TOSOH

Application Note

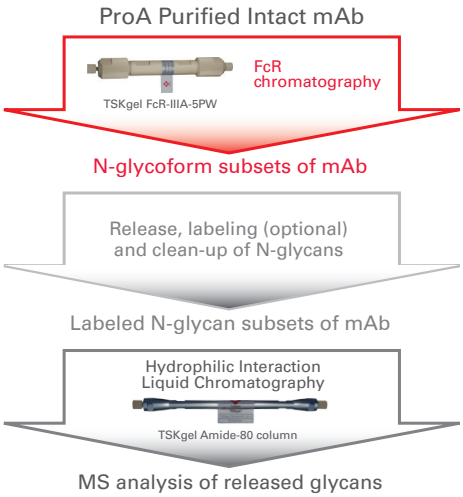
Monoclonal Antibody Characterization by semi-preparative FcR-Based Affinity Chromatography and HILIC-MS

Monoclonal antibodies (mAbs) are an important class of therapeutics with the immense capacity to treat multiple diseases. Due to the complex nature and glycan heterogeneity of these products, characterization and strict control of their critical quality attributes is necessary to maintain product quality and efficacy. The mAb glycans linked to the Asn-297 glycosylation site on the Fc region impact biologic activities such as antibody-dependent cellular cytotoxicity (ADCC) and stability.

The TSKgel® FcR-IIIA columns separate monoclonal antibodies into 3 subsets of affinity to the FcγRIIIA ligand: low, medium and high affinity. These correlate with different mAb glycoforms and their ADCC activity. To quantitate and elucidate the glycan profile of the different glycoforms separated by FcR-IIIA affinity, fractions can be analyzed by releasing and labeling the glycans before analysis on hydrophilic liquid interaction chromatography (HILIC) followed by mass spectrometry (MS).

TSKgel FcR-IIIA-5PW is a semi-preparative affinity column which immobilizes the recombinant FcγRIIIA ligand bonded to porous 10 µm polymethacrylate particles which can load up to 5 mg of mAb. It differs from the analytical column (TSKgel FcR-IIIA-NPR), which is based on non-porous material and is typically loaded with ≤50 µg of mAb. Therefore, the presented workflow benefits from the use of the semi-preparative TSKgel FcR-IIIA-5PW column as more sample can be collected at once (*Figure 1*).

Figure 1. Novel workflow for analysis of released glycans.



The added utility of this semi-preparative column allows for material collection in sufficient quantity for in-depth analysis of mAb glycoforms via enzymatic glycan release followed by HILIC-MS.

Material and Methods

Experimental FcR-IIIA Conditions

Column:	TSKgel FcR-IIIA-5PW, 10 µm, 7.8 mm ID × 7.5 cm(P/N 0023532)
Mobile phase:	A: 50 mmol/L citrate/NaOH, pH 6.0 B: 50 mmol/L citrate/NaOH, pH 4.0
Method:	Equilibrate: 5 CV MP A Wash: 4 CV 25% MP B Elution: linear gradient 25-90% B over 14 CV Hold 4 CV at 90% B and 100% B
Flow rate:	Equilibration, load, and wash steps: 0.5 mL/min Elution and hold steps: 0.25 mL/min
Instrument:	ÄKTA™ avant 25 FPLC
Detection:	UV @ 280 nm
Temperature:	ambient
Sample:	5 mg protein A-purified trastuzumab (Herceptin® biosimilar)

HILIC-MS Conditions

Column:	TSKgel Amide-80, 2 µm, 2.1 mm ID × 15 cm (P/N 0023456)
Mobile phase:	A: 50 mmol/L ammonium formate, pH 4.4 B: 100% acetonitrile
Gradient:	From 65-58% B in 35 min
Flow rate:	0.2 mL/min
Instrument:	Shimadzu Nexera® XR UHPLC
Detection:	Fluorescence: Ex 265 nm, Em 425 nm MS: SCIEX X500B Q-TOF, ESI positive, m/z 200-3500
Temperature:	50 °C
Sample:	5 µL for load sample and 10 µL from collected FcR-column elution peaks

