Fundamentals: Intact protein mass spectrometry - tips and best practices

SUMS Seminar Series
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…some labs for you!
Submit your questions!
Overview

- Brief history of intact protein analysis with mass spectrometry
- Advantages and limitations of current methods and instrumentation
  - Ion Formation: ESI vs MALDI
  - Sample preparation, introduction
  - Mass separation: Quadrupole, TOF, Orbitrap
  - Data processing: Deconvolution, Drug-Antibody-Ratio
- Resources for further study
2002 Nobel prize in Chemistry
"for the development of methods for identification and structure analyses of biological macromolecules"

Electrospray
Quadrupole
40 kDa protein
Published 1989

Soft Laser Desorption
Time of Flight
34 - 50 kDa proteins
Published 1989

Prize share: 1/2 awarded to Kurt Wüthrich "for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution."

In 2020, wide variety of MS applications for intact proteins

- **ESI**
  - Non-covalent interactions
  - Charge variant screening of biotherapeutics (with capillary electrophoresis)
  - Hydrogen-Deuterium Exchange studies (HDX)

- **ESI or MALDI**
  - Molecular weight confirmation:
    - purified proteins and commercial antibodies
    - proteins modified with dyes or drugs, characterize extent of labeling, Drug-Antibody-Ratio
  - Top down structural studies

- **MALDI**
  - Non-covalent interactions
  - Imaging
  - Non-defined-length-PEGylated proteins
Matrix Assisted Laser Desorption Ionization

- Mix protein sample and matrix of choice (ex. sinapic acid) on the metal target plate

- Pulsed laser generates a plume of matrix and analyte molecules

- Ionized sample molecules continue to the mass analyzer

MALDI-TOF/MS: example spectra for unusually large molecules


MALDI is a soft ionization technique

NIST-mAb on an AutoFlex MALDI-TOF, Bruker FlashNote, Aug 2018, FN-005
Electrospray ionization

- A solution containing the sample of interest is nebulized into fine droplets.
- The droplets dry, reduce in size, and divide into smaller droplets, eventually generating desolvated analyte ions with one or more residual charges.
- Proteins tend to form multiply charged ions during electrospray, facilitating analysis by mass spectrometry.

figure by Andreas Dahlin - ESI positive mode, CC BY 2.0, https://commons.wikimedia.org/w/index.php?curid=72802277
ESI/MS Protein mass spectrum – Mathematical deconvolution

$M_r = \text{mass of protein}$

$n = \# \text{ of charges on m/z}$

$m_p = \text{mass of H}^+ = 1.0073$

Given $m/z_1 = (M_r + n_1 m_p) / n_1$

and $n_2 = n_1 + 1$

Can rearrange to solve for $n_1$

$$n_1 = (m/z_2 - m_p) / (m/z_1 - m/z_2)$$

Example calculation:

• $m/z_1 = 3230.7$
• $m/z_2 = 3160.5$

$$n_1 = (3160.5 - 1.0073) / (3230.7 - 3160.5) = 45$$

$calcM_{r1} = (45 \times 3230.7) - (45 \times 1.0073) = 145336.2$

$calcM_{r2} = (46 \times 3160.5) - (46 \times 1.0073) = 145336.7$


Full scan MS spectrum of a 145 kDa protein
ESI/MS Protein mass spectrum – Computer assisted deconvolution

Setting the deconvolution range, artifacts

Raw data: Full scan mass spectrum

Processor data, deconvolution mass range

5 to 15 kDa

\[ m/z_1 = \frac{M_r + n_1 m_p}{n_1} \]

28 to 38 kDa

5 to 50 kDa
Real protein vs polymer

Even spacing indicates polymer ion series

ESI-MS of ion series found in Tween 80. Similar series observed for PEG.

Ion suppression

- ESI and MALDI ion formation processes are competitive - different compounds can have large variations in ionization efficiency
- Ion suppression can be caused by salt, detergents, polymers. Must separate the protein from these prior to the ionization step

Allowable concentrations of various salts and buffer components - guidance from the Harvard Center for Mass Spectrometry

<table>
<thead>
<tr>
<th>Surfactant, Buffer and Salt</th>
<th>Mw (g/mol)</th>
<th>MALDI (mM)</th>
<th>MALDI (wt.%</th>
<th>ESI (mM)</th>
<th>ESI (wt.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>121</td>
<td>100</td>
<td>1.0</td>
<td>n. a.</td>
<td>n. a.</td>
</tr>
<tr>
<td>HEPES</td>
<td>238</td>
<td>100</td>
<td>2.4</td>
<td>n. a.</td>
<td>n. a.</td>
</tr>
<tr>
<td>Glycerol</td>
<td>92</td>
<td>130</td>
<td>1.2</td>
<td>n. a.</td>
<td>n. a.</td>
</tr>
<tr>
<td>N-Octyl-β-glucopyranoside</td>
<td>292</td>
<td>3.4</td>
<td>0.1</td>
<td>3.4</td>
<td>0.1</td>
</tr>
<tr>
<td>n-Octyl sucrose</td>
<td>468</td>
<td>n. a.</td>
<td>n. a.</td>
<td>2.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

