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

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

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

- 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-acetyllactosamine (NANA)
- N-glycolylactosamine (NGNA)

* as used in the mAb-Glyco Chip database
Background

- **Glycan structure and nomenclature**

  - Trimannosyl is defined as the common core structure for all \textit{N-glycans}.
  - Glycans are defined using four numbers: \textit{ABCD}

  \begin{itemize}
  
  \item \textbf{A} \ldots the number of \textit{GlcNAc} (□) residues \textit{outside} the core
  \item \textbf{B} \ldots the presence (1) or absence (0) of \textit{fucose} (▲) \textit{within} the core
  \item \textbf{C} \ldots the number of \textit{galactose} (○) residues
  \item \textbf{D} \ldots is the number of terminal \textit{sialic acid residues} outside the core,

      an attached \textbf{A} defines the sialic residue(s) as \textit{NANA} (◊)
      an attached \textbf{G} as \textit{NGNA} (◊)
  \end{itemize}

* as used in the \textit{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 (◇)

* 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

**Workflow**

- Purification of MAb from bio-reactors or from cells, bio-liquids etc.
- Purified MAb in sample vials
- Analyze using protein chip and TOF or Q-TOF MS
- Analyze data and report validated results with MassHunter Qual and BioConfirm software

**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
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

- Antibody
  - Deglycosylation with PNGase F
  - Glycosylamines
    - Hydrolysis (-NH$_3$)
  - N-Glycan
    - MALDI-TOF/MS
    - LC/MS
    - HPLC/pulsed amperometric detector
Glycan Characterization
- Typical workflows of traditional analyses

Antibody
Deglycosylation with PNGase F

Glycosylamines
Hydrolysis (-NH$_3$)

N-Glycan

Reduktant

2-AB
Labelling agent

labelled N-Glycan

- HPLC / fluorescence detection
- CE/fluorescence detection

- MALDI-TOF/MS
- LC/MS
- HPLC/pulsed amperiometric detector
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

I  Complete analysis on an **integrated HPLC-Chip Design** and removal of labelling step

II **Automation** → reduce sources of errors

III **Fast analysis** → fast answers

IV 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
mAb-Glyco-Chip

- **Automated** on-Chip workflow: Glycan transfer
mAb-Glyco-Chip

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

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

(A) Oster Rotor
Out
Cap Pump → ER → Waste
In
Nana Pump → PGC → MS

(B) Oster Rotor
Out
Cap Pump → ER → Waste
In
Nana Pump → PGC → MS

(C) Oster Rotor
Out
Cap Pump → ER → Waste
In
Nana Pump → PGC → MS

(D) Oster Rotor
Out
Cap Pump → ER → Waste
In
Nana Pump → PGC → MS

(E) Oster Rotor
Out
Cap Pump → ER → Waste
In
Nana Pump → PGC → MS

**Identified N-Glycans**

1 min
6 s
4 min
1 min
6 min
12 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

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<th>Compound Label</th>
<th>RT</th>
<th>Mass</th>
<th>Name</th>
<th>Formula</th>
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<th>Diff (ppm)</th>
<th>Isomer(s) Present</th>
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</table>
Revolution in N-Glycan Characterization

- Fast analysis

Experimental Time

LC- or CE- Fluorescence
MALDI- MS

mAb-Glyco Chip MS
10-30 minutes

Experimental Time

2-4 days
3-8 hours

N-Glycan concentration
HPLC analysis
TOF MS Detector

mAb sample

PNGase F, enzymatic N-glycan release

4 minutes
6 seconds
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
mAb-Glyco-Chip Kit
– A complete turnkey solution for N-glycan characterization
mAb-Glyco-Chip Kit
– Chip Stability, Reproducibility and Lifetime

Long-term stability and robustness of the mAb-Glyco Chip: (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 Graf², 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: \( \text{Glycan} = \frac{G}{\sum (\text{all G’s})} \).
**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: Glycan = G / Σ (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

- 2-aminobenzoamide labelled
- LC – 2 h gradient
<table>
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<th>m/z</th>
<th>Glycan Identified</th>
<th>Alternative</th>
<th>Relative Ratio [%]</th>
<th>%RSD</th>
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<td>0.3</td>
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<td>1785.67</td>
<td>bG2</td>
<td>0.5</td>
<td>7.23</td>
<td></td>
<td>1809.58</td>
<td>bG2</td>
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<td>bNG1</td>
<td>0.1</td>
<td>19.62</td>
<td></td>
<td>1850.52</td>
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<td>1930.70</td>
<td>bG1SG</td>
<td>1,6 or 1,3</td>
<td>2.9</td>
<td>3.65</td>
<td>1930.7</td>
<td>bG1SG</td>
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<td>0.82</td>
<td>2092.76</td>
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<td>hG0M5</td>
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Comparsion 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/MS</th>
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<tbody>
<tr>
<td><strong>Quantification</strong></td>
<td>Linear range</td>
<td>10 fmol-1 pmol 100 X (+)</td>
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<tr>
<td></td>
<td>Minimal sample 0.6 mg mAb</td>
<td>50 ng mAb</td>
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<tr>
<td><strong>Reproducibility</strong></td>
<td>Intra day RSD (&gt;1% relative amt) 1-5%</td>
<td>1-5%</td>
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<tr>
<td></td>
<td>Inter day RSD (&gt;1% relative amt) 1-5%</td>
<td>1-5%</td>
</tr>
<tr>
<td><strong>Identification</strong></td>
<td># glycan identified Similar as LC-FLD</td>
<td>More glycans identified</td>
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<tr>
<td>Specificity</td>
<td>Isobenzoglycan 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
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) represent an important class within the wide range of immunosuppressants, and are deemed invaluable tools for the treatment of various immune-mediated conditions, such as cancer, transplant rejection, and autoimmune diseases. The quality and integrity of a glycoprotein are of vital importance for the success of immunotherapeutics. Understanding the glycosylation profile of mAbs is critical, as non-therapeutic glycoforms in the mAb can lead to lower antibody efficacy, plasma clearance, and re-sensitization. Additionally, differences in the length of the glycan chains and the presence of monomethyl or di-methylated N-acetylglucosamine residues in the N-linked glycosylation sites can have significant impact on immunosuppressive efficacy, plasma clearance, and re-sensitization. mAbs are typically produced using a myeloma cell line, which results in the production of a high percentage of glycoforms with complex glycan structures. The Agilent mAb-Glyco Chip Kit is designed to provide a rapid, high-throughput method enabling the characterization of glycoforms of mAbs and their effect on therapeutic and immunosuppressive efficacy.

Physicochemical analysis of glycosylation properties of mAbs, relevant to the efficacy, stability, and immunosuppressive activity, is critical for the development of novel mAbs. The Agilent mAb-Glyco Chip Kit can provide comprehensive analysis of mAbs with complex glycan structures, thereby enabling a high-throughput method for the characterization of mAbs

This Technical Overview describes:
- the instrumental setup of the Agilent 1200 Infinity HPLC ChipRF system
- the design of the Agilent mAb-Glyco Chip Kit
- the automated mAb-glycan characterization analysis workflow and data
- the scalability, reproducibility and robustness of the Agilent mAb-Glyco Chip Kit.