Glycoform Profiling from Therapeutic Antibodies at the Protein, Peptide and Cleaved Glycan Level using Mass Spectrometry

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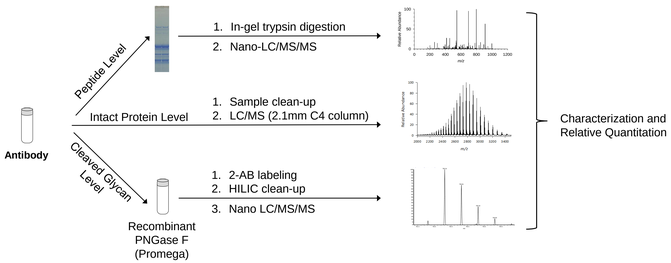
1. Abstract and Introduction

The glycans (oligosaccharides) attached to proteins play a crucial role in many recognition, signaling, and adhesion events within and between cells. Aberrations in the glycans have been implicated in many diseases including immune deficiencies, neurodegenerative diseases, hereditary disorders, cardiovascular diseases and cancer[1,2]. In depth studies of these glycan structures will potentially help improve disease markers and in turn, lead to development of novel clinical diagnostic technologies. Glycans are also found attached to almost all of the new biological protein drugs. The carbohydrate composition and the structure of the glycans are important for the safety and efficacy of these therapeutic proteins. Understanding exactly how the carbohydrates in therapeutic proteins modulate their functions in patients is key to the development of an optimal glycan profile, emphasizing the importance of performing glycan analysis. The development of novel analytical methods, strategies, and consumables will help achieve this. This presentation summarizes the glycoform profiling data from a therapeutic antibody at three different levels: intact protein, peptide (glycopeptides) and cleaved glycans using mass spectrometry (MS).

(1) Ann.Rev. Biochem. 2003, 72, 643-691. (2) Curr. Opin. Chem. Biol. 2009, 13, 601-607.

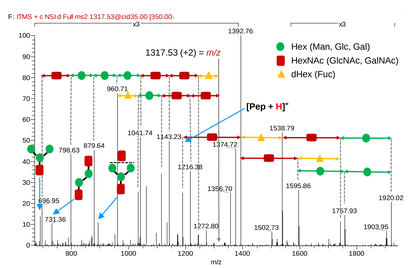
2. Glycan Analysis - Workflow



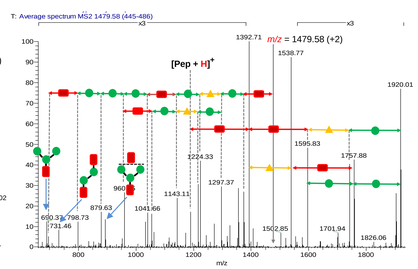
- **Peptide Level**: Light and heavy chains of the antibody were reduced and separated on a 10% Bis-Tris NuPage gel (Invitrogen). The heavy chain gel band was excised and processed by in-gel digestion using a robot (ProGest, DigiLab) and sequencing grade trypsin (Promega). Nano LC/MS/MS of the glycopeptides was acquired on an Orbitrap Velos Pro mass spectrometer (ThermoFisher) coupled to a nanoAcquity (Waters) LC system.
- **Intact Protein**: Intact antibody was directly loaded on a 2.1mm x 10cm XBridge C4 column (Waters) and LC/MS data were acquired on Q Exactive mass spectrometer (ThermoFisher) coupled to a nanoAcquity LC system.
- **Cleaved Glycan**: Antibody was treated with recombinant PNGase F (Promega) and the resultant cleaved glycans were isolated using a HILIC cartridge (Prozyme), dried and derivatized using 2-aminobenzamide (2-AB). The glycans were analyzed by nano LC/MS/MS using a Q Exactive coupled to a nanoAcquity LC system.

