Profiling Released High Mannose and Complex N-Glycan Structures from Monoclonal Antibodies Using Optimized Hydrophilic Interaction Chromatography

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INTRODUCTION

N-glycans of a therapeutic mAb can impact circulation half-life and efficacy, therefore, it is important that they be well characterized and monitored. A standard approach to the analysis of mAb N-glycans involves releasing N-glycans enzymatically, labeling to improve detectability, and profiling by hydrophilic interaction chromatography (HILIC)[1]. Recent developments in released N-glycan profiling, made possible by the novel *Rapi*Fluor-MS labeling reagent, have simplified the procedure, reduced the overall sample preparation times, and enabled unprecedented sensitivities for both fluorescence and mass spectrometric detection.[2] Yet, even with such capabilities, it might be necessary to tailor methods to ensure robust and optimal performance, particularly in the separation of glycans related to critical quality attributes.

This work shows the development of a HILIC method that optimizes the profiling of N-glycans commonly found on mAbs. The separation method was developed so as to improve the resolution of high mannose glycans, those terminated with N-glycolylneuraminic acid, and species containing alpha-linked galactose monosaccharides.

METHODS

Sample Description

The Waters Intact mAb Mass Check Standard (Part Number (p/n) 186006552) was reconstituted in water to a concentration of 2 mg/mL. N-glycans were released from a 15-µg aliquot of this murine mAb and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n176003606) following the instructions provided in its care and use manual (715004793). *Rapi*Fluor-MS-labeled N-glycans were prepared for injection at a concentration of 0.5 pmol/µL (as a mixture in a solvent composed of 90 µL SPE eluate, 100 µL dimethylformamide, and 210 µL acetonitrile).

*Rapi*Fluor-MS High Mannose Standard (p/n 186008317) was reconstituted in water to produce a 5 pmol/µL solution. A series of spiked samples were then prepared by mixing *Rapi*Fluor-MS-labeled glycans from Intact mAb Mass Check Standard with the RapiFluor-MS High Mannose N-Glycan Standard and water. In this way, four spiked samples containing 0.45 pmol/µL of sample-derived N-glycans were prepared along with varying concentrations of the high mannose glycans. Spiking produced samples with mannose-5 (M5) at a relative abundance ranging from approximately 0.2 to 2%.

LC Conditions for *Rapi*Fluor-MS Released N-Glycans

Chromatographic separations were performed using the following conditions, unless otherwise noted:

***Universal N-Glycan Profiling Method*:**

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10°C
Analytical column temp.:	60°C
Flow Rate:	0.4 mL/min
Injection volume:	10 µL DMF/ACN-diluted samples 1 µL for aqueous samples
Column:	ACQUITY UPLC Glycan BEH Amide, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Fluorescence Detection:	Ex 265 nm / Em 425 nm, 2 Hz
Mobile Phase A:	50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)
Mobile Phase B:	ACN (LC-MS grade)

Time	Flow Rate(mL/min)	%A	%B	Curve
0.0	0.4	25	75	-
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

***mAb N-Glycan Profiling Method*:**

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10°C
Analytical column temp.:	45°C
Flow Rate:	0.5 mL/min
Injection volume:	10 µL DMF/ACN-diluted samples 1 µL aqueous samples
Column:	ACQUITY UPLC Glycan BEH Amide 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Fluorescence Detection:	Ex 265 nm / Em 425 nm, 2 Hz
Mobile Phase A:	50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate, p/n 186007081)
Mobile Phase B:	ACN (LC-MS grade)

Time	Flow Rate (mL/min)	%A	%B	Curve
0.00	0.5	20	80	-
3.00	0.5	27	73	6
35.0	0.5	37	63	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	20	80	6
47.6	0.5	20	80	6
55.0	0.5	20	80	6

MS Conditions for *Rapi*Fluor-MS Released N-Glycans:

MS system:	Xevo G2-XS QTof	Desolvation temp.:	500 °C
Ionization mode:	ESI+	Source Offset:	50 V
Cone voltage:	75 V	Desolvation gas flow:	600 L/Hr
Analyzer mode:	Resolution (~40 K)	Capillary voltage:	2.2 kV
Source temp.:	120 °C	Acquisition:	700–2000 m/z 0.5 sec scan rate
Calibration:	NaI, 0.1 µg/µL from 100–2000 m/z		
Lockspray:	100 fmol/µL human Glu-fibrinopeptide B		
Data management:	MassLynx Software (V4.1), UNIFI (v1.7)		

More information can be found in application note:
Library Number: APNT134865659
Part Number: 720005516EN

