Higher throughput glycosylation analysis of biopharmaceuticals by mass spectrometry

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Center for Proteomics and Metabolomics

CASSS AT EUROPE, BARCELONA, MARCH 8, 2018
GLYCOSYLATION

- Highly prevalent co- and post-translational protein modification (PTM)
- Considered to be the most complex PTM
- More than 50% of human proteins are glycosylated

Why is analysis of glycosylation necessary?

- Protein-protein interaction
- Recognition
- Signaling
- Trafficking
- Age dependent
- Changes with disease
The most abundant monosaccharides in humans are:

- **Glucose**: 162.0528 Da
- **Galactose**: 162.0528 Da
- **Mannose**: 162.0528 Da
- **Fucose**: 146.0579 Da
- **N-Acetyl-D-galactosamine**: 203.0794 Da
- **N-Acetyl-D-glucosamine**: 203.0794 Da
- **N-Acetylneuraminic acid**: 291.0954 Da

**Glucose**

**Galactose**

**Mannose**

**Fucose**

**N-Acetyl-D-galactosamine**

**N-Acetyl-D-glucosamine**

**N-Acetylneuraminic acid**
## ANALYSIS APPROACHES

### INTACT GLYCOPROTEINS
- Different isoforms
- Protein specific
- Not site-specific
- Requires isolated proteins

### GLYCOPEPTIDES
- Site-specific
- Protein specific
- Fast increase of complexity of the data

### RELEASED GLYCANS
- Analysis of complex samples
- Not site-specific
- Not protein-specific
Workflow: MALDI-TOF-MS of N-glycans

• Largely automated sample preparation
• Differentiation of sialic acid linkages
• Mild procedure: assessment of glycan modifications (e.g. acetylation, phosphorylation)
• Fast and largely automated data processing
• Can be combined with reducing end labeling,
  • for CE-MS analysis or
  • negative-mode MALDI-MS analysis
Ethyl esterification for differentiation and stabilization of sialic acid linkages

α2,6

α2,3

EDC, EtOH, HOBt
1h at 37 °C

+28.032 Da

-18.011 Da
PNGase F release under slightly acidic conditions

Stimulating this reaction route by lowering the pH

- Stimulating this reaction route by lowering the pH
- core GlcNAc
- carbodiimide reagent
- aspartic acid peptide residue
- PNGase F
- core GlcNAc glycosylamine
- hydrolysis
- core GlcNAc
- core GlcNAc coupled to core GlcNAc

Gerda Vreeker g.c.m.vreeker@lumc.nl
Automated cotton HILIC solid phase extraction

MALDI-TOF-MS spectrum of N-glycans from human plasma

Bladergroen/Reiding, 2015, J. Proteome Res.
MALDI-FTICR-MS vs. MALDI-TOF-MS

<table>
<thead>
<tr>
<th></th>
<th>MALDI-TOF-MS</th>
<th>MALDI-FTICR-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>± 0.1 Da</td>
<td>± 0.001 Da</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>85 glycans detected</td>
<td>110 glycans detected</td>
</tr>
</tbody>
</table>

Gerda Vreeker  
g.c.m.vreeker@lumc.nl
Data preprocessing: MassyTools

Peak annotation on spectrum sums

Targeted extraction

Area calculation of individual isotopes

Subtract background from isotopes

Group isotopes

Quality control

Low total spectrum area in analytes

Low fraction of analytes above S/N value

Isotopic mismatch (quality score)

### Repeatability

#### MALDI-FTICR-MS

<table>
<thead>
<tr>
<th>Day</th>
<th>Plate</th>
<th>Intra-plate</th>
<th>Intra-day (day 1)</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>5.9% 14.2%</td>
<td>5.9% 14.2%</td>
<td>4.7% 9.8%</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>5.8% 17.1%</td>
<td>4.8% 9.2%</td>
<td>4.9% 9.9%</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>5.5% 14.2%</td>
<td>4.7% 9.8%</td>
<td>4.9% 9.9%</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4.8% 12.2%</td>
<td>4.7% 9.8%</td>
<td>4.9% 9.9%</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>4.7% 15.8%</td>
<td>4.7% 9.8%</td>
<td>4.9% 9.9%</td>
</tr>
<tr>
<td>Average all plates</td>
<td></td>
<td>5.3% 14.9%</td>
<td>5.3% 9.8%</td>
<td>4.9% 9.9%</td>
</tr>
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</table>

