# Introduction to Glycan Analysis

-Techniques in Glycobiology-

NHLBI CardioPEG – Gerald W. Hart, August 19, 2013

Funded by NHLBI P01HL107153

## Techniques in Glycobiology

<table>
<thead>
<tr>
<th>Week</th>
<th>Date</th>
<th>Title</th>
<th>Faculty Leader</th>
<th>Location</th>
<th>Lab/Lec</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8/19</td>
<td>Introduction to Glycoconjugate Analysis</td>
<td>Jerry Hart</td>
<td>Physiology 612</td>
<td>Lecture</td>
</tr>
<tr>
<td>2</td>
<td>8/21</td>
<td>Colorimetric Assays</td>
<td>Jerry Hart</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8/23</td>
<td>Release of glycans from proteins and peptide</td>
<td>Yarema and Zachara</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8/26</td>
<td>Monosaccharide &amp; Linkage Analysis</td>
<td>Natasha Zachara</td>
<td>Physiology 612</td>
<td>Lecture</td>
</tr>
<tr>
<td>3</td>
<td>8/28</td>
<td>Glyco-Enzyme Inhibition</td>
<td>Kevin Yarema</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8/30</td>
<td>Principles of labeling glycoconjugates</td>
<td>Kevin Yarema</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9/2</td>
<td>Labor Day</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>9/4</td>
<td>Compositional Analysis</td>
<td>Natasha Zachara</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9/6</td>
<td>O-GlcNAc modification</td>
<td>Natasha Zachara</td>
<td>Physiology 612</td>
<td>Lecture</td>
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<tr>
<td>5</td>
<td>9/9</td>
<td>Study of N-linked and O-linked glycans</td>
<td>Hui Zhang</td>
<td>Physiology 612</td>
<td>Lecture</td>
</tr>
<tr>
<td>5</td>
<td>9/11</td>
<td>Separation of oligosaccharides</td>
<td>Jerry Hart</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9/13</td>
<td>Linkage Analysis</td>
<td>Zachara and Hart</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>9/16</td>
<td>The O-GlcNAc modification</td>
<td>Natasha Zachara</td>
<td>Physiology 612</td>
<td>Lecture</td>
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<tr>
<td>5</td>
<td>9/18</td>
<td>Exoglycosidases to probe structure</td>
<td>Jerry Hart</td>
<td>Lab</td>
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<tr>
<td>6</td>
<td>9/20</td>
<td>O-GlcNAc</td>
<td>Zachara and Hart</td>
<td>Lab</td>
<td></td>
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<tr>
<td>6</td>
<td>9/22</td>
<td>Characterizing GPI anchors</td>
<td>Jerry Hart</td>
<td>Physiology 612</td>
<td>Lecture</td>
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<tr>
<td>6</td>
<td>9/25</td>
<td>Characterizing GPI anchors</td>
<td>Jerry Hart</td>
<td>Lab</td>
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<tr>
<td>7</td>
<td>9/27</td>
<td>Glycosyltransferases as probes</td>
<td>Jerry Hart</td>
<td>Lab</td>
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<td>7</td>
<td>9/30</td>
<td>Analysis of Proteoglycans and sGAGs</td>
<td>Jerry Hart</td>
<td>Physiology 612</td>
<td>Lecture</td>
</tr>
<tr>
<td>8</td>
<td>10/2</td>
<td>Glycan site-mapping</td>
<td>Van Eyk &amp; Zachara</td>
<td>Lab</td>
<td></td>
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<tr>
<td>8</td>
<td>10/4</td>
<td>Analysis of Proteoglycans and GAGs</td>
<td>Jerry Hart</td>
<td>Lab</td>
<td></td>
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<tr>
<td>8</td>
<td>10/7</td>
<td>Analysis of glycosphingolipids</td>
<td>Subroto Chatterjee</td>
<td>Physiology 612</td>
<td>Lecture</td>
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<tr>
<td>8</td>
<td>10/9</td>
<td>Analysis of glycosphingolipids</td>
<td>Subroto Chatterjee</td>
<td>Lab</td>
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<tr>
<td>8</td>
<td>10/11</td>
<td>Lectures in the analysis of glycans and glycoproteins</td>
<td>Hui Zhang</td>
<td>Lab</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>10/14</td>
<td>MS analysis of glycans/glycoproteins</td>
<td>Van Eyk and Zhang</td>
<td>Physiology 612</td>
<td>Lecture</td>
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<tr>
<td>10</td>
<td>10/16</td>
<td>Principles of NMR</td>
<td>Allen Bush</td>
<td>Lecture</td>
<td></td>
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<tr>
<td>10</td>
<td>10/18</td>
<td>NMR Workshop</td>
<td>Allen Bush</td>
<td>Workshop</td>
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<td>10</td>
<td>10/21</td>
<td>MS analysis of glycans/glycoproteins</td>
<td>Van Eyk and Zhang</td>
<td>Physiology 612</td>
<td>Lecture</td>
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<td>10</td>
<td>10/23</td>
<td>MS theory</td>
<td>Van Eyk and Zhang</td>
<td>Lab</td>
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<tr>
<td>10</td>
<td>10/25</td>
<td>MS analysis of glycans and glycoproteins</td>
<td>Van Eyk and Zhang</td>
<td>Lab</td>
<td></td>
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</tbody>
</table>

• Note: With the exception of the lecture on 10/16, lectures will be 90 minutes long and take place in Physiology 612. Lectures will be made available on Blackboard at the end of the course.

