High Throughput Accurate Mass Screening of Monoclonal Antibodies

Jim Blasberg
April 28, 2011
Analytical R&D
Analytical R&D Objectives

Serve as corporate center of excellence for mass spectrometry

Provide analytical support to Research Biotech and SAFC product development teams

Generate application data supporting marketing of new Biotech products and evaluation of new technologies for Business Development

Provide high-end analytical characterization of proteins and antibodies

Provide specialized testing in support of Quality Control

Lead and/or support troubleshooting and problem-solving initiatives in support of high-value products
Generalized Protein/Glycoprotein Characterization Workflow

 Bulk Sample

 Intact MW analysis with all PTM
 SDS-PAGE
 LC-MS

 Reduce Complexity

 Remove PTM
 Enzymatically
 Chemically

 Specific Structural Details

 Peptide/Glycopeptide Mapping
 LC-MS/MS

 Data Analysis

 Glycan Analysis
 MALDI/ESI
 HPAEC
 HPLC

 Correlate Data from All Methods

 Reduce & Separate
 SEC-MS

 Sialic Acid Analysis
 HPLC-FLD
 HPAEC,
 Colorimetry

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Why Study Intact Protein Mass?

Intact mass analysis can detect altered chemical and physical properties related to protein function

- Detection of PTMs
  - N/C-terminal variation
- Glycosylation state
  - 50 - 90% of proteins are glycosylated
- Confirm primary sequence
- Evaluate stability
  - Oxidation or degradation
Intact Protein Analysis – General Considerations

**ESI**

- We chose to focus on Electrospray Ionization or ESI as our technique of choice for intact protein analysis

Infusion or RP-HPLC sample introduction
Intact Protein Analysis – General Considerations

Deconvolution
• How we get from multiply charged to zero-charge accurate mass
• Time of Flight (TOF) mass analyzer – Waters LCT or QTof Premier
• Multiply charged ions centered around 1000 m/z
  – Good resolving power in this m/z range
  – Deconvolute to zero charge

A: The m/z values can be expressed as follows:

\[
m/z = \frac{MW + nH^+}{n}
\]

B: With 2 adjacent m/z values we can determine charge state and solve for MW
Intact Protein Analysis – General Considerations

Sample Introduction - ESI

- Direct Infusion - MS
  - Low throughput
  - Good approach for small numbers of relatively pure samples
  - Typically requires cleanup

- RP-HPLC - MS
  - Moderate throughput
  - Analyte dependent, MS compatible
  - Good for protein purity and intact

<table>
<thead>
<tr>
<th>Sample Prep Requirement</th>
<th>Infusion</th>
<th>RP-HPLC</th>
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<tbody>
<tr>
<td>Analyte Dependent</td>
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<td>MS Compatible</td>
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<td>Sample Load Requirement</td>
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<tr>
<td>Throughput</td>
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</table>
Intact Protein Analysis – Direct Infusion

Desalting and Direct Infusion

- Request for intact mass of single protein sample
- UF solvent exchange/desalting
- Zero charge accuracy within 0.01%

**Desalted BSA 1 mg/mL**

20110421_BSA_INFUSION_001 28 (0.519) M1 [Ev-72518,It25] (Gs,0.750,1322:1984,1.00,L50,R50); Cm (17:29) TOF MS ES+ 6.66e3

**Cysteinylated BSA**

Δ=119

**Deconvoluted Data Using MaxEnt1**
Intact Protein Analysis – RP-HPLC-MS

RP-HPLC of Intact Protein
- Purity and intact data – Murine EGF
- Generic 0.1% TFA/ACN gradient

**UV Trace**

**MS TIC**

Sum across peak including potential impurities
Intact Protein Analysis – RP-HPLC-MS

RP-HPLC of Intact Protein

- Purity and intact data – Murine EGF
- More than one isoform under main peak

Deconvoluted MS Data
Theory = 6039.7
Experimental = 6039.3

Minor Component B
6039-5925=114
Truncated N-terminal ASN?

EGF Sequence
NSDSECPPLSHDGYCLHDG
VCMYIEALDKYACNCVVGY
IGERCOYRDLKWWELR

A: 6039.32±0.08
B: 5925.13±0.06

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Intact Protein Analysis – RP-HPLC-MS

RP-HPLC of Intact Protein

- Purity and intact data – Murine EGF
- XICs demonstrate multiple isoforms are present

oxEGF-N

oxEGF

EGF

EGF-N

12-Jun-2008

061208_EGF_004 1: TOF MS ES+
1514.7 1.07e3
11.04 11.32

061208_EGF_004 1: TOF MS ES+
1510.8 7.43e3
11.26

061208_EGF_004 1: TOF MS ES+
1482.2 1.99e3
11.37

3% 10% 69% 18%
Intact Protein Analysis – SEC-MS

SEC-MS of Intact Protein
- Traditionally not MS compatible
- Developed MS compatible MP system, 45% ACN/0.1% TFA
- 3 – 300 x 7.8 mm columns in series

Peak | Analyte | MW
--- | --- | ---
1 | BSA | 66429
2 | Cytochrome C | 12327
3 | Aprotinin | 6511
4 | Insulin B | 3496
5 | LH-RH | 1183
6 | TRH | 362
7 | Phenylalanine | 165
Intact Protein Analysis – SEC-MS

SEC-MS of Intact Proteins
• Modify for general use, single column
  – Shorten run time
  – Divert small MW after analyte(s) elute
  – 8-minute run time, no re-equilibration required

![Graphs showing BSA Non-Reduced and BSA Reduced with SEC traces]
Intact Protein Analysis – SEC-MS

