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Capillary Chromatography for Therapeutic Proteins and Their Subunit Analysis Using MAbPac RP Column

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ABSTRACT

Purpose: A new capillary Thermo Scientific™ MAbPac™ RP column (4 µm, 0.15 mm x 150 mm) has been developed for the analyses of intact therapeutic proteins and their subunits. Owing to the increased sensitivity offered by capillary flow, this column can be utilized in the early stage of process development, where the scarcity of the sample possesses challenges.

Methods: Intact mAbs were buffer exchanged using 10k Da cut off centrifugal filters and analyzed. For the assessment of the ratio of fucosylated/afucosylated glycoforms, intact mAbs were digested with Endo S enzyme. To generate mAb subunits, mAbs were broken down into *scFc*, *LC* and *Fd'* portions by Ide S digestions followed by reduction and clean up. Intact proteins were separated analysed in normal mode at low resolution (17,500), while subunits were separated and analysed in protein mode at high resolution (140,000) using a Thermo Scientific™ UltiMate™ 3000 RSLCnano System coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer controlled by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) and Thermo Scientific™ Xcalibur™ software.

Results: Using Capillary MAbPac offers utmost sensitivity (using typically 10-20 ng sample) and supports all the intact protein workflows carried out in high flow mode.

INTRODUCTION

The state -of- art, mass spectrometry based analyses of therapeutic proteins and their subunits have become an important approach in biopharmaceutical industry. Post translational modifications such as glycosylation, glycation, C-terminal lysine truncation, deamidation, etc. can easily be analysed at both intact and subunit levels, with minimal sample preparation required.

In the first phase of the early stage of process development, the use of micro bioreactors substantially decreases cost during the optimization of different conditions (such as feeds volume, pH and temperature) to achieve the highest rate of cell growth and the desired protein attributes. However, due to the small volume of such micro bioreactors, the amount of sample that needs to be taken daily to monitor titer and protein attributes is therefore limited.

By the introduction of the capillary MAbPac RP column, these obstacles which otherwise slow down process development are overcome. Owing to the increased sensitivity provided by capillary flow, high-quality MS data can be obtained with a reduced mass of sample, due to minimal sample dispersion and improved desolvation in capillary chromatography. Also, researchers dealing with a scarce quantity of biological samples prepared from cells, tissues, etc. can also greatly benefit of using this column in discovery top down proteomics. In addition to the superior sensitivity, another hallmark of this column is attributed to high resolution protein separation attainable in a high throughput manner.

MATERIALS AND METHODS

Sample Preparation

Five mAbs (Trastuzumab Innovator and Biosimilar, Rituximab, NIST mAb and a Fab glycosylated mAb) were used in this work. Intact mAbs were analyzed after purification on a 10 kDa cut off filter to get rid of detergent and salt of the formulation buffer. Endo S digestion of intact mAbs was performed at a ratio of 20 µg mAb to 1 µL enzyme solution, the solution was incubated at 37 ºC for 30 min. To generate different subunits, mAbs were diluted with 1x PBS (pH 7.4) to 1 mg/mL. 100 µL of diluted mAb was digested with 1.5 µL of IdeS solution for 1h at 37 ºC. After the digestion was complete, 4.16 µL Bond-Breaker solution (500 mM) was added to the digested mAb solution and was further incubated at 50 ºC for 30 min to achieve complete reduction of disulfide bonds.

Table 1. Mass Spectrometric Conditions Used for All Experiments

	Intact mAbs (Normal mode)	Protein mode (Trapping gas 0.2)	Targeted MS^2 (4-plex)	Intact mAbs (High flow)
Scan range	1800-3800	700-3000	Fixed first mass at 200	1800-3800
Resolution (Full MS/MS^2)	17,500/n.a.	140,000/n.a.	n.a./140,000	17,500/n.a.
AGC, Full MS	3 x 10^6	3 x 10^6	2 x 10^5	3 x 10^6
Max injection time (Full MS,	200 ms	200 ms	200 ms	200 ms
Isolation window	n.a.	n.a.	6 m/z	n.a.
Microscans	10	5	5	10
Capillary temperature	300	300	300	300
SID (eV)	20	20	20	10
NCE (%)	n.a.	n.a.	20	n.a.

Data Analysis

MS data were processed using Thermo Scientific™ BioPharma Finder™ 3.2 software.

RESULTS

Figure 1. Effect of Flow Rate on Sensitivity/Glycoform Distribution

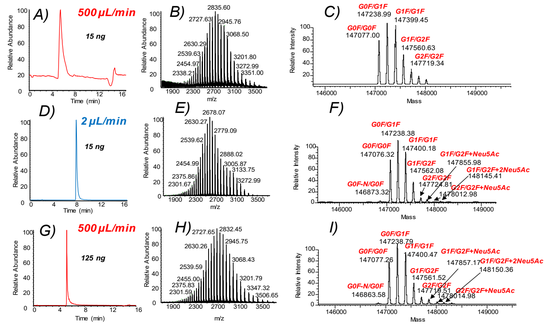
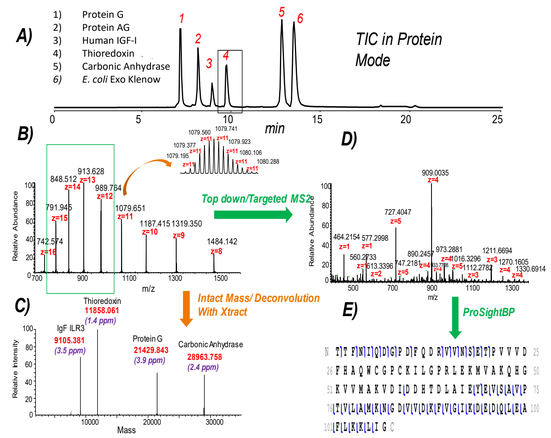


Figure 1/A and B compares *TIC*s of 15 ng injected mAb in high and low flow LC systems, sensitivity gain is 140 times more in capillary chromatography. On column dilution of small quantity of sample resulted in higher noise level in the spectrum (B) and biased glycoprofile distribution (C) after deconvolution. In the control experiment (G); injection of 125 ng in high flow yielded identical glycoform distribution (I) compared to the data obtained at 2 µL/min for 15 ng (F).