• Note: Laboratory classes will be scheduled from 1-6pm, but may run longer.
Topics

- Overview of Glycoproteomic and Glycomic Approaches
- Composition Analysis of Glycans. – Labs 8/21 & 9/4
- Radiochemical and Bioorthogonal Labelling of Glycans – Lab 8/30
- Detection of Glycoproteins in Polyacrylamide Gels
- Lectin Arrays to Analyze Glycoproteins – Lab. 10/11
- Antibody Detection of Specific Glycans in Gels
- Detection of Specific Glyosphingolipids on TLC.- Lab. 10/7 & 10/9
- Methods for Isolation of Glycoproteins.
- Enzymatic and Chemical Release of Glycans from Glycoproteins. – Lab 8/23
- Labeling Released Glycans – Lab 8/30
- Methods to Separate Released Glycans –Lab 9/11
- Glycosidases & Structural Analyses – Lab 9/18
- Methylation Analysis for Linkage Determination – Lab. 8/26
- MALDI-TOF Profiling of Glycans – Lab 10/14
- Nuclear Magnetic Resonance Spectroscopy – Labs 10/16 & 10/18
- GPI-Anchors & Glycosaminoglycans – Lab 9/25
- Glycosyltransferases As Probes- Lab 9/27

Types of Glycoproteomics – Require Different Methods:

- PROTEIN FOCUSED
  - PROTEIN ID + ID OF PREVIOUSLY GLYCOSYLATED SITES

- GLYCAN FOCUSED
  - CHARACTERISATION OF GLYCAN COMPOSITION AND STRUCTURE

- GLYCOPROTEIN FOCUSED
  - PROTEINS AND GLYCOSYATION INCLUDING SITE SPECIFIC DISTRIBUTION

Many Options for Glycoprotein Analyses:

Scheme 1 | Details of the major routes for glycoprotein, glycoconjugate and glycocalyx characterization. Monosaccharides can be obtained by hydrolysis from glycoprotein, glycopeptide or released glycans. Abbreviations: HPAC-PAE, high-performance anion exchange chromatography with pulsed amperometric detection; MEC, micellar electrokinetic capillary chromatography; DLS, dynamic light scattering; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; CE-MS, capillary electrophoresis with laser-induced fluorescence; HILIC, hydrophilic interaction liquid chromatography; WAX, weak anion exchange RP, reverse phase; ES-MS/MS, electrospray ionization tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; CID, collision induced dissociation; ETD, electron transfer dissociation; ECD, electron capture dissociation.
Rough Estimates of Glycans by Colorimetric Assays (Lab. #1)
Wed. August 21, 2013

Useful as a starting place to estimate the quantity of glycans in a tissue or cell.

- Phenol-Sulfuric Acid Assay for hexoses & pentoses.
- MBTH Assay for Hexosamines & Acetylhexosamines.
- Carbazole Assay for Uronic Acids
- BCA Assay for Reducing Sugars
- Analysis of Free Sialic Acids From Glycoconjugates by the Thiobarbituric acid assay.

Compositional Analysis by HPAEC-PAD

HPAEC-PAD = High Performance Anion Exchange Chromatography with Pulsed-Amperimetric Detection
Compositional Analysis by GC-MS of Alditol Acetates

Monosaccharides → Reduce → Peracytlate (acetic anhydride in pyridine) → GC-MS

1. Rhamnitol
2. Fucitol
3. Ribitol
4. Arabinitol
5. Mannitol
6. Galactitol
7. Glucitol
8. Inositol

Alditols → 1 peak per monosaccharide

Radiolabeling strategies for the detection of glycans

METABOLIC LABELING
Radiolabeled monosaccharides

35SO4
(GAGS)

Cells

Partially purified:
Glycoproteins
Proteoglycans
Glycolipids

Periodate oxidation/reduction with sodium [H]-borohydride
(sialic acid)

CHEMICAL LABELING

SEPARATION AND DETECTION
SDS PAGE or agarose

DETECTION BY AUTORADIOGRAPHY
TLC
Metabolic Labeling of Glycoproteins with Radioactive Sugars

Martin D. Snider

Nice discussion of issues:
1. Competition with glucose – need to compromise.
   - it is advisable to use these Precursors (open squares) in medium with reduced Glc (0.1 mg/ml). Lowering the Glc concentration further is not advisable because Glc starvation can affect glycan synthesis.
   - cells must not exhaust the supply of Glc or other nutrients in the medium.
   - “Catch 22” – need to lower glucose, but you don’t want to alter glycosylation!

Analysis of glycans using the bioorthogonal chemical reporter strategy

Scott T. Laughlin & Carolyn R. Bertozzi
VOL. 2 NO. 11 | 2007 | NATURE PROTOCOLS
Metabolic and covalent labeling of glycans for *in vivo* imaging of the glycome

Drawback is low efficiency of incorporation

The bioorthogonal chemical reporter strategy for imaging glycans.

Laughlin & Bertozzi 12–17 | PNAS | January 6, 2009 | vol. 106 | no. 1
Imaging of Glycans in Living Zebrafish!

