# 15th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry (Mass Spec 2018)

## Symposium Co-chairs:

Michael Boyne, *COUR Pharmaceuticals Development Company*
Richard Rogers, *Just Biotherapeutics*

September 9-12, 2018
Hilton San Francisco Union Square
San Francisco, CA USA

*Organized by*

CASSS

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# Welcome to the 15th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry

We are pleased to welcome you to the 15th Symposium on the Practical Application of Mass Spectrometry in the Biotechnology Industry. The focus of this Symposium is the application of mass spectrometry (MS) for product characterization, process monitoring, formulation development and release testing in the pharmaceutical industry. Since mass spectrometry is used routinely 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. Although most of the applications may deal with biopharmaceuticals (proteins, DNA, viral vectors), applications for conventional pharmaceuticals will also be discussed. The Symposium will be held over a period of two and a half days devoted to practical concerns in the use of MS within the biotechnology and pharmaceutical industries.

The success of this symposium will depend not only on our experienced and knowledgeable speakers and 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 and roundtable sessions that have been designed to stimulate the 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 program partners and exhibitors. Thank you to AbbVie, Inc., Agilent Technologies, Biogen, Bioinformatics Solutions, Inc., Bruker Daltonics, Inc., Charles River Laboratories, Eli Lilly and Company, Genedata Inc., Genentech, a Member of the Roche Group, GenNext Technologies, Inc., Genovis AB, Merck & Co., Inc., Pfizer, Inc., Protein Metrics Inc., Roche Diagnostics GmbH, Sanofi, SCIEX, Shimadzu Corporation, Thermo Fisher Scientific, Waters Corporation and YMC America, Inc. We are also thankful for the expert assistance and support of 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 2019!

# CASSS Mass Spec Student Travel Grants

CASSS is pleased to provide a limited number of student travel grants for PhD students and post-doctoral fellows who present applicable posters at the 15th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry (Mass Spec 2018). 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. Topics will include the utility of MS as an alternative to conventional assays such as peptide mapping, ion exchange, capillary electrophoresis, as well as for the analysis of process-related impurities, such as host cell proteins; introduction of MS in the QC laboratory, validation/transfer/compliance strategy, system suitability/assay acceptance criteria; expectations from regulatory agencies for MS based assays for product characterization, release and stability; technical challenges regarding quantitation, ionization, higher order structure analysis, etc.; applications of MS for high throughput screening of cell-lines, raw materials, in-process samples; and benchmarking of new technology.

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

CASSS has awarded student travel grants to the following individuals:

***in-vivo* Characterization of Antibodies Using a Nano-HPLC –MS/MS Approach**
Annika Doell, *University Duisburg-Essen, Germany*

**Reversible Self-association (RSA): A Stumbling Block for Developing High Concentration IgG1 mAb Formulations**
Yue (Martin) Hu, *University of Kansas, USA*

**The Organizing Committee gratefully acknowledges the following program partners for their generous support of this Symposium**

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# Media Program Partners

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Technology Networks

# Mass Spec 2018
# Scientific Program Summary

## Sunday, September 9, 2018

| | |
|---|---|
| 08:00 – 09:00 | **Breakfast (Full Day Short Course Attendees ONLY)** in Union Square 21 (4th Floor) |
| 08:00 – 13:30 | **Short Course Registration ONLY** in the Golden Gate Foyer (Lobby Level) |

**Short Course** in Union Square 23 & 24 (4th Floor)
Short Course Instructor: Anders Lund, *Synlogic, Cambridge, MA USA*

| | |
|---|---|
| 08:30 – 12:00 | **Fundamentals of Mass Spectrometry in the Analysis of Protein Therapeutics**<br>Anders Lund, *Synlogic, Cambridge, MA USA* |
| 12:00 – 13:00 | **Lunch (Full Day Short Course Attendees ONLY)** in Union Square 21 (4th Floor) |
| 13:00 – 17:00 | **Applications of Mass Spectrometry to Characterize Protein Therapeutics**<br>Anders Lund, *Synlogic, Cambridge, MA USA* |

## User Group Meetings

| | |
|---|---|
| 12:00 – 16:00 | **908 Devices User Group** in Union Square 3 & 4 (4th Floor) |
| 13:45 – 16:00 | **Agilent Technologies User Group** in Union Square 1 & 2 (4th Floor) |

# Monday, September 10, 2018

07:30 – 08:30 **Breakfast** in the Golden Gate Ballroom

07:30 – 17:45 **Registration for Mass Spec 2018** in the Golden Gate Foyer (Lobby Level)

08:30 – 08:45 **Welcome and Introductory Comments** in Plaza Room A
Michael Boyne, *COUR Pharmaceuticals Development Company, Northbrook, IL USA*

**Keynote I Session** in Plaza Room A
Session Chair: Michael Boyne, *COUR Pharmaceuticals Development Company, Northbrook, IL USA*

08:45 – 09:45 **Unraveling the Structural Heterogeneity of Recombinant Protein Therapeutics with High-resolution Mass Spectrometry: A Personal Journey**
Jason Rouse, *Pfizer, Inc., Andover, MA USA*

09:45 – 10:15 **Networking Break** – Visit the Exhibits and Posters in the Golden Gate Ballroom

**Process and Product Characterization Session** in Plaza Room A
Session Chairs: Yelena Lyubarskaya, *Sanofi, Westborough, MA USA*
and Sarah Rogstad, *CDER, FDA, Silver Spring, MD USA*

10:15 – 10:45 **Quantitative Analysis of Glycation and Its Impact on Antigen Binding**
Jingjie Mo, *Janssen R&D, LLC, Malvern, PA USA*

10:45 – 11:15 **Identification of Multiple Serine to Asparagine Sequence Variation Sites in an Intended Copy Product Of LUCENTIS® by Mass Spectrometry**
Francois Griaud, *Novartis Pharma AG, Basel, Switzerland*

11:15 – 11:45 **A Hybrid Intact MS and Peptide Mapping Approach for Elusive Sequence Variant Detection during Cell Line Development**
Thomas Slaney, *Bristol-Meyers Squibb Company, Pennington, NJ USA*

11:45 – 12:00 **Lunch for Technical Seminar Attendees** – Please take lunch and return to Plaza Room A for the "Lunch and Learn"

# Monday, September 10, 2018 continued

12:00 – 12:30 **Technical Seminar: Lunch and Learn**

**Characterization and Monitoring of Post Translational Modifications Using Dedicated MAM Software**
Zoe Zhang, *SCIEX, Redwood City, CA USA*

Sponsored by SCIEX **Plaza Room A**

12:30 – 13:00 **Technical Seminar: Lunch and Learn**

**Automated Analysis of MS Data from a Diverse Biomolecular Portfolio**
Jasmin Sydow-Andersen, *Novo Nordisk A/S, Måløv, Denmark*

Sponsored by Genedata Inc. **Plaza Room A**

13:00 – 14:30 **Poster Session I** in the Golden Gate Ballroom

**Antibody Conjugates Session** in Plaza Room A
Session Chairs: David Passmore, *RubrYc Therapeutics, Redwood City, CA USA*
and John Valliere-Douglass, *Seattle Genetics, Inc., Bothell, WA USA*

14:30 – 15:00 **Identification and Monitoring of Potential Critical Quality Attributes of an Antibody-drug Conjugate by Mass Spectrometry to Support Process Characterization**
Xuan Chen, *ImmunoGen, Inc., Waltham, MA USA*

15:00 – 15:30 **Heightened Characterization of ADCs: Overcoming Challenges to Support Process and Product Understanding**
Olga Friese, *Pfizer, Inc., Chesterfield, MO USA*

15:30 – 16:00 **An Automated Quantitative Mass Spectrometry Assay to Help Select Next-generation Anti-staphylococcus Aureus Antibody-antibiotic Conjugates by Evaluating Intra-cellular Retention Kinetics of Released Antibiotics**
Hilda Hernandez-Barry, *Genentech, a Member of the Roche Group, South San Francisco, CA USA*

16:00 – 16:15 **Moving Break –** Please make your way to the Franciscan Ballroom on the Ballroom Level

16:15 – 17:15 **Roundtable Session** in the Franciscan Ballroom

17:15 – 18:30 **Welcome Reception** in Grand Ballroom A

# Tuesday, September 11, 2018

07:30 – 08:30 **Breakfast** in the Golden Gate Ballroom

08:00 – 18:00 **Registration** in the Golden Gate Foyer

**Keynote II Session** in Plaza Room A
Session Chair: Christopher Yu, *Genentech, a Member of the Roche Group, South San Francisco, CA USA*

08:30 – 09:30 **Deciphering Protein Biology Using Mass Spectrometry**
Alma Burlingame, *University of California, San Francisco, San Francisco, CA USA*

09:30 – 10:00 **Networking Break** – Visit the Exhibits and Posters in the Golden Gate Ballroom

**Emerging Product Areas and Methods Session** in Plaza Room A
Session Chairs: Jason Rouse, *Pfizer, Inc., Andover, MA USA*
and Arjen Scholten, *Janssen Vaccines and Prevention, Leiden, Netherlands*

10:00 – 10:30 **Proteomic Characterization of Exosomes and Their Impurities**
Damian Houde, *Codiak BioSciences, Cambridge, MA USA*

10:30 – 11:00 **Mass Spectrometry in the CAR-T Development Lab**
Ken Prentice, *Juno Therapeutics, Seattle, WA USA*

11:00 – 11:30 **Assessment of Susceptible Chemical Modification Sites of Trastuzumab and Endogenous Human Immunoglobulins at Physiological Conditions**
Ingrid Grunert, *Roche Diagnostics GmbH, Penzberg, Germany*

11:30 – 11:45 **Lunch for Technical Seminar Attendees** – Please take lunch and return to Plaza Room A for the "Lunch and Learn"

11:45 – 12:15 **Technical Seminar: Lunch and Learn**

**How PASEF Scans Take Peptide-based Characterization Workflows to the Next Level**
Guillaume Tremintin, *Bruker Daltonics, Inc., San Jose, CA USA*

Sponsored by Bruker Daltonics, Inc. **Plaza Room A**

# Tuesday, September 11, 2018 continued

12:15 – 12:45 **Technical Seminar: Lunch and Learn**

**From Automated Sample Preparation to Streamlined Data Analysis: A Complete Analytical Solution for RapiFluor-MS Labeled N-linked Glycan Analysis**
Ying Qing Yu, *Waters Corporation, Milford, MA USA*

Sponsored by Waters Corporation **Plaza Room A**

12:45 – 13:00 **Mini Break**

**Young Scientist Session** in Plaza Room A
Session Chair: Steven Cohen, *SAC Analytical Consultants, Hopkinton, MA USA*

13:00 – 13:30 **Spatiotemporally-resolved Proteomics with Next-generation Enzymatic Proximity Labeling Methods**
Shuo Han, *Stanford University, Stanford, CA USA*

13:30 – 14:00 ***in-vivo* Characterization of Antibodies Using a Sensitive Nano-HPLC –MS/MS Approach**
Annika Doell, *University Duisburg-Essen, Essen, Germany*

14:00 – 14:30 **Reversible Self-association (RSA): A Stumbling Block for Developing High Concentration IgG1 mAb Formulations**
Yue (Martin) Hu, *University of Kansas, Lawrence, KS USA*

14:30 – 16:00 **Poster Session II** in the Golden Gate Ballroom

16:00 – 17:30

**Recombinant Protein Therapeutics Workshop: To Sequence or Not to Sequence**
in Plaza Room A
Introductory Speaker: Karen Lee, *Sanofi, Framingham, MA, USA*
Facilitator: Nadine Ritter, *Global Biotech Experts, LLC, Germantown, MD USA*
Workshop Chair: Anders Lund, *Synlogic, Cambridge, MA USA*

17:30 – 18:30 **Exhibitor Reception -** Visit the Exhibits in the Golden Gate Ballroom

# Wednesday, September 12, 2018

07:30 – 08:30 **Breakfast** in the Golden Gate Ballroom

08:00 – 16:15 **Registration** in the Golden Gate Foyer

**Keynote III Session** in Plaza Room A
Session Chair: Richard Rogers, *Just Biotherapeutics, Seattle, WA USA*

08:30 – 09:30 **Computational Strategies for Improving the Quantitative Analysis of Peptides by Tandem Mass Spectrometry**
Michael MacCoss, *University of Washington, Seattle, WA USA*

09:30 – 10:00 **Networking Break** - Visit the Exhibits and Posters in the Golden Gate Ballroom

**Mass Spec Software - Data Process and Analysis Session** in Plaza Room A
Session Chairs: Richard Rogers, *Just Biotherapeutics, Seattle, WA USA*
and Ingo Lindner, *Roche Diagnostics GmbH, Penzberg, Germany*

10:00 – 10:30 **Driving Efficiency in Pre-clinical Development with Automated Mass Spectrometry Analysis and Characterization of Novel Biologics**
Hirsh Nanda, *Janssen R&D, LLC, Spring House, PA USA*

10:30 – 11:00 **Quantifying Proteomes Using the Open-source Trans-proteomic Pipeline**
Michael Hoopmann, *Institute for Systems Biology, Seattle, WA USA*

11:00 – 11:30 **Seeing Things Differently: Innovation in Computational Mass Spectrometry**
Rob Smith, *University of Montana, Missoula, MT USA*

11:30 – 11:45 **Lunch for Technical Seminar Attendees** – Please take lunch and return to Plaza Room A for the "Lunch and Learn"

11:45 – 12:15 **Technical Seminar: Lunch and Learn**

**Molecular Characterization Using Native Chromatographies Coupled to Mass Spectrometry**
Wendy Sandoval, *Genentech, a Member of the Roche Group, South San Francisco, CA USA*

Sponsored by Protein Metrics Inc. **Plaza Room A**

# Wednesday, September 12, 2018 continued

12:15 – 12:45 **Technical Seminar: Lunch and Learn**

**Applications of Multi-Attribute Method (MAM) for Control of Critical Quality Attributes of Protein Therapeutics**
Da Ren, *Amgen Inc., Thousand Oaks, CA USA*

Sponsored by Thermo Fisher Scientific **Plaza Room A**

12:45 – 13:00 **Mini Break**

**CMC Strategy and the Use of Mass Spec in the QC Lab Session** in Plaza Room A
Session Chair: Frances Namuswe, *CDER, FDA, Silver Spring, MD USA*

13:00 – 13:30 **Multi-Attribute Method (MAM) Evaluations and Regulatory Considerations for Implementation**
Sarah Rogstad, *CDER, FDA, Silver Spring, MD USA*

13:30 – 14:00 **Interlaboratory Performance Metrics from the MAM Consortium New Peak Detection Round Robin Study**
Trina Mouchahoir, *NIST/IBBR, Rockville, MD USA*

14:00 – 14:30 **Technological Advances in Host Cell Protein Testing and Risk Management: Enhancing the Quality of Clinical Products**
Ned Mozier, *Pfizer, Inc., Chesterfield, MO USA*

14:30 – 15:00 **Panel Discussion**

15:00 – 15:10 **Poster Award Announcement** in Plaza Room A

15:10 – 15:20 **Closing Comments** in Plaza Room A
Richard Rogers, *Just Biotherapeutics, Seattle, WA USA*

# Oral Abstracts

**Unraveling the Structural Heterogeneity of Recombinant Protein Therapeutics with High-Resolution Mass Spectrometry: A Personal Journey**

Jason Rouse

*Pfizer, Inc., Andover, MA USA*

Protein mass spectrometry (MS) began in earnest thirty years ago with the introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These two emergent ionization techniques, along with evolving mass analyzer geometries, would rapidly revolutionize protein characterization, starting with molecular mass determination, primary sequence elucidation and post-translational modification identification. Protein MS techniques quickly expanded into proteomics for large-scale protein identification, higher-order structure assessments, and large protein complex elucidation. Over the past 25+ years, MALDI and ESI MS have been essential technologies in the biotech industry for the heightened characterization of recombinant proteins, protein conjugates, viral vectors, and vaccines (and more) – both qualitatively, in terms of molecular integrity and proteoform identification, and more recently, quantitatively, with multi-attribute method and proteomic analysis of sequence variants and residual host cell proteins. During the development of glycoprotein therapeutics, MS-based heightened characterization, in conjunction with orthogonal biochemical analytics, provides detailed information about product quality, specifically sequence integrity, N-/C-terminal heterogeneity, N-/O-linked glycosylation, unique post-translational and chemical modifications, degradation pathways, and structure-function relationships, affording assurance that the intended molecule was successfully manufactured to support regulatory submissions and subsequent clinical trials. Secondly, MS excels at detecting protein structural changes via molecular mass and profile shifts. MALDI MS was employed early-on for assessing the effects of process improvements on product quality. With the arrival of the high-resolution ESI-quadrupole time-of-flight (QTOF) platform in 2000, MS became empowered to officially support "comparability exercises" with heightened characterization endpoints. Specifically, pre-change and post-change batches were compared rigorously by ESI-QTOF MS at the intact, subunit, and/or proteolytic peptide levels to assess predetermined process improvements and/or manufacturing site changes. As part of the CASSS Mass Spec conference 15-year anniversary, I will share my personal journey over the past 25 years in characterizing glycoprotein therapeutics, specifically discussing interesting case studies, trends and on-going challenges.

**NOTES:**

**Quantitative Analysis of Glycation and Its Impact on Antigen Binding**

Jingjie Mo, Renzhe Jin, Qingrong Yan, Izabela Sokolowska, Michael J. Lewis, Ping Hu

*Janssen R&D, LLC, Malvern, PA USA*

Glycation has been observed in antibody therapeutics manufactured by the fed-batch fermentation process. It not only increases the heterogeneity of antibodies, but also potentially affects product safety and efficacy. In this study, non-glycated and glycated fractions enriched from a monoclonal antibody (mAb1) as well as glucose-stressed mAb1 were characterized using a variety of biochemical, biophysical and biological assays to determine the effects of glycation on the structure and function of mAb1. Glycation was detected at multiple lysine residues and reduced the antigen binding activity of mAb1. Heavy chain Lys100, which is located in the complementary-determining region of mAb1, had the highest levels of glycation in both stressed and unstressed samples, and glycation of this residue was likely responsible for the loss of antigen binding based on hydrogen/deuterium exchange mass spectrometry analysis. Peptide mapping and intact liquid chromatography-mass spectrometry (LC-MS) can both be used to monitor the glycation levels. Peptide mapping provides site specific glycation results, while intact LC-MS is a quicker and simpler method to quantitate the total glycation levels and is more useful for routine testing. Capillary isoelectric focusing (cIEF) can also be used to monitor glycation because glycation induces an acidic shift in the cIEF profile. As expected, total glycation measured by intact LC-MS correlated very well with the percentage of total acidic peaks or main peak measured by cIEF. In summary, we demonstrated that glycation can affect the function of a representative IgG1 mAb. The analytical characterization, as described here, should be generally applicable for other therapeutic mAbs.

**NOTES**:

**Identification of Multiple Serine to Asparagine Sequence Variation Sites in an Intended Copy Product of LUCENTIS® by Mass Spectrometry**

Francois Griaud

*Novartis Pharma AG, Basel, Switzerland*

Patent expiration of first-generation biologics and the high cost of innovative biologics are 2 drivers for the development of biosimilar products. There are, however, technical challenges to the production of exact copies of such large molecules. In this presentation, a head-to-head comparison between the originator anti-VEGF-A Fab product LUCENTIS® (ranibizumab) and an intended copy product using an integrated analytical approach will be described. Published and unpublished data will be presented. While no differences could be observed using size-exclusion chromatography, capillary electrophoresis-sodium dodecyl sulfate and potency assays, different acidic peaks were identified with cation ion exchange chromatography and capillary zone electrophoresis. Further investigation of the intact Fab, subunits and primary sequence with mass spectrometry demonstrated the presence of a modified light chain variant in the intended copy product batches. This variant was characterized with a mass increase of 27.01 Da compared to the originator sequence and its abundance was estimated in the range of 6–9% of the intended copy product light chain. MS/MS spectra interrogation confirmed that this modification relates to a serine to asparagine sequence variant found in the intended copy product light chain. We demonstrated that the integration of high-resolution and sensitive orthogonal technologies was beneficial to assess the similarity of an originator and an intended copy product.

**NOTES:**

**A Hybrid Intact MS and Peptide Mapping Approach for Elusive Sequence Variant Detection during Cell Line Development**

Thomas Slaney, Hangtian Song, Neil Hershey, Erik Langsdorf, Wei Wu, Li Tao

*Bristol-Myers Squibb Company, Pennington, NJ USA*

Mass spectrometry has become an indispensable technology in process development for biologics, mainly due to the comprehensive information it provides and the sensitivity it achieves. Nevertheless, the detection and elucidation of sequence variants in therapeutic proteins by mass spectrometry still presents a significant challenge, since variants can be generated by multiple mechanisms and are usually at low levels. Although highest sensitivity is typically obtained with peptide mapping assays, incomplete sequence coverage and atypical types of sequence variants still render some sequence variants undetected. We have therefore adopted a strategy combining intact and subunit mass analyses with peptide mapping to minimize chances of missing sequence variants during cell line development. The combination approach allowed us to detect uncommon sequence variants that would be hard to identify by regular peptide mapping analysis. For example, a Gln-to-Lys variant with a 0.04 Da mass shift was observed in peptide mapping but not intact MS, whereas a DNA frame shift mutation co-purified with our protein of interest was missed by routine peptide mapping searches but was readily detected by intact MS. Detailed description of the work flow and data analysis for these examples will be presented.

