N-Glykan-Charakterisierung therapeutischer monoklonaler Antikörper - neue und schnelle Lösungen zur N-Glykan Profilierung

Moritz Wagner
Waldbonn
Background

- What are glycans and why are they important?

Glycans are carbohydrates attached post-translationally to proteins
Glycans improve protein water solubility
Glycans protect proteins from clearance and degradation
Glycans mediate cellular recognition and signaling
Glycans are cell, species, and site specific.

Dall-Olio, Clin Mol Pathol. 1996
Background

- Glycan structure and nomenclature*

Glycan structure has a strong influence on antibody activity

**What is ADCC?**

ADCC: Antibody-Dependent Cell-Mediated Cytotoxicity

- **Tumor Lysis by ADCC**
- **Cytotoxicity**
- **Target Cell (Cancer Cell)**
- **Antibody**
- **Fc Receptor**
- **Effector Cell (NK cell & Monocyte)**

ADCC is a major mechanism for killing tumor cells by therapeutic antibodies.

- Low fucose content correlates to high ADCC function
  - 40X higher affinity/activity

  *Ferrara et al. J. Biol. Chem. 2006
  http://www.eurekah.com.cn/images/ADCC.jpg*

- Gal-alpha-gal linkage is a non-human glycan shown to trigger hypersensitivity


* as used in the mAb-Glyco Chip database
Background
- Glycan structure and nomenclature*

Trimannosyl core

Addition of sugars

Complex glycan profiles

- N-acetylgalactosamine (GlcNAc)
- Galactose (Gal)
- Fucose (Fuc)
- Mannose (Man)
- N-acetylneuramic Acid (NANA)
- N-glucosylneuramic Acid (NGNA)

* as used in the mAb-Glyco Chip database
Background
- Glycan structure and nomenclature*

- Trimannosyl is defined as the common core structure for all \(N\)-glycans
- Glycans are defined using four numbers: \(ABCD\)
  
  - **A** \ldots the number of GlcNAc (\(\square\)) residues **outside** the core
  - **B** \ldots the presence (1) or absence (0) of fucose (\(\bigtriangleup\)) **within** the core
  - **C** \ldots the number of galactose (\(\bigcirc\)) residues
  - **D** \ldots is the number of terminal sialic acid residues **outside** the core, an attached \(A\) defines the sialic residue(s) as \(\text{NANA} (\biglozenge)\)
    
    an attached \(G\) as \(\text{NGNA} (\biglozenge)\)

* as used in the mAb-Glyco Chip database
Background
- **Glycan structure and nomenclature**

- Trimannosyl is defined as the common core structure for all \( N \)-glycans
- Glycans are defined using four numbers: \( ABCD \)

\( A \) … the number of GlcNAc (□) residues **outside** the core
\( B \) … the presence (1) or absence (0) of fucose (▲) **within** the core
\( C \) … the number of galactose (●) residues
\( D \) … is the number of terminal **sialic acid residues** outside the core, an attached \( A \) defines the sialic residue(s) as NANA (◇) an attached \( G \) as NGNA (◇)

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<th>B</th>
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\= \(2020\ 0A\ 0G\)
\= \(3111\ 1A\ 0G\)
\= \(2122\ 0A\ 2G\)

* as used in the mAb-Glyco Chip database
Intact Protein Analysis

• Accurate mass measurements of intact proteins, whole subunits, or domains are useful for the rapid verification of sequence composition and identification of posttranslational modifications, such as *glycosylation*.

**Agilent QTOF/TOF MS**
• Accurate mass
• Resolution

**Protein Chip**
- **Trapping-column**: 40nL
- **Nano-column**: 43mm x 75 µm ID
- **Packing**: Zorbax SB C8, 5µm (300Å)
Intact Protein Analysis

- monoclonal antibody (mAb)

Mass spectrum of the intact mAb; 5-10 ng on column, compared to conventional µg-levels!