Zebrafish embryos metabolically labeled with Ac4GalNAz and reacted with Alexa Fluor 647-conjugated DIFO (DIFO-647) at 60 h postfertilization (hpf) followed by Alexa Fluor 488-conjugated DIFO (DIFO-488) at 63 hpf to detect newly synthesized glycans. Temporal resolution during development.

Detection of Glycoproteins in SDS-PAGE
## Detection of Glycoproteins in SDS-PAGE

### Procedures for Glycoprotein Detection and Characterization

<table>
<thead>
<tr>
<th>Presence/Absence vs Control</th>
<th>Detection</th>
<th>Analysis of Composition</th>
<th>Analysis of Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell culture</td>
<td>inhibition of N- or O-glycosylation</td>
<td>metabolic incorporation of H-sugars</td>
<td></td>
</tr>
<tr>
<td>sample preparation</td>
<td>chemical deglycosylation, enzymatic deglycosylation (endoglycosidase)</td>
<td>(exo)glycosidase treatment</td>
<td></td>
</tr>
<tr>
<td>separation by electrophoresis</td>
<td>after glycosylation inhibition or deglycosylation</td>
<td>lectin affinity electrophoresis - crossed immuno-electrophoresis</td>
<td></td>
</tr>
</tbody>
</table>

### Post-Electrophoresis Procedures

- Stain of untreated proteins
  - Alcian Blue
  - Stains-All
  - Reaction after periodate oxidation
    - PAS
    - Derivatized hydrazides
      - (biotin, digoxigenin)
      - Pro-Q Emerald®
- Lectin affinity blotting
  - on the sugar chains chemically or enzymatically removed from the protein
  - Sequential enzymatic degradation and HPLC
  - MS

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**Early work on RBC Surface Glycoproteins**

*periodic acid-Schiff (PAS) staining*

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**Proteomics 2006, 6, 5385–5408**
Selective Staining of Glycoproteins on 2D-Gels:

Pro-Q Emerald 488 glycoprotein stain – Proprietary dye reacts with periodate reacted glycans.


Lectin Blotting is a Powerful Tool to Detect Glycoproteins:

Table 1: The binding specificity of the lectins used in this study

<table>
<thead>
<tr>
<th>Lectin name</th>
<th>Full name of lectin</th>
<th>Population variation</th>
<th>Primary mono-oligosaccharide preference</th>
<th>Primary binding specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAL</td>
<td>Alliaceae</td>
<td>50-Fold</td>
<td>Fucose</td>
<td>Fucose (α-1,6) N-acetylglucosamine or fucose (α-1,3) N-acetylglucosamine residues [37]</td>
</tr>
<tr>
<td>UEA I</td>
<td>Ulex europaeus I</td>
<td>30-Fold</td>
<td>Fucose</td>
<td>Fucose (α-1,2) galactosyl (β-1,3) N-acetylglucosamine (β-1,6) and other oligosaccharides residues [36]</td>
</tr>
<tr>
<td>JAC</td>
<td>Jacalin lectin</td>
<td>4-Fold</td>
<td>Galactose</td>
<td>O-linked galactosyl (β-1,3) N-acetylglucosamine, including the mono- or di-oligomannose forms [59, 60]</td>
</tr>
<tr>
<td>MAE I</td>
<td>Maackia amurensis I lectin</td>
<td>1600-Fold</td>
<td>Galactose</td>
<td>Static acid (α-2,3) galactosyl (β-1,4) N-acetylglucosamine residues, tolerating substitution of N-acetylglucosamine with sialic acid at the 3-position of galactose [51, 52]</td>
</tr>
<tr>
<td>PSA</td>
<td>Phaseolus vulgaris agglutinin</td>
<td>7-Fold</td>
<td>Manose</td>
<td>a-Manose-containing oligosaccharides with an N-acetylglucosamine-linked α-flour residuo included in the sequence [19, 48, 49]</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
<td>4-Fold</td>
<td>Manose and glucose</td>
<td>Internal and nonreducing terminal α-mannose residues but also binds nonreducing terminal α-glucose and α-N-acetylglucosamine residues [51, 47, 50]</td>
</tr>
<tr>
<td>GNL</td>
<td>Galanthus nivalis lectin</td>
<td>25-Fold</td>
<td>Manose</td>
<td>(α-1,3) Manose residues, and unlike most manose-binding lectin, it does not bind high-mannose residues [69]</td>
</tr>
<tr>
<td>HBL</td>
<td>Helix pomatia lectin</td>
<td>1500-Fold</td>
<td>Manose</td>
<td>(α-1,3)- and (α-1,6)-linked polylaminosaccharide structures and does not recognize mannos to be at the nonreducing terminus [51]</td>
</tr>
<tr>
<td>RPL</td>
<td>Ricinus communis agglutinin</td>
<td>20-Fold</td>
<td>N-Acetylglucosamine</td>
<td>Ricinus lectin and other bind oligosaccharides with a terminal α-N-acetylglucosamine [31, 52]</td>
</tr>
<tr>
<td>VVA</td>
<td>Ficus carica lectin</td>
<td>1000-Fold</td>
<td>N-Acetylglucosamine</td>
<td>α- or β-linked terminal N-acetylglucosamine, especially a single α-N-acetylglucosamine residue linked to α-mannose or fucose (25, 56)</td>
</tr>
<tr>
<td>DSL</td>
<td>Dillanthus flavonoides lectin</td>
<td>185-Fold</td>
<td>N-Acetylglucosamine</td>
<td>(β-1,4)-linked terminal N-acetylglucosamine oligomers, preferring chitobiose or chitotriose over a single N-acetylglucosamine residue and also binds well to N-acetylglucosamine and oligomers containing repeating β-acylglucosamine linkages [37, 60]</td>
</tr>
<tr>
<td>LEL</td>
<td>Lens culinaris lectin</td>
<td>9000-Fold</td>
<td>N-Acetylglucosamine</td>
<td>(β-1,3)-linked N-acetylglucosamine oligomers performing tetras and pentas of b-linked sugar [57, 58]</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
<td>15-Fold</td>
<td>N-Acetylglucosamine</td>
<td>Terminal β-1,3-linked N-acetylglucosamine, galactosyl (β-1,4) N-acetylglucosamine (β-1,3) α-linked sugars of large oligosaccharides and also binds sugars to (α-2,3), and (α-2,4)-linked terminal N-acetylglucosamine acid sugars (α-2,3) galactosyl (β-1,3) N-acetylglucosamine (α-2,6) N-acetylglucosamine and other (α-2,3)-linked static acid residues [67]</td>
</tr>
<tr>
<td>MAE II</td>
<td>Maackia amurensis II lectin</td>
<td>700-Fold</td>
<td>Static acid</td>
<td>Static acid attached to a terminal galactose in (α-2,6) linkage and to a lesser degree (α-2,3) linkage [55]</td>
</tr>
<tr>
<td>SNA</td>
<td>Sorbus nigra lectin</td>
<td>15-Fold</td>
<td>Static acid</td>
<td>Static acid attached to a terminal galactose in (α-2,6) linkage and to a lesser degree (α-2,3) linkage [55]</td>
</tr>
</tbody>
</table>
Detection of Glycoproteins by Glycan-Specific Antibodies:
Eg. Pan-Specific antibody to O-GlcNAc