3. Peptide Level Glycan Analysis

- MS/MS data from tryptic peptides are used to characterize the glycoforms. Annotated MS/MS spectra of the G0F and G2F glycans attached to the Fc N297 in peptide EEQYNSTYR are shown below. Note product ion data are dominated by glycan fragmentation with no peptide backbone fragmentation.

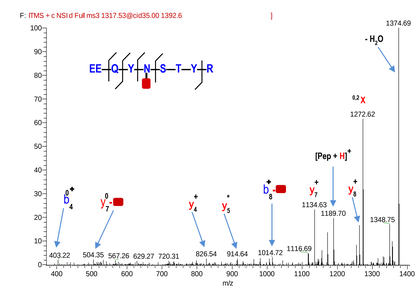


MS/MS m/z 1317.53 (+2): G0F Peptide - $(GlcNAc)_2(Fuc)(Man)_3(GalNAc)_2$

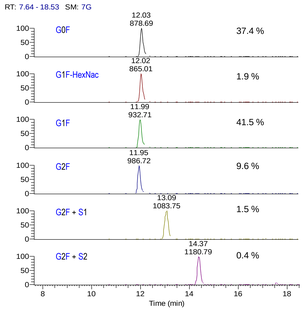


MS/MS m/z 1479.58 (+2): G2F Peptide - $(GlcNAc)_2(Fuc)(Man)_3(GalNAc)_2(Gal)_2$

- The minimally modified product ion in each case (peptide + HexNAc) is selected for MS/MS/MS in order to confirm the site of attachment as N297. An example MS/MS/MS spectrum for the G0F glycoform is shown below.
- Multiple glycoforms attached the EEQYNSTYR peptide were identified and characterized. The +3 charge state was used to reconstruct high resolution accurate mass (HR/AM) selected ion chromatograms, several of these are shown below along with the corresponding relative percentage abundances based on the sum of all peak areas.



MS/MS/MS spectrum of EEQYNSTYR-HexNAc *m/z* =1392.70



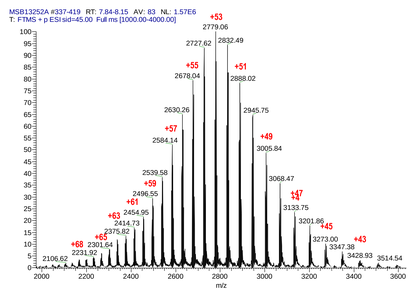
HR/AM (±15 ppm) Quantitation of Selected Glycopeptides

- Glycoforms observed and their relative abundances are summarized below. These represent the sum of both the full (EEQYNSTYR) and missed cleaved (TKPREEQYNSTYR) peak areas. It was noted that the relative distribution was slightly different for each version, this should be considered carefully when utilizing peptide-level data.

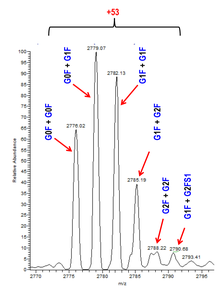
Glycoform	Observed m/z (+3) Th.	Theoretical m/z (+3) Th.	Error (ppm)	% Abundance
M5	802.6494	802.6498	0.53	1.55
M6	856.6672	856.6674	0.26	0.47
G0-HexNAc	762.3074	762.3078	0.47	0.19
G0	830.0003	830.0009	0.71	1.27
G1	884.0175	884.0185	1.12	1.08
G1-HexNAc	816.3259	816.3254	0.67	0.10
G2	938.0345	938.0361	1.70	0.52
G0F-HexNAcHex	756.9759	756.9761	0.29	0.30
G0F-HexNAc	810.9937	810.9937	0.03	2.24
G0F	878.6873	878.6869	0.51	37.37
G1F-HexNAc	865.0120	865.0113	0.78	1.89
G1F	932.7052	932.7045	0.80	41.54
G2F	986.7216	986.7221	0.46	9.59
G2F + S1	1083.7533	1083.7539	0.51	1.49
G2F + S2	1180.7856	1180.7857	0.05	0.38

4. Intact Protein Level Glycan Analysis

- The summed LC/MS spectrum below shows the charge envelope from +45 to +70. The expanded region of the most intense charge state (+53) shows six different glycoform combinations for the two heavy chains: G0F+G0F, G0F+G1F, G1F+G1F, G1F+G2F, G2F+G2F and G1F+G2FS1.

