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Technical note

Rapid quantitation of monoclonal antibody N-glyco-occupancy and afucosylation using mass spectrometry

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ABSTRACT

N-glyco-occupancy and afucoslyation level are two important quality attributes associated with N-glycosylation of therapeutic monoclonal antibodies (mAbs). We report here a fast mass spectrometry-based workflow for quantification of N-glycan site-occupancy and afucoslyation level of mAbs with improved throughput, precision, sensitivity and robustness. This method uses the deglycosylation after the first GlcNAc and inter-chain reduction of the mAbs, followed by liquid chromatography/mass spectrometry (LC-MS) analysis. The entire process can be completed within one hour, which provides a rapid quantitation of N-glyco-occupancy and afucosylation to support high-throughput cell line selection and process development for mAb biopharmaceuticals.

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N-linked glycosylation of recombinant therapeutic monoclonal antibodies (mAbs) is known to impact on mAb structure, stability, and biological function [1,2]. The quality attributes associated with N-linked glycosylation are usually characterized in detail and monitored carefully during lead selection, cell line screening and process development for a mAb biopharmaceutical program [3]. The levels of N-glycan site-occupancy and afucoslyation level, in particular, can affect the effector functions of mAbs [4–6] and are among the most critical quality attributes to screen during the mAb development.

The common method for determination of N-glycan site-occupancy is peptide mapping of PNGase F deglycosylated protein, in which the Asp/Asn ratio of the N-glycosylation site indicates the glycosylation site-occupancy. The afucosylation is commonly determined by summing up the level of individual afucosylated glycan peaks from traditional N-glycan characterization method, which usually involves enzymatic release of glycans, followed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), or fluorescence labeling of released glycans followed by electrophoresis, e.g. capillary zone electrophoresis of APTS-labeled glycan; or chromatography separation techniques, such as hydrophilic interaction (HILIC) and ion exchange (IEC) of 2-aminobenzamide (2-AB) labeled N-glycan [7]. These approaches although providing detailed information of individual N-glycans, are usually too time-consuming and labor-intensive to support fast analyses of large number of samples, common at early stages of drug development, during lead molecule selection, cell line screening and process development. Therefore, a method capable of fast and accurate determination of N-glyco-occupancy and afucoslyation level for large number of samples is required to meet the demand in early development of mAbs.

Herein we report a mass spectrometry-based workflow, which includes first treatment of mAb by GlycINATOR enzyme (Genovis, Sweden) to cleave between the two GlcNAc in the N-glycan core, followed by partial reduction and liquid chromatography/mass spectrometry (LC-MS) analysis of heavy chain and light chain species. GlycINATOR eliminates the glycoform heterogeneity and converts the glycosylated heavy chain species into simple forms, one attached with a GlcNAc and the other with a core-fucosylated GlcNAc, which in addition to the aglycosylated heavy chain become the three major forms of the mAb heavy chain in the final mixture for LC-MS analysis. Removing the N-glycosylation diversity allows this method to report total %glyco-occupancy and %afucosylation level in one analysis. Compared to the traditional method combining multiple low-level glycan peaks to report %afucosylation, this method provides a more accurate measurement when