Deconvolution indicates 3 main species (A-C) of the mAb analysed:

A. Intact mAb with a pair of G0F glycans
B. mAb with one G0F glycan
C. mAb devoid of any N-linked glycan
D. Indication of „exchange“ of one G0F against a G1F

Calculated mass (A) = 148812.81
Measured mass (A) = 148811.95
Mass Accuracy = 5.7 ppm!
**Intact Protein Analysis**

- **monoclonal antibody (mAb) after PNGase F deglycosylation**

**Incubation w/ PNGase F: 37°C over night**

**Mass spectrum:** Well defined charge species with minimal adducts satellites

A. Result from deconvolution of deglycosylated mAb

B. mAb with one G0F glycan

**Calculated mass** (A) = 145923.24

**Measured mass** (A) = 145924.41

**Mass Accuracy** = 8 ppm
mAb-Glyco Chip Kit
Agilent HPLC-Chip/MS
– Portfolio w/ 12 different chips + custom chip

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<td>Custom Chip Special SPQ Number</td>
<td>Design your own chip and ask for a special quote</td>
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Agilent Technologies
mAb-Glyco Chip Kit
- Infinitely faster N-glycan characterization

- Up to 100 x faster than existing methods!
  - for the characterization of N-glycans on monoclonal antibodies

- Automated turnkey solution!
  - for on-chip deglycosylation, N-glycan separation and high sensitivity TOF/QTOF detection

- Integrated comprehensive data processing!
  - for automated glycan identification, quantification and reporting
Glycan Characterization

- Typical workflows of traditional analyses

[Diagram showing the process of deglycosylation with PNGase F, followed by hydrolysis to form N-Glycan, and then analysis using MALDI-TOF/MS, LC/MS, and HPLC/pulsed amperometric detector.]
Glycan Characterization

- Typical workflows of traditional analyses
**Glycan Characterization**

- **Typical workflows of traditional analyses**

  - Complex workflows!
  - Each step = error source!
  - Time consuming = ½ - 4 days!
  - Introduces a bottleneck during development phase of mAbs!
mAb-Glyco Chip Kit

- **Goals**

1. **Complete analysis on an integrated HPLC-Chip Design** and removal of labelling step
2. **Automation** → reduce sources of errors
3. **Fast analysis** → fast answers
4. **Develop turnkey solution** for N-glycan profiling
mAb-Glyco-Chip
– Integrated HPLC-Chip design

(a) Enzyme reactor packed with immobilized PNGase F beads for on-chip deglycosylation of mAbs
(b) PGC-enrich and (c) analytical columns for trapping and the separation of cleaved N-glycans
(d) Electrospray tip for direct transfer to (Q)TOF detection
(e) The mAb-Glyco Chip utilizes the unique rotor-in-rotor design of the chip cube!
mAb-Glyco-Chip

Automated on-Chip workflow: Sample injection
mAb-Glyco-Chip

- **Automated on-Chip workflow: Enzyme reactor fill**

(A) Outer Rotor
   - Cap Pump
   - Enzyme Reactor Fill

(B) Inner Rotor
   - Nano Pump
   - Sample Injection

Capillary pump pressure [bar]
Nanoflow pump gradient [%B]
mAb-Glyco-Chip

**Automated on-Chip workflow: Deglycosylation**

(A) Outer Rotor
- Cap Pump
- ER
- Waste
- Sample Injection

(B) Outer Rotor
- Cap Pump
- ER
- Waste
- Enzyme Reactor Fill

(C) Outer Rotor
- Cap Pump
- ER
- Waste
- Deglycosylation

Capillary pump pressure [bar]

Nanoflow pump gradient [%B]

1 min 6 s 4 min
mAb-Glyco-Chip

- Automated on-Chip workflow: Glycan transfer

(A) Outer Rotor
- Cap Pump → ER → Waste
- Inner Rotor
- Nano Pump → PGC → MS

(B) Outer Rotor
- Cap Pump → ER → Waste
- Inner Rotor
- Nano Pump → PGC → MS

(C) Outer Rotor
- Cap Pump → ER → Waste
- Inner Rotor
- Nano Pump → PGC → Deglycosylation → MS

(D) Outer Rotor
- Cap Pump → ER → PGC → Waste
- Inner Rotor
- Nano Pump → PGC → Glycan Transfer → MS

Capillary pump pressure [bar]
Nanoflow pump gradient [%B]
mAb-Glyco-Chip

- **Automated on-Chip workflow: Glycan separation/detection**

(A) Outer Rotor: Cap Pump → ER → Waste
   Inner Rotor: Nano Pump → PGC → MS
   Sample Injection

(B) Outer Rotor: Cap Pump → ER → Waste
   Inner Rotor: Nano Pump → PGC → MS
   Enzyme Reactor Fill

(C) Outer Rotor: Cap Pump → ER → Waste
   Inner Rotor: Nano Pump → PGC → MS
   Deglycosylation

(D) Outer Rotor: Cap Pump → ER → PGC → Waste
   Inner Rotor: Nano Pump → PGC → MS
   Glycan Transfer

