Direct Analysis of Heterogeneous Biotherapeutics

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CASSS: Thermo Fisher Scientific Lunch Seminar
Intact Mass Measurement Approaches for Biotherapeutics

- Denaturing MS
- Native MS
- Hyphenated nMS
- Charge reduction (PTCR)
- Charge Detection MS (DMT)

Molecular Complexity
Ease of analysis
Biological representation
Glycoproteins as biotherapeutics

- Glycosylation is present on >50% of all human proteins
  - Role of glycosylation in the cell: Fc effector function, protein structure, more...
- >40% approved biotherapeutics are glycosylated
  - IgG-based and Fc-fusion biologics
- Glycosylation can affect pharmacological properties of biotherapeutics:
  - potency, stability, bioavailability, solubility, and immunogenicity.

- A single glycosylation site on a protein can produce vast molecular heterogeneity that precludes direct analysis by mass spectrometry (MS), the predominant analytical tool for glycoprotein characterization.
Strategies to investigate glycoproteins by MS

Ease of analysis

Glycan Comp
- Released glycans
- Total glycoforms

Site Occupancy
- Percentage PTM for each N-motif position

Site Mapping
- Which glycoforms are present on what sites

Intact MS
- Nature of PTMs on molecule and complexity
Increasingly complex modalities require adaptive analytical strategies

Antibody/Fab Derivatives
Conjugated Formats
Non-covalent complexes/membrane proteins

Fc Fusion proteins
Intact Mass Measurement Approaches for Biotherapeutics

Charge reduction (PTCR)

Single Charge Detection (DMT)

What can I do with this complicated mess?
Proton Transfer Charge Reduction (PTCR)

- Charge (z) is determined based on the spacing between the adjacent charge states.
- PTR may *create* charge state distributions.
Proton Transfer Charge Reduction (PTCR) on resolved charge states

Before PTCR

After PTCR

Perfluoroperhydrophenanthrene

323 m/z

970 m/z
PTCR on unresolved charge states

*ASMS 2019: Orbitrap Eclipse with extended mass range (8k) and PTCR*
UNIGLAMS: Universal Intact Glycoprotein Analysis by Mass Spectrometry

Overlapping windows of PTCR spectra are acquired and stitched together for deconvolution

Example: m/z 4000-7000, 30th step, 60th isolation, 10th overlap, 100us scans, PTCR4
UNIGLAMS Workflow

SPECTRUM

ORBITRAP MS

SOURCE

ISOLATION

PTCR

Spectrum

Luis Schachner
UNIGLAMS Workflow

SOURCE

ISOLATION

PTCR

ORBITRAP MS

Spectrum

Luis Schachner
UNIGLAMS Workflow

SOURCE  ISOLATION  PTCR  ORBITRAP MS

Spectrum

Luis Schachner
UNIGLAMS workflow

Glycoprotein

Desalt & buffer exchange to 100mM Ammonium acetate

Nano capillary infusion to mass spectrometer

MS\textsuperscript{1} to determine m/z envelope range for acquisition and optimum SID

Narrow MS\textsuperscript{2} isolation at apex to confirm ion signal

Increase PTCR in MS\textsuperscript{2} isolation range to establish appropriate duration

Analyze & annotate
Ovalbumin contains a single N-linked glycan yet has multiple proteoforms

MS1

- N-linked glycosylation (N292)
- N-acetylation (G1)
- Phosphorylation mono-

UNIGLAMS

15
Ovalbumin contains a single N-linked glycan yet has multiple proteoforms
Utility of PTCR is limited by Eclipse m/z range

Eclipse Orbitrap
m/z 8000 @ PTR 10ms or less for a 100kDa protein

Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer
m/z 16,000 @ PTR 20ms (or more)
Charge reduced segmented isolation resolved proteoforms for identification

**20k ligand scTrimer fused to mulgG2A**

- Each ligand subunit has 1 N-linked glycan (on NVT)
- GSS linkers of varying lengths (why there are multiple samples)
- Unresolvable by native MS or SEC-MS, even after N&O degly
- Expected MW = ~160 kDa
A Phase IIb heterogeneous glycosylated cytokine Fusion protein

- CHO derived cytokine Fc-fusion protein
- Two IL-22 cytokines fused with the Fc portion of an IgG to prolong half-life
- Aglycosylated Fc to minimize effector function (N to G)
- Heavily glycosylated cytokines
  - 8 N-glycosylation sites
  - > 60 N-Glycans identified

Michelle Irwin Beardsley & IL22Fc dev team
Stepping through glycoforms with UNIGLAMS

MS1

Ion isolation + PTCR

MS2

Isolation 100 Th window

PTR (@15ms) 50 Th segments

5600-6700

Deconvolution
Stepping through Spectra on Orbitrap Ascend

m/z 5950 segment
1uScan (IIT 16ms)

m/z 6000 segment
1uScan (IIT 16ms)

Deconvolved Single Scans
>160 glycoforms identified by BPF from UNIGLAMS on cytokine fusion protein

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<th>Modification</th>
<th>Average Mass (Da)</th>
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Challenges with Ion Isolation

Ion Trap Isolation
100 Th

Quadrupole Isolation
~20 Th

m/z 5600
UNIGLAMS with Quadrupole isolation
Separation of four Sialic acid fractions with UNIGLAMS
Automating UNIGLAMS

➢ Determine m/z range by MS1
➢ Determine optimal PTCR parameters

➢ Method Wizard

➢ Step by 1 Th, can do 1 or more uScans
➢ 6 min run time for 1uS @ res 15000
Intact Mass Measurement Approaches for Biotherapeutics

Charge reduction (PTCR)

Direct Mass Technology (DMT)
Direct Mass Technology (DMT)

a.k.a. CD-MS, single particle MS, individual ion MS
DMT workflow

Step 1. Enable Direct Mass technology mode

Step 2. Single ion injection at each m/z

Step 3. Establish calibration curve

Step 4. Collect scans of individual ions

Step 5. Obtain charge state and m/z

Step 6.

$\text{m/z} \times z \rightarrow m$ for each ion

MW of heterogenous molecule

Some Applications of DMT

- Membrane protein in nanodisc
- 5 min collection per CE
- Low concentration BsAb (100ug/ml)

with Michael Marty
DMT on the low SA Fraction: a cautionary tale of oversampling?
DMT on the low SA Fraction: a more conservative analysis
No resolution to the resolution issue

➢ MW of SA15 shifts higher compared to MW of SA8
Two approaches to improve spatial resolution of heterogeneous biomolecules

**UNIGLAMS**

- Small window isolation followed by charge reduction.
- Multiple scan windows may be merged together after deconvolution to reveal proteoforms present.
- Ideal for glycoproteins and other biomolecules with overlapping charge states.

**DMT**

- Single particle detection at high resolution.
- No deconvolution needed (so complexity not an issue).
- Useful for large molecules or complexes, membrane proteins and oligomeric structures.
Thank you!

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