**NOTES:**

**Identification and Monitoring of Potential Critical Quality Attributes of an Antibody-drug Conjugate by Mass Spectrometry to Support Process Characterization**

Xuan Chen

*ImmunoGen Inc., Waltham, MA USA*

Antibody-drug conjugates (ADCs) are cancer-targeting biotherapeutic molecules that are composed of monoclonal antibodies linked to small molecule cytotoxic payload.Mirvetuximab soravtansine our lead compound, also known as IMGN853 , consists of the maytansinoid DM4 payload conjugated to M9346A antibody via sulfo-SPDB linker.

One of the important steps of drug development and preparation for regulatory filings is the establishment of a robust manufacturing process for the ADCs and their components (e.g., antibody, linker, cytotoxic payload). Assessment of the criticality of quality attributes is essential for planning process characterization activities. Structure-activity relationship (SAR) studies provide information about the biological impacts of product variants. SAR studies were performed by generating and/or isolating variants that were analyzed by physicochemical methods and their bioactivities were monitored using in vitro binding and cell-based assays. Mass spectrometry played a key role in identifying antibody and ADC product variants.

Process characterization studies evaluate the impact of process parameters on critical quality attributes with the intent of identifying critical process parameters. These studies use design-of-experiment approaches and generate a large number of samples from the multiple manufacturing process steps. Several attributes of the process characterization samples (e.g., oxidation, deamidation, trisulfides, and conjugation sites) were monitored by mass spectrometry. During the talk, the mass spectrometric methods and results will be presented and their importance on the development of consistent manufacturing processes will be discussed.

**NOTES:**

**Heightened Characterization of ADCs: Overcoming Challenges to Support Process and Product Understanding**

Olga Friese[1], Jacquelynn Smith[1], Paul Brown[1], Andrew Dawdy[1], Thomas Powers[1], Jason Rouse[2]

*[1]Pfizer, Inc., Chesterfield, MO USA, [2]Pfizer, Inc., Andover, MA USA*

The development of analytical methods for the characterization of antibody drug conjugates (ADCs) presents significant challenges given the complexity of ADCs resulting from the addition of the drug payloads to already complex antibodies. Mass spectrometry is used for in-depth characterization of ADCs to determine the level and sites of drug conjugation and heterogeneities present due to the conjugation chemistry. The typical MS approach includes mass analysis of both the intact and de-N-glycosylated 4-chain ADC, and two or three-part subunit/domain analysis followed by proteolytic mapping. These MS-based analyses are used to confirm the sequence fidelity of the ADC as well as extent and integrity of conjugation. Several challenges that were encountered during heightened characterization of ADCs by LC/MS will be presented. ESI MS analysis of many intact ADCs remains challenging due to extensive conjugation with hydrophobic linker-payloads, non-covalent subunits, and/or chemically unstable linkers. However, further method development to optimize the mobile phases during LC separation, ESI source and ion transfer parameters allow establishment of reliable MS-based assays for accurate drug-to-antibody ratio determination. Denaturing conditions of typical LC/MS analyses impede the successful detection of intact, 4-chain ADCs generated via cysteine site-directed chemistry approaches where hinge region disulfide bonds are partially reduced. However, this class of ADCs is detected intact reliably under non-denaturing SEC/MS conditions, also referred to as native MS. For ADCs with acid labile linkers such as one used for conjugation of calicheamicin, a careful selection of mobile phase composition is critical to retain intact linker-payload during LC/MS analysis. Increasing pH of the mobile phase prevented cleavage of a labile bond in the linker moiety and resulted in retention of the intact linker-payload. In-source fragmentation was also observed with typical ESI source parameters during intact ADC mass analysis for a particular surface accessible linker-payload moiety conjugated to the heavy chain C-terminal.

**NOTES:**

**An Automated Quantitative Mass Spectrometry Assay to Help Select Next-generation Anti-staphylococcus Aureus Antibody-antibiotic Conjugates by Evaluating Intra-cellular Retention Kinetics of Released Antibiotics**

Hilda Hernandez-Barry, Kimberly Kajihara, Daniel Tran, Martine Darwish, Richard Vandlen, Leanna Staben, Thomas Pillow, Wouter Hazenbos, Kelly Loyet

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

Methicillin-resistant Staphylococcus aureus is the leading cause of hospital-acquired infections, but it has become more difficult to treat due to evolved resistance to antimicrobial drugs and intracellular bacterial reserves in phagolysosomes. For this purpose, a new kind of ADC therapeutic has recently emerged, namely antibody-antibiotic conjugates (AACs). An AAC combines the binding specificity of an antibody with an antibiotic and can deliver the bactericidal activity needed for complete killing. However, this AAC has no direct antibacterial activity on *S. aureus* until the AAC opsonized bacteria is taken up by the host cells and the intracellular proteases act on the cathepsin cleavable linker and release the antibiotic in its active form.

Kinetics and concentration of free drug released intracellularly are a couple of key parameters that contribute to the efficacy of an AAC. To this end, we have developed an in vitro cell-based LC/MS drug release automated assay in a 96-well format in order to measure the intracellular concentration (ICC) of released antibiotic at specific time-points. This quantitative LC/MS assay employs a QTRAP® 6500 Sciex instrument in MRM mode for detection of the antibiotics of interest. The ICC has been determined for numerous AAC candidates in order to select an AAC that is both retained in the cell and efficacious at killing bacteria. When the ICC is greater than the minimum efficacious concentration to eliminate S. aureus at pH 5 (MEC5), it is a reasonable predictor of AAC potency. Thus, using ICC and MEC5 data together with catalytic cleavage and potency assays, we can more fully characterize the fate and action of the AAC payload release and ultimately guide the design of more effective and targeted AAC therapeutics.

**NOTES:**

**Deciphering Protein Biology Using Mass Spectrometry**

Alma Burlingame

*University of California, San Francisco, San Francisco, CA USA*

The number of human genes is relatively small and known, and the sequencing at both the DNA and RNA levels is now routine. However, protein studies at the detailed molecular level provide experimental challenges of daunting complexity by comparison.

Topics of current interest in protein biology include:

(1) direct measurement of nascent protein synthesis;

(2) modulation of protein function by posttranslational processes; and

(3) studies of the interactions of proteins and the architecture of protein complexes.

This presentation will discuss examples from these areas of our research where mass spectrometry has played a key role.

Acknowledgement:

Financial support has been provided by UCSF Program for Breakthrough Biomedical Research, the Adelson Medical Research Foundation and HHMI.

**NOTES:**

**Proteomic Characterization of Exosomes and Their Impurities**

Damian Houde, Rane Harrison

*Codiak BioSciences, Cambridge, MA USA*

Extracellular vesicles (EVs) are a heterogeneous population of nano-sized cell-derived membrane vesicles that are actively released into the extracellular space. Once released, EVs perform critical roles in intercellular communication by transferring their biological content (i.e., proteins, lipids, nucleic acids, and other compounds) between cells. Consequently, and because EVs are nontoxic and nonimmunogenic, they have become an attractive option for delivering pharmaceutical and biopharmaceutical payloads for a variety of medical conditions. However, given their inherent heterogeneity, EVs are often difficult to purify and characterize. Here, EVs from a human cell-line were purified by two different methods. The EV samples were then extensively characterized by proteomic analysis, which enabled the identification of proteins, their content, and supported the comparison of different isolated populations. These data were critical for the identification of EV associated proteins and proteinaceous particulate impurities from the host cell line, which helped drive improvements to the EV purification process.

**NOTES:**

**Mass Spectrometry in the CAR-T Development Lab**

Ken Prentice

*Juno Therapeutics, Seattle, WA USA*

Cell based therapeutics have been hailed as the third pillar of medicine and there has been rapid acceptance of this class of drugs in both academic and industry settings in the last few years. Specifically chimeric antigen receptor expressing t-cells (CAR T) have garnered significant attention with the recent FDA approval of the first cell-based immunotherapies for oncology. Cells and cell based therapeutics have been traditionally analyzed using immunochemistry (i.e. flow cytometry) or genomic (i.e. PCR/NGS) based assays. While mass spectrometry has had limited use in this class of therapies, most applications have focused on target discovery. This talk will focus on mass spectrometry as a powerful analytical tool for structural elucidation and process development support of CAR T products. Applications encompassing raw materials, cell culture, and CAR T protein detection and characterization will be discussed.

**NOTES:**

**Assessment of Susceptible Chemical Modification Sites of Trastuzumab and Endogenous Human Immunoglobulins at Physiological Conditions**

Ingrid Grunert

*Roche Diagnostics GmbH, Penzberg, Germany*

The quality control testing of chemical degradations in the bio-pharmaceutical industry is currently under controversial debate. Here we have systematically applied in vitro and in vivo stress conditions to investigate the influence of protein degradation on structure-function. Extensive purification and characterization enabled identification and functional assessment of the physiological degradation of chemical modification sites in the variable complementarity-determining regions (CDRs) and conserved region of trastuzumab. We demonstrate that the degradation of the solvent accessible residues located in the CDR and the conserved fragment crystallizable region (Fc) occurs faster in vivo (within days) compared to the levels observed for bio-process and real-time storage conditions. These results hence question the rationality of extreme monitoring of low level alterations in such chemical modifications as critical patient safety parameters in product quality control testing, given that these modifications merely mirror the natural/physiological aging process of endogenous antibodies.

**NOTES:**

**Spatiotemporally-resolved Proteomics with Next-generation Enzymatic Proximity Labeling Methods**

Shuo Han

*Stanford University, Stanford, CA USA*

The ability to characterize endogenous proteins – their structures, localization, trafficking, and function – within the native context of living cells is necessary to advance our understanding of cellular processes and pathologies. Traditional methods to study the protein constituents of subcellular compartments or their respective interactions are limited, and often require the use of perturbing conditions such recombinant protein tags, cellular lysis and fractionation. We will describe two recent technologies based on genetically-targetable enzymes that catalyze proximity-dependent biotinylation of endogenous proteins in living cells, which can be subsequently enriched and identified using mass spectrometry. Engineered ascorbate peroxidase (APEX) has been used to gain insight into the proteomic compositions of various cellular structures and macromolecular complexes that are traditionally inaccessible, including the synaptic clefts and several mitochondrial sub-compartments. In addition, APEX can also biotinylate RNAs for spatial transcriptomic analysis. BioID is a complementary method to APEX that utilizes a promiscuous biotin ligase to perform proximity-dependent labeling. However, while APEX labeling requires 1 minute, BioID requires long labeling periods of 18-24 hours. Using directed evolution, our lab has developed TurboID: a smaller, faster promiscuous biotin ligase that catalyzes proximity labeling upon 10-minute addition of biotin. Together, these new methods will enable scientists to map proteomes of organelles and signaling pathways, with high spatiotemporal resolution, in a wide range of cell types and model organisms.

**NOTES:**

## *in-vivo* Characterization of Antibodies using a Sensitive Nano-HPLC–MS/MS Approach

Annika Doell[1], Markus Hollmann[2], Oliver Schmitz[1]

[1]*University Duisburg-Essen, Essen, Germany,* [2]*AbbVie Deutschland GmbH & Co KG, Ludwigshafen, Germany*

Biotherapeutics are products of genetically engineered cells, and include hormones, regulatory peptides and proteins. In particular, therapeutic proteins have become increasingly important in the treatment of numerous severe diseases. Antibodies are an example of therapeutic proteins, which are used in medicine for treatment of e.g. autoimmune diseases as well as different types of cancer.

The analysis of protein modifications on peptide level using liquid chromatography coupled to mass spectrometry (LC-MS) is a common approach to characterize protein therapeutics. While protein characterization of *in-vitro* samples is performed routinely, the analysis of protein therapeutics after *in-vivo* administration has recently gained significant attention. There is a profound interest in getting an understanding of what happens to the biotherapeutic molecules *in-vivo* and correlating critical quality attributes with immunogenicity and bioavailability. With the Open Flow Microperfusion (OFM) approach, subcutaneously applied antibodies are extracted via probes from the interstitial fluid. Those samples are then analyzed using the sensitive Nano-HPLC-MS/MS approach.

The combination of the above-mentioned methods can potentially provide new insights into the field of antibody modifications that may occur in the subcutaneous layer. A deeper understanding of metabolization mechanisms after subcutaneous administration would bring a remarkable benefit for the patient's safety and also the development of biopharmaceutics.

**NOTES:**

**Reversible Self-association (RSA): A Stumbling Block for Developing High Concentration IgG1 mAb Formulations**

Yue (Martin) Hu

*University of Kansas, Lawrence, KS USA*

Monoclonal antibodies (mAbs) have become a class of drugs of high importance for treating numerous human diseases. Subcutaneous (SC) administration is an increasingly common and convenient route for patients to be able to do home-based treatments. Since mAbs often require high mg/kg dosing and SC injections are limited to a small injection volume (~1.5 mL), this necessitates the development of high-concentration mAb formulations. High concentration mAb solutions pose many pharmaceutical challenges including physical instability during manufacturing, storage, and delivery. In addition, reversible self-association (RSA) has emerged as an important formulation challenge in terms of significantly increasing solution viscosity, turbidity and even phase separation. In this work, four different human immunoglobulin G1 molecules were obtained from MedImmune with varying solution properties at high concentration. By evaluating mAbs with both "good" and "bad" solution properties at different protein concentrations in a single formulation buffer, the goal is to use hydrogen-deuterium exchange mass spectrometry (HX-MS) to identify peptide segments involved in the protein-protein interactions (PPIs) of the mAbs. These results will help to identify patterns that may lead to the identification of RSA "hot-spots" for high concentration mAb formulation issues. First, intact mass analysis and peptide mapping were performed to confirm sequences and post-translational modifications of different mAbs, trying to identify important chemical modification, truncation, glycosylation, and get high peptide coverage on the sequence. Second, HX-MS experiments were performed on mAbs solutions (in D2O) using lyophilization-reconstitution approach to obtain site-specific information on the regions of "bad" mAbs (peptide segments) that are primarily responsible for RSA at high concentrations. In addition, excipient effects on mAbs RSA were also examined by HX-MS on their ability of promoting or disrupting protein interactions. In terms of future work, HX-MS and biophysical data will be correlated to obtain a more comprehensive understanding of RSA behaviours of the four mAbs, and to determine if common motifs are present that may correspond to molecular "hot spots" of PPIs, and see if a particular RSA effect on solution correlates with a particular molecular hot spot sequence.

**NOTES:**

**Computational Strategies for Improving the Quantitative Analysis of Peptides by Tandem Mass Spectrometry**

Michael MacCoss

*University of Washington, Seattle, WA USA*

Proteomics technology has improved dramatically over the last decade. The technology developments have largely been directed around instrument hardware, where instruments have been developed that scan faster, are more sensitive, and have greater mass measurement accuracy. However, the basic workflow has remained largely unchanged -- mass spectrometers are directed toward the acquisition of tandem mass spectra on the most abundant molecular species eluting from a chromatography column. More recently, efforts have been focused on the acquisition of mass spectrometry data on target peptides of interest. With improvements in instrument hardware and instrument control software, the practical experimental difference between a targeted and discovery proteomics is beginning to become blurred. These analyses are a significant change from the traditional proteomics workflow and have required the development of novel computational strategies to analyze, visualize, and interpret these data. We will present work illustrating our efforts in the development of targeted proteomics and provide a vision for challenges that still need to be overcome before these analyses become routine and replace more traditional discovery proteomics methodology.

**NOTES:**

**Driving Efficiency in Pre-clinical Development with Automated Mass Spectrometry Analysis and Characterization of Novel Biologics**

Hirsh Nanda[1], Bo Zhai[1], Andy Mahan[1], Eric Carlson[2], Yong Kil[2], Li Jing[2], Andrew Nichols[2]

*[1]Janssen R&D, LLC, Spring House, PA USA, [2]Protein Metrics Inc., San Carlos, CA USA*

Large molecule therapeutics are susceptible to numerous posttranslational modifications and other variants which may have detrimental effects on efficacy, stability or immunogenicity. Mass spectrometry-based peptide mapping provides information on many important molecule attributes such as posttranslational modification, clipping, glycosylation and sequence variants. As biotherapeutic portfolios progress from mAbs to more complicated architectures (e.g. multi-specifics, scaffold proteins, ADCs and antigens) both the challenges and the demands for MS based characterization has only grown. Given the complexity of analysis, data processing often limits the throughput of the assays as it requires multiple software packages and extensive manual interpretation. To manage characterization and PTM analysis of multiple candidate molecules with fast turn-around times, an automated end-to-end data analysis pipeline has been developed to accelerate clone selection and developability characterization within pre-clinical development. The pipeline is a vendor agnostic end-to-end solution, which provides a UI for efficient data validation and report generation. Based on the Byonic MS/MS search engine and Byologic software (Protein Metrics) for label-free quantitation, search parameters were designed to detect in a single run for PTMs (oxidation, deamidation, isomerization), glycans, N/C-terminal heterogeneity such as unprocessed signal peptide or clipping as well as sequence variants. Examples from non-platform therapeutics will be presented and a comparison to other software solutions will be discussed.

**NOTES:**

**Quantifying Proteomes Using the Open-source Trans-proteomic Pipeline**

Michael Hoopmann[1], Jason Winget[2], Luis Mendoza[1], Robert Moritz[1]

*[1]Institute for Systems Biology, Seattle, WA USA, [2]Proctor & Gamble, Mason, OH USA*

The Trans-Proteomic Pipeline (TPP) is a suite of open-source, freely available software tools for the analysis of proteomes using mass spectrometry. Highly modular and customizable, the TPP is both a complete toolset and also capable of interfacing with many popular data analysis tools. While several quantitative proteomics workflows have been supported since its inception, recent developments have resulted in new tools for label-free quantitation of proteins from shotgun mass spectrometry. Here we present StPeter, a tool capable of implementing both Normalized Spectral Abundance Factor and Normalized Spectral Index quantification metrics. The tool has been seamlessly integrated into the TPP for reproducibility and ease of use. We demonstrate the use of the TPP to obtain quantitative protein results from shotgun mass spectra, and compare the quantitative methods of StPeter to many state-of-the-art stand-alone tools and packages. We also demonstrate that the software is computationally efficient and supports data from a variety of instrument platforms and experimental designs. An obvious advantage of using the TPP is it negates the need to manage inputs and outputs between the many software utilities required to perform quantitative analyses, dramatically streamlining quantitative workflows and simplifying user interactions. Results can be viewed within the TPP graphical user interfaces and exported in standard formats for downstream statistical analysis. The TPP is freely available at http://www.tppms.org and additional information about StPeter can be found in DOI:10.1021/acs.jproteome.7b00786

**NOTES:**

**Seeing Things Differently: Innovation in Computational Mass Spectrometry**

Rob Smith

*University of Montana, Missoula, MT USA*

By many accounts, innovation in mass spectrometry data processing has lagged far behind innovation in instrumentation. In this talk, we suggest strategies for accelerating innovation in computational mass spectrometry through questioning both what current limitations are and why they exist. The impact of greater problem understanding on innovation is explored, from explicit identification of problem assumptions to creating non-ambiguous vocabularies for describing problems in greater detail and accuracy. In aggregate, these approaches provide a practical pathway to greater innovation in mass spectrometry data processing.

**NOTES:**

**Multi-attribute Method (MAM) Evaluation and Regulatory Considerations for Implementation**

Sarah Rogstad

*CDER, FDA, Silver Spring, MD USA*

Currently, mass spectrometry (MS) is primarily used for drug substance characterization in the protein therapeutic field. However, there has been a recent push toward the use of MS-based methods for quality control (QC) purposes, collectively known as multi-attribute methods (MAM). As these methods have not been used previously in this context, new regulatory questions must be addressed prior to their full implementation. Such questions include whether these methods are fit for purpose, the extent of the capabilities of these methods, the ability of technicians to make quick pass/fail decisions, and how to implement and assess appropriate system suitability, method validation and comparisons with traditional methods. FDA has identified four major points to consider for MAM implementation: method validation, performance comparisons to traditional methods, capabilities and specificities of new peak detection, and risk assessment. A research program was developed to address these considerations to better assess the applicability of method. An in-house MAM platform was developed for the relative quantitation of specific product quality attributes (PQAs) of rituximab, which was chosen as a model protein. Samples included a range of expiration dates for both US approved and unapproved products. Analysis included method development, forced degradation, system suitability assessment, and comparisons to traditional methods. The study found high reproducibility between users with varying experience levels. However, higher coefficient of variation (CV) values (>15%) were found for lower abundance PQAs (<5%). These data were found to be generally consistent with data from released glycan HILIC profiling analyses. During a forced degradation study with US approved rituximab (40 °C/75% RH for 28 days), oxidation and deamidation were found to increase linearly over the time course while C-terminal lysine clipping, N-terminal pyroglutamination and glycosylation did not show significant changes over time. Using the new peak detection feature of the method, one peak, that was not specifically targeted in the analysis, was found to increase over the time course (> 10-fold change in area, peak intensity threshold of 0.1% of the TIC). This peak was determined to be an isoaspartic acid modification. Additional studies may be needed to further examine the efficacy of the new peak detection feature. Forced degradation results were also compared to results from orthogonal methods. Charge variant analysis showed an increase in acidic peaks over time. MAM deamidation data was found to have a linear correlation ($R2 = 0.94$) with the acidic peak data. Further assessment and statistical analyses will be conducted on this relationship as well as on the clipped variant data. System suitability testing approaches were also assessed.