RESULTS AND DISCUSSION

A Universal N-Glycan Profiling Method

The N-Glycan Profiling HILIC method described in the GlycoWorks *Rapi*Fluor-MS Kit user's manual was developed to be applied to a wide array of *Rapi*Fluor-MS labeled N-glycans, from small biantennary structures to highly sialylated, tetraantennary species. However, this Universal N-Glycan Profiling Method is not optimal for many specific N-glycan profiles. For example, the Universal N-Glycan Profiling Method produces chromatograms for N-glycans from a murine mAb with at least 13 different peaks **(Table 1, Figures 1A and 1B)**, but several of these are only partially resolved and require the use of MS detection and an analysis of extracted ion chromatograms in order to be monitored more precisely **(Figures 1C and 1D)**. Notable critical pairs exhibiting at least partial co-elution include M5/A2G1 and FA2G2Sg1/FA2G2Ga2. Given the significance of monitoring M5 and immunogenic glycans, like those containing the noted N-glycolyl neuraminic acid (Sg) and alpha-linked galactose monosaccharides, these separations were optimized to increase resolution.

	Glycan	Mi (Da)	2+	3+
1	A2	1627.66	814.84	543.56
2	FA2	1773.72	887.87	592.25
3	M5	1545.61	773.81	516.10
4	FA1G1	1732.69	867.35	578.57
5	A2G1	1789.71	895.86	597.58
6	FA2G1	1935.77	968.89	646.26
7	FA2G1	1935.77	968.89	646.26
8	FA2G2	2097.82	1049.92	700.28
9	FA2G1Ga1	2097.82	1049.92	700.28
10 & 11	FA2G2Ga1	2259.88	1130.95	754.30
12	FA2G2Sg1	2404.92	1203.46	802.65
13	FA2G2Ga2	2421.93	1211.97	808.32

Table 1. *Rapi*Fluor-MS labeled N-glycans from the Intact mAb Mass Check Standard.

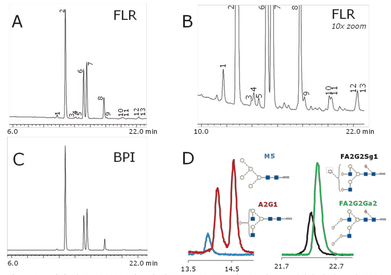
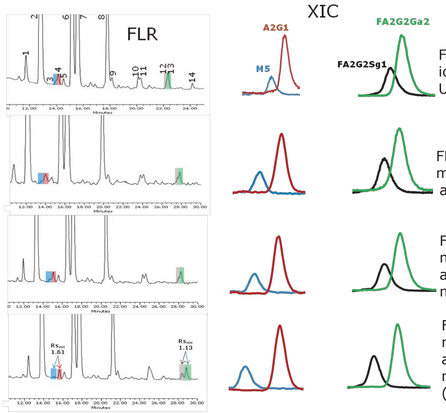


Figure 1. (A) Fluorescence (FLR) chromatogram of *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard obtained using the Universal N-Glycan Profiling Method. (B) Fluorescence chromatogram 10x zoom (C) Base peak intensity (BPI) (D) Extracted Ion Chromatograms (XICs) of critical pairs to highlight issues with partial co-elution.

Developing a Higher Resolution mAb N-Glycan Analysis Method

The co-elution of critical glycan pairs necessitated the development of an LC method specifically tailored for N-glycans released from mAbs.



Fluorescence (FLR) chromatogram and extracted ion chromatograms (XICs) obtained with the Universal N-Glycan Profiling Method

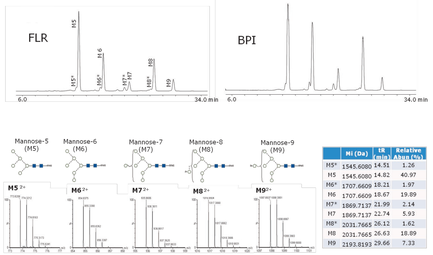
FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60°C column temperature, and a 35 minute gradient from 26 to 37% H_2O

FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60°C column temperature, and a two-step gradient of 20 to 27% H_2O in 3.2 min followed by 27 to 37% H_2O in 31.8 min.

FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 45°C column temperature, and a two-step gradient of 20 to 27% H_2O in 3.2 min followed by 27 to 37% H_2O in 31.8 min (mAb N-Glycan Profiling Method).