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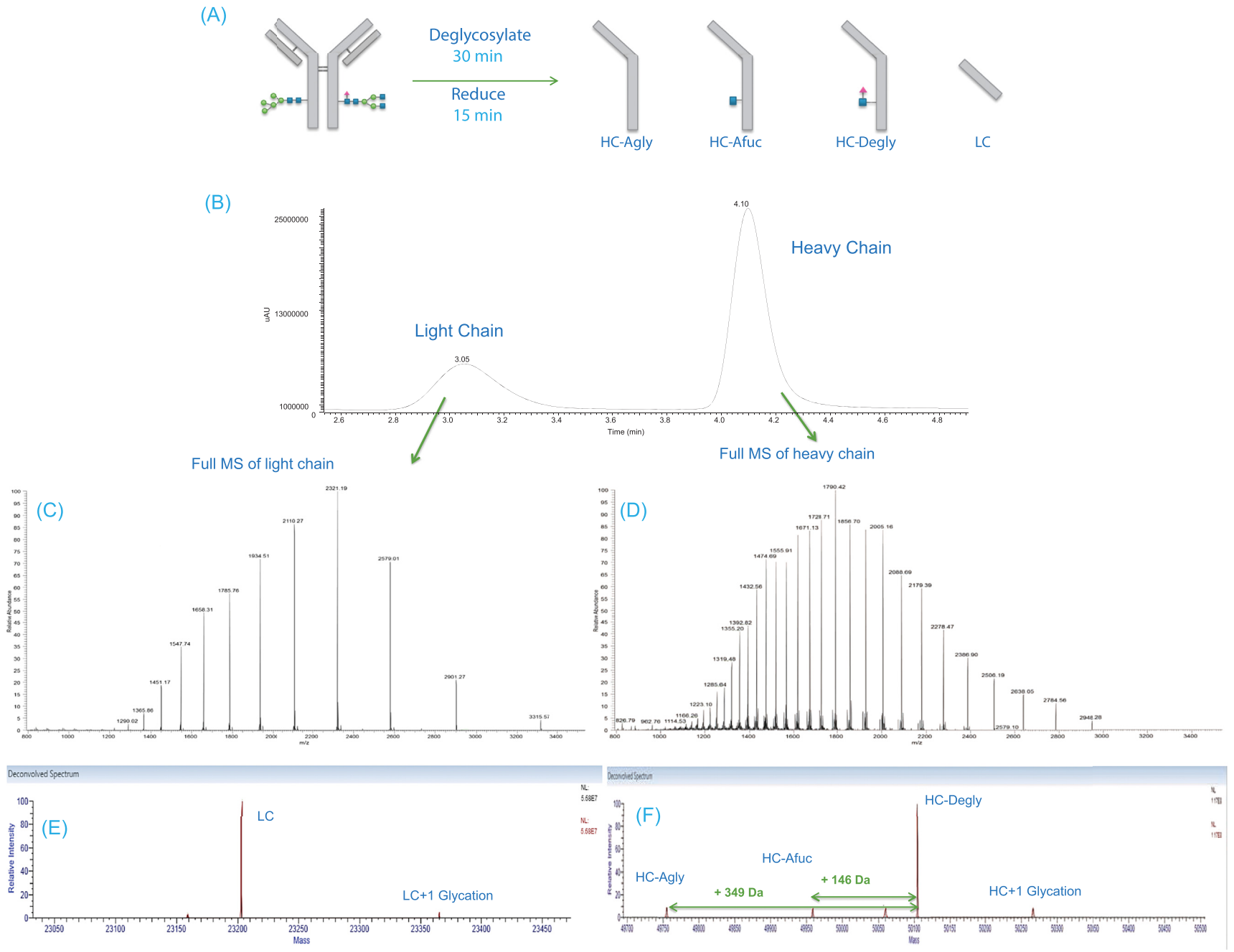


Fig. 1. (A) Diagram of sample preparation of mAb1. (B) Total ion chromatogram of the light chain and heavy chain. (C) Mass spectrum of the light chain. (D) Mass spectrum of the heavy chain. (E) Deconvoluted spectrum of the light chain. (F) Deconvoluted spectrum of the heavy chain.

these multiple peaks are consolidated into one peak for measurement.