Detection of Glycosphingolipids by Antibodies After TLC:

1. TLC
   separation of GSLs

2. Immunostaining
   sequential multicolor staining
   1. Fast Red
   2. SCIP
   3. DAB
   chromogenic substrate
   enzyme
   secondary antibody
   GSLs
   glass slide
   inactivation

3. Mass spectrometry
   mass analyzer
   IR-laser

Anal. Chem. 2009, 81, 9481–9492
Multiple immunostaining combined with multicoloring of TLC-separated neutral GSLs from human erythrocytes

Typical Protocol Used to Analyze Glycoproteins:

1. 1D-, 2D-gels
2. Protease
3. Enrichment of glycopeptides
4. Fractionation by HPLC
5. Deglycosylation
6. ESI-MS and -MS/MS
7. MALDI-MS and -MS/MS
8. Assignment of glycosylation site and glycan sequence

Affinity methods for isolation of glycoproteins.

Unnatural sugars used in the isolation of glycoproteins.
Options for Releasing Glycans From Proteins:

- **N-glycans**
  - Enzymatic
    - N-glycosidase F (PNGase F) clearing every N-glycan except those having α(1–3)-linked fucose on the reducing-terminal GlcNAc [7]
    - PNGase A liberating any N-glycan [7]
    - Endoglycosidases or Glycoamidases such as Endoglycosidase H [11]
  - Chemical
    - Hydroxyamidase: Glycans are liberated using anhydrous hydrazine at 90 °C for 4 hours [18]
    - O-glycanase [7]

- **O-glycans**
  - Enzymatic
    - Hydrazinolysis: Glycans are liberated using anhydrous hydrazine specifically at 60 °C for 5-6 hours [7;19]
    - Reductive allyl-catalyzed β-elimination by use of alkaline borohydride
    - Non-reductive β-elimination [14;15]
    - Alternatives releasing agents as trifluoroethane-sulfonic acid [16] or various amines [17]
  - Chemical

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### Table 12.4.1 Enzymes Described in This Unit

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Indications and uses</th>
<th>Monitorsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo D</td>
<td>Transient appearance of highly processed, sensitive forms prior to addition of GlcNAc by GlcNAc transferase I</td>
<td>Cis to medial Golgi</td>
</tr>
<tr>
<td>Endo F₂</td>
<td>Presence of biantennary chains ± core fucose</td>
<td>Medial Golgi</td>
</tr>
<tr>
<td>Endo F₃</td>
<td>Presence of core fucosylated biantennary α chains and/or trisaccharide chains ± core fucosylation</td>
<td>Medial Golgi</td>
</tr>
<tr>
<td>Endo-β-galactosidase</td>
<td>Presence of poly-N-acetyllactosamines</td>
<td>Trans-Golgi and TGN</td>
</tr>
<tr>
<td>Endo H</td>
<td>Conversion of high mannose to complex type N-linked chains</td>
<td>Cis to medial Golgi</td>
</tr>
<tr>
<td>O-Glycosidase</td>
<td>Presence of Galβ1,3GalNAc-α Thr/Ser O-linked chains</td>
<td>Cis to medial Golgi</td>
</tr>
<tr>
<td>PNGase F</td>
<td>Presence of N-linked chains; cleaves nearly all N-linked chains; only enzyme that cleaves tetraantennary chains</td>
<td>Medial Golgi</td>
</tr>
<tr>
<td>Sialidase</td>
<td>Acquisition of static acids</td>
<td>Trans-Golgi and TGN</td>
</tr>
<tr>
<td>O-Sialoglycoprotease</td>
<td>Presence of mucin-like proteins with cluster of sialylated glycans</td>
<td>Trans-Golgi and TGN</td>
</tr>
</tbody>
</table>