**NOTES:**

**Interlaboratory Performance Metrics from the MAM Consortium New Peak Detection Round Robin Study**

Trina Mouchahoir[1], John Schiel[1], Richard Rogers[2]

*[1]NIST/IBBR, Rockville, MD USA, [2]Just Biotherapeutics, Seattle, WA USA*

The Multi-attribute Method (MAM) is an emerging application of ultrahigh-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) useful for simultaneous monitoring of multiple biopharmaceutical product quality attributes. The MAM Consortium was initially formed as a venue to share regulatory experiences, harmonize best practices, and generate innovative methodology to facilitate widespread integration. Its members recently contributed to a new peak detection (NPD) inter-laboratory study to evaluate performance metrics and reproducibility of the MAM utilizing pre-digested samples of the NISTmAb RM 8671. Evaluation of the data focused on attribute analytics as well as the NPD component of MAM. The initial results provide a global overview of the current state of the industry as well as valuable insight regarding the robustness and reproducibility of the MAM NPD platform.

**NOTES:**

**Technological Advances in Host Cell Protein Testing and Risk Management: Enhancing the Quality of Clinical Products**

Ned Mozier

*Pfizer, Inc., Chesterfield, MO USA*

Improvements in databases and mass spectrometry (MS) technology have radically changed the landscape for HCP testing and impurity management. The oft debated question as to whether MS has a function in the quality control testing laboratory is no longer hindered by its capabilities, although this remains a strategic decision for corporations. The power of MS for identification of HCPs is now common in development, especially in regard to process development and for risk management. The immunoassay remains the bedrock by which most quality decisions are made, but increasingly MS is being used as an orthogonal method. This has enabled more useful risk analysis and intelligent decision-making. Together, immunoassay and MS are the most important tools for managing the risk of impurities such as HCPs and have led to higher quality products. How various technologies are applied during the development paradigm has evolved and been accelerated with the advent of biosimilars. This presentation will review the known and unknown risks of HCPs and provide cases where individual proteins have been shown to affect clinical trials, portfolio decisions and how the technological innovations have influenced this direction.

**NOTES:**

**NOTES:**

# Recombinant Protein Therapeutics Workshop: To Sequence or Not to Sequence

**Tuesday, September 11**
**16:00 – 17:30**
**Plaza Room A**

**Workshop Chair:**
Anders Lund, *Synlogic, Cambridge, MA USA*

**Facilitator:**
Nadine Ritter, *Global Biotech Experts, LLC, Germantown, MD USA*

**Speaker:**
Karen Lee, *Sanofi, Framingham, MA USA*

**Scribes:**
Steve Cohen, *SAC Analytical Consultants, Hopkinton, MA USA*
Yelena Lyubarskaya, *Sanofi, Westborough, MA USA*

Our industry is constantly changing and evolving. This workshop is focused on the use of LCMS and peptide mapping in the primary characterization of protein and peptide biologics.

One of our goals for this meeting is to write a "peptide mapping best practices" document. This document will be used to help discern the guidance around LCMS analysis of biopharmaceutical products, specifically protein based biotherapeutics. To help, we invited all Mass Spec 2018 attendees to contribute by filling out a survey. We are trying to recognize if there are industry best practices around protein characterization and protein lot release.

As a preface to this workshop, ICH guidelines Q5B and Q6B for novel molecular entities describe the analysis of the expression constructs, specifically "Segments of the expression construct should be analyzed using nucleic acid techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the final product." Given this statement, how do we as an industry apply "other tests"? Please participate in the discussion – ask QUESTIONS, add your observations! Our goal is to use the data from the survey (and the discussion from the workshop) to compile a best practices document to be published late 2018, or early 2019 by the CASSS Mass Spec Organizing Committee.

**NOTES:**

**NOTES:**

# Roundtable Discussion

## Monday, September 10
## 16:15 – 17:15

There are 15 roundtable topics. The plan is for these to be active discussions, not presentations or lectures. To create useful discussion, we are going to try and limit each topic to 10 attendees. **Seating will be on a first come, first serve basis**. These discussions will include a facilitator, whose role is to help assist the discussion and ensure a lively exchange, and a scribe, whose role is to make general, anonymous notes about the discussion that will be posted on the Mass Spec 2018 website.

Listed below is a quick view of the Roundtable Topics, Facilitators and Scribes:

**Table 1: Challenges During Regulatory Submissions**
Facilitator: Ramsey Saleem, *Amgen Inc.*
Scribe: John Valliere-Douglass, *Seattle Genetics, Inc.*

**Table 2: Process Analytics Technology and Mass Spectrometry**
Facilitator: Ananya Dubey, *Waters Corporation*
Scribe: Nalini Sadagopan, *Agilent Technologies*

**Table 3: MS in the Biomanufacturing Environment**
Facilitator: Hirsh Nanda, *Janssen R&D LLC*
Scribe: Sean McCarthy, *SCIEX*

**Table 4: Host Cell Protein Analysis**
Facilitator: Annemiek Verwilligen, *Janssen Vaccines and Prevention*
Scribe: Sarah Rogstad, *CDER, FDA*

**Table 5: Automation**
Facilitator: Sreekanth Suravajjala, *Amgen Inc.*
Scribe: Kristin Boggio, *Pfizer, Inc.*

**Table 6: *in vivo* Biotransformation Analysis Strategies and Real Lab Experiences**
Facilitator: Olga Friese, *Pfizer, Inc.*
Scribe: Yelena Lyubarskaya, *Sanofi*

**Table 7: Comparability**
Facilitator: Karen Lee, *Sanofi*
Scribe: Jason Rouse, *Pfizer, Inc.*

**Table 8: MS/MS Sequencing of Proteins, Do We Need to Do It and When?**
Facilitator: Bruno Genet, *Sanofi*
Scribe: Anders Lund, *Synlogic*

**Table 9: Characterization of Antibody Drug Conjugates (ADCs)**
Facilitator: Midori Greenwood-Goodwin, *Genentech, a Member of the Roche Group*
Scribe: Sunnie Kim, *Seattle Genetics, Inc.*

**Table 10: Forced Degradation**
Facilitator: Tom Chen, *Bayer*
Scribe: Jason Gilmore, *Seattle Genetics, Inc.*

**Table 11: Deep Dive into Genesis of Trisulfide Formation and Analysis By MS**
Facilitator: Zhiqi Hao, *Genentech, a Member of the Roche Group*
Scribe: Delia Li, *Genentech, a Member of the Roche Group*

**Table 12: Intact Analysis/Top Down Analysis**
Facilitator: Srikanth Kotapati, *Bristol-Myers Squibb Company*
Scribe: David Passmore, *RubrYc Therapeutics Inc.*

**Table 13: System Suitability Strategies for Mass Spectrometers and LC/MS Methods**
Facilitator: Ying Zhang, *Pfizer, Inc.*
Scribe: Frances Namuswe, *CDER, FDA*

**Table 14: Challenges for Bi-specifics/Tri-specifics**
Facilitator: Greg Staples, *Agilent Technologies*
Scribe: Nicole Liu, *Genentech, a Member of the Roche Group*

**Table 15: Software**
Facilitator: Joe Shambaugh, *Genedata, Inc.*
Scribe: Brian Gau, *Pfizer, Inc.*

# Technical Seminars

**Technical Seminar: Lunch and Learn**
**Monday, September 10**
**12:00 – 12:30**
**Plaza Room A**

**Characterization and Monitoring of Post Translational Modifications Using Dedicated MAM Software**

Fan Zhang[2], Zoe Zhang[2], Sean McCarthy[1]

*[1]SCIEX Framingham, MA USA, [2]SCIEX Redwood City, CA USA*

Development and production of biopharmaceuticals are complex and challenging. Frequently the assessment of post-translational modifications including deamidation, oxidation, glycosylation, glycation among many others must be assessed to understand their impact on safety and efficacy of the final product. As the molecular complexity and the challenges for commercialization increases, there is a need for enhanced analytical approaches to detect and track a wide range of post-translational modifications throughout the development of biotherapeutics. Traditionally, multiple analytical techniques have been required to assess the full range of biopharmaceutical product attributes; However, the application of mass spectrometry has recently been applied for simultaneous detection and quantification of a wide range of molecular properties which can supplement or potentially replace traditional orthogonal assays.

Peptide mapping with mass spectrometry is a common assay for assessment of post-translational modifications. Recently the concept of a Multiple Attribute Methodology (MAM) has been introduced which places greater rigor on the use of LC-MS peptide map experiments as an orthogonal approach for characterization and monitoring of biopharmaceutical attributes. The range of modifications that can be identified and monitored using this approach is extensive. In addition, the ability to localize modifications is enhanced using an LC-MS approach compared to indirect measurements which may provide greater confidence in the correlation of characterization data with downstream outcomes. In addition to tracking the therapeutic molecule itself, an LC-MS approach using MAM may be used to detect specified and un-specified impurities related to the biotherapeutic production providing a purity assessment.

Presented here is the use of High-Resolution Accurate Mass data processed using BioPharmaView™ 3.0 Software for characterization and quantification of a range of post-translational modifications of a biotherapeutic. We will also discuss the use of this solution for quantification of specified impurities and highlight the ability to detect un-specified impurities using new peak detection.

**NOTES:**

**Technical Seminar: Lunch and Learn**
**Monday, September 10**
**12:30 – 13:00**
**Plaza Room A**

**Automated Analysis of MS Data from a Diverse Biomolecular Portfolio**

Jasmin Sydow-Andersen

*Novo Nordisk A/S, Måløvm, Denmark*

The wide range of vendor-specific raw data formats as well as a large variety of potential modifications to be analyzed can make MS characterization of a diverse biomolecular portfolio challenging. At Novo Nordisk, we analyze LC-MS/MS data sets ranging from small peptides to large protein complexes derived from our portfolio comprising GLP-1, insulin, coagulation factors, and antibodies. This portfolio of different proteins represents a significant analytical challenge, as they all potentially carry different kinds of modifications (γ-carboxylations, disulfide bonds, glycans, etc.), which in turn require specific MS methods.

Peptides and proteins are analyzed using many different MS instruments from different vendors, each with their own specific data formats, software tools, and applications. While accelerating data processing is important, because complex stability studies contain numerous data sets, harmonization of analyses from different applications and instruments on one platform can also contribute substantially to increased productivity. Both these factors are addressed by Genedata Expressionist; which not only automates data processing using a workflow-based approach, but also supports a wide variety of data sources and allows implementation of any MS application or method.

Here we show how these concepts come together in the analysis of comprehensive data sets and discuss the challenges associated with current methods. We focus on a workflow for peptide map evaluation of a model antibody; which uses chemical-noise reduction, retention-time alignment, peak detection, and isotope clustering as powerful data processing tools to obtain high-quality results. Charge and adduct grouping can be used to provide highly accurate peptide quantification, and a range of filters can be applied to focus on certain areas of molecules, such as antibody CDRs.

**NOTES:**

**Technical Seminar: Lunch and Learn**
**Tuesday, September 11**
**11:45 – 12:15**
**Plaza Room A**

**How PASEF Scans Take Peptide-based Characterization Workflows to the Next Level**

Guillaume Tremintin

*Bruker Daltonics, Inc., San Jose, CA USA*

This seminar will focus on the benefits that can be derived from PASEF MS/MS scans (Parallel Accumulation and Serial Fragmentation) for the rapid and sensitive analysis of protein digests. The implementation of trapped ion mobility (TIMS) on the Bruker timsTOF Pro enables a new scan mode, PASEF, which dramatically improves MS/MS speed (>100 Hz) and sensitivity at the same time. This enables high throughput proteomics with >1400 proteins identified from a HeLa digest with a 5 min gradient.

The examples presented will cover how traditional peptide mapping tasks such as primary sequence verification and PTM analysis can be significantly accelerated with much less material. In addition, the improved sensitivity can be utilized to improve the confidence of host protein identification (HCP). HCP coverage in the ppm range can be achieved with short gradients, while longer methods yield unprecedented identification rates from a simple 1D-LC setup.

**NOTES:**

**Technical Seminar: Lunch and Learn**
**Tuesday, September 11**
**12:15 – 12:45**
**Plaza Room A**

## From Automated Sample Preparation to Streamlined Data Analysis: A Complete Analytical Solution for RapiFluor-MS Labeled N-linked Glycan Analysis

Ying Qing Yu

*Waters Corporation, Milford, MA USA*

Glycosylation is an important attribute of biopharmaceutical products to monitor from development through manufacturing. However, glycosylation analysis has traditionally been a time-consuming process with long sample preparation protocols and manual interpretation of the data. To address the challenges associated with glycan analysis, we have developed a streamlined analytical solution that covers the entire analysis from automated sample preparation to data analysis. In this presentation, we will discuss the recent implementation of automation platforms including Hamilton, Tecan, and Andrew Alliance for the GlycoWorks N-linked Glycans sample preparation kit with RapiFluor-MS (RFMS) labeling chemistry. The combination of automated sample preparation with Waters rapid RFMS labeling technology provides a scalable yet flexible solution that offers significant time savings and increased data consistency in the analysis of glycans.

To support the streamlined sample preparation, Waters has developed an efficient analytical data processing workflow for structural assignment of RFMS labeled N-glycans using the compliant-ready UNIFI Scientific Information System. Following HILIC-UPLC/FLR/MS analyses, complementary FLR and MS data is processed simultaneously in an automated fashion to expedite the task of structural assignment and relative glycoform quantification. Using the integrated glycan structural library, a search based on calibrated retention time in glucose units (GU) and accurate mass are used in the identification and structural elucidation of released glycans. Data generated from mAbs and fusion proteins are discussed to show the full capability of this enabling analytical workflow.

**NOTES:**

**Technical Seminar: Lunch and Learn**
**Wednesday, September 12**
**11:45 – 12:15**
**Plaza Room A**

**Molecular Characterization Using Native Chromatographies Coupled to Mass Spectrometry**

Wendy Sandoval

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

We employ native chromatographies coupled to high resolution mass spectrometry to probe the extent and impact of minor antibody variants, post-translational modifications and monitor correct assembly. Intact mass analysis of separated moieties allows for a facile and complete description of the molecule in question. Charge variant native mass spectrometry (CVMS), an integrated native ion exchange mass spectrometry-based charge variant analytical approach that delivers detailed molecular information in a single, semi-automated analysis will be presented. Characterization of variants such as deamidation, which are traditionally unattainable by intact mass due to their minimal molecular weight differences, were measured unambiguously by mass and retention time to allow confident MS1 identification. An example of ligand screening using native online size separation (SEC-MS) will be provided in which non-covalent protein-small molecule interactions are interrogated and affinity ranked. Although chromatographic separation offers the time resolution of species, a key component of the analysis is the ability of intact mass deconvolution algorithms to accurately report and quantify the species present.

**NOTES:**

**Technical Seminar: Lunch and Learn**
**Wednesday, September 12**
**12:15 – 12:45**
**Plaza Room A**

## Applications of Multi-Attribute Method (MAM) for Control of Critical Quality Attributes of Protein Therapeutics

Da Ren

*Amgen Inc., Thousand Oaks, CA USA*

Abstract not available at time of print.

**NOTES:**

# Poster Abstracts

# ADC and Bispecifics

**P-200-M**

**Overcoming Unusual Challenges in the Characterization of Monoclonal Antibodies by Mass Spectrometry**

Bruno Genet, Séverine Clavier, Armelle Martelet, Nelly Lechat

*Sanofi, Vitry sur seine, France*

Different modalities of monoclonal antibodies are pushing the limits of characterization by mass spectrometry. With this new class of biomolecules, new critical quality attributes need to be carefully monitored to ensure product quality as efficacy and safety, in particular absence of immunogenicity. From developability in early phase, during process development and routine monitoring in first clinical phases, Mass Spectrometry is more widely used to monitor post translational modifications or to identify and monitor unwanted HCPs.

Several case studies will illustrate the first stages of development of a monoclonal antibody and the characterization of unusual modifications (such as sulfation, additional glycosylation). The enzymatic desulfation of the antibody allowed to generate different levels of sulfation to monitor the impact on biological activities.The oxidation need to be carefully monitored as all the components of a formulation buffer can impact its level. Finally, a presence of an additional glycosylation on one chain complexify the global pattern and need a monitoring of separate glycoforms on each chains.

In addition to traditional methods (intact mass in denaturing conditions and peptide mapping), the use of non-denaturing native SEC-MS method for the investigation of structural heterogeneity complete the toolbox for the in-depth characterization of atypical behavior.

**NOTES:**

**P-201-T**

## A New LC-MS Approach for Enhancing Subunit-Level Profiling of mAbs and ADCs

Xiaoxiao Liu[1], Jennifer Nguyen[1], Jacquelynn Smith[2], Olga Friese[2], Jason Rouse[3], Daniel Walsh[2], Ximo Zhang[1], Nilini Ranbaduge[1], Matthew Lauber[1]

*[1]Waters Corporation, Milford, MA USA, [2]Pfizer, Inc., Chesterfield, MO USA, [5]Pfizer, Inc., Andover, MA USA*

Protein reversed phase chromatography is heavily dependent on the conditions under which it is performed. Methods employing polymeric columns and trifluoroacetic acid (TFA) have been preferred by chromatographers but are inherently restricted to low pressure, low throughput analyses and compromised MS detection. Accordingly, a novel LC-MS platform has been developed for the subunit profiling of mAb-based therapeutics, and it includes three critical breakthroughs: a new particle technology to afford increased throughput, a unique high coverage phenyl surface to lessen ion pairing dependence, and a more MS-friendly mobile phase system based on highly purified difluoroacetic acid (DFA).

Our investigations have shown that it is possible to achieve higher resolution separations of mAb subunits when DFA is used in place of TFA. Along with a newly developed column technology based on an optimized superficially porous particle and novel phenyl surface chemistry, it has thus been possible to achieve unprecedented resolution and to accelerate analyses via the use of high flow rates. In addition, a 4-fold increase in MS signal has been observed when 0.1% DFA is used in place of 0.1% TFA. The newly developed phenyl-based stationary phase, used in combination with DFA mobile phases, has also dramatically improved the recovery of challenging samples, such as ADC subunit digests. Ultimately, it is now possible to envision a new platform method where unforeseen levels of detail can be observed with high fidelity using 15 minute or shorter LC-MS runs.

**NOTES:**

**P-202-M**

**Identifying and Characterizing Bispecific-related Impurities Using Intact Mass Analysis**

Agatha Wieczorek

*Amgen Inc., Los Angeles, CA USA*

Therapeutic bispecific antibodies (B-Abs) can target two pathways implicated in disease. In general, a B-Ab has two different light chains (LC1 and LC2), and two different heavy chains (HC1 and HC2) that ideally correctly pair via engineered charge pair mutations. Because of the challenges associated with multi-chain expression and assembly, product related variants exist for bispecifics that are not encountered in a monovalent antibody. These include half molecules, mis-paired light chains, and homodimers. Due to the complexity and co-elution of the product related impurities, the bispecific antibody herein was not able to leverage platform methods; primarily by size exclusion chromatography (SEC) or reduced and non-reduced SDS based capillary electrophoresis (CE-SDS). However, cation exchange chromatography (CEX) was heavily utilized and found to be an ideal means of separating impurities by exploiting localized surface charges. Additionally, intact mass methods were used to identify and characterize the product related attributes. Extensive characterization using mass spectrometry demonstrated the removal of these impurities throughout the process and in the final product. Data obtained by the CEX and MS methods was able to verify that 1) varying cell culture temperature conditions resulted in the formation of different mis-pairs (HC1:LC1 homodimer and LC2:HC2 half mAb) in the aggregate species, and 2) mRNA expression levels for each culture condition confirmed increased expression of LC1 in the varying culture conditions. In summary, we present a mass spectrometry based screening strategy to ensure proper molecule assembly, and subsequently characterize and control product related impurities during manufacturing, purification and release testing. Additionally, varying cell culture temperature conditions resulted in the formation of different mis-pairs. RNA expression levels for each culture confirmed increased expression of LC1 in the culture conditions where increased HC1:LC1 homodimer was observed, suggesting different cell culture conditions can affect chain expression and subsequent molecule assembly.

**NOTES:**

**NOTES:**

# CMC Strategy and the Use of Mass Spec in the QC Lab

**P-203-T**

**Streamlined MAM Analysis for New Peak Detection, Comprehensive Data Inspection, and Confident Reporting**

Eric Carlson, Rose Lawler, Ilker Sen, Michelle English, Kossi Lekpor

*Protein Metrics Inc., San Carlos, CA USA*

Mass spectrometry plays an increasingly important role in the characterization and comparison of innovator protein products as well as biosimilar samples. Recently, there is increasing interest in relying on mass spectrometry to apply Multi-Attribute Methods (MAM) to characterize biotherapeutics in a consistent manner with the aim of replacing (or at a minimum supplementing) other traditional analyses. In this poster, we show an example of Protein Metrics Byos™ which is able to perform fully and semi-automated quality control (QC) ready data analysis for MAM applications. Byos is used to generate a clear list of product quality attributes (PQAs) which is presented to an analyst for inspection and curation. Byos then uses the curated PQA list to annotate a reference chromatogram. The annotated chromatogram is used as a basis to compare to additional files for new peak detection, quantitative comparison and comprehensive reports. There are also options for full automation of the process. Results show MAM analysis of a stressed sample and highlight the results of new peak detection and differential quantification of PQAs.