…and many more

Offline sample cleanup/desalting techniques

Options
- Dilution
- Dialysis / Buffer exchange
- Zip-tip
- MWCO filters, Desalting spin columns
e.g. Bio-Rad Micro Biospin P-6 gel columns, remove small molecules, salt, MWCO 6000

Drawbacks
- >10uL of sample and/or high concentrations
- Time and sample for method development to minimize protein loss due to
  - precipitation
  - failure to stick to/elute from media
  - decomposition
- Not practical when sample limited and no prior optimization
Online desalting: separation in time

Via analytical scale LC-ESI/MS

- Robust - protein is maintained in a reliable buffer system until injected on the column
- Predictable sample requirements - One injection consumes 2 to 5 uL of a 20 uM solution; ~1 ug of a single proteoform on column
- Reverse phase chromatography
  - Waters BioResolve RP mAb Polyphenyl 450A 2.7u 100x2.1mm (previously used Zorbax Diphenyl)*
  - Waters MassPREP™ Micro Desalting Columns (2.1 x 5 mm, P/N 186004032)
  - C4, C8, PLRP-S

Other On-line Separation Techniques for ESI/MS

- Size Exclusion
- Capillary Electrophoresis

MALDI vs Electrospray Ionization

MALDI Pros
- Fast / Flexible sample analysis time: Potential to acquire data in minutes as well as to revisit the sample days later
- Straightforward spectral interpretation

MALDI Cons
- Desalting samples is important for data quality
- Cleanup methods (zip-tip, dialysis, spin columns) can be sample and time consuming, and difficult to automate for small sample batches
- Art of sample preparation: inter- and intra-lab method transfer can be challenging

Electrospray Pros
- Online desalting minimizes sample loss, dilution
- LC sample introduction methods are relatively robust, reproducible, automated.
- Transferring methods is relatively straightforward

Electrospray Cons
- Longer run times, 10 to 40 minutes per sample plus a blank to check for carryover
- Post analysis data processing with spectral deconvolution software
Frequently asked questions about intact protein LC/MS at SUMS

How large can the protein be?
○ Depends upon the scientific question you want to answer

How much protein do you need?
○ Usually about 10uL of a 20uM solution, but it depends upon sample heterogeneity / number of proteoforms

Was my reaction successful? I am binding a dye/drug molecule of molecular weight x to my protein. Can your mass spectrometer resolve the compounds of interest?
○ Protein MW
○ Modifier MW
○ Sample heterogeneity / number and relative abundance of proteoforms

How accurate is the reported MW?
Time of Flight mass spectrometer

Resolving power \( R = \frac{m}{\Delta m} \)

- \( m \): mass of the ion
- \( \Delta m \): peak width at 50% peak height (\( R_{\text{FWHM}} \))
  minimum between adjacent peaks at 10% valley (\( R_{10\%} \))

Simulated spectrum: \( \text{C}_{33}\text{H}_{66}\text{N}_{12}\text{O}_{12}\text{S}_{2} \)
\( m = 1007.4, \Delta m = 0.1 \)
\( R = \frac{1007.4}{0.1} = 10,074 \)
ESI/MS Protein mass spectrum – multiply charged ions

Full scan MS spectrum of a 10 kDa protein

\[ R = \frac{2550.3}{0.25} = 10,201.3 \]
Quadrupole mass spectrometer

Simulated spectrum: $\text{C}_{43}\text{H}_{66}\text{N}_{12}\text{O}_{12}\text{S}_{2}$

$m = 1006$, $\Delta m = 1$

$R = \frac{1006}{1} = 1,006$
Myoglobin mass spectrum acquired on a Waters single quadrupole mass spectrometer

Full scan mass spectrum

Deconvolution results from Protein Metrics Intact software
Resolving Power and Intact Protein analysis

Simulated myoglobin mass spectra:
\[ C_{769}H_{1212}N_{210}O_{218}S_2 \]

- Resolving power = 1000 (quadrupole)
- Resolving power = 10,000 (older TOF)
- Resolving power = 50,000 (newer TOF up to 80,000)
- Resolving power = 250,000 (newer Orbitrap not shown)

Monoisotopic MW = 16940.96
Average MW = 16951.48

Resolving power becomes more important as sample complexity increases

LC-ESI/MS of large proteins: How large?

Example of Waters MassPrep antibody standard run on the Bruker micrO-TOF-QII

Full scan mass spectrum

Deconvoluted with Bruker MaxEnt software
How much protein do you need?

We usually ask for 20uL of a 20uM solution. For 150 kDa proteins that is about 1mg/mL. For precious samples, we can work with 10uL.