aAbbreviation: TGN, trans-Golgi network

*Current Protocols in Protein Science 12.4.1-12.4.25, November 2010*
PNGase F works best on Denatured Proteins or Glycopeptides, But Does Not Release All N-Glycans Equally.

(PNGase A from Almonds cleaves all N-Glycans as long as peptide has COOH and amino terminus)

A. PNGase F can cleave when an α-1–6 Fucose is on the core GlcNAc

B. PNGase F can not cleave when an α-1–3 Fucose is on the core GlcNAc

Several Endo-Glycosidases For Releasing N-Glycans Are Available Commerically

PNGase F useful in Site Mapping by MS

Sigma-Aldrich Chemical Deglycosylation Strategies
Unlike PNGase F, O-Glycosidase is Highly Specific:

Enzymatic De-Glycosylation of O-Glycopeptides Requires Multiple Enzymes.
Alkaline β-Elimination releases Ser/Thr-linked Sugars:

$[^3\text{H}]\text{Gal}$ $\xrightarrow{\beta\text{-Elimination}}$ $[^3\text{H}]\text{Gal(β1-4)GlcNAcitol}$

Often done with borohydride to prevent ‘peeling’.
Replacement of O-GlcNAc with DTT
Using β-elimination/michael addition

BEMAD Site Mapping

Control Cells
Performic acid treatment
Alkaline Phosphatase
BEMAD
Add Controls
O-GlcNAc-peptides
O-phosphate-peptides
<--Trypsin-->

Experimental Cells
Alkaline Phosphatase
BEMAD
Heavy DTT
Light DTT

Mix

Heavy and Light DTT labeled O-GlcNAc-peptides
Thiol Enrichment
LC-MS/MS

Heavy and Light DTT labeled O-phosphate-peptides
Thiol Enrichment
LC-MS/MS

Strategy for O-GlcNAc/O-Phosphate site mapping

Alkaline induced β-Elimination

Michael Addition

O-GlcNAc
(Serine-O-GlcNAc)

O
H
C
CH2
N
H
C
O

(dehydroalanine)

PSVPVSerGSAPGR

O-GlcNAc

BAP

1. Provides tag for Affinity enrichment
2. Tag is stable in mass spectrometer

MALDI-TOF

BEMAD Site Mapping

Combination of Enzymatic Tagging with ‘Click-It’ Chemistry & Solid-Phase BEMAD to Map Sites:

Hydrazine hydrolysis has been found to be effective in the complete release of unreduced O- and N-linked oligosaccharides. –Destroys the Peptide!

Sigma-Aldrich Chemical Deglycosylation Strategies

Anhydrous Hydrazine = Rocket Fuel!!!
Strategies for the analysis of released glycoprotein-glycans.

Periodate Oxidation to Label Glycans:

Labeling of glycans.

A 2-Aminobenzoic acid (2-AA) labeling via reductive amination,

B 1-phenyl-3-methyl-5-pyrazolone labeling via a Michael-type addition,

C labeling with phenylhydrazide, and

D glycan permethylation
### Table 47.1: Separation techniques and their acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Technique</th>
<th>Description</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACE</td>
<td>fluorophore-assisted carbohydrate electrophoresis</td>
<td>gel-electrophoresis-based chromatographic technique for separating samples derivatized with an anionic fluorophore</td>
<td>separation, identification, and quantification of labeled mono- and oligosaccharides</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
<td>gas-phase chromatographic technique for separating volatile derivatized samples</td>
<td>sugar composition and linkage analysis; usually interfaced with MS</td>
</tr>
<tr>
<td>HPAC</td>
<td>high-performance anion-exchange chromatography</td>
<td>chromatographic separation technique carried out at high pH</td>
<td>separation, identification, and quantification of mono- and oligosaccharides without derivatization</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
<td>chromatographic technique for separating charged molecules</td>
<td>separation, identification, and quantification of charged glycans; sometimes interfaced with MS</td>
</tr>
<tr>
<td>HPTLC</td>
<td>high-performance thin-layer chromatography</td>
<td>chromatographic technique for analytical separations</td>
<td>glycolipid characterization</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
<td>gel electrophoresis technique for separation of proteins according to molecular weight</td>
<td>glycoprotein characterization</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
<td>1D- and 2D-NMR spectroscopy</td>
<td>number and anomeric configuration of monosaccharides in a glycan</td>
</tr>
<tr>
<td>CONE</td>
<td>correlation spectroscopy</td>
<td>2D-NMR spectra; cross-peaks indicate protons joined by few bonds</td>
<td>identity and anomeric configuration of monosaccharides in a glycan</td>
</tr>
<tr>
<td>TOCSY</td>
<td>total correlation spectroscopy</td>
<td>2D-NMR spectra; cross-peaks define whole spin system (e.g., one monosaccharide residue)</td>
<td>identity and anomeric configuration of monosaccharides in a glycan</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear Overhauser effect spectroscopy</td>
<td>2D-NMR spectra; cross-peaks indicate protons close in space</td>
<td>sequence analysis, conformational analysis</td>
</tr>
<tr>
<td>ROESY</td>
<td>rotating-frame NOESY</td>
<td>2D-NMR spectra; cross-peaks indicate protons close in space; better than NOESY for oligosaccharides</td>
<td>sequence analysis, conformational analysis</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond spectroscopy</td>
<td>2D-NMR spectra; cross-peaks indicate proton and C, N, or P atom linked by few bonds</td>
<td>assignment of NMR signals to atoms in structure; sequence and substitution analysis</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single–quantum coherence spectroscopy</td>
<td>2D-NMR spectra; cross-peaks indicate proton and C, N, or P atom linked by one bond</td>
<td>assignment of NMR signals to atoms in structure</td>
</tr>
</tbody>
</table>
### Tools of Glycome Profiling