**NOTES:**

**P-204-M**

## A Quantitative MS-based Multiple-attribute Method (MAM) Approach for New Biological Entities and Biosimilar Candidates Presented by a Full Method Qualification for an IgG1 Market Product

Anke Schnabel, Gerhard Körting, Julia Brückner, Yvonne Jasper, Heiner Falkenberg, Roland Moussa

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Detailed characterization including assessment of critical quality attributes of biopharmaceuticals is essential across process development, manufacturing and release. The multiple-attribute method (MAM) Peptide Mapping with quantitative LC-ESI-MS provides detailed and site-specific information on product quality attributes (PQAs), like amino acid sequence, terminal processing, truncation, deamidation, oxidation or glycosylation. The MAM approach has been proven to be a powerful tool during development and production of new biological entities and biosimilar candidates and has the potential to replace conventional protein analytical methods for characterization and quantification of PQAs. Thereby, the MAM approach has to meet various demands in the different development phases regarding sample throughput, standardization, and quality level with the ultimate discipline GMP-release testing. In originator monitoring and subsequent biosimilarity exercise the MAM needs to be qualified according to current guidelines with special regards to the comparability assessment and robustness.

We present the application of MAM in IgG1 biosimilar development using Thermo Scientific Q Exactive instrumentation and the software solution (Protein Metrics Inc.) for fast processing of post translational modification (PTM) levels, terminal variants and N-glycosylation profile. Based on a case study the principle of a molecule-specific method qualification of a MAM peptide mapping MS approach will be introduced including the evaluation of the quality parameters: analyte autosampler stability, repeatability, intermediated precision, linearity and specificity of the method using appropriate stress samples.

The MAM approach offers the technical potential for validation and usage as GMP release testing for biopharmaceuticals. However, multiple challenges need to be solved to use MS in fully compliant routine analyses, such as the variability for low-level impurities, and GMP-compliant software for data acquisition and automated data processing.

**NOTES:**

**P-205-T**

## A Method for the Rapid Identification of Monoclonal Antibodies Using a Waters Acquity® QDa®

Nicole Stephens

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

In this study we utilized the Waters Acquity® QDa® mass detector to develop a rapid, QC friendly peptide mapping ID method with mass detection. By using instruments employing Empower software, we were able to develop detection and reporting solutions to augment the already established UV peptide mapping method. As an initial step, we developed a Lys-C peptide mapping method employing reversed phase UHPLC followed by MS1 mass detection. For product identification, mAb specific Empower processing methods were developed utilizing 3-4 unique identifying peptides along with 5-6 system suitability peptides. The Empower processing methods use retention time, peak area and m/z (multiple charge states when possible) for the identification of a peptide. Building product specific processing methods allows for automated processing of data without analyst manipulation. Finally, an Empower report method was created for easy visualization of peak identifications and confirmation of product identification.

**NOTES:**

**P-206-M**

## Application of an MS Method for Critical Quality Attributes Characterization of Therapeutic Antibody and Biosimilar Product

Terry Zhang, Haichuan lIU, Stephane Houel, Michael Blank, Aaron Bailey, Jonathan Josephs, Jennifer Sutton

*Thermo Fisher Scientific, San Jose, CA USA*

Recombinant monoclonal antibodies (mAb) and derivatives have become the fastest growing class of human therapeutics since 1980s. As an element of regulated manufacturing process, the determination and monitoring of critical quality attributes (CQAs) for a biotherapeutic compound is essential for lot release and acceptance with regulatory agencies. Traditionally, a combination of multiple separation techniques including HILIC, SEC, CEX, and RP-HPLC in conjunction with UV spectroscopy and NMR is used for attribute analysis and purity assessment. A multi-attribute method (MAM), developed recently by Rich Rogers, has gained increasing interest in biopharmaceutical industry. The MAM depends upon high resolution and accurate mass (HRAM) mass spectrometry to provide quantitative information for CQAs monitoring and new peak detection. The patent expiration of first generation mAb therapeutics provides opportunity for biosimilar mAbs to enter biotherapeutics market. FDA expects that side-by-side comparison analysis of suitable attributes of the biosimilar and innovator reference products should be performed. In this study, the MAM was used to compare differences in selected CQAs of a biosimilar and the innovator product of Trastuzumab.

Therapeutic antibodies, Trastuzumab and its biosimilar, were reduced, alkylated and digested with trypsin. The peptides were separated and eluted using a Vanquish Horizon uHPLC system (Thermo Scientific) with an Accucore C18 column. The MS and MS/MS data were acquired using a Chromeleon-controlled Q Exactive Plus (Thermo Scientific) with Biopharma option The BioPharma Finder 3.0 was used for antibody peptide mapping and CQAs selection. The selected CQAs were imported into Chromeleon for CQAs quantiation and comparison between the innovator and biosimilar products. The preliminary results showed that the percentages of some of the CQAs were very similar between the biosimilar and innovator products, whereas other CQAs (e.g. glycosylation) showed differences between the two products.

**NOTES:**

# Data Process and Analysis

**P-207-T**

**Supporting Bioprocess Development and Method Transfer using Multi Attribute Monitoring Methods**

Robert Birdsall, Weibin Chen, Ximo Zhang, Ying Qing Yu

*Waters Corporation, Milford, MA USA*

As part of a sound pharmaceutical quality system (ICH Q10), an increasing trend of MS-based techniques in lab settings traditionally associated with optical-based assays has been observed in an effort to improve and expand the body of knowledge surrounding a drug product. This has facilitated the introduction of LCMS-based peptide mapping methods for semi-targeted monitoring of biotherapeutic protein attributes or "Multi-Attribute Monitoring" (MAM) workflows in an effort to reduce redundant assays and increase productivity. Challenges associated with these approaches include managing extensive data sets for identification of PQAs, determining assay criteria for CQA's as methods migrate from development to manufacturing environments, and assessing platform suitability for deployment into regulated development and QC/lot release roles.

In this study, we have generated a data set representative of a process development setting to demonstrate how these challenges can be addressed with a single Chromatography Data Software (CDS) platform. A peptide map (N=48) of a therapeutic mAb was acquired with 19 CQA candidates monitored using an LCMS platform incorporating GXP practices. Thresholding of data using CV and % Area (% modification) as screening parameters was used for efficient data visualization for rapid identification of process deviations in existing PQA's across 1800 data result sets. Successful method migration to a manufacturing environment using the same LCMS-based CDS platform is demonstrated with the incorporation of system suitability to assess system readiness in a regulated environment. Using targeted monitoring, CQA abundance of a biological replicate (N=3) representing a release assay was quickly assessed using pass/fail criteria integrated into reporting for efficient review of data and lot release. Results from this study have been compiled to enable data-based discussions of fit-for-purpose MS for implementing peptide map based attribute monitoring in process development and regulated environments as part of life cycle management.

**NOTES:**

**P-208-M**

## Towards the Comprehensive Mass Spectrometry-based Biopharmaceutical Analysis using a Novel Software Suite

Lin He, Yi Liu, Weiping Sun, Di Wang, Tom Yang, Lei Xin, Wen Zhang, Qingyang Li, Baozhen Shan

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High-resolution mass spectrometry (MS) is widely used for the characterization of therapeutic proteins, e.g., monoclonal antibodies (mAb), in both discovery and development stages. More specifically, MS is an effective method to measure the molecular weight of intact proteins, retrieve amino acid sequence information, peptide mapping, and identification of post-translational modifications (PTMs) and sequence variants. Deep studies also involve disulfide bond linkage analysis and glycan profiling. However, the higher performance of emerging instruments outstrips the capacity of the data analysis. A fully equipped analytical pipeline handling the aforementioned tasks is highly demanded by both industrial and academic communities. Here we propose a software suite, PEAKS Pharma, for readily transit the experimental data to comprehensive knowledge.

The proposed software suite includes three main functions: 1) intact mass spectrum deconvolution for molecular weight analysis, 2) complete protein de novo sequencing for accurate protein sequence retrieval, and 3) protein sequence validation, including peptide mapping and protein characterization.

The evaluation of the software suite was performed on NIST mAb data generated from different labs for different purposes. The result showed that 1) our intact mass analysis module could efficiently deconvolute the intact mass spectra and retrieve accurate molecular masses, 2) with the multiple enzyme digestion approaches, our complete protein de novo sequencing module could give the accurate amino acid sequences of both heavy and light chains, and 3) using the tryptic digest data, our peptide mapping could get >97% sequence coverage, as well as the expected disulfide bond linked peptides and N-linked glycan profiling result. Furthermore, the peptide features without acquisition of tandem mass spectra could also be automatically and accurately matched to the given proteins in the peptide mapping analysis, according to the accurate precursor mass and retention time.

**NOTES:**

**P-209-T**

## An Automated Approach for Comprehensive Characterization and Quantification of Low-abundance Sequence Variants in a Standard Monoclonal Antibody

Peter Haberl[1], Albert van Wyk[2], Maurizio Bronzetti[3], Aude Tartiere[4], Joe Shambaugh[3], Arnd Brandenburg[6]

*[1]Genedata GmbH, Munich, Germany, [2]Genedata Limited, Cambridge, United Kingdom, [3]Genedata, Inc., Lexington, MA USA, [4]Genedata, Inc., San Francisco, CA USA, [6]Genedata AG, Basel, Basel, Switzerland*

In the production of biopharmaceuticals, sequence variants (SVs) are protein species that contain unintended changes to the target amino acid sequence. The presence of SVs can adversely affect the safety and efficacy of biopharmaceutical preparations, and therefore an analytical method that enables sensitive detection of SVs would represent a significant step toward ensuring product quality. Mass spectrometric methods enable identification of SVs, but the minimization of false positives remains a challenge during data analysis. We present an automated processing workflow for LC-MS/MS data that provides comprehensive characterization and sensitive quantification of sequence variants and describe an effective strategy to minimize the number of false positive and false negative identifications.

Traditional approaches to SV identification rely solely on the efficacy of search engines. Using the dedicated software platform Genedata Expressionist® (Genedata AG, Basel, Switzerland), we developed a data analysis strategy that incorporated a systematic reduction of the space searched for SV candidates. Briefly, all peptides (both unmodified and with expected post-translational modifications) belonging to the target mAb sequence were identified with high confidence and excluded from the search space before SV analysis. This reduction of the search space dramatically reduced the number of false positive identifications. Subsequent steps in the automated workflow were created to search for specific groups of misincorporations; such as Phe→Tyr and those occurring within under-alkylated peptides. These targeted searches were key to identifying variants that might otherwise have been overseen (false negatives). Applied to an NIST antibody sample, our workflow generated only a few dozen SV candidates. Investigation of these potential SVs — through manual validation of MS/MS spectra — required significantly less time than would have been required to investigate the large number of candidates generated using traditional approaches.

**NOTES:**

**P-210-M**

## A New N-glycan Workflow in Byos

Elaine Sun[1], Marshall Bern[2], Mario DiPaola[1], Andrew Hanneman[1]

*[1]Charles River Laboratories, Woburn, MA USA, [2]Protein Metrics Inc., San Jose, CA USA*

The analysis of released N-linked glycans from next-generation biotherapeutic glycoproteins is challenging due to numerous factors including multiple glycosylation sites, highly-branched structures, isomers, and a potential for motifs not commonly encountered with monoclonal antibodies like phosphorylation, NeuGc, and polylactosamine repeats. Here, we describe efficiency-enhancing new software tools designed to support data processing for analyzing released N-glycans, achieving dual goals of quantitation, and of mass spectrometry-based characterization. The processing workflow includes automatic integration of HILIC fluorescence chromatograms (FC), association of FC peak quantification with mass spectrometry data, and quick query of an N-glycan database to provide peak-by-peak compositional analysis supporting structural interpretation. To further enhance the confident identification of N-glycan structures including highly-branched and uncommon motifs, we are building MS/MS spectrum matching tools to support both high-energy and low-energy CID. We match observed MS/MS peaks to theoretical fragment ions, and store well-identified m/z-corrected MS/MS spectra in spectral libraries for future matching of known N-glycans. The workflow will also detect new and high-fold-change elution peaks in comparison projects, and flag incompletely identified elution peaks for further elucidation using multiple levels of MS, exoglycosidase sequencing, or other methods.

**NOTES:**

**P-211-T**

## High Throughput LC-MS Analysis of Intact Light and Heavy Chain Supporting mAb Screening and Cell Line Characterization

Elaine Sun[1], Ahmet Cansizoglu[1], Andrew Hanneman[1], Mario DiPaola[1], Marshall Bern[2]

*[1]Charles River Laboratories, Woburn, MA USA, [2]Protein Metrics Inc., San Jose, CA USA*

LC-MS of reduced mAbs to provide intact molecular mass determination is a widely used characterization tool supporting monoclonal antibody drug development, where it is used to assess for the correct amino acid sequences of light chain and heavy chains, as well as any post-translational modifications including glycosylation. Molecular weight confirmation of LC/HC is also useful in support of cell line development; however, higher sample throughput is required. Here we present high-throughput LC-MS mass analysis of mAb light/heavy chain by employing the Intact Mass software program from Protein Metrics Incorporated, where the time-consuming data analysis bottleneck has effectively been removed. Using batch processing to support high-throughput, Q-TOF LC-MS data from more than 100 mAb samples was quickly analyzed with results consistently showing comparable data quality to a more traditional low-throughput characterization work flow, including high mass accuracy, identification of major and minor species, and a simple data reporting structure.

**NOTES:**

**P-212-M**

## Four Levels of Approach for Comprehensive Glycosylation Profiling of Monoclonal Antibodies

David Wong

*Agilent Technologies, Santa Clara, CA USA*

Protein glycosylation is one of the major post-translational modifications (PTMs) of mAbs that play an important role in many biological processes. The distribution and composition of the glycans bound to the mAb molecules can influence therapeutic efficacy and immunogenicity. Consistent glycosylation-associated quality control of therapeutic mAbs has become a high priority in pharmaceutical bioprocessing. In this study, we demonstrated all four levels of LC/MS workflows (Intact mAb, mAb subunits, glycopeptides and released glycans) that utilize an automated liquid-handling robot for sample preparation, a UHPLC system, an accurate mass LC/Q-TOF system, and automated data processing using BioConfirm software for glycan/glycoform quantitative analysis.

The intact mAb workflow provided rapid assessment of the major glycoforms of the mAb. We can perform a relative quantitation comparison on the selected glycoforms among different samples. The mAb subunits analysis workflow after complete protein reduction offered detailed quantitative information about individual glycans such as G0F, G1F, and G2F. Our results showed that the relative quantitation (%) results of the glycoforms from intact mAb were comparable to the results from the NISTmAb subunit workflow. The glycopeptide analysis through peptide mapping allowed relative quantitation of glycans and also offered N-glycosylation site(s) information. Finally, the released glycan workflow with both fluorescence and mass spectrometric detection provided high analytical sensitivity and the best quantitation. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans was used for identification using our proprietary Find-by-Formula algorithm. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F, was comparable between the fluorescent and MS data. Overall, our complete automated workflow solution dramatically improves not only productivity by allowing convenient sample preparation and streamlined data acquisition, but also accuracy in mAb glycan quantitation data analysis.

**NOTES:**

# Emerging Product Areas

**P-213-T**

**Assessing Higher Order Structure Similarity with HDX-MS**

Asish Chakraborty, Ying Qing Yu, Weibin Chen

*Waters Corporation, Milford, MA USA*

Hydrogen deuterium exchange mass spectrometry (HDX-MS) is a key technology that provides critical information on both protein conformation and dynamics. The automation of sample handling, data collection, and data analysis has catalyzed the expansion of HDX-MS from academia into the world of biopharmaceuticals. Ease of use, sensitivity, speed, and resolution in the study of the higher order structure of proteins and protein complexes make this technique a uniquely valuable tool in the biophysical toolbox.

In recent years, HDX-MS has become an indispensable tool in the biopharmaceutical industry to answer important questions concerning biotherapeutic structure, stability, and interactions. In this study, we used HDX-MS to compare the higher order structure similarity between innovator and biosimilar versions of a mAb. Our results show that the structures of the innovator and biosimilar mAbs are comparable. However, minute differences were observed in the Fc-$C_H2$ domain and a few overlapping peptides attracted our attention in this region. Details will be discussed in the presentation.

**NOTES:**

**P-214-M**

## Advanced MS-based Evaluation of Biologic Compatibility with Intravenous-administered Small Molecule Medications

Dongdong Wang[1], Annie He[1], Wanlu Qu[1], Nazila Salamat-Miller[2], Paul A. Salinas[2], Jennifer S. Chadwick[1], Shiaw-Lin Wu[1]

*[1]BioAnalytix Inc., Cambridge, MA USA, [2]Shire, Lexington, MA USA*

A recent trend in biologic therapeutics has been the development of increasingly potent molecules, including growth factors, enzymes, antibody-drug conjugates and bispecifics, which are dosed in clinical settings at very low concentrations (<10 mg/mL). However, knowledge of chemical compatibility of intravenous (IV) biologic drugs co-administered with small molecule drugs at such low concentration is limited. As such, there is a significant need for approaches that enable accurate detection and effective characterization of drug-drug compatibility at low concentrations. In the present study, a robust sample preparation method with hybrid mass spectrometry was developed to assess quantitation levels and characterize integrity at very low levels (<10 mg/mL) of rhIGFBP-3/rhIGF-1, a heterodimeric biologic complex intended for preventing complications of prematurity in preterm infants. The method was further developed to determine relative amounts of low-abundance variants, specifically oxidized species and fragments, when prepared as an admixture with several small-molecule drugs, including furosemide, caffeine citrate, ampicillin, penicillin and vancomycin, which vary widely in pH and formulation and have a diverse set of chemical properties. No increase in oxidation or fragmentation levels for rhIGF-1/rhIGFBP-3 was observed post mixing and acceptable recovery of biologic was observed with most co-mixed drugs. Lower biologic recovery and drug-modified rhIGFBP-3 was observed with ampicillin and penicillin. The results of this study demonstrate accurate quantitation can be reproducibly achieved for low concentration biotherapeutics (LOQ at 3 mg/mL) and associated variants (present at ~1% of the biotherapeutic level) when admixed with massive excess of small molecule drugs, further providing valuable information about the compatibility and stability of the active biologic at clinically relevant concentrations.

**NOTES:**

**P-215-T**

## Flash Oxidation (FOX): A New and Improved Platform for Biopharmaceutical Hydroxyl Radical Protein Foot-printing Higher Order Structural Analysis

Scot Weinberger[1], Joshua Sharp[2], Ronald Orlando[2]

*[1]GenNext Technologies, Inc., Montara, CA USA, [2]University of Mississippi, Oxford, MS USA*

Hydroxyl Radical Protein Foot-printing (HRPF) is an emerging and promising higher order structural (HOS) analysis technique that involves the irreversible labeling of a protein's exterior by reaction with hydroxyl radicals followed with MS analysis to identify the outer portions of the protein by its increased mass. HRPF has been used to detect: defects in protein HOS and function; monoclonal antibody production (Mab) defects; the interplay of Mab HOS and drug function; as well as biosimilar failure and storage induced defects for innovator products. Academic laboratories have demonstrated the utility of HRPF for HOS analysis, however adoption in pharma is slow, and only in laboratories that have hired researchers from or closely collaborate with HRPF academic labs. We have identified barriers that have limited the adoption of HRPF in biopharma. These include: 1) the use of dangerous and expensive lasers that demand substantial safety precautions; 2) the irreproducibility of HRPF caused by background scavenging of OH radicals that limit comparative studies; and 3) the absence of data processing tools to facilitate HRPF data analysis. *As such, there are no commercial HRPF sample preparation devices, despite the demonstrated need for its HOS analytical power.*

The FOX System replaces expensive and hazardous UV lasers with proprietary plasma lamp technology, creating a facile and safe bench-top means to perform biopharmaceutical HRPF HOS analysis. Real-time measurement of effective hydroxyl radical yield provides reproducible labeling irrespective of background scavenging. Moreover, our FoxWare data processing software processes results in minutes, in lieu of days as presently experienced. This poster describes the FOX system platform and demonstrates utility in protein HOS analysis when compared to the current-day, laser-based approach.

**NOTES:**

**P-216-M**

## Universal S-Trap Sample Processing: Standardized Reproducible Sample Workup from Sub-ug to Multi mg Scales for all Sample Types

John Wilson

*Cold Spring Harbor Laboratory, New York, NY USA*

Variability in sample preparation has long hampered bottom-up proteomics. Proteins' vastly different solubilities and myriad extraction protocols yield very different data depending on what proteins are [not] extracted. S-Trap sample processing solves this problem by integrating: 1) complete protein solubilization with 5% SDS; 2) total protein denaturation (pH < 1 and > 70% organic); 3) simultaneous sample concentration/cleanup; and 4) rapid reactor-type proteolytic digestion.

S-Traps were obtained from ProtiFi (www.protifi.com, Huntington NY). S-Trap micros were used from sub-ug to 100 ug max, S-Trap minis from 100 – 300 ug, S-Trap midis > 300 ug, and 96 well plates for high throughput applications. Briefly, all samples were extracted with 5% SDS, fully denatured, captured on the S-Trap where they were cleaned of all contaminants including Laemmli sample buffer, PEG, detergents, urea, reduction and alkylation reagents, etc. and digested.