The sample preparation protocol is demonstrated in Fig 1A. The mAb samples are first treated with GlycINATOR for 30 min at 37 °C and next reduced by dithiothreitol (DTT) at a final concentration of 15 mM for 15 min at 37 °C in 100 mM Tris buffer, pH 7.5. The samples are then analyzed using an ultra-high performance liquid chromatography (1290 Infinity Binary UHPLC System, Agilent) in line with an Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). Two micrograms of each sample is injected onto a phenyl column (TSKgel Phenyl-5PW, 10 μm, 1000 Å, 2 × 75 mm, Tosoh Bioscience) equilibrated at column temperature of 55 °C. Separation of the heavy chain and light chain was achieved by a gradient of 0.03% trifluoroacetic acid (TFA)/water (mobile phase A) and 0.024%TFA/acetonitrile (ACN) (mobile phase B) at a flow rate of 0.4 mL/min. The separation is carried out using a step-gradient, which is described below. The gradient starts at 10% mobile phase B during loading and remains at 10% B for 1.5 min. It then runs up to 29% B in 0.1 min and stays for 1.5 min, after that, changes to 32% in 0.1 min and stays for 1.5 min, followed by increasing to 80% B in 0.5 min and staying for 0.5 min, before decreases to 10% B for re-equilibration of the column for 2.5 min. Mass spectrometric settings include capillary voltage of 4 kV, capillary temperature of 350 °C, S-lens RF level 50, and an auxiliary gas heater temperature of 50 °C. The MS raw data are processed using Protein Deconvolution 3.0 (Thermo Fisher Scientific). In the total ion chromatogram shown in Fig. 1B, the peaks at retention time 3.1 min and 4.1 min correspond to light chain and heavy chain, respectively. The raw spectra of light chain and heavy chain are shown in Fig. 1C and D, respectively. The deconvoluted mass spectra shown in Fig. 1E and F confirmed the identity of the light chain and heavy chain. As shown in Fig. 1F, the dominant peak corresponds to heavy chain with a core-fucosylated GlcNAc, whereas the peak with a 146 Da lower mass corresponds to the afucosylated form represented by heavy chain with the GlcNAc only, and a peak with 349 Da lower mass corresponding to the aglycosylated heavy chain. A low-level singly glycated peak was observed for the dominated heavy chain peak. A "-44Da" artifact peak was commonly observed for the dominant peak in the intact mass analysis using Exactive Plus MS in our lab across multiple mAbs. Intensities of deconvoluted peaks (excluding the −44Da artifact peak) were used to calculate the relative level of afucosylation and N-glyco-occupancy of the mAbs. The simplified spectrum allows an easy peak assignment and quick quantitation of the peaks using a simple Excel worksheet.

The enzyme activity and specificity have been well characterized and reported by the vendor [8]. GlycINATOR was found to effectively hydrolyze complex N-linked glycans on antibodies [9]. Sample reduction was performed under a non-denaturing condition to avoid denaturant adduct formation during LC-MS analysis. However, the optimal final DTT concentration to achieve complete partial reduction and avoid over reduction or disulfide scrambling under the non-denaturing condition appears to be antibody dependent. The DTT concentration used in our lab typically ranges from 10 mM to 50 mM for most antibodies.

The light chain and the heavy chain are well separated by LC (Fig. 1B) and each monitored using their individual set of MS settings optimized for their sensitivity, particularly the in-source collision-induced dissociation (SID) setting. The SID voltage needs to be optimized to sufficiently remove the protein adducts but not to result in fragmentation of the protein or loss of fragile modifications. The SID value typically used in our lab ranges 45–60 for heavy chain and 20–30 for light chain. The common MS settings for heavy chain and light chain peaks include m/z scan range from 800 to 3500, microscans of 10, mass resolution of 17,500, and maximum injection time of 50 ms.

The method is compared with a hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FL) N-glycan method for quantitation of afucosylation and the results are shown in Supplemental Fig. 1. The results from these two methods showed reasonable correlation. The HILIC method with florescence detection can provide levels of individual glycoforms. However, sum of individual low-level peaks results in artificially higher quantitation levels; in the meantime the method suffers from high method variability due to many low-level peaks present around the LOQ of the HILIC-FL method. This intact mass method can be easily adopted for rapid determination of the afucosylation and N-glyco-occupancy levels for any antibodies which are not well characterized yet during early development stage.

The method was also assessed for its repeatability. A total of 35 repeated analyses of separate preparations of the same sample were found with an RSD of 6.8% for N-glyco-occupancy and 7.8% for afucosylation, shown in Supplement Fig. 2. Linearity of the method was examined using a dilution series samples prepared by mixing aglycosylation and afucoslyation enriched (>40%) and depleted (<5%) samples. The measured aglycosylation and afucosylation values are plotted against the mixing ratio and shown in Supplemental Fig. 3. The data demonstrated a good linearity of this assay in a range of 0.0%–8.0% for aglycosylation with R^2 value at 0.9890, and a range of 4.7%–43.6% with R^2 value at 0.9992 for afucosylation.

In summary, a fast LC-MS method is developed for targeted quantitation of N-glyco-occupancy and afucosylation of monoclonal antibodies. The analysis including sample preparation, LC-MS as well as data analysis can be completed within one hour for one sample and is highly adaptable for high-throughput analysis of large number of samples. The method demonstrated decent repeatability as well as a good linearity over a wide range of afucosylation and N-glyco-occupancy levels. This method is established in our lab as a generic method for measurement of N-glyco-occupancy and afucosylation and has been used to support high-throughput cell line screening and process development for multiple monoclonal antibodies.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.06.029.

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