<table>
<thead>
<tr>
<th>Glycome profiling tool</th>
<th>Sensitivity</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary electrophoresis (CE)</td>
<td>fmol range</td>
<td>High-throughput</td>
<td>Indirect structural information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Short analysis time</td>
<td>Labeling is necessary</td>
</tr>
<tr>
<td>Liquid chromatography (LC): High-performance liquid chromatography</td>
<td>pmol–fmol range</td>
<td>High dynamic range</td>
<td>No available databases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Separation of isomers</td>
<td>Small sample volume</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Available databases due to high reproducibility</td>
<td>Hypersensitive with MS more difficult compared to LC with MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Easy hypersensitive with MS</td>
<td></td>
</tr>
<tr>
<td>High-pH anion-exchange chromatography</td>
<td>nmol–pmol range</td>
<td>High dynamic range</td>
<td>Low to medium throughput</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Separation of isomers</td>
<td>Labeling is not strictly necessary, but advantageous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No labeling is necessary</td>
<td>Time-consuming</td>
</tr>
<tr>
<td>Mass spectrometry (MS): Matrix-assisted laser desorption ionization</td>
<td>pmol–fmol range</td>
<td>High resolution</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No labeling necessary</td>
<td>Sensitive to sample contaminants</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tolerates more sample contaminants than ESI</td>
<td>No separation of isomers and isotopic structures</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chemical modifications needed to improve ionization and stability of labile glycan modifications</td>
</tr>
<tr>
<td>Electrospray ionization (ESI)</td>
<td></td>
<td>High resolution</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No labeling necessary</td>
<td>Sensitive to sample contaminants</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soft ionization, allowing labile glycan modifications intact</td>
<td>No separation of isomers and isotopic structures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reality coupled to CE and LC</td>
<td>Complex data profile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower throughput than MALDI</td>
</tr>
<tr>
<td>Chip-ESI</td>
<td></td>
<td>High-resolution</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High-throughput microchips</td>
<td>Sample clean-up necessary in case of high-throughput microchips</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CE and LC microchips</td>
<td>Complex data profile</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Online sample treatments</td>
<td></td>
</tr>
<tr>
<td>Microarray</td>
<td>pmol–fmol range</td>
<td>High-throughput</td>
<td>Labeling is necessary</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Screening of living cells</td>
<td>Limited structural information</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simultaneous analysis of various classes of carbohydrates</td>
<td>Qualification issues</td>
</tr>
</tbody>
</table>

### Normal-Phase Chromatography of Underivatized Glycans: A Type of HILIC

![Normal Phase Chromatography](image)

Fig. 1. Elution pattern of Fraction 1c (100 µg) on 5 µm Lichrosorb NH2.
Analysis of N-linked oligosaccharides from human "1-acid Glycoprotein - 2-AB-labeled oligosaccharides

Normal Phase Chromatography of 2-AB labeled Glycans:

Figure 2. HPLC Profile of the 2-AB labeled N-linked glycan library obtained from Fetuin.

Figure 3. Separation of partially hydrolyzed 2-AB labeled dextran on normal phase HPLC. The numbers indicate glucose units (gu).

Glycoprotein Analysis Manual Sigma-Aldrich
Comparison of fetuin digested with PNGaseF and fetuin that has undergone ammonia-based β-elimination at both 40°C and 60°C.

Reverse-phase HPLC with fluorescence detection of 2-AB-labeled glycans

L. R. Ruhaak & G. Zauner & C. Huht & C. Bruggink & A. M. Deelder & M. Wuhrer
Anal Bioanal Chem (2010) 397:3457-3481
Analysis of 2-AA-labeled total plasma N-glycans by matrix assisted laser desorption/ionization (MALDI)–Fourier transform ion cyclotron resonance (FTICR)–MS

A natural glycan microarray approach with reductively aminated glycans

L. R. Ruhaak & G. Zauner & C. Huhn & C. Bruggink & A. M. Deelder & M. Wuhrer
Ion-Exchange Chrom. – Separate Glycans by Charge.

DEAE-Sephadex Separation of Glycans with 1, 2 or 3 Sialic acids

Size After Desialylation

Gel Filtration Chromatography – Separate Oligosaccharides by Size.