S-Trap sample processing allowed unbiased protein analysis including poorly soluble proteins (membrane proteins, chromatin and muscle, etc.) and was successfully applied without alteration to all samples including tissues, FFPE blocks, cell lines, biological fluids, etc. Using only standard lab equipment, S-Traps enabled reproducible sample processing with < 10% CVs from protein to peptides in around 1 – 2 hrs from sub-microgram to multi-milligram scales.

S-Trap processing is highly reproducible (CVs typically < 10%) and independent of the properties of the proteins under study. Without change, its harmonized protocol is applicable to all kinds of samples from liquid biopsies (serum and dried blood spots [DBS]) to FFPE blocks to tissues or cell lines. S-Trap processing increases protein recovery, affording analysis of very small sample quantities, and can enable simultaneous proteomics and metabolomics.

**NOTES:**

# Intact/Subunit/Top-down Analysis

**P-217-T**

## Assessing Biosimilarity of an Intact Monoclonal Antibody Drug by Simultaneously Monitoring Charge Heterogeneity and Glycoform Profile using Orbitrap Native LC-MS

Aaron Bailey[1], Roberto Gamez[1], Guanghui Han[2], Jennifer Sutton[1], Terry Zhang[1], Stephane Houel[1], Shanhua Lin[4], Kate Southwick[1], Wendy Sandoval[2], Jonathan Josephs[1]

*[1]Thermo Fisher Scientific, San Jose, CA USA, [2]Genentech, a Member of the Roche Group, South San Francisco, CA USA, [4]Thermo Fisher Scientific, Sunnyvale, CA USA*

Manufacturing of innovator biologics can be accurately mimicked to produce generic "biosimilar" drug products. In order to satisfy safety and efficacy requirements, a biosimilar drug must be reasonably comparable to an innovator. Comparability is directly assessed by measuring a panel of critical quality attributes (CQAs). Multi-attribute method (MAM) has been demonstrated to measure several CQAs simultaneously using LC-MS peptide mapping data. Additional CQAs, such as charge variants and intact glycoform profile are conventionally monitored at the intact level using ion exchange chromatography (IEC) or capillary electrophoresis (CE). Our IEC-MS platform combines charge variant separation with native protein accurate mass measurement to allow isoform-specific monitoring of multiple CQAs of intact therapeutic proteins.

Intact trastuzumab was separated by IEC (ProPac WCX-10) directly coupled to MS. Mobile phases consisted of aqueous 50 mM ammonium acetate using a pH gradient from 6.8 to 10.1. LC-MS was accomplished using a Vanquish H-Class UHPLC system with a variable wavelength detector directly connected to a Thermo Scientific™ Q Exactive™ HF-X Orbitrap™ mass spectrometer with BioPharma Option. Native LC-MS raw data were analyzed using a time-resolved deconvolution approach utilizing Sliding Window and ReSpect algorithms in Thermo Scientific™ BioPharma Finder™ software. Thermo Scientific Chromeleon software was used to integrate XIC peak areas and relatively compare CQAs of innovator and biosimilar drug samples.

Time-resolved deconvolution analysis of the innovator drug resulted in identification of several specific isoforms comprised of differential N-glycosylation in combination with a variety of low level charge-imparting PTMs, such as deamidation, C-terminal lysine removal, or sialylation of N-glycans. We observed marked differences in glycoform profile and charge variant isoforms when comparing innovator and biosimilar. These differences observed were consistent with a comparison of these same two samples via MAM Orbitrap peptide mapping analysis.

**NOTES:**

**P-218-M**

## Structural Analysis of the Multimers of Bovine Serum Albumin by Ion Mobility-mass Spectrometry

Rebecca Glaskin[1], Dawn Stickle[2]

*[1]Agilent Technologies, Lexington, MA USA, [2]Agilent Technologies, Odessa, FL USA*

Proteins have dynamic structures that often adopt multiple conformational states dependent on their function. Mass Spectrometry (MS) is a powerful tool for the analytical characterization of biomolecules that provides sensitivity, speed, small sample consumption, and ability to analyze complex mixtures. When coupled to ion mobility spectrometry (IMS), characterization of both mass and molecular structure of these biomolecules can be obtained to probe the conformation states these proteins can adopt. This can reveal information in regards to subunit composition as well as the overall topology of protein complexed, even examine the range of conformation states protein complexes can adopt and allows the detection of protein assemblies in a single spectrum.

An Agilent G1992A nano-electrospray ionization source was coupled to an Agilent 6560 IM-QTOF instrument where 20 $mg \bullet mL^{-1}$ of Bovine Serum Albumin (BSA) was infused at a flow rate of 16 $L \bullet hr^{-1}$ from one of the following solution conditions: 150 mM ammonium acetate, 0.1% formic acid (by volume), and 50:50:0.1 water/acetonitrile/formic acid (by volume).

With the additional dimension of separation provided by the drift tube, we utilize the fact that different charge states fall along trendlines based on the increased force larger charge states experience as they travel through the drift tube. These different trendlines allow for increased selectivity in the detection of the various multimers of BSA. The resulting multidimensional distributions obtained from these three different solutions show the formation of different multimers. The 150 mM ammonium acetate solution displayed the largest multimeric complexes compared to the other two solutions. As the solution conditions changes, the observed multimers decrease to predominantly monomer in the 50:50:0.1 water/acetonitrile/formic acid solution. Comparisons of the resulting maximum entropy deconvolution and two-dimensional plots of drift time vs $m/z$ show insight into how BSA monomers combine to form these multimeric species.

**NOTES:**

**P-219-T**

## Middle-down Analyses of Unmodified and Stressed Monoclonal Antibodies using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer

Stephane Houel[1], Romain Huguet[2], Jennifer Sutton[2], Aaron Bailey[2], Vlad Zabrouskov[2], Jonathan Josephs[2]

*[1]Thermo Fisher Scientific, Cambridge, MA USA, [2]Thermo Fisher Scientific, San Jose, CA USA*

Peptide mapping is the gold standard for in-depth characterization of biotherapeutics, but the sample preparation, LC runtime and data analysis can be time consuming. Considering that subunit mass analysis is a common assay in the biopharmaceutical industry, getting sequence information using the same sample preparation and LC settings would be advantageous. Automated data processing has historically been one of the bottlenecks for middle-down experiments; however, dedicated software developed for biotherapeutics characterization is available today. Finally, high sequence coverage for middle-down experiments on biotherapeutics is imperative. In this study, we show how to achieve high sequence coverage on mAbs and pinpoint sites of oxidation resulting from oxidative stress.

Mabs were digested with IdeS followed by denaturation and reduction. Data were acquired using a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer coupled to a Thermo Scientific™ Vanquish™ Horizon UHPLC system. Targeted MS/MS spectra were collected by isolating and combining multiple charge states. Higher-energy Collision Dissociation (HCD), Electron Transfer Dissociation (ETD) and Ultra-Violet Photo-Dissociation (UVPD) fragmentation techniques were used and the resulting LC-MS/MS data were searched with Thermo Scientific™ Biopharma Finder™ 3.0 software.

High sequence coverage was obtained by combining multiple modes of fragmentation even when subunit chromatographic peak widths were only around 30 seconds. These results suggest that middle-down experiments could be a valuable alternative assay to peptide mapping for fast amino acid sequence confirmation.

The subunit mass analysis of mAbs stressed with hydrogen peroxide, showed that up to two sites could be oxidized at the same time. Multiplexing different charge states representing different level of oxidation of a stressed mAb can be a strategy to quickly identify sites of oxidation and can provide unique information.

**NOTES:**

**P-220-M**

## Application of a Domain-specific Free Thiol Method to Monoclonal Antibody Characterization

Yi Pu, Rachel Chen, Tai Nguyen, Chongfeng Xu, Zoran Sosic, Tyler Carlage, Li Zang

*Biogen, Cambridge, MA USA*

Characterization of free thiol variants in antibody therapeutics is important for biopharmaceutical development, as the presence of free thiols may have an impact on aggregate formation, structural and thermal stability, as well as antigen-binding potency. Most current methods for free thiol quantification involves labeling of free thiol groups by different tagging molecules followed by UV, fluorescence or mass spectrometry (MS) detection. Here, we optimized a label-free liquid chromatography (LC)-UV/MS method for free thiol quantification at a subunit level and studied free thiol variants in several monoclonal antibodies (mAbs) with different processes, formulations and stability, with a focus on understanding of possible correlations between free thiols and aggregates.

The subunit free thiol separation and quantification was based on a combined proteolysis method, which generated three antibody fragments, Fab, single chain Fc and Hinge. The free thiol forms could be separated from their corresponding subunits using reserved-phase LC and subsequently detected by UV and MS. This method was successfully applied to various in-house IgG1 mAbs for characterization of domain-specific free thiol variants. The quantification results were compared with two orthogonal approaches, Ellman’s assay and bottom-up peptide mapping. Ellman's assay measured total free thiol content in intact antibodies, while bottom-up approach, with site-specific free thiol information, was achieved by differential alkylation of cysteine residues using isotope labeling followed by peptide mapping.

Due to the potential roles free thiols play in the formation of disulfide-linked aggregates, we applied these methods to understand correlations between free thiol content and the aggregate formation tendency in antibodies via analysis of a variety of samples with elevated levels of aggregates, different formulations and from process changes. The insights provided by this study as well as a discussion of the various free thiol methods will be presented.

**NOTES:**

**P-221-T**

## Rapid On-column Monoclonal Antibody Subunit Generation for Automated Glycan Analysis

Kevin Cook[1], Stephan Björk[2], Maria Nordgren[2], Helén Nyhlén[2], Jonathan Sjögren[2], Fredrik Olsson[2]

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Antibody subunit analysis using IdeS enzymatic digestion and LC-MS has become a widely accepted analytical strategy for rapid characterization of therapeutic antibodies and related products. The IdeS enzyme specifically digests IgG just below the hinge, generating F(ab')2 and Fc/2 fragments. The IdeS based middle-level LC-MS workflow enables analysis of multiple antibody quality attributes such as glycosylation, oxidation, and c-terminal lysine clipping. Detailed knowledge of post-translational modifications is required for development and manufacturing of biopharmaceuticals.

Here we present a solution for rapid and consistent subunit generation facilitated by on-column digestion of a monoclonal antibody. We developed and characterized a HPLC column with immobilized IdeS for automated middle-level workflows and tested the robustness by studying the Fc-glycan profile of a therapeutic antibody over time. The IdeS column showed consistent digestion over prolonged time periods at 37°C and allowed for more than 200 injections of samples with relevant antibody concentrations without noticing significant reduction of digestion performance. The subunit enzyme column could be used with standard HPLC-MS setup and potential be connected directly to a bioreactor. Initially the column was tested for rapid Fc-glycan determination as the first of many possible critical quality attributes that could be monitored automatically by this online digestion method. Additionally, automated antibody multi attribute method analysis could be developed using the HPLC IdeS column reducing sample handling errors and increasing throughput.

**NOTES:**

**P-222-M**

## Quantitation of Intact Therapeutic Protein in Plasma Matrix by LC/MS

Yihan Li, Ian Moore, Fan Zhang, Sahana Mollah, Lei Xiong

*SCIEX, Redwood City, CA USA*

LC/MS based quantitation of intact therapeutic proteins using HRMS has become a significant new workflow in biopharmaceutical development. Here we present a workflow to quantify intact therapeutic proteins in plasma matrix, including target protein enrichment, LC/MS data acquisition and data analysis.

Immunocapture workflow using magnetic beads was utilized to capture trastuzumab from 50 mL of plasma standards. After target capture, plasma was removed and the beads were washed twice. 0.5% formic acid was added to elute trastuzumab from beads. The eluents were analyzed with a 10 min gradient in SCIEX ExionLC™ AD system coupled to SCIEX X500B QTOF system. Data was acquired in TOF-MS mode and processed in PeakView®.

To reduce interference from matrix proteins and enable quantitation of low-concentration samples, all the data files were reconstructed with Bio Tools micro-application in a none commercial version of PeakView® prior to quantitative analysis: raw data was converted into .wiff format; then every two TOF-MS spectra in the converted file were averaged and deconvoluted (input m/z range 2200-3600, output mass range 130-170 kDa, resolution 5000); the original spectra in the converted file were replaced by the corresponding deconvoluted data to form a reconstructed .wiff file. The deconvoluted data of trastuzumab revealed three major components: 148056.08 (with two units of G0F), 148218.22 (with G0F and G1F) and 148380.36 Da (with G0F and G2F). Extracted ion chromatograms were generated with these three masses with a tolerance window of ±2 Da. A gaussian smooth was performed with a smoothing width of 1 point. Peaks were automatically selected in PeakView® and the calculated peak areas were used for quantitation. The linear dynamic range of quantitation is 100 ng/mL to 50000 ng/mL. This workflow could serve as a generic quantitation method for intact therapeutic proteins in complex biological matrix.

**NOTES:**

# LC and LC/MS

**P-223-T**

**A Fit-for-purpose Accurate Mass MS for Routine Biotherapeutic Analysis**

Henry Shion[1], Jonathan Pugh[2], Jing Fang[1], William Alley[1], Robert Lewis[2], Ying Qing Yu[1], John Gebler[1], Scott Berger[1], Weibin Chen[1]

*[1]Waters Corporation, Milford, MA USA, [2]Waters Corporation, Wilmslow, United Kingdom*

The adoption of accurate mass MS for routine analysis has been hindered by instrument size, operation complexity, hardware robustness, and reproducibility. Yet, there is a need for this approach; for example, efficient data collection and analysis of quality-relevant information within the pharmaceutical industry. Here, we describe the performance evaluation of a prototype small-footprint time-of-flight MS that could overcome many of the above challenges.

For intact mass analysis, a dilution series for both trastuzumab and NIST mAb established a limit of detection (LOD) and intra-system LOD reproducibility. Results were comparable to other high-resolution MS systems. More structurally complex proteins (infliximab, trastuzumab emtansine, and EPOs), were analyzed and data demonstrated the greater heterogeneity of these molecules. The average drug-to-antibody ratio (DAR) for a cysteine-conjugated ADC agreed with values from commercial MS systems and hydrophobic interaction chromatography (HIC).

NIST mAb subunits showed good mass accuracy. Low oxidation levels were observed for both subunits after forced degradation experiments.

Peptide mapping of the NIST mAb was used to evaluate system robustness. A high sequence coverage was determined through precursor accurate masses and identification of fragment ions.

Glycan analysis was performed on the NIST mAb. All major glycans were correctly identified as well as many lower abudance glycans, including some potentially immunogenic glycans.

Several key biopharmaceutical experiments were performed on a small-footprint prototype TOF MS instrument. The data generated for all experiments (intact mAb mass, peptide mapping, glycan analysis) were very comparable to currently available commercial high resolution MS instrumentation. Thus, these data show great promise that this instrument could be in appropriate choice for routine MS-based biopharmaceutical analyses.

A prototype small-footprint oa-TOF MS provides fit-for-purpose data for multiple experiment types relevant to routine biotherapeutic analysis.

**NOTES:**

**P-224-M**

## Mass Spectrometric Investigations to Dissect N-terminal Heterogeneity of Protein Therapeutics

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Therapeutics are susceptible to enzymatic and non-enzymatic fragmentation specific to structural motifs within the protein. Any significant clipped species and the analytical methods used to monitor the purity and integrity become part of the critical quality attributes of the product or process. Herein we describe several mass spectrometry methods to identify and quantify the levels of the clipped species. Our first approach involves an unbiased high throughput screening approach to understand potential *N*-terminal clipping by combing TMPP or dimethyl labeling with intact mass analysis and short gradient tryptic peptide mapping. The ability to apply multiple dissociation methods in tandem with the ability to trigger these MS events based on reporters (TRT)[1] is essential to selectively sequence peptides specific to TMPP labeled peptides. We show the utility of this approach in a bench mark study where a clipped species was spiked in at predefined ratios TMPP-labeled and analyzed by intact mass and tryptic peptide mapping. The methodology is then applied to monitor potential degradation products over the course of bioreactor expression study conducted over a period of 14 days. Clipping is further validated by examining the host cell proteome in the context of identifying and finding associations between the putative enzyme and clipped site. Our second approach is a label-free quantitative (LFQ) approach to detect non-enzymatic clipped species during full developability undergoing stress conditions of bispecific mAbs. A novel search strategy is used for detecting non-enzymatic clipping while LFQ is used to obtain occupancy of the clipped species by measuring the peak areas of the unclipped peptide counterpart between release material where clipping is absent, and thermally or high pH stressed samples where clipping is present. We report on the accuracy of the occupancy over the typical %ratio estimates for clipping and compare with intact-MS, UV-based, and stable isotope dilution estimates.

**NOTES:**

**P-225-T**

## Bottom Up Approach with Low pH Digestion for Differentiation of Monoclonal Antibody Therapeutics using High Resolution Accurate Mass LC-MS

Lianji Jin

*ORA, FDA, Cincinnati, OH USA*

The Forensic Chemistry Center is an FDA laboratory that conducts forensic analyses of multiple categories of drug products. As monoclonal antibody (mAb) therapeutics become more common in the treatment of a variety of medical conditions, rapid and in-depth screening methodologies for analyzing these products are needed to protect consumers from suspected counterfeits, unapproved or misbranded products. Bottom up protein analysis is commonly used since it can yield a lot of information about the protein from the enzymatic digestion. The sample preparation artifacts typically associated with this approach were addressed by others using a low pH digestion procedure that minimizes oxidation and deamidation reactions. In this work, bottom up approach with the low pH digestion was evaluated for differentiating mAb therapeutics at the peptide level using two different high-resolution accurate mass LC-MS systems: Orbitrap Elite and Q-Exactive. Sequence coverage was examined for five IgG subtype mAb therapeutics: two reference materials and three therapeutics products (denosumab, tocilizumab and infliximab). Extensive sequence coverage for both heavy and light chains was obtained for each mAb using either LC-MS system. A few abundant glycopeptides were also detected, consistent with previous findings during comprehensive Fc N-glycosylation profiling, which allow the peptide with the N-glycosylation site counted toward the overall sequence coverage. Tocilizumab sequence discrepancy present in public domain was pinpointed at the peptide level combined with our previous findings at the intact, subunit and subdomain levels. Similarly, infliximab full sequence is deduced using the partially available public sequence information. Additionally, minor peptide charge variants were readily resolved which helped with determining the heterogeneity of the N- and C-terminal ends. Bottom up approach with a low pH digestion was well suited for differentiating five mAb therapeutics at the peptide level with increased resolution.

**NOTES:**

**P-226-M**

## A 2D LC-MS/MS Strategy for Reliable Detection of 10-ppm Level Residual Host Cell Proteins in Therapeutic Antibodies

Delia Li, Feng Yang, Donald Walker, Jeannine Schoenfelder, Joseph Carver, Reed Harris, Christopher Yu, David Michels, Alice Zhang

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One of the challenges for producing biotherapeutics is clearing host cell proteins (HCPs) during the purification process. HCPs in the final product can pose an immunogenicity risk in patients or potentially impact the efficacy and toxicity of the biologic, and therefore residual HCPs must be monitored. While ELISA is currently most commonly used, methodologies employing LC-MS/MS have been increasingly used to identify and quantify residual HCPs in biopharmaceutical products.

We developed an approach to improve the sensitivity and reliability for low-level HCP detection that uses a high pH-low pH two-dimensional reversed phase LC-MS/MS in conjunction with offline fraction concatenation. Proof-of-concept was established using a model of seven proteins, spanning a size range of 29-78 kDa, spiked into a purified antibody product to simulate the presence of low-level HCPs. Our approach enabled consistent identification of all seven proteins at 10 ppm with 100% success rate following LC-MS/MS analysis of six concatenated fractions across multiple analysts, column lots and injection loads.

For the first time, we demonstrate an effective LC-MS/MS strategy that has not only high sensitivity, but also high reliability for HCP detection. The method performance has high impact on pharmaceutical company practices in using advanced LC-MS/MS technology to ensure product quality and patient safety.

**NOTES:**

**P-227-T**

## A Plate-based Immunoaffinity LC-MS/MS Assay for Universal Quantification of Monoclonal Antibodies in Animal Serum

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Monoclonal antibodies are the largest class of biotherapeutics approved for a variety of clinical indications, particularly in oncology and autoimmune diseases. During preclinical drug development, assays are required to understand the absorption, distribution, metabolism and excretion of monoclonal antibodies, which is crucial for their design and selection. Hereby, we demonstrate a robust, high-throughput workflow for quantification of human IgG1 antibodies in animal sera by LC-MS/MS utilizing a stable isotope labeled universal monoclonal antibody internal standard which is introduced prior to immunoaffinity enrichment and tryptic digestion on an easy-to-use 96-well plate format. The novel aspects of this workflow are the use of a microtiter plate format without magnetic beads and the low sample volume requirements.