(E) Outer Rotor: Cap Pump → ER → Waste
   Inner Rotor: Nano Pump → PGC → MS
   Glycan Separation/Detection

Capillary pump pressure [bar]
Nanoflow pump gradient [%B]

Enzyme Reactor
PGC Enrichment Column
Waste
PGC Analytical Column
ESI-MS
Sample In (Capillary Pump)
Nanoflow Pump
mAb-Glyco-Chip

- **Automated on-Chip workflow: Fast analysis**

(A) Outer Rotor
Cap Pump → ER → Waste

Inner Rotor
Nano Pump → PGC → PGC → MS
Sample Injection

(B) Outer Rotor
Cap Pump → ER → Waste

Inner Rotor
Nano Pump → PGC → PGC → MS
Enzyme Reactor Fill

(C) Outer Rotor
Cap Pump → ER → Waste

Inner Rotor
Nano Pump → PGC → PGC → MS
Deglycosylation

(D) Outer Rotor
Cap Pump → ER → PGC → Waste

Inner Rotor
Nano Pump → PGC → MS
Glycan Transfer

(E) Outer Rotor
Cap Pump → ER → Waste

Inner Rotor
Nano Pump → PGC → PGC → MS
Glycan Separation/Detection

Capillary pump pressure [bar]
Nanoflow pump gradient [%B]

12 min

1 min → 6 s → 4 min → 1 min → 6 min
Data Processing

- **Fast analysis**: Answers in minutes

- Data evaluation occurs with Mass Hunter Qualitative Software
- Molecular Feature Extractor to extract compounds from TIC's.
- Match compounds w/ accurate mass and structure glycan database
- (A) Identified glycans hits
- (B) LC-Chromatograms
- (C) Assigned glycan structures
- (D) Corresponding mass spectra
- (E) List of m/z-values
Data Processing

- **Fast analysis:** Answers in minutes

**Reporting**

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<tr>
<th>Compound Label</th>
<th>RT</th>
<th>Mass</th>
<th>Name</th>
<th>Formula</th>
<th>Tgt Mass</th>
<th>Diff (ppm)</th>
<th>Isomer(s) Present</th>
<th>Total Volume</th>
<th>Total Vol%</th>
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</tbody>
</table>
Revolution in N-Glycan Characterization

- Fast analysis

PNGase F, enzymatic N-glycan release

N-Glycan concentration

HPLC analysis

TOF MS Detector

mAb sample

4 minutes

6 seconds

Experimental Time

LC- or CE- Fluorescence

MALDI MS

mAb-Glyco Chip MS

10-30 minutes

3-8 hours

2-4 days
mAb-Glyco Chip Reagent Pack
– Completeness: Includes all chemicals and reagents needed

• System Conditioning Reagent
• Deglycosylation buffers = loading mobile phase
• A: Glycan-std’s to verify chromatographic performance
• B: mAb-std to verify function of enzyme reactor

![Image of mAb-Glyco Chip Reagent Pack]

![Graphs showing chromatographic performance and mAb sample analysis]
mAb-Glyco-Chip Kit
– A complete **turnkey solution** for N-glycan characterization

Reagent Pack

mAb-Glyco Chip

Complete turnkey solution

Reporting Templates

Accurate Mass Glycan Database

Structure Viewer
mAb-Glyco-Chip Kit
– Chip Stability, Reproducibility and Lifetime

(A) Extracted glycan pattern of the analysed antibody at injection number 1 and 200; (B) Relative glycan ratio as function of number of injections performed (4 most intense N-glycans). Sample: IgG from bovine serum (Sigma), 75 ng on-column.

Note: Chip must not dry out; store it wet at -20°C!
High Throughput Native Glycan Profiling
by MALDI-MS and Chip-based LC ESI-MS

Poster: ASMS 2010: Dayin Lin¹, Heidi Zhang², Christian Graf¹, Lukas Trojer¹, Kurt Forrer², and Tom van de Goor¹
¹Agilent Technologies, inc. Waldbronn, 76337 Germany
²Novartis Biologics, Basel, CH-4057 Switzerland
Workflow 1
– *N-glycan analysis using MALDI-MS*

- N-glycans released offline using PNGase F.
- Glycans purification using hypercarb PGC column.
- Neutral & acidic glycans separated & analyzed in pos & neg ion mode, respectively.
- Glycan identity established by comparing measured MW w/ theoretical values.
- Glycan distribution estimated by intensitites: $\text{Glycan} = \frac{G}{\Sigma (\text{all } G)'s}$. 
Workflow 2