Fig. 1. Sequential exoglycosidase digestions of oligosaccharides A, B, and C. The radioactive sugars were subjected to Bio-Gel P-4 column chromatography, and the radioactivity in each tube (3 ml/tube) was determined with a liquid scintillation spectrometer. The black arrows at the top of the figure indicate the elution positions of glucose oligomers (numbers indicate glucose units), and the white arrows indicate the positions of standard oligosaccharides: I, Gal$_1$-GlcNAc$_1$, Man$_3$, GlcNAc$_1$-Fuc$_1$-GlcNAc$_1$; II, GlcNAc$_1$-Man$_2$, GlcNAc$_1$-Fuc$_1$-GlcNAc$_1$; III, Man$_2$, GlcNAc$_1$-Fuc$_1$-GlcNAc$_1$; IV, GlcNAc$_1$-Fuc$_1$-GlcNAc$_1$; V, Fuc$_1$; VI, N-acetylglucosaminyl. A, oligosaccharides A (----), B (---), and C (--.--); B, oligosaccharides A (----), B (---), and C (--.--); C, the radioactive peaks in (B) digested with β-N-acetylhexosaminidase; D, the radioactive peak in (C) incubated with α-mannosidase; E, the radioactive peak in (D) digested with β-mannosidase; F, the radioactive peak in (E) incubated with β-N-acetylhexosaminidase; G, the radioactive peak in (F) digested with α-fucosidase. Conditions for exoglycosidase digestion are described in "EXPERIMENTAL PROCEDURES."

J. Biochem. 88, 819-827 (1980)
Dionex with Pulsed-Amperometric Detection – A Breakthrough in the mid-80s

At pH 13 Carbohydrates ionize and Become Charged molecules.

Fig. 1. Separation of neutral oligosaccharides using HPAE-PAD. A mixture of oligosaccharides (=1 nmol each) were chromatographed on a Dionex CarboPac PA-1 column (4.6 × 250 mm) and detected by PAD. Gradient 1 was used to elute the larger oligosaccharides and is shown by the dashed line.

Derivatization of Glycans for Electrophoresis

8-amino-1,3,6-pyrenetrisulfonic acid

Fig. 1. Reductive amination of glucose with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS).
Capillary Electrophoresis of Glycans

Figure 1. Process for preparing and labelling oligosaccharides for analysis.

Fluorophore-Assisted Carbohydrate Electrophoresis:

Purified Glycoprotein

N-Glycanase Digestion \( \rightarrow \) 2 hours to overnight

Free Oligosaccharides

Label with fluorophore \( \rightarrow \) 3 hours to overnight

Labeled Oligosaccharides

Load onto FACE Gel \( \rightarrow \) 30 minutes

Electrophoresis \( \rightarrow \) 1 - 1 1/2 hours

FACE Imaging and Data Analysis

From Glyko
Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

Hydrophilic Interaction Liquid Chromatography (HILIC)
(Formally called "Normal Phase" Chromatography)
HPLC-HILIC profile of N-glycans released from heavy chain human serum IgG
Glycosidases used for structural analysis

Glycosidases remove sugars

- α-Mannosidase
- β-Mannosidase
- α-Fucosidase

High mannose- or hybrid-type N-glycan

Neuraminidase
- β-Galactosidase
- β-N-Acetylgalactosaminidase

Exoglycosidases remove terminal sugars
Endoglycosidases remove glycans

Must have this residue to be cleaved by Endo H

Endoglycosidase H (Endo H)

Exoglycosidase Sequencing using HILIC of 2AB-Labeled Glycans

Figure 4 | Detailed structural analysis of human serum IgG using exoglycosidase sequencing. (a) Cleavage sites of commonly used exoglycosidases for glycan fingerprinting. ABD: Arthrobacter ureafaciens glycosidase (EC 3.2.1.18) releases β-GlcNAc/Neu5Ac linked nonreducing terminal N-acetylglucosamine (Neu5Ac, NeuAc) and N-acetylgalactosamine (NANA, Neu5Gc). AAB: Streptococcus pneumoniae mannosidase (EC 3.2.1.18) releases N-acetyl-α-mannosidase (NANA and NAGNA). STG: Streptococcus thermophilus β-galactosidase (EC 3.2.1.23) hydrolyzes nonreducing terminal galactose with β(1→4) and β(1→6) linkages. SPG: S. pneumoniae α-galactosidase (EC 3.2.1.23) hydrolyzes nonreducing terminal galactose (β-1→4) (Kagoo, AMF: aldonolactosidase (EC 3.2.1.31) releases α(1→6)-linked nonreducing terminal galactose residues except core α(1→6) fucose. BFP: bovine kidney α-L-fucosidase (EC 3.2.1.51) releases α(1→2)-linked nonreducing terminal fucose residues more efficiently than α(2→3)-linked fucose. CLU: B. subtilis α-L-fucosidase (EC 3.2.1.51) will digest α(1→6)-linked GlcNAc to mannose but not a branching GlcNAc (α-1→4) linked to mannose. (b) Representative HILIC profiles of 2AB-labeled N-glycans from human IgG before (UND) and after exoglycosidase digestion. For the purpose of clarity, only the sequential digestion of two pools is shown with black arrows. NAs in glycans will elute can be obtained reproducibly. A combination of the specificity of the exoglycosidases used and a knowledge of the incremental value of each monosaccharide facilitates the analysis of the glycan structure sequence and intact assignment.
A simple example of methylation analysis, showing a structural motif that may be found in the polysaccharide glycogen.