Biotinylated anti-human Fc capture antibody was added to the wells of a streptavidin-coated plate and incubated for 30 min at room temperature. To each well, 10 ng of SIL internal standard and 5 mL of sample/standard were added with subsequent incubation at room temperature for 2 hours. After washing, the contents of each well were digested by trypsin at 60 °C for 2 hours. Three tryptic peptides from the conserved IgG1 antibody heavy chain region, along with the corresponding tryptic stable isotope labeled internal standard peptides, were monitored in each sample. Assay parameters were developed using a generic monoclonal human antibody. The linear assay range was established in the range of 0.1 to 12.5 µg/ml using a 5 µl serum sample volume. The calibration curves of triplicate injections of standards prepared in cynomolgus monkey serum show a linear curve with regression coefficient of >0.99, %CV values of <20%, and accuracies ranging from 85-115%. The upper assay range could be extended to 200 ug/mL using a non-linear 5PL regression analysis. Universal assay performance was verified with several biotherapeutic antibodies, including adalimumab, infliximab, rituximab and cetuximab.

**NOTES:**

**P-228-T**

**Biotherapeutic Protein Analysis by MS-compatible Size Exclusion Chromatography**

Veronica Qin

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High molecular weight (HMW) aggregates and low molecular weight (LMW) fragments of biotherapeutic proteins may be induced by light, temperature fluctuations, pH changes and other stress conditions such as mechanical forces. Close monitoring of these size variants is critical because immunogenic responses and differences in pharmacokinetics or potency could arise due to the existence of these species in the drug product.

In this presentation, size exclusion chromatography (SEC)-UV and SEC coupled to mass spectrometry were applied to analyze the size variants derived from therapeutic proteins, and MS compatible mobile phases under non-denaturing conditions were explored. LC/MS conditions and mobile phase composition were optimized to achieve best resolution and MS sensitivity. This SEC-MS method provided high resolution and MS sensitivity of the size variants including HWM aggregates, monomer and LMW species, and with shorter analysis time compared to conventional SEC-HPLC method. With deconvoluted ESI mass spectrum molecular weight of these species was determined.

**NOTES:**

**P-229-M**

## High-throughput Screening of Antibodies using RapidFire Mass Spectrometry for Clone Selection of Bispecific Antibodies

William Sawyer

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With high sensitivity and throughput, ELISA and FRET assays are commonly used to detect and quantitate biotherapeutics. However, challenges associated with assay development time, sample specificity, detection interference have led to a major drive in developing high throughput mass spectrometry. With recent new advances, high-throughput label-free screening is now realized. Indeed, the Agilent RapidFire, a trap-n-elute liquid chromatography system coupled to mass spectrometry, has been routinely utilized for screening small molecule and peptide libraries. However, there are relatively few reported applications using this system to screen protein drugs such as the emerging bispecific antibody therapeutics. In our application, bispecific IgGs were generated through coexpressing two different light and heavy chains in a single host cell. While this strategy is potentially more efficient, unwanted mispaired IgG species can be produced in addition to the desired bispecific IgG. The goal is to rapidly screen through hundreds to thousands of clones through characterizing and quantitating bispecific antibodies to select for the best pair match. In order to achieve the desired throughput, we have developed a RapidFire-MS method towards this purpose. A variety of chromatography resins were evaluated and loading and elution parameters were optimized. Here, we report a high-throughput RapidFire-TOF platform for detecting and characterizing antibodies at low nanomolar range at a rate of 25 seconds per sample. This platform enables direct analysis of bi-specific antibodies and potential impurities in cell culture media for clone selection.

**NOTES:**

**P-230-T**

## Hydroxyl Radical Protein Footprinting for Measurement of Protein-Ligand Interactions in gp120

Joshua Sharp[1], Sandeep K. Misra[1], Amika Sood[2], Paulo A. Soares[3], Vitor H. Pomin[1], Robert J. Woods[2]

*[1]University of Mississippi, Oxford, MS USA, [2]Complex Carbohydrate Research Center, Athens, GA USA, [3]Institute of Medical Biochemistry Leopoldo de Meis, Rio de Janeiro, Rio de Janeiro, Brazil*

Experimentally measuring sites of ligand binding in proteins can be challenging, especially when the protein and/or the ligand is not a homogeneous entity but exists as a mixture or in multiple proteoforms/glycoforms. Here, we present recent results using a technology known as hydroxyl radical protein footprinting (HRPF) by Fast Photochemical Oxidation of Proteins (FPOP). This method uses *in situ* hydroxyl radical-protein chemistry to covalently label amino acid side chains, with the chemistry completed on the order of microseconds. Once the protein surface is labeled, the protein is processed with a standard bottom-up proteomics workflow, and the amount of oxidation at each amino acid side chain is measured by bottom-up LC-MS/MS. This analysis measures changes in the protein surface due to structural events based on changes in the apparent rate of oxidation by the radical when comparing two or more structural states (e.g. ligand-bound versus ligand-free).

We use HRPF by FPOP to determine the sites of binding for a model heparin pentasaccharide (fondaparinux) on HIV gp120 from the JR-FL strain. Heparan sulfate is known to play an essential role in viral adhesion and entry, so understanding how gp120 interacts with heparan sulfate at a molecular level is important. Additionally, we demonstrate how HRPF data can be coupled with computational methods such as blind and targeted ligand docking approaches to both cross-validate the results, as well as to provide atomic-resolution models that are supported by empirical HRPF data. Using these methods, we identify two sites of heparan sulfate binding for JR-FL gp120: the V3 loop and the N-/C-terminal tail domain. Molecular docking identifies basic residues that modulate the interaction and provide a molecular model to test for disruption of the interaction. These data demonstrate the usefulness of HRPF by FPOP to analyze complex protein-ligand interactions.

**NOTES:**

**P-231-M**

## Host Cell Protein Analysis of Biopharmaceuticals Using Automated Sample Preparation and LC-MS/MS

Linfeng Wu, Shuai Wu

*Agilent Technologies, Santa Clara, CA USA*

Biopharmaceuticals such as monoclonal antibody (mAb) have been growing fast in the past few years. Since biopharmaceuticals are generated from biological sources, some of the low-level host cell proteins (HCPs) can remain in the final products even after multiple purification steps. Due to their potential to affect product safety and efficacy, identification and quantification of HCPs in drug product are required by regulatory.

In this study, a workflow coupling automated sample preparation and LC-MS/MS analysis was demonstrated. A CHO cell-cultured purified IgG1 mAb spiked with UPS2 proteomic standards were used to evaluate the whole workflow. Digested samples were fractionated on cartridges using a sample preparation robot followed by LC-MS/MS analysis using a LC-QTOF for protein discovery and semi-quantification analysis. These results were also compared to samples without fractionation. The digest sample was also subjected to quantification analysis using LC-MRM assay coupled with stable isotope dilution method on a triple quadrupole (TQ) to evaluate the platform.

Our results show that all the spiking proteins above 2 ppm were identified with high confidence using the sample automation robot and LC-QTOF system. Peptides from HCPs could be quantified at sub-ppm level with excellent precision and accuracy using the LC-TQ system.

**NOTES:**

**P-232-T**

## Ultra-sensitive Quantification of Monoclonal Antibodies and ADCs in Mouse Plasma using Trap-Elute MicroLC-MS/MS Method

Lei Xiong, Ji Jiang, Remco van Soest

*SCIEX, Redwood City, CA USA*

Quantification of monocolonal antibodies (mAbs) and antibody-drug conjugates (ADCs) in biological fluids plays critical roles cross multiple stages of the biotherapeutic development. Nowadays, LC-MS has been routinely adopted for biologics quantitation serving as the orthogonal technology to the traditional ligand binding assays (LBAs). As the amount of biological sample that can be collected from a small animal is limited, sensitivity improvement of the biologics quantitation assay has called specific importance in LC-MS method development. The implementation of micro flow chromatographic technique and immunoaffinity based sample preparation method provides significant improvement on assay sensitivity. MicroLC provides multiple fold boost on signal intensity, while immunoaffinity based sample preparation dramatically improves the sample cleanness, thereby reducing baseline interference. Herein we introduce a hybrid LBA/microLC-MS/MS workflow for ultra-sensitive quantification of Trastuzumab Emtansine and SILuLite SigmaMab in mouse plasma.

Immunocapture workflow using magnetic beads was utilized to capture mAb and ADC from 25 mL of plasma. The analytes were eluted from the beads and digested by trypsin/lysineC. The digested samples were then subjected to LC-MS/MS analysis with MRM mode. In order to achieve the desired assay sensitivity with limited sample, a 5 µL/min HPLC flow rate was applied for improved ionization efficiency; a trap-elute LC profile was used to increase sample injection volume and shorten sample loading time. The MRM parameters for signature peptides were extensively optimized.

With the optimized method condition, the presented micro-flow assay achieved a LLOQ of 1 ng/mL for trastuzumab emtansine quantification and 2 ng/mL for SILuLite SigmaMab in mouse plasma. The assay accuracies are 85-115% and CV%s are below 15% for all tested samples. The calibration curves cover 4-4.5 orders of magnitude and display regression coefficients (r) > 0.995 using a weighting of $1/x^2$.

**NOTES:**

**P-233-M**

## Assessment of the Impact of IgG Antibody Post-translational Modifications (PTMs) on the Neonatal Fc Receptor (FcRn) Binding Using a Rapid Affinity Chromatography- Mass Spectrometry Method

Xueqing Zhao

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The neonatal Fc Receptor (FcRn) is critical to protect IgG from lysosomal degradation and to regulate the IgG half-life in circulation. The presence of PTMs in the Fc region of an IgG may affect FcRn-IgG interaction and thereby influence the pharmacokinetic properties of an IgG *in vivo*. We have developed an online two-dimension liquid chromatography (2D-LC) –mass spectrometry (MS) method to quickly assess the effect of PTMs on the binding between the antibody and FcRn. The first-dimension affinity separation is conducted on a stationary phase containing immobilized FcRn (Roche Diagnostics, Germany). Antibodies and antibody PTM variants, bound to the FcRn affinity column are eluted from the column using a pH gradient, which mimics the physiological FcRn-IgG binding and dissociation process. The eluted antibody species of interest can be sequentially fractionated, desalted and further separated online with the second-dimension reverse phase column and then analyzed directly by MS. With the 2D-LC setup, sample handling is minimized, and potential contamination and sample loss are also reduced.

This novel approach has been successfully applied to characterize monoclonal antibodies and their oxidation variants. Compared to the native antibodies, decreased binding was observed for antibodies exhibiting high levels of oxidation; this result has been confirmed by Biacore assay. This FcRn 2D-LC-MS method can simultaneously evaluate FcRn-IgG binding, while identifying critical PTMs on the separated variants that can influence FcRn binding.

**NOTES:**

**NOTES:**

# Process and Product Characterization

**P-234-T**

## Epitope Mapping of Diphtheria Toxin by Hydrogen Deuterium Exchange Mass Spectrometry (HDXMS)

Shaolong Zhu[1], Peter Liuni[2], Derek Wilson[2], Andrew James[1]

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Hydrogen Deuterium Exchange Mass Spectrometry (HDXMS) has emerged as a critical tool for characterizing protein conformation, protein-protein and protein-ligand interactions. This technology has been adopted in biopharmaceutical settings for evaluating protein stability, structure, and for analysis of antigen/antibody epitopes. In this study, HDXMS was implemented to conduct epitope mapping of diphtheria toxin (DTx) against two monoclonal antibodies (mAbs) in an effort to better understand mAb/antigen interactions during development of next-generation antigenicity/potency assays. DTx is composed of three domains: 1) Receptor (R) domain for entry into the cell through receptor-mediated endocytosis. 2) catalytic (C) domain for inhibiting protein synthesis through ADP ribosylation of elongation factor 2. 3) translocation (T) domain for insertion and creation of pore into the endosome facilitating the transfer of C domain into the cytosol.

Waters Synapt G2-S coupled to a nanoAcquity UPLC system with HDX module was used to perform all the HDX time course experiments. 10µM of free DTx and 10µM:20µM (DTx:mAb) of the complex was allowed to mix with deuterated buffers (90%) at pH/pD 7.5 for times ranging from 2 min to an hour. The reaction was quenched at pH 2.5 with subsequent digestion of deuterated protein in pepsin/protease XIII (1:1) column.

HDXMS profiles comparing free DTx, and two DTx-mAbs complexes, yielded approximately 90% sequence coverage. HDX data for one mAb showed reduction in deuterium uptake spanning the T and R domains whereas the other mAb showed reduction of deuterium uptake in the C domain partly encompassing separate regions of the catalytic loop. In both instances, the epitopes were conformational. Taken together, our HDX results are consistent with western blot experiments conducted to corroborate DTx domain recognition by the selected mAbs.

**NOTES:**

**P-235-M**

## Identification and Quantification of Glycans from Proteins with Multiple Glycosylation Sites by Automated LC-MS Data Processing

Sven Bahrke

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Glycosylation is a critical quality attribute of therapeutic proteins for development of innovator biotherapeutics as well as biosimilars. LC-MS based methods are powerful tools for glycoprofiling. As manual evaluation of the LC-MS data is time consuming and prone to errors automation of the data processing appears to be an advantageous improvement.

The present study demonstrates the versatility of an automated solution for glycan characterization of commercial therapeutic proteins comprising different numbers of glycosylation sites (2 - 8). Data processing time, regulatory considerations and quality (total assigned peak area and the number of false annotations) are evaluated. Products resulting from different rodent and human cell lines were investigated.

N-glycans were released enzymatically from the proteins by N-glycanase F and subsequently labeled with fluorescent dyes. Data were recorded by using an HILIC-UPLC-FLD-ESI-QTOF MS/MS system. Recorded data were automatically processed (GlycoFiler) including integration of fluorescence data, deconvolution of MS/MS spectra and matching to a glycan library, calculation of glycan parameters of biologically relevant quality attributes like core fucosylated glycans, high mannose type structures, mono- to tetrasialylated glycans etc. With each data acquisition a set of standards is run to prove validity of the analyses, automation features comparison of the samples to the standards.

The absolute number of N-glycan structures automatically detected and quantified ranged from 46 (Trastuzumab) to 368 (EGFR) depending on the number of N-glycosylation sites and the expression cell line.

Automated structure identification is based on an MS/MS spectra library comprising characteristic fragments for isobaric structures like core vs. antennary fucose.

Increasing complexity of the N-glycosylation neither compromises speed of the automated data processing, ranging from 2 – 4 minutes, nor quality of the analyses in comparison to manual data evaluation: The non-assigned peak area ranged from 1-2%, false positives were below 2%. Replicate analyses resulted in standard deviations of < 2%.

**NOTES:**

**P-236-T**

## Biotherapeutic Thermal Stability and Aggregation Tracked Using Ion Mobility Spectrometry

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*IonDx, Monterey, CA USA*

We introduce a new type of ion mobility spectrometry to investigate variations in the higher-order structure (HOS), aggregation and thermal stability of biotherapeutic drugs. A first technical benefit of our ion mobility measurements is the capability to study M(1+) ions that have not been previously distorted during the electrospray process. We show the variation in CCS of 6 antibodies that were electrosprayed but prevented from passing through high charge states and therefore assumed to be in a near native conformation. In contrast, CCS data for electrosprayed biotherapeutics measured using ion mobility-mass spectrometry show CCS increasing with the number of charges. The large number of charges on an electrosprayed protein stretches its conformation, thus leading to CCS values that are distorted. Our technique eliminates the coulombic distortion problem. A second technical benefit of our technology is a capability for measuring the thermal stability of biotherapeutics. Our method connects forced thermal degradation with our ion mobility spectrometer and monitors the concentration of M(1+) ions as a function of sample temperature. We present thermograms for 6 antibodies including NISTmAb and use these plots to calculate melting point temperatures. The thermograms lead to melting point temperatures that are similar to Tm values derived from differential scanning calorimetry (DSC). The biggest benefit of this method for determining Tm, is the sample requirement – only 0.5 ug of sample is needed compared to mg for DSC, thus allowing thermal stability tests to be run at all stages of product development, including early stages when bacterial expression vectors are used before manufacturers switch to mammalian expression systems. The simplicity of this methodology opens the possibility for real-time process monitoring.

**NOTES:**

**P-237-M**

## Refinement of the Multi-attribute Method to Monitor Product Quality Attributes in Biotherapeutics

Kristin Boggio[1], Carly Daniels[2], Andrew Dawdy[2], Anastasiya Manuilov[1], Wenqin Ni[1], Himakshi Patel[1], Thomas Powers[2], David Ripley[1], Joshua Woods[2], Ying Zhang[1], Olga Friese[2], Matt Thompson[1], Keith Johnson[1], Justin Sperry[2], Jason Rouse[1]

*[1]Pfizer, Inc., Andover, MA USA, [2]Pfizer, Inc., Chesterfield, MO USA*

Biotherapeutics, with modalities ranging from monoclonal antibodies to gene therapies, comprise a large portion of pharmaceutical development pipelines. Development of biotherapeutics requires a thorough understanding of product quality attributes (PQAs) to ensure that the product meets desired quality, safety, and efficacy profiles. Conventionally, an array of chromatographic, electrophoretic, and ELISA-based methods are used to monitor a wide range of PQAs including product-related structural heterogeneity such as glycosylation, charge isoforms, fragmentation, aggregation, and oxidation. Execution of multiple methods, however, is time consuming, costly, and often provides an indirect measure of biologically relevant PQAs. We have incorporated the mass spectrometry (MS)-based multi-attribute method (MAM) at various stages of the non-GMP product development lifecycle to monitor multiple PQAs simultaneously within a single experiment, to simplify analyses.

MAM involves low-artifact LC/MS-peptide mapping with a high-resolution mass spectrometer and bioinformatics with new peak detection (Biopharma Finder and Chromeleon (Thermo Scientific)). Data analysis using Biopharma Finder allows for the identification of PQAs, while Chromeleon allows for semi-automated monitoring and quantitation of known PQAs and the detection of new peaks. In this presentation, a Design of Experiment (DoE) based refinement of the MAM digestion method was performed across a panel of IgG monoclonal antibodies (IgG1, IgG2, and IgG4) by optimizing reduction/alkylation conditions, digestion time, buffer exchange, protein concentration, and choice of enzyme, to produce a robust method. ANOVA analysis of identified PQAs revealed that reduction/alkylation conditions were robust and didn't greatly impact results, while protein concentration and choice of digestion enzyme had a greater impact on observed digestion efficiency.

**NOTES:**

**P-238-T**

## Mass Spec Approaches to Detect Clipped Forms of Target-related CHO Homolog in a High Concentration rhumAb X

Scott Chamberlain[1], Viswanatham Katta[2]

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When a therapeutic antibody (rhumAb X) targeted to a human 5k peptide (hu-pep5k) was expressed in CHO cells, it is possible that a CHO homolog of peptide (CHO-pep5k), if present, gets copurified. It is challenging to detect and measure such low-level impurities in the antibody formulated at ~150 mg/mL. The technique must be 'blind' to the antibody and/or the impurity has to be enriched in some fashion for the impurity peptide to be detected, characterized and amounts measured.

At the exploratory stage, MALDI-TOFMS turned out to be uniquely capable of detecting different low-level clipped forms of the CHO-pep5k. It provided high sensitivity for low MW peptides even in the presence of large excess of the antibody and formulation components. The spectra showed intense signals from low mass peptides (<3kDa) that could be identified by TOF-TOF method to be clipped forms 11-33, 11-34 of the homolog CHO-pep5k. A simplified sample prep with 4HCCA matrix allowed generation of linear maldi-tof spectra of the peptide impurities and intact antibody from the same spot. LC/MS/MS (Q-Exactive) allowed injection of larger volumes of supernatant after precipitating the antibody. This data verified the presence of the 11-33 and 11-34 forms and also showed other minor clipped forms. Spiking experiments with N15-labeled CHO-pep5k indicated the intact peptide to be at lower levels than some of the clipped forms. All the observed clipped forms contain the binding epitope of the target for this antibody. The observed cleavage sites are attributable to a less specific enzyme known to play a role in CHO-pep5k formation.

**NOTES:**

**P-239-M**

## Development of a Procedure to Minimize Oxidation and Deamidation During Protease Digestion

Ken Chanthamontri, Amber Henry, Pegah Jalili, Kevin Ray

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Non-enzymatic deamidation and oxidation is a major source of recombinant monoclonal antibody instability and microheterogeneity. In order to obtain an accurate assessment of site-specific Asparagine (Asn) deamidation and Methionine (Met) oxidation, it is critical to reduce artifactual modifications that occur during sample preparation steps. However, typical protein digestion workflows often introduce a significant amount of artifacts such as deamidation and oxidation. In this study we have developed an optimized protocol utilizing an improved digestion buffer which allows efficient protein digestion in less than 6 hours with minimized artificial modifications such as deamidation and oxidation.

The optimized digestion protocol was used to investigate two case studies. During the first case study, a model monoclonal antibody known to be particularly susceptible to oxidation during downstream processing was analyzed using the optimized workflow. The levels of oxidation and deamidation were compared in stressed, protein A-purified samples using the standard purification procedure and control samples. The results show that the average level of oxidation was 4% for control antibody sample and increased to 12% and 11.3% for purified sample and stressed sample, respectively. A second case study was performed to compare our optimized protocol with a protocol recently published by NIST. The levels of deamidation, oxidation under-alkylation, and missed cleavages were calculated for both protocols using peak areas from extracted ion chromatograms. Our results indicate that the levels of deamidation and oxidation, missed cleavages, and under-alkylation were similar for both protocols *e.g.* the average amount of oxidation of six Met containing peptides for triplicate digestions was observed to be 1.8% and 1.9% for the NIST protocol and our digestion protocol, respectively. The average amount of deamidation of 13 Asn containing peptides was 0.3% and 0.2% for the NIST protocol and our protocol, respectively.