#### MALDI-TOF-MS

<table>
<thead>
<tr>
<th>Day</th>
<th>Plate</th>
<th>Intra-plate</th>
<th>Intra-day (day 1)</th>
<th>Inter-day</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4.1% 9.2%</td>
<td>5.9% 14.2%</td>
<td>5.9% 14.2%</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4.8% 8.9%</td>
<td>4.8% 8.8%</td>
<td>4.9% 9.2%</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>4.4% 8.8%</td>
<td>4.7% 8.8%</td>
<td>4.9% 9.2%</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4.2% 9.6%</td>
<td>4.7% 9.6%</td>
<td>4.9% 9.2%</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>4.4% 8.5%</td>
<td>4.7% 9.6%</td>
<td>4.9% 9.2%</td>
</tr>
<tr>
<td>Average all plates</td>
<td></td>
<td>4.3% 9.0%</td>
<td>5.3% 14.2%</td>
<td>4.9% 14.2%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycan compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5N4F1, H5N4E1, H5N4E2, H5N5F1, H5N5F1E1, H5N5F2E1, H5N5F2E2, H5N5F2E3, H7N6E2, H7N6E3, H7N6L1E2, H7N6L2E2</td>
</tr>
</tbody>
</table>

### Table

<table>
<thead>
<tr>
<th>Plate</th>
<th>Most abundant peak (H5N4E2)</th>
<th>Most abundant 10 peaks</th>
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</thead>
<tbody>
<tr>
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<td>4.1% 9.2%</td>
<td>5.9% 15.3%</td>
</tr>
<tr>
<td>2</td>
<td>4.8% 8.9%</td>
<td>5.8% 17.1%</td>
</tr>
<tr>
<td>3</td>
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<td>4.8% 14.2%</td>
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</tr>
<tr>
<td>Average all plates</td>
<td>4.3% 9.0%</td>
<td>5.3% 14.9%</td>
</tr>
</tbody>
</table>
Derived traits

MALDI-FTICR-MS

MALDI-TOF-MS

General Fucosylation Bisection Sialylation α2,3-sialylation α2,6-sialylation
**INSTRUMENTATION - CESI-MS(/MS)**

**CE** separates ions based on their electrophoretic mobility with the use of an applied voltage. **ESI** introduces protonated ions into the MS, the MS sorts and separates the ions according to their mass and charge in vacuum.
TPNG PROFILE WITH CESI-MS
BARE-FUSED SILICA CAPILLARY

Injection: 5 psi 60sec
40 nL

BFS capillary – 90 cm, 30µm id, BGE composition: 10 % AA, capillary coolant: 20° C, voltage: 20 kV, injection sample: 5 psi, 60 sec (40 nL)
**TPNG PROFILE WITH CESI-MS BARE-FUSED SILICA CAPILLARY**

**Injection:** 5 psi 60 sec 40 nL

BFS capillary – 90 cm, 30µm id, BGE composition: 10 % AA, capillary coolant: 20° C, voltage: 20 kV, injection sample: 5 psi, 60 sec (40 nL)

*Kammeijer, G.S.M. et al. Manuscript in preparation*
Bare-Fused Silica Capillary
Injection: 5 psi 60 sec (40 nL)

Dynamic Coated Neutral Capillary
Injection: 5 psi 60 sec (40 nL)
Increasing sensitivity of glycan/glycopeptide analysis with CESI-MS

Significant increase in sensitivity with nano-LC-MS in combination with acetonitrile enriched nitrogen (DEN)- gas

The solvent vapor acts as a dopant for enrichment. The charge state of multiple charged ions are modified optimizing the signal intensity.

Improvement factor of approximately one order of magnitude

Application Note # LCMS-93 amaZon speed ETD: Exploring glycopeptides in protein mixtures using Fragment Triggered ETD and CaptiveSpray nanoBooster-2014

INCREASING SENSITIVITY OF GLYCAN/GLYCOPEPTIDE ANALYSIS WITH CESI-MS

Applicable on CESI-MS?

INCREASING SENSITIVITY OF GLYCAN ANALYSIS WITH CESI-MS

Background MS signal

**A – CESI-MS**

An overall lower background observed especially in the higher mass region

**C – CESI-MS with DEN-gas**
INCREASING SENSITIVITY OF GLYCAN ANALYSIS WITH CESI-MS

Improvement (total plasma N-glycome)

CE-ESI-MS (N=3)
CE-ESI-MS with DEN-gas (N=3)

Relative area

Mannose
Galactose
Fucose
N-Acetylgalactosamine
α2,3 N-Acetylenuraminic acid
α2,6 N-Acetylenuraminic acid


3/21/2018
INCREASING SENSITIVITY OF GLYCAN ANALYSIS WITH CESI-MS

Improvement (total plasma N-glycome)

- CE-ESI-MS (N=3)
- CE-ESI-MS with DEN-gas (N=3)

S/N

Mannose
Galactose
Fucose
N-Acetylglicosamine
α2,3 N-Acetyleneuraminic acid
α2,6 N-Acetyleneuraminic acid