Using the *Rapi*Fluor-MS High Mannose Standard to Demonstrate System Suitability

The resolution gains afforded by the new mAb N-Glycan Profiling Method allows for better monitoring of high mannose structures. To demonstrate this, the *Rapi*Fluor-MS labeled N-glycans from a murine mAb were spiked with varying concentrations of *Rapi*Fluor-MS labeled high mannose glycans (**Figure 3**). Four *Rapi*Fluor-MS labeled glycan samples were prepared with M5 relative abundances ranging from 0.2 to 2.0% and analyzed as illustrated in (**Figure 4A**). In these samples, M5, M6, and M8 are readily detected, while M7 and M9 are not due to their lower relative abundances in the spiking standard. The high resolution of the method allows for better integration. This can be clearly demonstrated by plotting the fluorescence peak areas of M5, M6, and M8 as functions of the spiking level. The linearity of these data ($R^2 \geq 0.974$) underscores the suitability of this technique for monitoring high mannose structures (**Figure 4B**). This also demonstrates that this particular mAb is effectively free of high mannose species and that the M5 previously monitored during the development of the separation is near the limit of quantitation of this method.



	Mi (Da)	LR Amt.	Relative Amt. (%)
M5[5]	1545.6080	14.51	1.26
M5	1545.6080	14.82	40.97
M6[6]	1707.6609	18.21	1.97
M6	1707.6609	18.67	19.89
M7[7]	1869.7137	21.99	2.14
M7	1869.7137	22.74	5.93
M8[8]	2031.7666	26.12	1.62
M8	2031.7666	26.63	18.89
M9	2193.8193	29.66	7.33

Figure 3. Analysis of the Waters *Rapi*Fluor-MS High Mannose Standard

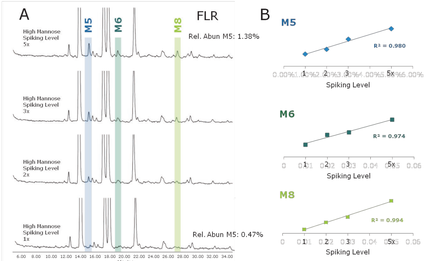


Figure 4. (A) FLR chromatogram obtained for *Rapi*Fluor-MS N-glycans prepared from Intact mAb Mass Check Standard spiked with *Rapi*Fluor-MS High Mannose Standard. (B) Peak area of high mannose structures. Separations were performed using the mAb N-Glycan Profiling Method.

GU Values from the Universal N-Glycan Profiling Method Versus the mAb N-Glycan Profiling Method

GU values can be used along with the mAb N-Glycan Profiling Method as replacements to standard retention times to improve the robustness of data reporting. It is important to recognize that GU values, regardless of labeling strategy, are method specific. To assign GU values, a dextran ladder, consisting of glucose multimers of increasing length, is used as an external calibrant.[3] The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values. GU values obtained using the Universal N-Glycan Profiling Method and the mAb N-Glycan Profiling Method are provided **(Table 2)**. Differences between the methods led to shifts in the retention times of the individual glucose multimers. Clearly, it is important to give consideration to how GU values are generated and how they are to be used.

Glycan	Universal N-Glycan Profiling GU	mAb N-Glycan Profiling GU
A2	5.49	5.54
FA2	5.82	5.91
M5	6.19	6.24
FA1G1 + A2G1	6.23	6.37
A2G1	6.38	6.49
FA2G1	6.69	6.72
FA2G1	6.85	6.86
FA2G2	7.43	7.69
FA2G1Ga1	7.55	7.81
FA2G2Ga1	8.25	8.57
FA2G2Ga1	8.30	8.60
FA2G2Sg1	9.06	9.39
FA2G2Ga2	9.11	9.49
FA2G2Ga1Sg1	9.88	10.25

Table 2 Glucose unit values for the *Rapi*Fluor-MS labeled N-glycans from the Intact mAb Mass Check Standard and the *Rapi*Fluor-MS High Mannose Standard, as obtained with the Universal N-Glycan Profiling Method versus the mAb N-Glycan Profiling Method.

CONCLUSIONS

By labeling with *Rapi*Fluor-MS, mAb N-glycans can be analyzed with high sensitivity using both fluorescence and MS detection. Through adjusting the sample loading condition, gradient steepness, flow rate, and separation temperature of the HILIC separation, it is also possible to improve the profiling of certain mAb-derived glycan structures, including Man5/A2G1+FA1G1 and FA2G2Sg1/FA2G2Ga2 critical pairs. These method improvements facilitate being able to more accurately monitor high mannose structures.

References

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3. Campbell, M. P.; Royle, L.; Radcliffe, C. M.; Dwek, R. A.; Rudd, P. M., GlycoBase and autoGU: tools for HPLC-based glycan analysis. *Bioinformatics* **2008,** *24* (9), 1214-6.

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