**NOTES:**

**P-240-T**

## Metabolomic Profiling of Fed-batch CHO Cell Culture Samples Using LC-MS and GC-MS Methods

Rachel Chen, Amr Ali, Zoran Sosic, Li Zang

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Most of recombinant therapeutic proteins are currently produced by mammalian cell culture processes, such as Chinese Hamster Ovary (CHO) cell line. Process engineering efforts, including optimization of culture media, feeding strategies and culture conditions, were extensively applied to increase the productivity and improve product quality of therapeutic proteins. To better understand the underlying cellular metabolism associated with a cell culture process change, metabolomics analysis using three methodologies, including non-targeted LC-MS and GC-MS methods as well as a targeted LC-MS quantitation method, was performed on fed-batch CHO cell culture samples and the results were compared.

Non-targeted LC-MS and GC-MS methods were employed for metabolite detection from the spent media taken from bioreactors at different time points. The non-targeted LC-MS method, which involved hydrophilic interaction chromatography (HILIC) separation coupled with MS analysis on an LTQ-Orbitap, identified ~140 compounds, through accurate mass and MS2 fragmentation matching. GC-MS analysis on a TOF MS instrument identified similar number of compounds through library mass spectrum matching. In addition, a targeted LC-MS method using MRM-based quantitation strategy (developed by Shimadzu) was employed for the monitoring of 95 metabolites that are commonly observed in cell culture media.

The non-targeted LC-MS and GC-MS methods provided complementary compound coverage from the cell culture analysis. LC-MS method identified most amino acids, their derivatives and degradants, dipeptides, vitamins, nucleotides, etc., while GC-MS analysis detected other compound classes, including carbohydrates, sugar phosphates, hydroxyl acids, and fatty acids. The targeted LC-MS quantitation method captured the relative abundance change of major culture medium components and cellular metabolites in a high-throughput fashion. A principle component analysis (PCA) of the data revealed a significantly different metabolomic profile under the selected culture condition. The differentially expressed compounds were mapped to KEGG pathways, which allowed for a better understanding of cell culture metabolism affected by the process change.

**NOTES:**

**P-241-M**

## Robust Automated Platform for Routine Analysis and Investigations Regarding Metabolites in Cell Culture using the Waters Quadrupole Dalton (QDa®)

Scott Corley, Eike Zimmermann

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We present a novel robust method for the routine analysis of metabolites in cell culture using the Waters Quadrupole Dalton (QDa®) single quadrupole mass spectrometer. The method is comprehensive with single analysis detection of amino acids, vitamins, organic acids, glucose, and other small molecules. This method represents an improvement upon UPLC methods typically used to monitor metabolites, such as the Waters AccQTag Ultra method to monitor amino acids and UV methods to monitor organic acids. These improvements include quantification of a broader range of metabolites in a single assay, a linear range of quantification over 2 orders of magnitude through the use of internally labeled standards (ILS), streamlined sample preparation including a consistent single dilution, elimination of any derivatization, and automation using a TECAN liquid handling robot. The method is robust enough to allow automated data processing, saving additional analyst efforts. We have demonstrated excellent comparability to typical techniques used to monitor cell culture media. The use of a comprehensive metabolite method in-house delivers our upstream colleagues faster process understanding and expedites process improvements. Finally, the use of the QDa represents an alternative to numerous reports of higher resolution mass spectrometry methods to monitor metabolites, where the QDa is an easy to use, lower cost addition amenable to most analytical labs in biopharma where operation does not require expertise in mass spectrometry. Additionally, we will present a case study, where the QDa was used to confirm addition of lipid metabolites into media designed to increase specific productivity in our cell line.

**NOTES:**

**P-242-T**

## Identification, Quantification and Monitoring of Low-Abundance Host Cell Proteins During Monoclonal Antibody Purification

Catalin Doneanu

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In recent years, LC/MS-based assays have been adopted as orthogonal techniques to ELISA for HCP analysis due to their flexibility and sensitivity. Here we explored the capabilities of a single dimension chromatographic assay, coupled with mass spectrometry using two different data independent acquisition modes for detection of peptide precursors and their fragments. While in MSE mode all peptide ions produced by the electrospray source are transmitted by the quadrupole analyzer, in SONAR mode the quadrupole slides over the mass range of interest during the time required for recording a single MS-spectrum by the TOF-analyzer. Co-eluting precursor ions with different m/z are separated during the rapid quadrupole scan and their corresponding fragmentation spectra are acquired using an identical quadrupole separation. Mass spectra are recorded with high-resolution (~ 25,000) for precursors/fragments. The first step of the HCP identification and quantification workflow is the HCP Discovery Assay performed in SONAR mode using extensive (90 min) peptide separations. Following data processing with Progenesis QI for proteomics 4.0, the HCPs are identified by a proteome-wide database search. In addition, SONAR MS/MS fragmentation spectra can be assembled into spectral libraries, containing peptide precursors, charge states and retention times. In the second step of the HCP workflow, additional HCP samples resulted from the purification of the same biopharmaceutical, are analyzed by higher–throughput HCP Monitoring assays employing data-independent MSE acquisition with 30 min peptide separations. The LC/MSE dataset is then searched against the spectral library for HCP identification and quantification at every step during biopharmaceutical purification. An antibody product initially purified by Protein A affinity chromatography was further purified by SCX (strong-cation exchange) chromatography using different elution conditions. Two HCPs and 4 spiked protein standards were identified across 5 mAb preparations (one Protein A and 4 SCX fractions). The sensitivity of both assays (Discovery and Monitoring) was 10 ppm.

**NOTES:**

**P-243-M**

## Leveraging Proteomics to Improve CHO Bioreactor Productivity

Brian Gau[1], Revati Wani[1], James Guthrie[1], Peter Slade[1], Olga Friese[1], Jason Rouse[2]

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Increasing cell culture productivity is an imperative for economical biotherapeutic drug manufacture. With late stage projects, the bioprocess levers for productivity are limited to exogenous controls, such as media constituent levels, feed frequency, and pH. Historically these have been optimized by design-of-experiment studies on smaller-scale bioreactors. While such an empirical approach is powerful, it provides little molecular insight as to how the most important changes mediate improved productivity. Proteomics can illuminate this underlying biochemistry by highlighting the multitude of host cell proteins—in addition to the expression target—that are down- or up-regulated.

We report the highlights of a proteomics study of two treatments which gave substantial improvement in mAb production in CHO cells. Nocodazole is a drug used to arrest mitosis. Treatment of bioreactor cultures with Nocodazole resulted in doubling of the Qp , relative to that of the control, 72 h after inoculation at Day 5. This trend was accelerated at higher pH. 27 cell pellet and spent media samples were trypsin-digested and analyzed by reversed-phase LC-MS/MS using a long-gradient. The Orbitrap Elite ultrahigh-resolution mass spectrometer ensured accurate HCP ID, with MS/MS spectra matched to peptides by a Mascot search of the NCBInr and Pfizer databases. Relative quantitation of HCPs was based on component peptide peak areas. Protein annotation and interaction data were collated using String-db.org and pathway databases. We found that Nocodazole treatment promotes increases in expression of proteins associated with oxidative phosphorylation, fuel metabolism, and protein maturation in the ER, and decreases in expression of proteins associated with the cell cycle.

**NOTES:**

**P-244-T**

## Automated MS Analysis for High-volume Quality Control of Recombinant Protein Variants

Albrecht Gruhler[1], Arnd Brandenburg[2]

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Samples from a variant screen containing hundreds of ~40 kDa proteins were prepared in 96-well plates, analyzed by Size Exclusion (SE)-UHPLC, and characterized by LC-MS intact mass analysis. SE-UHPLC was used to determine the protein concentration and the amount of high- and low-molecular-weight protein. Subsequently, each sample was subjected to LC-MS analysis using a Bruker maXis II instrument. Collected MS data were analyzed using an automated batch processing workflow running on the Genedata Expressionist MS data processing platform.

Briefly, data processing comprised loading raw data files, generating average spectra for the RT ranges in which proteins eluted, deconvolution over the relevant mass ranges, and identification of different species through mapping to the protein's sequence. Deconvolution preserved the isotopic structure of the peaks and allowed highly accurate calculation of the monoisotopic masses of the isotope clusters. Automatic protein mapping, which took several known post-translational modifications into account, was performed for each sample. Finally, a report listing all identified protein peaks and the highest-intensity unidentified protein peaks was generated.

In this way, we could efficiently and reproducibly process the ~50 GB of MS data generated during the analysis of each 96-well plate.

**NOTES:**

**P-245-M**

## Amino Acid Profiling to Improve Production and Quality Targets of Therapeutic Monoclonal Antibodies using Rapid, High Throughput Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry

Diksha Gupta, Brian Lee, Daniel Tengco, SJ Diong, Vangipuram Rangan, Gavin Dollinger

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Amino acids are the basic supplements of cell culture feeds with a known effect on productivity, therefore, cell culture feeds optimization is a critical step. Using a microfluidic capillary electrophoresis (CE)-MS platform (ZipChip, 908 Devices) we can rapidly (<2 min) analyze amino acids directly from cell culture with virtually no sample preparation. This strategy aims to identify the most effective amino acids and related concentrations for target product enhancement. Here we demonstrate rapid, high throughput screening of amino acids for the optimization of feed strategy in both hybridoma and CHO cells.

**NOTES:**

**P-246-T**

## O-Glycosylation in a CDR of an IgG1 Antibody -Structural Characterization of a Rare Variant Using High Resolution LC-MS Analysis

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During clinical product development, it is critical to characterize unknown product variants, gain knowledge regarding potential impact on safety and potency, and develop an appropriate control strategy to ensure adequate product quality. Recently, an IgG1 antibody in early stage development was found to contain an elevated level of acidic charge variants. In this study, comprehensive analysis using mass spectrometry and other analytical tools was performed to identify and characterize the variants.

Liquid chromatography-mass spectrometry (LC-MS) analysis of the intact protein revealed that the product contains a variant 947 Da larger than the expected mass. With peptide map analysis, it was determined that the unknown modification is located in one of the light chain complementarity determining regions (CDRs). Further detailed characterization was performed with a unique data-dependent LC-MS/MS method using two different fragmentation mechanisms, higher-energy collision dissociation (HCD) and electron transfer dissociation (ETD). Data generated with this method provided complete structural information, including the sequence of the modified peptide, the modification attachment site, and the structure of the modification. The results indicated that one of the serine residues of the CDR-containing peptide was modified with negatively charged, sialic acid-containing *O*-glycans. More than 80% of the determined *O*-glycan species is Hex1HexNAc1NeuAc2, which contains two sialic acid residues.

This identification was confirmed by sequential treatment of the variant with sialidase and *O*-glycanase enzymes, which removed specific components of the glycan, followed by LC-MS analysis. The impact of the variant on bioactivity was assessed in an antigen-binding assay using a sample enriched with the variant and the results were used to establish the control strategy for the product.

**NOTES:**

**P-247-M**

## Development of a Targeted nanoLC-MS/MS Method for Quantitation of Residual Toxins from Bordetella Pertussis

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Pertussis, also known as whooping cough, is a highly contagious respiratory disease caused by infection by Bordetella pertussis (*B. pertussis*). Pertussis pathogenesis is driven by cell-surface adhesion proteins and secreted toxins; some of which have been harnessed for their immunogenic properties as purified components in acellular vaccines. Three of these virulence factors, tracheal cytotoxin (TCT peptide), adenylate cyclase toxin (ACT) and dermonecrotic toxin (DNT), are toxins with potential for co-purification and thus must be monitored as process-related impurities. Residual toxins are traditionally detected using animal test or cell-based approaches. Like all biologically-driven methods, these assays are subject to variability from matrix effects and are unable to match the specificity provided by targeted mass spectrometry (MS)-based workflows.

This work describes the development of a targeted MS-based analysis for sensitive and selective detection of residual TCT, ACT and DNT from *B. pertussis.* In silico tryptic digests of ACT/DNT protein sequences were used to generate synthetic peptide libraries comprised of candidate peptides for detecting low concentrations of ACT/DNT in biological samples purified from *B. pertussis*. Candidate peptides were screened by nano LC-MS/MS on a high resolution, high mass (Q-Exactive HF) instrument with peptide performance assessed at both the $MS^1$ and $MS^2$ level. Parallel reaction monitoring (PRM) and data dependent acquisitions (DDA) were used to establish specificity, linearity and reproducibility of precursor and fragment ion responses for targeted, analyte-specific fragmentation patterns. Collectively, the results demonstrate that a synthetic peptide screening approach can be used to develop a targeted method for detecting residual toxins from *B. pertussis*. The targeted method will be evaluated as an in vitro strategy for quantitation of residual protein toxins in biological matrixes.

**NOTES:**

**P-248-T**

## Employing MS-based Multi-attribute Methods (MAMs) for Automated Quality Monitoring of Biotherapeutics

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Production of biopharmaceuticals requires costly process monitoring strategies and quality systems to ensure final product quality. Critical quality attributes (CQAs) — typically measured using a variety of analytical techniques — are used to analyze biopharmaceuticals before release. However, CQAs are generally related to physical properties of biopharmaceuticals and do not characterize the product at the molecular level. Many biopharmaceutical producers are exploring MS-based multi-attribute methods (MAMs), which enable measurement of multiple quality attributes in a single test using a single technology and provide detailed insights at the molecular level. Such methods are especially attractive because they offer the potential of reducing development and manufacturing costs while at the same time, increasing product quality and safety. We present an automated MAM processing workflow using the Genedata Expressionist® software platform (Genedata AG, Basel, Switzerland) for automated quality monitoring of biotherapeutics. In an initial step, a system suitability test provides confirmation that the performance of the analytical instrumentation — and the quality of the data that it delivers — is adequate for the intended analyses. Dedicated data processing workflows are tailored to measure the CQAs for a given biomolecule. Optimized data processing applied to large data sets and execution times scaled linearly with the number of samples. Multiple quality attributes — such as the presence, location, and relative ratios of amino acid modifications in the target molecule — could be determined from a single data set. Comparison of test and reference samples enabled the identification of potential contaminants. A data analysis workflow used to monitor a bioreactor in real time demonstrated that an implementation of the MAM approach created using the software platform was amenable to automation. The availability of a compliance module offering GxP functionalities — such as audit trails, electronic signatures, and data- and user-management — facilitates the deployment of such MAM implementations in regulated environments.

**NOTES:**

**P-249-M**

## Unexpected Asp-isomerization Behavior in Therapeutic Proteins: Connecting Primary Structure with Higher Order Structure and Dynamics

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The complexity of protein-based therapeutics contributes to their overall liability to chemical modification and degradation that can negatively impact the shelf-life of drug products and significantly increases burden during manufacturing process development. In discovery and early development stages of research and development, multiple candidates are evaluated for potency and stability; however, timeframes are often very short for making decisions on optimal molecules. One of the more challenging modifications, Asp isomerization, results in only a slight local re-arrangement of the poly-peptide chain and is often only apparent after prolonged stability testing. Yet iso-Asp modifications can have a deleterious effect on biological activity, especially when located in the active CDR region. In an effort to determine accelerated stress conditions that predict long-term stability behavior, three therapeutic mAbs containing 'DS', 'DG' & 'DD' isomerization motifs were exposed to varying pH and temperature conditions. Mass-spectrometry which has established itself as an exquisite tool for identifying protein modifications was used to monitor and quantify the chromatographically separated iso-species. Asp isomerization is known to be catalyzed under low pH conditions, however surprisingly for one mAb, a significantly faster rate of modification was detected under high pH conditions. Biophysical characterization of the molecule at the different pH suggested a correlation between higher order protein structure and primary structure modification. Molecular dynamics simulation of the fab with residue protonation states corresponding to high and low pH showed that the CDR loop containing the Asp site was substantially more flexible at high pH. The simulations suggest that the atypical pH dependence of Asp isomerization is due to pH-dependent flexibility of the CDR loop. The results of this study suggest that accelerated stress studies complimented with molecular simulation can provide predictive and mechanistic insight into long-term stability of protein therapeutics.

**NOTES:**

**P-250-T**

## Characterization and Monitoring of Post Translational Modifications Using Dedicated MAM Software

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Development and production of biopharmaceuticals are complex and challenging. Frequently the assessment of post-translational modifications including deamidation, oxidation, glycosylation, glycation among many others must be assessed to understand their impact on safety and efficacy of the final product. As the molecular complexity and the challenges for commercialization increases, there is a need for enhanced analytical approaches to detect and track a wide range of post-translational modifications throughout the development of biotherapeutics. Traditionally, multiple analytical techniques have been required to assess the full range of biopharmaceutical product attributes; However, the application of mass spectrometry has recently been applied for simultaneous detection and quantification of a wide range of molecular properties which can supplement or potentially replace traditional orthogonal assays.

Peptide mapping with mass spectrometry is a common assay for assessment of post-translational modifications. Recently the concept of a Multiple Attribute Methodology (MAM) has been introduced which places greater rigor on the use of LC-MS peptide map experiments as an orthogonal approach for characterization and monitoring of biopharmaceutical attributes. The range of modifications that can be identified and monitored using this approach is extensive. In addition, the ability to localize modifications is enhanced using an LC-MS approach compared to indirect measurements which may provide greater confidence in the correlation of characterization data with downstream outcomes. In addition to tracking the therapeutic molecule itself, an LC-MS approach using MAM may be used to detect specified and un-specified impurities related to the biotherapeutic production providing a purity assessment.

Presented here is the use of High-Resolution Accurate Mass data processed using BioPharmaView™ 3.0 Software for characterization and quantification of a range of post-translational modifications of a biotherapeutic. We will also discuss the use of this solution for quantification of specified impurities and highlight the ability to detect un-specified impurities using new peak detection.

**NOTES:**

**P-251-M**

## Pharma Industry Case Studies with a Focus on Therapeutic Protein Development and Characterization

Michael Molhoj

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In the pharmaceutical industry, mass spectrometry goes hand-in-hand with therapeutic protein development and characterization. The talk will include real-world applications of mass spectrometry and among other topics include case studies on the detection and verification (and challenges) of rarely reported PTMs like pSer, 4-Hyp, sulfation, and succinimide cleavage during product characterization; differences or no differences between early-stage HEK and late-stage CHO cells derived materials; novel verification of hinge region *O*-glycosylation site by MS; how we approach preventing PTMs; development of our domain crossover bispecific antibody formats (CrossMab CH1-CL, VH-VL, and Fab) to ensure correct light chain assembly and prevent side products; subunit generation and analysis for functional analysis; and next level online therapeutic protein characterization using a flexible custom-made multidimensional liquid chromatography (MS/MS) system.

**NOTES:**

**P-252-T**

## LC-MS Based Characterization of an eNIST-Fab Fragment Produced in Escherichia coli

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The NIST monoclonal antibody (NISTmAb) reference material, RM 8671, provides a representative test molecule for performing system suitability tests, assisting in method qualification, and evaluating method/instrument performance and variability. For example, the Fab fragment of the NISTmAb RM 8671 (NIST-Fab) has been prepared and distributed for interlaboratory determination of higher order structure using techniques such as hydrogen-deuterium exchange mass spectrometry (HDX-MS) and nuclear magnetic resonance spectroscopy (NMR). Stable isotopically labelled versions of the NIST-Fab are required to facilitate NMR resonance assignment, provide contrast for small angle neutron scattering, and serve as internal standards for quantitative mass spectrometry. Mammalian cell expression, however, is not traditionally used as a practical or cost effective means of obtaining isotopically labeled mAbs; isotopically labeled proteins are commonly expressed in *Escherichia coli* (*E. coli*) gown in minimal media.

A recently developed *E. coli* strain was engineered with the intention of enhancing the fidelity of correct disulfide bond formation; this *E. coli* strain was subsequently used to produce a prototype Fab fragment from the NISTmAb, denoted as eNIST-Fab. Herein we utilize LC-MS based strategies for characterization of the eNIST-Fab prototype and subsequent comparison to the Fab fragment of the NISTmAb 8671. Although mAbs expressed in *E. coli* are not glycosylated, many features in the critical Fab fragment are shared with their mammalian counterparts. Product quality of the eNIST-Fab and evaluation of molecular sameness to the NIST-Fab are critical for assuring suitability of eNIST-Fab for intended use in the development of improved structural and biophysical techniques.

**NOTES:**

**P-253-M**

## Using Mass Spectrometry to Identify Host Cell Hydrolases in Biopharmaceuticals

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The importance of biopharmaceuticals in the treatment of severe diseases is steadily increasing. The bioprocess leading to high quality pharmaceuticals is complex and consists of a wide range of different steps and techniques. In the first part of biopharmaceutical production, the product of interest is expressed in a suitable host cell. The obtained cell culture fluid still contains– beside the desired therapeutic product – plenty of host cell impurities. The vast majority of these protein impurities are removed during the chromatographic purification steps applied in the downstream process. However, some host cell proteins are still present at the final process stage, e.g. due to the ability to interact with the antibody under the conditions applied. The remaining host cell proteins can adversely affect product quality due to different reasons. For example, hydrolases can either degrade the therapeutic product (proteases) or polysorbates (lipases) which are widely used to stabilize biopharmaceutical products like therapeutic antibodies.