3x

**GLYCOMICS**
**LOD DETERMINATION**

1. **Sample**
2. **PNGase F release**
3. **Derivatization**
   - $\alpha_2,3$ N-Acetylneuraminic acid vs $\alpha_2,6$ N-Acetylneuraminic acid
4. **Purification and enrichment - Cotton HILIC**
5. **Labeling**
   - Addition of label with cationic charge
6. **CESI-MS analysis**

## LOD – EXPERIMENTAL SET-UP
### 2 GLYCAN STANDARDS

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>MALDI-TOF-MS</th>
<th>CESI-MS with DEN-gas shows a ~100x higher sensitivity compared to MALDI-TOF-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount used</td>
<td><strong>Concentration</strong></td>
</tr>
<tr>
<td><strong>Dilution series</strong></td>
<td><strong>Volume 2 µL</strong></td>
<td><strong>CESI-MS</strong></td>
</tr>
<tr>
<td>1</td>
<td>2000 fmol</td>
<td>1000 fmol/µL</td>
</tr>
<tr>
<td>10</td>
<td>200 fmol</td>
<td>100 fmol/µL</td>
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<tr>
<td>20</td>
<td>100 fmol</td>
<td>50 fmol/µL</td>
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<tr>
<td>100</td>
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<td>200</td>
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<td>1000</td>
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<td>2000</td>
<td>1 fmol</td>
<td>0.5 fmol/µL</td>
</tr>
<tr>
<td>10000</td>
<td>0.2 fmol</td>
<td>0.1 fmol/µL</td>
</tr>
</tbody>
</table>
TPNG PROFILE WITH CESI-MS– NEUTRALS

Dynamic neutrally coated BFS capillary, 90 cm 30µm id, BGE: 10 % AA, capillary coolant: 25°C, voltage: 20 kV, injection sample: 5 psi, 60 sec (40 nL)

Static neutrally coated BFS capillary, 90 cm 30µm id, BGE: 10 % AA, capillary coolant: 25°C, voltage: 20 kV, injection sample: 1 psi, 60 sec (9 nL)
TPNG PROFILE WITH CESI-MS– NEUTRALS

Dynamic Coated Neutral Capillary Injection: 5 psi 60sec (40 nL)

Static Coated Neutral Capillary Injection: 1 psi 60sec (9 nL)
ZERO-FLOW PRINCIPLE

Intens. $\times 10^6$

Time [min]

0 20 40 60 80 100

0 2 4 6 8

0 1 2 3

0 2 4 6

10 min

20 min

30 min

50 min

EFFECT OF “ZERO-FLOW” ON THE SEPARATION OF ISOMERIC N-GLYCANS

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Intensity x10^5</th>
<th>Time (min)</th>
<th>Intensity x10^5</th>
<th>Time (min)</th>
<th>Intensity x10^5</th>
<th>Time (min)</th>
<th>Intensity x10^5</th>
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<td>50</td>
<td></td>
<td>50</td>
<td></td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

**HIGH MANNOSE**

**GALACTOSYLATION**

VS

VS
EFFECT OF “ZERO-FLOW” ON THE SEPARATION OF ISOMERIC N-GLYCANS

VS

BISECTION/GALACTOSYLATION

VS

VS

SIALYLATION

0 min
10 min
20 min
30 min
50 min

0 min
10 min
20 min
30 min
50 min

0 min
10 min
20 min
30 min
50 min

0 min
10 min
20 min
30 min
50 min

0 min
10 min
20 min
30 min
50 min

0 min
10 min
20 min
30 min
50 min

0 min
10 min
20 min
30 min
50 min
GLYCOPEPTIDE / N-GLYCAN ANALYSIS WITH CESI-MS

- Glycan analysis in positive ionization mode is possible after derivatization and labeling
- CESI-MS found to be ~100 times more sensitive than MALDI-TOF-MS
- Minor sample preparation is needed
- CESI-MS shows great potential for characterization of N-linked glycosylation in complex mixtures
- Usage of static coated neutral capillary shows high potential for N-glycan analysis
  - Isomeric separation visible for several N-glycan species
Analysis of immunoglobulin G Fc variants

→ get an integrated view of Fc modifications

→ Middle down approach:
  - focus on glycosylation of IgG allotypes
  - interactions of the glycosylation with other post translational modifications

(Vidarsson et al., 2014, *Frontiers in Immunology*)
Middle-down workflow

1. Human plasma Allotypes IVig
2. CaptureSelect™ FcXL beads
3. Incubate 1h shaking
4. Wash and add buffer+IdeS
5. Digest with IdeS O/N at 37°C
6. Collect Fab in flowthrough
7. Add FA to elute Fc
8. Collect eluate
9. Dry samples in vacuum centrifuge
10. Collect single Fc chains