– *N*-glycan analysis using mAb-Glyco-Chip

- 100 ng intact mAB directly loaded onto Agilent mAb-Glyco-Chip.
- Glycan cleavage online: 4 min incubation time.
- Released glycans captured by PGC trap column.
- After washing, trap column switched online with the analytical PGC-column.
- Formic acid/ACN linear nano-gradient (5 min) was used for separation.
- MS: Agilent QTOF 6520 mass spectrometer.
Results

- *N*-glycan analysis of “Protein B” using MALD-TOF MS

- MALDI-TOF spectra for unlabeled neutral glycans (top) and acidic glycans (bottom)

- Glycan distribution estimated by intensitites: \( \text{Glycan} = \frac{G}{\Sigma (\text{all } G's)} \)
Results

– N-glycan analysis of “Protein B” using mAb-Glyco-Chip

- PNGase F → online 4 min
- Neutral glycosylamines
- Acidic and hydroxyl forms
- Glycan distribution estimated by peak areas:

\[
\text{Glycan} = \frac{G}{\Sigma \text{all G’s}}
\]
Results

- N-glycan analysis of “Protein B” using Normal Phase LC / FLD

![Graph showing N-glycan analysis](image)

- 2-aminobenzoamide labelled
- LC – 2 h gradient
# Result Summary

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<th>m/z</th>
<th>Glycan Identified</th>
<th>Alternative</th>
<th>Relative Ratio [%]</th>
<th>%RSD</th>
<th>m/z</th>
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**FLD 2-AB glycan**

**Agilent Technologies**
Comparison Between On-chip Deglycosylation and Conventional Workflows

<table>
<thead>
<tr>
<th>High Throughput Parameters for mAb Glycan Analysis</th>
<th>MALDI MS</th>
<th>LC Chip/MP</th>
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<tbody>
<tr>
<td><strong>Quantification</strong></td>
<td>Linear range</td>
<td>10 fmol-1 pmol 100 X (+) 200 fmol-20 pmol 100 X (-)</td>
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<tr>
<td><strong>Reproducibility</strong></td>
<td>Minimal Sample</td>
<td>0.0 mg mAb</td>
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<tr>
<td>Intra day RSD (&gt;1% relative amt)</td>
<td>1~5%</td>
<td>1~5%</td>
</tr>
<tr>
<td>Inter day RSD (&gt;1% relative amt)</td>
<td>1~5%</td>
<td>1~5%</td>
</tr>
<tr>
<td><strong>Identification</strong></td>
<td># glycan identified</td>
<td>Similar as LC-FLD</td>
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<tr>
<td>Specificity</td>
<td>Isobaric bG1 isomers, ionic adducts</td>
<td>Isomer can be separated</td>
</tr>
<tr>
<td>Glycan characterization</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

- LC-Chip/MS method is more sensitive and provides more glycan identification, especially for acidic glycans. It can also separate isomers.
- LC-Chip/MS is much faster for single or smaller batch of sample(s).

ASMS 2010 poster Thp556, D. Lin etc., Agilent Technologies and Novartis Biologics
Conclusion
– *mAb-Glyco Chip Kit*

• Up to 100 x faster than existing methods for the characterization of *N*-glycans on monoclonal antibodies

• An automated turnkey solution for on-chip deglycosylation, *N*-glycan separation and high sensitivity TOF/QTOF detection

• Integrated comprehensive data processing for automated glycan identification, quantification and reporting

• Provides a robust workflow solution that aids in removing a major bottleneck during the development phase of mAb-based biotech drugs enabling the analyst to provide answers fast
Technical Overview

More Details

The Agilent mAb-Glyco Chip Kit for rapid and fully automated characterization of N-linked glycans from monoclonal antibodies

Technical Overview

Introduction
Monoclonal antibodies (mAbs) are essential in various industries and play a critical role in the development of innovative therapies. The characterization of glycans is essential for understanding the biological activity of mAbs, particularly in downstream processes such as purification, formulation, and storage. The Agilent mAb-Glyco Chip Kit offers a streamlined workflow for the rapid and automated characterization of N-linked glycans from mAbs.

This Technical Overview describes:
- The instrumental setup of the Agilent mAb-Glyco Chip system
- The design of the Agilent mAb-Glyco Chip kit
- The automated glycan cleanup, precipitation, and detection process
- The fully automated and high-throughput analysis of the Agilent mAb-Glyco Chip

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