In order to clarify the identity of host cell proteins impurities, mass spectrometry is the method of choice. However, the sensitivity of this analysis is hampered by the tremendous excess of the desired antibody over the host cell impurities. Hence, we focused on two different strategies to enhance the level of MS-based host cell protein identifications: MS analysis of antibody-depleted process samples and enrichment of low abundant protein impurities. Towards the latter approach, unknown host cell proteins were chromatographically enriched via a particular resin. Furthermore, current enrichment strategies focus on host cell proteins interacting with the therapeutic antibody as well as on fishing enzymes containing an active serine as found in the majority of hydrolases.

**NOTES:**

**P-254-T**

## High Throughput CE-based N-Glycan Analysis for Clone Screening in Biotherapeutic Protein Production

Amita Goel[1], Ruchika Srivastava[1], Thiago Millen[1], Nikhil Goel[1], Nicy Varghese[1], Divya Goel[1], John Yan[2], Tom Rice[2], Jim Torrence[2], Aled Jones[2], Ted Haxo[2]

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The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics, making glycan characterization an essential part of the development process. Glycan profiling is becoming more common and increasingly necessary in the early stages of biotherapeutic cell line development. Cell culture process optimization conditions calls for continuous assessment of the glycan profile. This requires significantly increased throughput for sample preparation, analytical instrumentation, data processing and expertise in glycan characterization. Unfortunately, these factors can cause a bottleneck to results when using traditional technologies.

Here we present a streamlined glycan screening solution that combines rapid sample preparation and analysis with a simplified data processing approach. The sample preparation includes a 5-minute deglycosylation step to release N-glycans, followed by glycan labeling and cleanup, and may be completed in under 1 hour. Labeled N-glycans are separated using a small and user-friendly capillary electrophoresis (CE) instrument, with a run time of 2 minutes per sample. This complete and focused approach allows for the preliminary screening of hundreds of single clones and cell culture conditions, enabling the selection of top candidates based on the desired N-glycan profile. Top clone candidates and optimized cell culture conditions can be efficiently selected for higher resolution profiling such as LC-FLR and LC-MS.

**NOTES:**

**P-255-M**

## Routine Analysis of Host Cell Proteins in Antibodies using PASEF

Stuart Pengelley[1], Guillaume Tremintin[2], Waltraud Evers[1], Mike Greig[3], Detlev Suckau[1]

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Therapeutic antibodies are manufactured in cell culture and downstream purification steps are applied to remove host cell proteins (HCPs). Since residual HCPs in monoclonal antibodies (mAbs) can cause immunogenic reactions or cause product degradation, there is a requirement to monitor these impurities during purification and in the final product. Bottom-up mass spectrometry approaches have already been established for analyzing HCPs but the low abundance of these proteins, in the region of 1 – 100 ppm in relation to the mAb, still present a significant challenge. Here, PASEF (parallel accumulation and serial fragmentation) scans, implemented on the timsTOF PRO QTOF, have been used to separate ions by m/z and mobility to detect residual HCPs in a single experiment using a standard LC-MS configuration.

The NISTmAb Reference Material 8671 and the Universal Proteomics Standard (UPS1, Sigma) were reduced using DTT in TFE solution and alkylated with iodacetamide prior to overnight digestion with trypsin (Promega). Peptides were separated with an analytical HPLC platform (Elute UHPLC, Bruker) and a nano hPLC platform (nanoElute, Bruker) coupled to a timsTOF Pro mass spectrometer (Bruker) using the PASEF scan mode. The detection limit for spiked protein as well as actual NISTmAb HCPs was evaluated.

The increased sensitivity obtained with the PASEF scan mode allowed improving on prior results both in terms of number of proteins identified and number of peptides identified for the detected HCPs. Over 200 HCPs could be identified.

**NOTES:**

**P-256-T**

**Rapid Identity Assays for mAb Development, Production Control and Release**

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During biopharmaceutical development and production there is a need for fast analysis return times to accelerate decision making and reduce costs. We utilized rapid protein digest methods and automated MALDI-TOF sample analysis with a software workflow to compare measurements against a target attribute profile. This comparison was used in clone selection workflows to screen glycan profiles in intact Fc-domains and to provide antibody identities rapidly, based on differentiating abundant peptides in peptide mass fingerprints.

Several antibodies were used in this study either as standard materials (NIST mAb) or as commercial formulations (Adalimumab, Trastuzumab. Panitumumab, Cetuximab and Natalizumab). For clone selection they were digested using IdeS (Genovis), diluted into DHAP or sinapinic acid MALDI matrix and the 2+ charge state was used for Fc-glycan profiling in linear mode MALDI-MS analysis. For rapid release identity testing they were incubated in 25% trifluoroethanol/ 5mM DTT (5min, 60°C) digested using trypsin/Lys-C (Promega), 5min after dilution with digestion buffer. Samples were MALDI analyzed in reflector mode using CCA matrix. Automatically acquired spectra were analyzed in BioPharma Compass 3.0 (Bruker). Antibody identity was confirmed based on the peptide profile similarity while rapid glycan profiling was based on Fc-linked glycan analysis.

These protocols allowed achieving 15min return times from intact antibody sample to automatic identity confirmation based on trypsin/Lys-C digests and of 30 min for Fc-glycoprofiling samples. This shows the potential of MALDI as versatile platform to accelerate characterization and identification tasks to support mAb development.

**NOTES:**

**P-257-M**

**Identification of Adeno-associated Virus Capsid Proteins Using ZipChip CE/MS**

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A simple and rapid identity test of adeno-associated virus (AAV) serotypes is important for supporting the AAV gene therapy development, as it relates to efficacy and safety of an AAV and is also recommended for AAV vector product release. However, the commonly used mass spectrometry-based identity tests by either MS peptide mapping or RP-LC/MS intact mass method have the limitations of extensive sample preparation, and relatively large amount of material needed due to low protein sample concentration. Herein, we describe a simple and novel microfluidic ZipChip CE/MS method used to characterize AAV capsid proteins and identify the AAV serotype. The analysis consumed 5 nL of sample, which is 10,000 times less than the amount for RP-LC/MS intact mass analysis. The three capsid proteins of either wild-type AAV2 or an AAV2 mutant with three amino acid mutations were separated and identified within 4 minutes directly from a polysorbate-containing formulation buffer. This rapid method can be suitable to confirm AAV serotype identity or monitor potential capsid protein heterogeneity to support the recombinant AAV gene therapy development.

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**P-258-T**

## Direct Characterization of Charge Isoforms by Online Cation-exchange HPLC/Mass Spectrometry

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Monoclonal antibody (mAb) products derived from mammalian cells are heterogeneous in their biochemical properties due to both intracellular posttranslational modifications (PTMs) as well as chemical changes induced by extracellular processes. Regulatory guidelines require characterization of primary sequence and PTMs present on mAbs to ensure acceptable quality, safety and efficacy profiles. MAb charge isoforms are characterized and monitored during product development lifecycle for consistency as well as evaluated for potential impact to biological activity. Common chromatographic and electrophoretic methods used to analyze charge isoforms include ion exchange chromatography (CEX-HPLC and AEX-HPLC), isoelectric focusing (IEF), capillary zone electrophoresis (CZE), and imaged capillary electrophoresis (iCE). In these methods, charge isoforms are generally classified as either acidic or basic species relative to the major species.

For mAbs, characterization of acidic and basic species involves CEX-HPLC and fractionation of the peak of interest, and then analysis by intact, subunit, and peptide mapping via liquid chromatography/mass spectrometry (LC/MS). The procedure is tedious, time consuming, and due to extensive sample manipulation, has the potential to introduce artifactual modifications that confound the interpretation of each species. Direct characterization of the charge heterogeneity profile by LC/MS through early process and product development activities, stability studies, and forced degradation studies would provide better understanding of charge isoforms present on the molecule in terms of pathways and kinetics. A CEX-HPLC/MS method using pH gradient elution with volatile, low ionic strength mobile phases coupled to a high-resolution mass spectrometer was evaluated. The method facilitated separation, identification, and quantitation of multiple common mAb charge-altering modifications (sialylated N-glycans, glycation, C-terminal lysine, C-terminal amidation, and N-terminal extensions) in a single experiment without the time-consuming fractionation of individual species. CEX-HPLC/MS provided rapid, comprehensive charge isoform monitoring and enhanced product and process understanding at an early stage of the development lifecycle.

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# Young Scientist

**P-259-M**

**Elucidating Disulfide Bonds in Biologics Through Semi-specific Proteolysis with Thermolysin and LC-MS/MS Analysis**

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Identification and characterization of disulfide bonds is necessary to assess protein quality, structure, and function. Established methods for identifying disulfide bonds in proteins typically use highly specific proteolytic enzymes, such as trypsin, followed by LC-MS/MS and identification algorithms. Although effective, there are significant dead-times in these workflows owing to lengthy digestion. Additionally, antibody therapeutics are often resistant to proteolysis and may require additional denaturation steps prior to digestion. We explored alternative semi-specific proteases to a) reduce sample handling steps, b) reduce digestion time, and c) increase confidence in assignments through assignment of overlapping digest products. Thermolysin (*Geobacillus stearothermophilus*), a semi-specific thermostable protease, functions optimally between 70-90 °C which is above the melting temperature of many antibodies. Thus our high temperature digestion alleviates the need for denaturants and helps facilitate rapid proteolysis.

While our thermolysin digestion protocols performed well experimentally, they presented bioinformatics challenges. Although semi-specific, thermolysin is thought to be more specific than other enzymes utilized in mass spectrometry, such as pepsin. A challenge in assigning disulfide bonds in non-specific and semi-specific digestions is the explosion in permutations of possible sequences to be cross-linked. This large search space presents a practical challenge due to an exponential increase in algorithm search time and decreasing confidence in assignments.

To address this challenge, we utilized Protein Metrics Software for facile iterative generation of defined enzymatic models for thermolysin digestion. Once digestion models were defined for each protein substrate, disulfide bonds can be assigned in an automated fashion. Methodologies and data presented are for trastuzumab (trade name Herceptin), a well-characterized monoclonal antibody (mAb) standard whose structural integrity relies on the correct arrangement of 16 disulfide linkages.

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# Late Breaking

**LB-20-M**

## Microchip Capillary Electrophoresis-ESI-MS for Rapid, Multi-level Analysis of Complex Proteins

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Mass spec characterization of proteins often requires multiple "levels" of analysis to generate a complete picture of the molecule. These levels are commonly referred to as top-down, middle-down, and bottom-up. While LC-MS methods have been developed for each of these levels, those methods typically require completely different columns and mobile phases for the different levels; and the methods typically yield relatively poor results for middle and top down analysis. Microchip CE-ESI-MS can achieve excellent performance for all of these levels, using the same exact experimental conditions. This enables rapid, back-to-back multilevel characterization of a protein, with no down-time between samples. This presentation will demonstrate that capability for a variety of proteins, including complex glycoproteins and monoclonal antibodies.

All work was performed using a commercially available microfluidic CE-ESI system (ZipChip, 908 Devices Inc.), attached to an orbitrap mass spectrometer (Thermo). The microfluidic devices utilized a covalently attached, neutral polymer surface coating to prevent analyte interactions and suppress electroosmotic flow. Proteins were analyzed at the top-down level by simply diluting to an appropriate concentration before loading into the microchip. Middle-down analysis was performed on monoclonal antibodies by reducing them with DTT and subjecting them to limited proteolysis using the IDeS enzyme. Bottom-up analysis utilized a standard enzymatic digestion method. All samples were analyzed using the same microfluidic device (ZipChip HR) and the same background electrolyte (methanol/water/formic acid, pH 2.2).

To achieve successful analysis at all three structural levels, a BGE with a relatively low pH was chosen. This BGE is not necessarily optimal for all levels of analysis of all proteins, but we found that it provided good results in all cases. Of particular note is the fact that this BGE fully denatures the structure of intact mAbs; so while we have previously demonstrated baseline resolution of intact mAb charge variants by microchip CE-MS, the work presented here did not yield resolution of intact charge variants. In this denaturing BGE we observe just a single mAb peak, but the electrophoretic separation still functions to electrokinetically "desalt" the sample. This yields a clean mass spectrum with a simple dilute and shoot method. Middle-down analysis of mAbs yields separation of three main segments of the molecule: the free light chain (LC), and the Fc and Fd domains of the heavy chain. We also observe separation of variant forms of each of these segments. Top-down analysis of smaller proteins using this method yields similar separation of variants. The separation of sialic acid variants of alpha-1-acid glycoprotein will be presented as an example. For middle-down analysis of mAbs and top-down analysis of smaller

proteins, we will present results for fragmentation via ETD, CID, and HCD. Of particular interest is the ability to achieve sufficient fragmentation in short enough times to be compatible with narrow microchip CE-MS peaks. Results that address this topic will be presented. Bottom-up analysis for a variety of proteins will also be presented. Results will focus on the ability to rapidly characterize protein structure and determine biologically relevant information.

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**LB-21-T**

## A Mass Spectrometry based "*ELISA*" Assay for Multi-attribute. Vaccine Potency Testing. A Mass Spectrometry based "ELISA" Assay for Vaccine Potency Testing Abrogates Antibody Dependency

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*[1] Janssen Vaccines and Prevention, Leiden, Netherlands, [2] Janssen Pharmaceutica NV, Beerse, Belgium*

At Janssen we are developing a candidate tetravalent HIV vaccine based on the AdVac® platform. The HIV vaccine contains four different are live, harmless, adenoviruses which are each genetically modified to encode for a different mosaic protein. These so-called transgenes consist of a variety of HIV antigens to protect against the multitude of HIV subtypes and clades that have been identified worldwide. Upon vaccination, the adenoviruses infect the human cells, which then express the desired mosaic antigens to elicit a broad, protective immune response against HIV. Potency testing of such vaccines is therefore expected to include a quantitative determination of transgene protein expression after infection of an appropriate cell substrate. The *in vitro* potency test is traditionally a cell-based assay, of which the result would be based on an ELISA read-out. In the case of our candidate tetravalent HIV vaccine, four selective antibodies would be needed for quantification of the expression of each of the four mosaic proteins. As these proteins have sequences with over 90% homology, generation of such antibodies is very challenging, and time consuming.

To overcome this problem, we developed a mass spectrometry potency method which allows for quantification of the expression of the four mosaic antigens in one single analysis. The advantage of mass spectrometry is that detection is based on monitoring the mass, retention time and fragmentation behavior of unique transgene specific peptides (proteotypic peptides). Selective detection by antibody binding – such as in ELISA – may not even be possible in the case of highly homologous proteins, whereas for MS detection a difference of one amino acid is already selective enough. Quantification of the proteotypic peptides, which each represent one of the four mosaic proteins, allowed us to determine their expression levels simultaneously. This MS-based "ELISA" assay can be performed directly in crude harvest cell lysate, without prior enrichment or use of immune reagents. In adherence with the USP guidance for industry the relative potency for the four different mosai proteins is determined by parallel line analysis versus a reference standard. The "MS-ELISA" is a promising assay platform, which can potentially be widely applied in the field of biologics and vaccines manufacturing and testing; even in a GMP environment.

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**LB-22-M**

## High-Resolution Hydroxyl Radical Protein Footprinting Introduction and Workflow

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Hydroxyl radical protein footprinting (HRF) with mass spectrometry (MS) is a recognized approach for assessing structure of proteins. Hydroxyl radicals react with a wide variety of side-chains resulting in their oxidation. Advances in liquid chromatography and MS approaches permit an examination of the labeling of individual residues, transforming the approach to high-resolution. Overall, the technology can provide an accurate measures of side-chain solvent accessibility in a wide range of interesting and useful contexts for the study of protein structure and dynamics in both academia and industry. Here we demonstrate the extension of the HRF workflow to include single residue examination, showcasing the importance of individual labels as significant telltales of local conformational change to a complex.

The typical workflow for high-resolution HRF is illustrated here by examination of a free antigen and an antigen-FAb complex. All samples are exposed to hydroxyl radicals for 0-800 microseconds of the x-ray beam. The amount of exposure for both samples is assessed using fluorophore Alexa-488. After exposure, the samples are subjected to deglycosylation using PNGase F. Deglycosylated samples are precipitated with 10% trichloroacetic acid (TCA)/acetone, reduced and alkylated with 10 mM iodoacetamide and 25 mM of DTT respectively, and digested with trypsin. The MS data are acquired using high-resolution LC-MS and analyzed, resulting in dose response plots for each peptide. Peptides from the antigen that show changes in modification against the complex are further analyzed for labeling at each residue.

HRF introduces side-chain oxidative modifications resulting in specific mass shifts at the specific site, which are identified from the tandem MS. To quantify the extent of modification, the selected ion chromatograms (SIC) are extracted from MS1 and integrated for the unmodified and all modified forms of peptide ion. These peak area values are used to characterize reaction kinetics in the form of dose response (DR) plots for each peptide or residue that measure the loss of unmodified as a function of the X-ray exposure. All modified peptide products for a given peptide in which modification occurs at the different residues are separated by LC due to their different hydrophobicity and quantified by MS enhancing resolution.

The solvent exposed regions of antigen are modified, and their rates of modification are compared for free antigen against the complex. Reduction in the rate of modification in the antigen-FAb complex (called rate constant, RC) indicate protection of the region to hydroxyl radical attack and potential locations of the binding interface. These protected regions are further analyzed for the extent of modification at each labeled residue. The residues within these regions that showed protection upon complex formation are candidates for the mutagenesis experiments and potential targets for mAb design.

The protection ratio (PR) is calculated as ratio of RCAntigen/RCComplex. The PR value for given peptide < 1 suggests that corresponding region experienced gain in solvent accessibility, PR□1 indicates that solvent accessibility of region remains unchanged, while PR>1 suggests that corresponding region exhibits protection from the solvent as a function of complex formation. Peptides with the highest PR value are examined individually as these residues are proven to be the binding interface between antibody and antigen. Importantly, the HRF technique identifies conformational epitopes. We will report several examples of epitope or paratope identification in the presentation.

High-resolution HRF provide specific residues that are candidates for mutagenesis experiments and potential targets for mAb design**.**

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**LB-23-T**

## Profiling the N-Glycosylation of Biotheraputic IgGs with HILIC-MRM Analysis of Trypsin Digested Culture Media

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The ability to accurately quantitate the glycan chains attached to glycoproteins has wide-ranging implications. Numerous studies over the past 40 years have demonstrated that abnormal glycosylation occurs in virtually all types of human cancers and demonstrate the potential of using glycan markers in either a diagnostic or a prognostic manner. The glycosylation on recombinant protein therapeutics is also known to have profound effects, with one of the better-known examples being the increased serum half-life of erythropoietin (EPO) resulting from glycoengineering. Hence, the quantification of glycoprotein glycans play important roles from the discovery of new diagnostic/prognostic markers to the development of therapeutic agents.

The focus of this presentation is the evaluation of an IgG with isotopically labeled glycans as an internal standard for the relative and absolute quantitation of N-linked glycans attached to human IgGs. We have developed a HILIC-MRM protocol that permits us to monitor 36 glycoforms attached to the conserved N-linked glycosylation site in the FC region of human IgGs. This procedure can be applied to the analysis of IgGs in cell culture media without the need for extensive sample cleanup and involves minimal sample processing. Essentially, the sample is spiked with an internal standard consisting of an isotopically labeled IgG. The material is then reduced, alkylated, digested with trypsin, and analyzed by HILIC-MRM. The internal standard allows for both absolute and relative quantitation across the multiple samples and reduces the experimental accuracy to <10%. To demonstrate utility our HILIC-MRM approach, we have performed a time course experiment to evaluate how glycosylation changes over the course of an expression and compared glycosylation profiles obtained from IgGs expressed under different conditions.

We have also compared the results obtained from the HILIC-MRM method to the standard purify/release/analyze approach. We have demonstrated that HILIC-MRM provides similar glycan profiles to those achieved with the standard approach. However, there are several notable exceptions, including the following. 1) The HILIC-MRM approach has a lower limit of detection compared to LC-MS analysis of released glycans, which provides for a larger linear dynamic range. 2) Several of the low-level species detected by LC-MS analysis of the released glycans appear to result from a contaminant glycoprotein, not the IgG under investigation. These contaminating glycans do not affect the HILIC-MRM approach because the glycans are left attached to the peptide, which serves as an identifier for the glycan's source glycoprotein. Information on the glycoprotein of origin is lost when the glycans are released from the peptide backbone. 3) The reproducibility of the HILIC-MRM approach was found to be superior, presumably resulting from reducing the number of sample handling steps.

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**LB-25-T**

## The Impact of Covalent Modifications on Antibody Aggregation

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Protein aggregation is a complex phenomenon that impacts biopharmaceutical development in virtually all stages. There are multiple pathways that lead to aggregation and all mechanisms of aggregation are not well defined yet control of this event is critical. In this study we attempt to decipher whether covalent modifications play a role in the stability or aggregation propensity of representative antibodies. We selected a panel of four antibodies, two IgG2's and two IgG1's for this study. They were selected based on two main criteria: the presence of conserved framework sequences and minimal non-germline mutations, and the presence of a minimal number of hotspots for glycation, methionine oxidation, and deamidation in the variable domains. This would allow us to focus on conserved Fc hotspots. The four molecules were then subjected to forced degradation to maximize glycation, oxidation or deamidation with the caveat being that the degree of modification may be beyond normal physiological ranges. Biophysical analyses and peptide mapping were performed on the degraded material to confirm conformation and quantify the degree of modification after treatment. The degraded molecules were then placed on stability to monitor levels of aggregation.

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