Together with Govert Somsen, Andrea Gargano and Guusje van Schaick, VU University Amsterdam

(Bondt et al., 2014, Molecular & Cellular Proteomics)
CESI-MS of human plasma-derived IgG Fc portions

Single Fc chain from single-donor human plasma

CE conditions: PEI-coated capillary; BGE, 20% Acetic Acid + 10% MeOH; separation, -20 kV, 20 °C

BPE

EIEs

IgG1

IgG2

IgG3

IgG4
## CESI-MS of human plasma-derived IgG Fc portions

### Allotypes of single-donor samples

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Allotype</th>
<th>Fc/2 mass, G1F (non reduced + Lysine clipped)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nomenclature</td>
<td>Position and amino acid</td>
</tr>
<tr>
<td>IgG1</td>
<td>IGHG1*03</td>
<td>356 358 422 431</td>
</tr>
<tr>
<td>IgG2</td>
<td>IGHG2*02</td>
<td>282 378</td>
</tr>
<tr>
<td>IgG3</td>
<td>IGHG3*06/07</td>
<td>379 385 392 409 419 422 435 436</td>
</tr>
<tr>
<td>IgG4</td>
<td>IGHG4*01</td>
<td>309 422</td>
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</tbody>
</table>

## Donor 1

![BPE graph for Donor 1](image)

**Intensity x10^4**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intensity</strong></td>
<td>23.5</td>
<td>22.5</td>
<td>21.5</td>
<td>20.5</td>
<td>19.5</td>
<td>18.5</td>
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</table>

## Donor 2

![BPE graph for Donor 2](image)

**Intensity x10^4**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
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</thead>
<tbody>
<tr>
<td><strong>Intensity</strong></td>
<td>25.5</td>
<td>24.5</td>
<td>23.5</td>
<td>22.5</td>
<td>21.5</td>
<td>20.5</td>
</tr>
</tbody>
</table>
HILIC-MS of human plasma

Donor 1

Allotypes: IGHG1*03
IGHG2*02

Donor 2

Allotypes: IGHG1*03 & 07
IGHG2*02

HILIC conditions: amideHILIC column; solvents, A: 98% ACN, 2% water, 0.1% TFA, B: 10% 2-propanol, 2% ACN, 0.1% TFA
HILIC-MS of human plasma

Donor 1

Allotypes:
- IGHG1*03
- IGHG2*02 & 06

Donor 2

Allotypes:
- IGHG1*03 & 07
- IGHG2*02

HILIC conditions: amideHILIC column; solvents, A: 98% ACN, 2% water, 0.1% TFA, B: 10% 2-propanol, 2% ACN, 0.1% TFA
HILIC-MS of IVIg

BPC

Chinese

Dutch

IGHG1*01

IGHG1*03

m/z

Intensity x10^6

Time [min]

+20

1239.6091
1252.4764
1262.6264
1270.7293
1278.8330
1286.9379
1293.3924
1301.5443

1245.7634
1260.9808
1272.4266
1277.1852
1285.3373
1308.0417

0.25
0.50
0.75
1.00
1.25
1.50

0.25
0.50
0.75
1.00
1.25

BPC

T.P.Senard@lumc.nl

21/03/2018
HILIC-MS of an allotype standard mAb Fc portion

**IGHG1*03**

Anti-RhD, produced in HEK cells, purification via protein A/G affinity column

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**Fc/2 mass (non reduced + Lysine clipped)**

<table>
<thead>
<tr>
<th>Theoretical mass</th>
<th>Experimental mass</th>
<th>Δ (Da)</th>
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<tbody>
<tr>
<td>25232.24</td>
<td>25231.47</td>
<td>0.77</td>
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<tr>
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<td>25247.46</td>
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<td>25410.50</td>
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</tr>
<tr>
<td>25588.53</td>
<td>25588.54</td>
<td>-0.01</td>
</tr>
</tbody>
</table>
Conclusions

• MALDI-MS of sialic acid-stabilized glycans is powerful for the high-sensitivity N-glycosylation analysis of large numbers of complex samples.
  • Sensitivity can be further boosted by approx. 100x by switching to CESI-MS after a one-pot reducing-end labeling.
  • Enhanced isomer separation is obtained with use of static neutrally coated capillaries and by initially applying a zero-flow regime.

• Human IgGs show allotype and PTM diversity which can be resolved by CESI-MS and HILIC-MS of Fc portions

• For higher throughput glycomics analysis of complex samples it is important to have suitable data preprocessing methods that are fast, standardized and partially automated.
ACKNOWLEDGEMENTS

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- Karli Reiding
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- Isabelle Kohler
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- Andrea Gargano
- Guusje van Schaick
- Govert Somsen

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