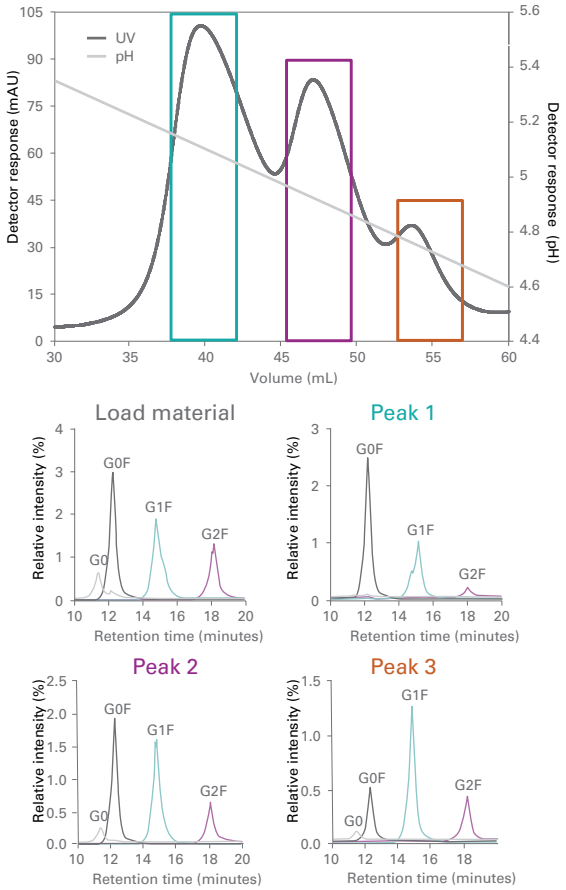
Table 1. Specifications and operating conditions.

MS Conditions:			
Source gas 1	60 psi	Spray voltage	5000 V
Source gas 2	60 psi	Declustering potential	20 + 0V
Curtain gas	45 psi	Collision energy	7 + 0V
CAD gas	7 psi	Source temperature	450 °C
Accumulation time	0.5 sec	Bins to sum	4

Results

Figure 2 illustrates protein A-purified trastuzumab analyzed on the TSKgel FcR-IIIA-5PW semi-preparative column. This peak profile is comparable to the analytical TSKgel FcR-IIIA-NPR (not shown), showing low affinity first, then mid and high affinity as pH decreases. Glycans were released and labeled from the collected peaks 1, 2, and 3 and injected onto a TSKgel Amide-80 HILIC column connected to MS for quantitative glycan analysis.

Figure 2. Elution profile of Herceptin biosimilar.



As demonstrated in *Figure 3*, use of the TSKgel Amide-80 column with mass spectrometry confirms that mAb glycoforms with the highest affinity to FcγRIIIA-ligand (peak 3) also contain the highest amount of galactose in their N-glycan structure (G1F and G2F glycan notations). Peak 2 shows a higher level of G1F relative to peak 1, and peak 1 contains a greater abundance of fucosylated glycans without terminal galactose (G0F).

Figure 3. HILIC-MS: Relative abundance of 6 different N-glycans within the 3 peaks from fractions collected by TSKgel FcR-IIIA-5PW.

Glycan	Structure	Load Material (%)
G2		0.00 ± 0.00
G2F		2.06 ± 0.01
G1		0.16 ± 0.01
G1F		26.76 ± 0.29
G0		1.15 ± 0.03
G0F		69.87 ± 0.34

N-acetylglucosamine (GlcNAc)
Fucose
Mannose
Galactose

Glycan	Peak 1 (%)
G2	0.00 ± 0.00
G2F	0.00 ± 0.00
G1	0.00 ± 0.00
G1F	7.60 ± 1.12
G0	0.00 ± 0.00
G0F	92.40 ± 1.12

Glycan	Peak 2 (%)
G2	0.00 ± 0.00
G2F	2.89 ± 0.11
G1	0.00 ± 0.00
G1F	40.56 ± 0.37
G0	2.05 ± 0.48
G0F	54.51 ± 0.27

Glycan	Peak 3 (%)
G2	0.00 ± 0.00
G2F	5.47 ± 1.10
G1	0.00 ± 0.00
G1F	71.69 ± 1.16
G0	2.29 ± 0.40
G0F	20.55 ± 0.44

Conclusion

This two-step workflow, consisting of the combination of semi-preparative FcR-IIIA affinity chromatography and HILIC separation, allows for the rapid screening of upstream and downstream mAb products. Utilizing HILIC-MS to confirm the presence and relative quantity of N-glycans in different mAb glycoforms permits in-depth characterization of mAbs. This type of analysis can be conducted on almost any mass spectrometer instrument, therefore bypassing the need for high-resolution equipment. The added utility to use the same sample material for orthogonal chromatography methods is a novel benefit for drug development and quality control. Additional advantages to this workflow include the ability to monitor FcγRIIIA affinity and relative ADCC activity without the need for a costly, labor-intensive and time-consuming bioassay.