and favorable reaction conditions the combination of IdeS with high-resolution mass spectrometry allows for rapid characterization of many critical quality attributes.

IdeS digests antibody molecules just below the hinge region. In order to carefully examine the hinge region characteristics and cover the full Fc fragment using the same characterization approach it is desirable to cleave the IgG molecule above the hinge region with very high specificity and at one single cleavage site. Preferably using an enzyme similar to IdeS that is efficient at mild non-reducing conditions. In this study we have identified and characterized a cysteine protease, which have shown a very high specificity when digesting human IgG1. The enzyme digests human IgG1 above hinge generating two Fab fragments and one Fc fragment. Combining this new enzyme with IdeS enzyme allows for specific isolation of the complete hinge region of IgG1 in a non-reduced state with very high yield and accuracy.

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