Figure 2. Intact Mass and Top Down Strategies, injected 20 ng



TIC displayed by Figure 2/A shows baseline separation of intact proteins. Of the 6 proteins, 4 can be isotopically resolved (B). Averaging multiple scans over a time window of 6-15 min, followed by deconvolution allows to identify highly accurate masses (C). Targeted MS^2 by spectral multiplexing results in a fragment ion spectrum (D), these ions are assigned to the sequence of protein as shown by the fragment map (E).

Figure 3. Intact mAbs, TICs are acquired in Normal Mode, injected 15 ng

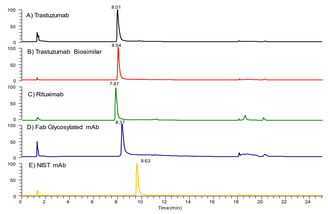
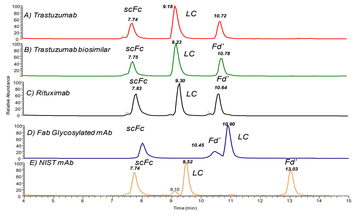


Figure 3 shows TICs of intact mAbs. While Trastuzumab innovator and biosimilar elute at the same time, the most hydrophobic mAb is NIST mAb, eluting later than the other four mAbs. Surprisingly, the *Fab* glycosylated mAb is the second most hydrophobic mAb even though this is the only mAb with two glycosylation sites, heavily occupied with hydrophilic N-glycans.

Figure 4. Subunits of 5 mAbs in Protein Mode, injected 15 ng



The retention time of the Fd' portion of NIST mAb evidences that the highest retention time (most hydrophobic) of NIST mAb can be attributed to the increased hydrophobicity of the Fd' subunit. Regarding Fab glycosylated mAb,, the chromatographic behavior of subunits shows, the LC elutes far later indicating this part to be responsible for the increased hydrophobicity.

Figure 5. Glycoform distribution of Innovator A) and Biosimilar Trastuzumab and after *Endo S* Digestion (C and D), Intact Workflow at 17,500 Resolution

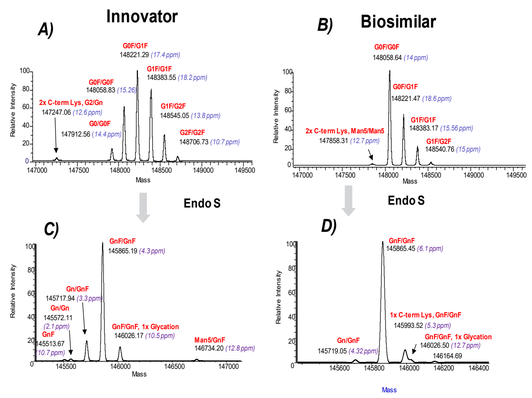


Figure 5/A and B compares glycoform distributions of intact Trastuzumab innovator and biosimilar. Trastuzumab innovator shows a higher level of galactosylation than the biosimilar. Digestion with EndoS (C and D) reveals the presence of non-enzymatic glycation at *146026* in both spectra. Its level is three times higher in the innovator than in the biosimilar. The high intensity of Gn/GnF glycoform in the innovator clearly confirms higher level of afucosylated glycans deposited in the Fc domain of the innovator Trastuzumab.

Figure 6. Glycoform distribution of scFc Subunit of Innovator and Biosimilar Trastuzumab, Intact Workflow at *140,000* Resolution

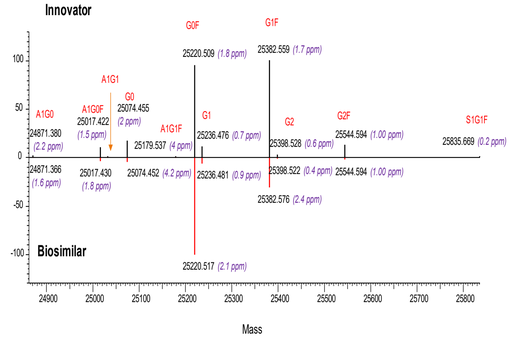


Figure 6 shows the mirror image of scFc glycoforms of innovator and biosimilar Trastuzumab. As shown by this comparison, 11 glycoforms can be distinguished. Their identification relies on the highly accurate (< 5 ppm) masses obtained after deconvolution. Major afucosylated glycoforms, such as G0, G1 and G2 are more abundant in the innovator.

CONCLUSIONS

- Capillary MAbPac RP column offers unprecedented sensitivity and excellent selectivity for the MS analyses of intact mAb and their subunits
- The column supports all the intact protein workflows such as intact mass and top down approaches
- Highly accurate, in depth analyses can be attained using low quantity (typical loading capacity 10-20 ng) samples

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