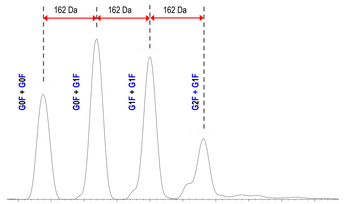


LC/MS spectrum of intact antibody; charge states are denoted in red.

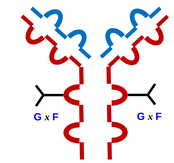


Expanded m/z region of +53 charge state.

- The baseline resolved deconvoluted MS spectrum below shows four glycoform pairs: G0F+G0F, G0F+G1F, G1F+G1F, G1F+G2F. Note that only the intact protein level data allows the observation of both homodimer and heterodimer heavy chain glycan populations (cartoon below left). This information is lost at both peptide and cleaved glycan level once the antibody is reduced. However, the number of glycoforms is less than at the peptide level.
- The relative quantitative distribution of glycoform pairs can be estimated based on the deconvoluted peak intensities.



The deconvoluted mass spectrum using MagTran software.

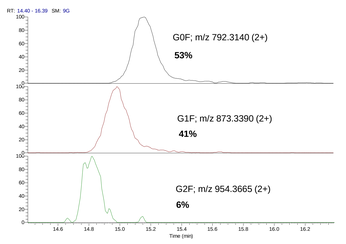


(*x* = 0-2)

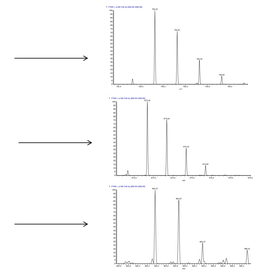
The glycans on the antibody attached to N297 of CH2 domain.

5. Cleaved Glycan Level Analysis

- PNGase F cleaved, 2-AB derivatized glycans were analyzed by LC/MS/MS. The HR/AM LC/MS data for the three major forms (G0F, G1F and G2F) are shown below with relative abundances indicated based on the sum of peak areas. The full MS spectrum of the doubly charged ions in each case are shown:



HR/AM (±15 ppm) Quantitation of Selected Glycans



Expanded mass spectra of G0F, G1F and G2F glycans

- Multiple glycans can be detected using this method, similar to glycopeptide level analysis. Site specificity is lost for antibodies or biologics with multiple modification sites.

6. Summary

- Glycans attached to a therapeutic antibody were identified, characterized, and quantified at peptide, intact protein, and cleaved-glycan level.
- Protein level analysis allows for heterodimer populations to be determined (when the two heavy chains have two different glycans). This information is lost once the antibody is reduced when using peptide and cleaved glycan level. Protein level analysis provides information on a limited number of glycoforms (lower sensitivity) and does not show site-specific information.
- Peptide level analysis allows for a large number of glycoforms to be characterized, with relative quantitation based on HR/AM LC/MS data. This showed that G0F, G1F and G2F comprise 89% of the total glycan population at N297 with their ratio determined to be 37%:42%:10%. Peptide level analysis also allows for multiple N- or O-glycan sites to be independently characterized and quantitated. Missed cleavages around the glycosylated site must be considered when interpreting the quantitative data. These analyses require only minimal amounts of starting material (1µg).
- Cleaved glycan analysis allows for the total glycan population to be analyzed in a single pool. This approach indicate that G0F:G1F:G2F are at 53%:41%:6% relative ratio.
- Data presented here suggests that multiple approaches provide complimentary information when characterizing the glycoforms of biologics.

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