Complementary Glycan Quantitation Strategies Based on High Sensitivity NanoLC/MS

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Introduction

Glycosylation is a ubiquitous post-translational modification (PTM), with glycans playing important roles in diseases and homeostasis. Monoclonal antibodies (mAbs), therapeutic proteins for treatments of cancers, need fine structural elucidation and improved analytics because of their biopharmaceutical presence. These mAbs are inherently heterogeneous proteins and have various protein modifications possible. The most important modification for these large glycoproteins is N-glycosylation on their Fc region. There are several analytical solutions possible for the identification and quantitation of glycans.

![Diagram of IgG structure](image)

Figure 1. A crystal structure of IgG1 is shown (1HZH.pdb). At right, a schematic diagram indicates different regions of interest for biochemical characterization.

Our samples include several human immunoglobulin Gs (IgGs) as well as the glycoprotein prostate specific antigen (PSA). IgG1 has at least two N-glycosylation sites. PSA is singly glycosylated at Asn69. We investigate two types of PSA using methods applied to IgG. In this work, we compare several quantitative techniques; including microfluidic based on-line deglycosylation and integrated nanoLC/MS analysis of the released glycans, nanoLC/MS analysis of intact glycoprotein and/or glycoprotein fragments, integrated enrichment of glycopeptides on nanoLC/MS, as well as UHPLC-FLD analysis of off-line released derivatized glycans.

Experimental

Protein samples were prepared by desalting, reduction by DTT, alkylation using iodoacetamide, and/or digestion (including the enzymes trypsin, PNGaseF, or IdeS), followed by mass spectrometry analysis using an Agilent 6224 TOF (or Agilent 6520 accurate mass Q-TOF) LC/MS with HPLC Chip Cube Interface. Purified intact IgG and PSA were analyzed with no sample preparation. N-glycan LC/MS measurements were performed on a mAb-Glyco Chip, which includes a PNGaseF reactor for on-line digestion. Off-line native or denaturing PNGaseF digests were also prepared for both IgG and PSA. Reductive amination using 2-aminobenzamide (2-AB) was tediously performed for glycan quantitation by LC-fluorescence detection (LC-FLD).

![Diagram of NanoLC/MS setup](image)

Figure 2. NanoLC/MS experiments provide complementary glycoprotein information, like the pieces of a puzzle.

![Diagram of HPLC-Chip setup](image)

Figure 3. Schematic flow diagram of an HPLC-Chip. A sample enrichment column, LC separation column and a nanoES tip is integrated on such chip. All LC/MS was performed on-chip. Depending on the sample, reverse phase C18, PLRP-S, C8, HILIC, and porous graphitized carbon (PGC) may be packed into the enrichment and analytical columns.

Symbolic Representation of Monosaccharides

- N-acetylgalactosamine (GalNAc)
- N-Glycan Example: 4132 2A 0G
- AB CD xA yG
- Mannose (Man)
- Galactose (Gal)
- Fucose (Fuc)
- N-acetylneuraminic acid (NeuAc)
- N-glycolneuraminic acid (NeuGc)
- N-acetylgalactosamine (GalNAc)
- N-Glycan Example: 4132 2A 0G
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![Diagram of flow diagram and two valve positions](image)

Figure 4. Flow diagram and the two valve positions of a mAb-Glyco chip with integrated on-chip deglycosylation.

Agilent Technologies
Monoclonal Antibody Results and Discussion

Intact IgG Analysis The fastest and easiest way to characterize IgG is to perform LC/MS without any sample treatment. HPLC Chip/MS offers high sensitivity detection down to 10 ng level. Integrated sample enrichment column enables online desalting. The newly developed deconvolution algorithm offered by MassHunter s/w significantly improved resolution. However, due to large molecular weight of 150 kDa and high heterogeneity, only limited glycosylation information can be obtained. PNGaseF treatment also may indicate other PTMs and protein core MW can be obtained by removal of N-glycans.

Analysis of the Fc Fragment The ~23 kDa Fc fragment can be readily analyzed by LC/MS after the IgG is proteolysed with IdeS. The smaller (relative to intact IgG) MW allows heterogeneity of the Fc fragment to be revealed. Relative quantitation of various glycoforms can be obtained as the Fc fragment dominates ionization efficiency. Better than 5 ng sensitivity was achieved. No isoform information can be obtained with this approach.

Glycopeptide Analysis IgG is digested by trypsin and analyzed with HPLC Chip/MS. Using reverse phase separation (left) glycopeptides are coeluted with other peptides and are often difficult to detect due to charge suppression. Using a HILIC trapping column (right), glycopeptides can be selectively enriched and then analyzed with reverse phase separation.

HPLC-Chip/MS of Released Glycans IgG can be deglycosylated, and the released glycans can be trapped and separated using a mAb-glyco chip. Glycan quantitation data can be generated with a predefined data analysis method and a glycan compound database within MassHunter (at top right). Relative quantitation of the various glycans is shown on the top left. This approach offers direct measurement of released glycans with minimum sample pretreatment.

Alternatively, HPLC-Chip/MS analysis can be performed on an in-solution deglycosylated sample (Samples 2 and 3) using a PGC chip. LC/MS runs of the digests from two IgGs indicate quickly that the proteins’ glycosylation from hybridoma cells (Sample 3) is more complex with more sialylation, including NeuGc, and more branching than its human cell version (Sample 2) despite a similar amino acid sequence.

UHPLC-FLD of Released Glycans Traditionally, glycan quantitation is done with LC-FLD (or CE-LIF) analysis of derivatized, released glycans. The entire sample preparation workflow is tedious and time consuming. There is no positive structure confirmation using fluorescence detector and quantitation of co-eluting peaks is not possible.
Prostate Specific Antigen Results and Discussion

Intact PSA Analysis

It is possible to determine PSA glycosylation due to its single glycosylation site and moderate protein MW. The glycan profiles of two PSA samples are compared in this experiment. Standard PSA (top left) is more highly sialylated than the High Isoform PSA (top right). The bar chart (bottom) shows the glycosylation differences between the two samples (green = standard PSA).

Glycopeptide Analysis

For PSA, glycopeptide analysis showed heterogeneity of the peptide in addition to complex glycosylation. Glycoforms from different peptides were summed.

Conclusion

- Technologies for the analysis of glycans have advanced over the past several years. Various analytical strategies can be applied to glycan quantitation, such as the analysis of intact protein, protein fragments, glycopeptides and free released glycans, etc.
- MS based detection methods offer significantly more information than traditional fluorescence based detection. NanoLC/MS, including microfluidic chip based using HPLC-Chip, is often the only viable option due to its high sensitivity. Traditional high flow LC/MS with detection sensitivity in the range of over 10 pmol is only useful when large amount of purified glycoprotein is available.
- Each of these analytical strategies offer unique benefits and often can not be replaced by other techniques. Comprehensive analysis using multiple methods may be required, depending on the nature of glycoprotein sample such as MW, number of glycosylation site, protein heterogeneity from glycosylation and other PTMs.

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