Methylation Analysis is Used to Determine Linkages.
Enzymes or NMR are used to Determine Anomericity.

Methyl Ether is Acid Stable

100% RXN

ReductionEliminates Anomeric Mixtures

Data from a glycomics study of N-glycans from mouse kidney.

Assumptions Based Upon Known Pathways
Typically Confirmed by MS/MS and enzymes.
Example of MALDI-TOF Profiling of Glycans

1H-NMR spectrum of a mixture of two trisialyl triantennary N-glycans

NMR is the most powerful method for glycan structure determination.

Provided 2 things are True:

1. Enough Material
2. Oligosaccharide is pure!
Chapter 47, Figure 5

NMR and MS data used in the determination of the structure of the complex pneumococcal capsular polysaccharide 17F

Figure 6 MS5 of the monosialo fragment Tri(OH)3Neu1, m/z 962, CE = 31, 28, 26, 28. MS conditions were as described for Figure 1. The oligosaccharide structure and fragmentation pathway are shown at the top of the figure.

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Complete Structural Analyses of Glycans Requires Several Approaches: MS, NMR, Enzymes, Fractionation.

Ion Trap MS
A Powerful tool For Structural Determination of Glycans.

Figure 6 MS5 of the monosialo fragment Tri(OH)3Neu1, m/z 962, CE = 31, 28, 26, 28. MS conditions were as described for Figure 1. The oligosaccharide structure and fragmentation pathway are shown at the top of the figure.
GPI-Anchored Proteins Are Extracted into the Detergent Phase Using Two-Phase Separation with TritonX-114:

**FIG. 1.** Flow diagram for the purification of GPI-anchored proteins from *T. minuta*. Numbers in parentheses show typical protein recovery, as percentage of total recovered protein, in the different fractions.

**Basic Structure of GPI-Anchors:**

Strategy for Analysis of GPI-Anchors:

Chemical and enzymatic reactions of GPI anchors
Characterization of Glycosaminoglycans:

Bacterial Eliminases Are Powerful Tools:

1. Sequential Degradation Followed by Gel Filtration.

2. Other Separation Methods.

FACE Analysis of GAG-Derived Disaccharides:

From: Methods in Molecular Biology, Vol. 171: Proteoglycan Protocols
Edited by: R. V. Iozzo © Humana Press Inc., Totowa, NJ
HPLC separation of CS-derived saturated and unsaturated disaccharides labeled with 2AB

Glycosyltransferases as Probes:

Fig. 1. Distribution of Galβ1-4GlcNAc residues on different cell types compared per cell equivalent. Thymocytes, splenocytes, splenic T-cells, splenic B-cells, EL-4 cells, and AKTIB 1b cells were exsialylated with various amounts of added α-2,3-sialyltransferase (0.77 U/ml) and CMP-[3H]NeuAc (0.25 μmol). Macromolecular [3H]NeuAc transferred to cell surface Galβ1-4GlcNAc-residue-containing glycoproteins was compared on a per cell basis (-Sialidase, upper panel). The same cell types were pretreated with sialidase as described under Materials and Methods and then exsialylated and the macromolecular [3H]NeuAc incorporated was compared (-Sialidase, lower panel). In each case, endogenous incorporation of [3H]NeuAc was subtracted (see Results).

CELLULAR IMMUNOLOGY 125, 337–353 (1990)
**Fig. 5.** Immunochemical identification of specific Galβ1-4GlcNAc-containing surface molecules. Thymocytes (Thym) and splenocytes (Splen) were either pretreated (+) or not treated (−) with sialidase before being exsialylated. After solubilization in Nonidet-P40, lysates were precleared and incubated overnight at 4°C with monoclonal antibodies coupled to Sepharose 4B (2 mg/ml). Immunoprecipitates were washed and eluted with SDS sample buffer. Eluted antigens were subjected to electrophoresis on either 7.5% (for T200 and LFA-1), or a 12.5% SDS-polyacrylamide gel (for Thy-1.2). The molecular weight standards are as listed for Fig. 4.

**CELLULAR IMMUNOLOGY 125, 337–353 (1990)**

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**Fig. 2.** Autoradiograph of exsialylated thymocytes. Thymocytes either were sialidase-treated (+NANAse) or were mock treated (−NANAse) before sialylation with the β-galactoside α-2,6-sialyltransferase as described under Materials and Methods. After incubation for 4 h at 4°C, the cells were harvested and prepared for autoradiography as described. The thymocytes remained greater than 90% viable as determined by trypan blue exclusion. The magnification is 400×.

**ANALYTICAL BIOCHEMISTRY 163, 123–135 (1987)**
Conclusions:

1. There are now a multitude of powerful methods to analyze glycoconjugates.

2. However, most of the existing technology requires a fairly high level of skill and knowledge in analytical chemistry.

3. Existing Technology is still unable to completely define the molecular species of a glycoprotein with more than one glycosylation site.

4. Major advances in Separation Technologies and in Mass Spectrometry have had a huge impact upon the field.