Sample heterogeneity challenges:
• multiple proteoforms reduce the effective concentration
• Resolving power sufficient to separate multiply charged ions?
Waters Intact mAb Check Standard

Intact mouse IgG1 protein
Fully characterized, available as a quality control to verify instrument performance

Example deconvoluted mass spectrum
Deconvoluted mass spectrum from micro-TOF-QII data

NIST Monoclonal Antibody Reference Material 8671

“RM 8671 was received as a bulk substance prepared using mammalian cell culture and downstream processing. Multiple bulk substance containers were homogenized to form the 14HB batch. Aliquots of 1 L each were made from the 14HB and each was diluted, vialled, and analyzed as a separate lot. The reference values of each lot of RM 8671 have been shown to be statistically equivalent, however each lot will have its own unique Report of Investigation. The lot listed on this page is the one that will be shipped.”

Deconvoluted mass spectrum - Bruker Maxis Q-TOF data

NIST mAb reference material is used for inter- and intra-laboratory method validation

*https://www-s.nist.gov/srmors/view_detail.cfm?srn=8671
Improving data for heterogeneous samples

**Break the protein into simpler pieces**
- Deglycosylation (NEB Rapid PNGase F nonreducing)
- IdeS/IdeZ to cleave at the hinge
- Disulfide bond reduction (TCEP or DTT)
- Deglycosylation and disulfide bond reduction (NEB Rapid PNGase F reducing)

**Separate proteoforms before mass analysis**
- Chromatography: RP-HPLC can separation reduced heavy and light chains
- Ion mobility (IMS-MS): separate light and heavy chains after ionization but before mass analysis
- Capillary electrophoresis (charge variant analysis): C-terminal lysines, deamidations

**Shift the charge envelope to higher m/z values** (Native mass spectrometry)
Waters Intact mAb Check Standard, reduced with TCEP

Reduced with Pierce TCEP-HCL No Weigh format

BioResolve RP mAb Polyphenyl 450A 2.7u 100x2.1mm from Waters, column temperature 50C, flow rate 0.3ml/min, injection volume 2uL (2ug on column)
Waters Intact mAb Check Standard, reduced with TCEP

Full scan mass spectra

Deconvoluted with Bruker MaxEnt software

Stanford University
Was my reaction successful, can you distinguish labeled vs. unlabeled?

Simulated
\( \text{C}_{6472}\text{H}_{9940}\text{N}_{1698}\text{O}_{2008}\text{S}_{52} \)

\( R = 10,000 \)

\( M_{\text{mono}} = 145239 \)

\( M_{\text{avg}} = 145329 \)

Simulated
\( \text{C}_{6472}\text{H}_{9940}\text{N}_{1698}\text{O}_{2008}\text{S}_{52} \)

\( R = 2,000,000 \) (for illustration only)

How large is the mass shift of interest?

How abundant is the mass shift of interest?
Was my reaction successful, can you distinguish labeled vs unlabeled?
Qualitative Analysis - Deconvolution
How accurate is the reported MW?

The accuracy of the molecular weight reported in the deconvoluted mass spectrum will depend upon instrument tuning, calibration and **spectral purity**.

More resolution usually facilitates better mass assignments due to reduced spectral interference.

In general
Micro-TOF-QII (Time of Flight mass spectrometer):
  Typical mass accuracy of 20ppm or
  • +/- 3 Da for a 150kDa protein
  • +/- 0.3 Da for a 15kDa protein
DAR calculation example

Drug Load Distribution = \( \frac{\text{pkArea}_n}{\text{pkArea}_{\text{sum}}} \)

Weighted average DAR = \( \sum (\text{DLD})_n \times n \)

A brief note about protein analysis under reduced vs native conditions

ESI/MS of reduced proteins:
- Generic methods, source conditions
- More sites for protonation, better sensitivity
- Charge envelope shifted to lower mass range, accessible to most instruments

ESI/MS of proteins in native (native-like) conditions
- More time and sample consumption to optimize source parameters
- Generally requires higher sample concentration
- Charge envelope shifted to higher mass range, TOF or high end Orbitrap
- Potential for research projects to investigate non-covalent interactions
Zip-Chip for analysis of reduced and native proteins

- Infusion mode – robust nanospray sample introduction for optimization or analysis of pure sample
- CE/MS
  - alternate chromatographic separation mode (charge variant analysis)
  - No carryover
  - Very fast sample run times (under 10 minutes)
- Can be configured with an autosampler for automated sample analysis or in Manual mode to recover unused sample post-analysis
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Opportunities for further study

- Mass Spectrometry: A Textbook, Jurgen Gross


- Pharmaceutical and BioScience Society workshop Sep 17, 2020:
  
  Regulated Large Molecule Bioanalysis: Fundamentals, the New FDA Guidance and Beyond
Acknowledgements

Stanford Dean of Research

Vincent and Stella Coates Foundation

And of course…
Questions
Stay tuned
More Fundamentals webinars on Thursdays, noon-1pm

April 16, 2020
New proteomic approaches and essential data handling tips
Speakers: Kratika Singhal, MS; Rowan Matney, BA

April 23, 2020
Measuring concentrations of small molecules using mass spectrometry – theory and practice, part II
Speaker: Karolina Krasinska, MS

April 30, 2020
Peptide quantitation strategies
Speakers: Fang Liu, PhD; Beryl Xia, PhD

May 7, 2020
Native protein approaches
Speaker: Beryl Xia, PhD

Details & registration: mass-spec.stanford.edu/events