SEC-MS of Reduced Antibody

- Optimize for sample load, 2.0 and 4.6 mm id
Antibody Analysis – HT Development

SAFC Interests – Glycoprotein and Protein Quality

• Media Development

• Raw Material Characterization

• Cell Line Engineering

• Ultimately the ability to tune glycoprofile

Standard workflows not amenable to high throughput
Antibody Analysis – HT Development

Typical Mammalian Antibody Glycan Structures

- Galactose
- N-Acetylglucosamine
- Mannose
- Fucose
Antibody Analysis – HT Development

SEC-MS Intact Protein Methodology
• Analysis of intact and reduced mAb

MS TIC
Intact Antibody

Raw Spectra

Deconvoluted Data

G0F/G0F
627

G0F/G1F

G0F/G2F
G1F/G1F

Heavy Chain

Deglycosylated

Intact Antibody

G0F
185000

G1F

G2F

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Antibody Analysis – HT Development

Option 1 – Direct from media

Transfer clarified media to 96-well plate

Reduce direct In-media

Analyze by SEC-MS

Process Data in BPL

• Fast and simple workflow
• Spiked pristine media ok
• Real samples precipitated and gave no signal in SEC-MS
Antibody Analysis – HT Development

Option 2 – 96 well UF membrane

1. Transfer clarified media to a 96-well UF plate and wash to remove low MW
2. Reconstitute and reduce
3. Analyze by SEC-MS
4. Process Data in BPL

- Two more steps than Option 1
- Still fast and simple
- Spiked pristine media showed only polymer, likely Pluronic
Antibody Analysis – HT Development

Option 3 – Protein-A purification

- Transfer Protein-A resin to a 96-well filter plate
- Wash
- Transfer clarified media to the 96-well filter plate
- Equilibrate/Wash
- Process Data in BPL
- Analyze by SEC-MS
- Reduce
- Add elution buffer equilibrate and collect

- Several more steps than Option 1 or 2
- A bit more involved but still reasonable time-wise, 2-3 hrs/plate
- To-date has been successful for numerous media samples of suitable titer
HT Application – Clone Screening

Purification Procedure
- 50-µL P3476 Protein A Agarose Fast Flow per well, wash w/EQ buffer
- 750-µL clarified spent media containing target IgG preferably <100 µg/mL
- Equilibrate 10-min, wash 2x w/EQ, add 100-µL ELUT buffer equilibrate 10-min
- Collect eluted antibody in a 96-well plate
- Add 5-µL 1M ABC and 1M DTT, incubate 0.5 hr at RT

SEC-MS Procedure
- Waters Acquity UPLC
- Column: Toso Haas TSK Gel SW3000XL, 300 x 2.0 mm, 4 µm
- MP: 0.1% TFA 45% CH₃CN
- Flow Rate: 0.125 ml/min
- Inj. Vol.: up to 20 µL
- Run time: 8 minutes
- Flow Divert: 4.6-7.9 min
- MS: Waters QToF Premier (ESI+)
- Capillary: 3.2 kV
- Sample Cone: 40.0 V
- RF Lens: 600.0 V
- Extraction Cone: 3.0 V
- Desolvation: 240 °C
- Source: 120 °C
- Scan Range: 400-4000
HT Application – Clone Screening

Purification Procedure

- Typical Plate Layout
- Real throughput
  - 84 spent media samples complete overnight
  - Data analysis day 2

<table>
<thead>
<tr>
<th>Purified mAb Reference</th>
<th>Spent Media Control</th>
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<tr>
<td><strong>Clone Screening Assay – Day 7</strong></td>
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<tr>
<td>Biological duplicate samples</td>
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<tr>
<td><strong>Clone Screening Assay – Day 10</strong></td>
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HT Application – BPL Output

BiopharmLynx Data Processing

- Automated deconvolution of data
- Set up BPL method with appropriate sequence and modifications
- Automated deconvolution of 96 samples in <20 min
- Manual inspection of results with final summary in Excel
HT Application – Reproducibility

Protein-A + SEC-MS + Data Processing
• Holistic assay variability evaluation
• Coaching expectations

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</tr>
<tr>
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HT Application – Clone Screening

Clone Screen Data Processing

• Evaluate glycoprofile of various clones
• Biological replicates look good
• Discovered widely variable clonal Man5 levels
HT Application – Clone Screening

Man5 Content

- Export data from BPL to Excel
- Average biological replicates
- Normalize Man5 to G0F and compare Man5 relative abundance
Generalized Protein/Glycoprotein Characterization Workflow

- **Bulk Sample**
  - Intact MW analysis with all PTM
    - SDS-PAGE
    - LC-MS

- **Reduce & Separate**
  - SEC-MS

- **Reduce Complexity**
  - Remove PTM
    - Enzymatically
    - Chemically

- **Specific Structural Details**
  - Peptide/Glycopeptide Mapping
    - LC-MS/MS

- **Data Analysis**
  - Glycan Analysis
    - MALDI/ESI
    - HPAEC
    - HPLC
  - Correlate Data from All Methods

- **Sialic Acid Analysis**
  - HPLC-FLD
  - HPAEC, Colorimetry

- **Generalized Protein/Glycoprotein Characterization Workflow**
HT Application – Clone Screening

Man5 Confirmation

- Confirm Man5 by glycopeptide analysis on the LTQ-FT
- Tryptic peptide EEQYNSTYR-Man5 (2405.9349)
HT Application – Media Component Titration

Component Titration Experiment – Product Quality Impact
- Same Protein-A - SEC-MS - BPL workflow
Acknowledgements

Sigma Analytical R&D
- Kevin Ray
- Ben Cutak
- Jim Walters
- Gordon Nicol
- Janet Irungu
- Mark Angeles

SAFC Biosciences
- Nan Lin
- Joaquina Mascarenhas
- Andrew Christie
- Chas Hernandez

Supelco
- Tracy Ascah