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Note d'application

Applying a Novel Glycan Tagging Reagent, *Rapi*Fluor-MS, and an Integrated UPLC-FLR/QTof MS System for Low Abundant N-Glycan Analysis

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Abstract

This application note describes a novel *Rapi*Fluor-MS labeling chemistry for rapid glycan sample preparation, and a UPLC-FLR/QTof MS system controlled by UNIFI Scientific Information System which shows a improved FLR and MS sensitivity from the *Rapi*Fluor-MS label and the QTof MS with StepWave Technology allow confident identification and characterization of minor but critical glycoforms from mAbs.

Benefits

- A novel glycan labeling reagent, *Rapi*Fluor-MS, significantly enhances both FLR and MS signals. Improvement from MS detection allows better detection for minor glycan forms.
- The Xevo G2-XS QTof Mass Spectrometer combines an off-axis ion guide, StepWave, with a novel collision cell design to provide significant increases in sensitivity for *Rapi*Fluor-MS labeled glycans.

Introduction

UPLC-FLR/MS(MS) analysis of released N-glycans labeled with a fluorescent tag has become routine with high-performance LC and MS instrumentations. Glycans labeled with commonly used fluorescent tags, such as 2-AB and 2-AA, can be detected by fluorescent (FLR) detection with ultra-high sensitivity. Unlike an FLR detector, mass spectrometry is known to be less sensitive to detect native or tagged glycans, especially low abundant ones, due to their poor ESI performance. The limited dynamic range of this approach has restricted the use of this combined workflow for glycan characterization.

To overcome the low MS ionization efficiency associated with conventional labels and confidently assign lower-level glycans, a novel tag, *Rapi*Fluor-MS has been developed by Waters. *Rapi*Fluor-MS contains a rapid tagging reactive group, an efficient fluorophore, and a functional group that imparts high ionization efficiency.[1] Complete tagging of glycans can be achieved in less than 5 minutes using this novel reagent.

Initial results with this glycan label show significant enhancement in both FLR and MS(MS) signals compared to 2-AB.[1] The increased sensitivity enables the detection and identification of very low level glycans, at 0.1%, with sufficient MS signal. In this study, we demonstrate the benefits of combining *Rapi*Fluor-MS with an integrated UPLC-FLR/QTof MS system for detailed characterization of the minor glycoforms from the human IgG and mouse IgG1 samples.

Experimental

Sample preparation

The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit Care and Use manual (p/n 715004793en) < https://www.waters.com/waters/support.htm?lid=134829211&type=USRM> contains a detailed sample preparation procedure for the deglycosylation of N glycans from biotherapeutics, followed by the *Rapi*Fluor-MS labeling step and glycan extraction using an SPE device. The entire sample preparation procedure took 30 minutes.

LC conditions

All chromatographic mobile phases are prepared using LC-MS compatible solvents and reagents.

System:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC FLR
Column:	ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm
Column temp.:	60 °C
Mobile phase A:	50 mM ammonium formate (pH 4.4)
Mobile phase B:	100% acetonitrile

UPLC HILIC LC gradient table:

Time(min)	Flow rate(mL/min)	%A	%B	Curve
0.0	0.4	25	75	6
40.0	0.4	49	51	6
41.5	0.2	100	0	6
44.5	0.2	100	0	6
48.1	0.2	25	75	6
52.6	0.4	25	75	6

Time(min)	Flow rate(mL/min)	%A	%B	Curve
60.0	0.4	25	75	6

FLR settings:

MS conditions

System:	Xevo G2-XS QTof MS: ESI+ in sensitivity mode (resolution ~ 30,000)
Capillary voltage:	3.0 kV
Cone voltage:	80 V
Source temp.:	120 °C

Desolvation temp.:	300 °C
Desolvation gas flow:	800 L/h

LockSpray

Capillary voltage:	3.0 V
Cone voltage:	40 V
Scan time:	0.5 s
Interval:	20 s

GFP solubilized in 0.1% formic acid with 50:50 (MeCN: H_2O) at 200 fmol/µL was infused, *m/z* = 785.8421 (z = 2) was used for lock mass calibration.

Collision induced dissociation

MS/MS analyses were performed in continuum mode from 100–2000 *m/z* with collision induced dissociation (CID) to generate glycan fragmentation data. Ions with 2+and 3+ charge states were selected for fragmentation. Customized collision energy tables that were charge state and mass specific were used for optimized fragmentations; the approximated CE range was between 15 to 40 eV. Data Dependent Acquisition (DDA) was used with duty cycle times of 1.6 sec and 0.5 sec for MS and MS/MS modes. The two most abundant precursors were selected for fragmentation.

Data management

UNIFI Scientific Information System v1.7.1

Figure 1. Biopharmaceutical System Solution with UNIFI for glycan analysis.

Results and Discussion

Previous work showed that the *Rapi*Fluor-MS labeling reagent improves N-glycan MS ionization in positive ion mode. More than two order of magnitude MS sensitivity increase was observed when compared to 2-AB label.[1] Combined with highly sensitive Xevo G2-XS QTof Mass Spectrometer, we are now able to detect minor glycoforms with high confidence.

Figure 2 shows an example of analyzing the *Rapi*Fluor-MS labeled N-Glycans released from 0.5 µg of human IgG on UPLC-FLR/QTof MS system. Comparable FLR and MS response across a broad range of glycans was easily

achieved.

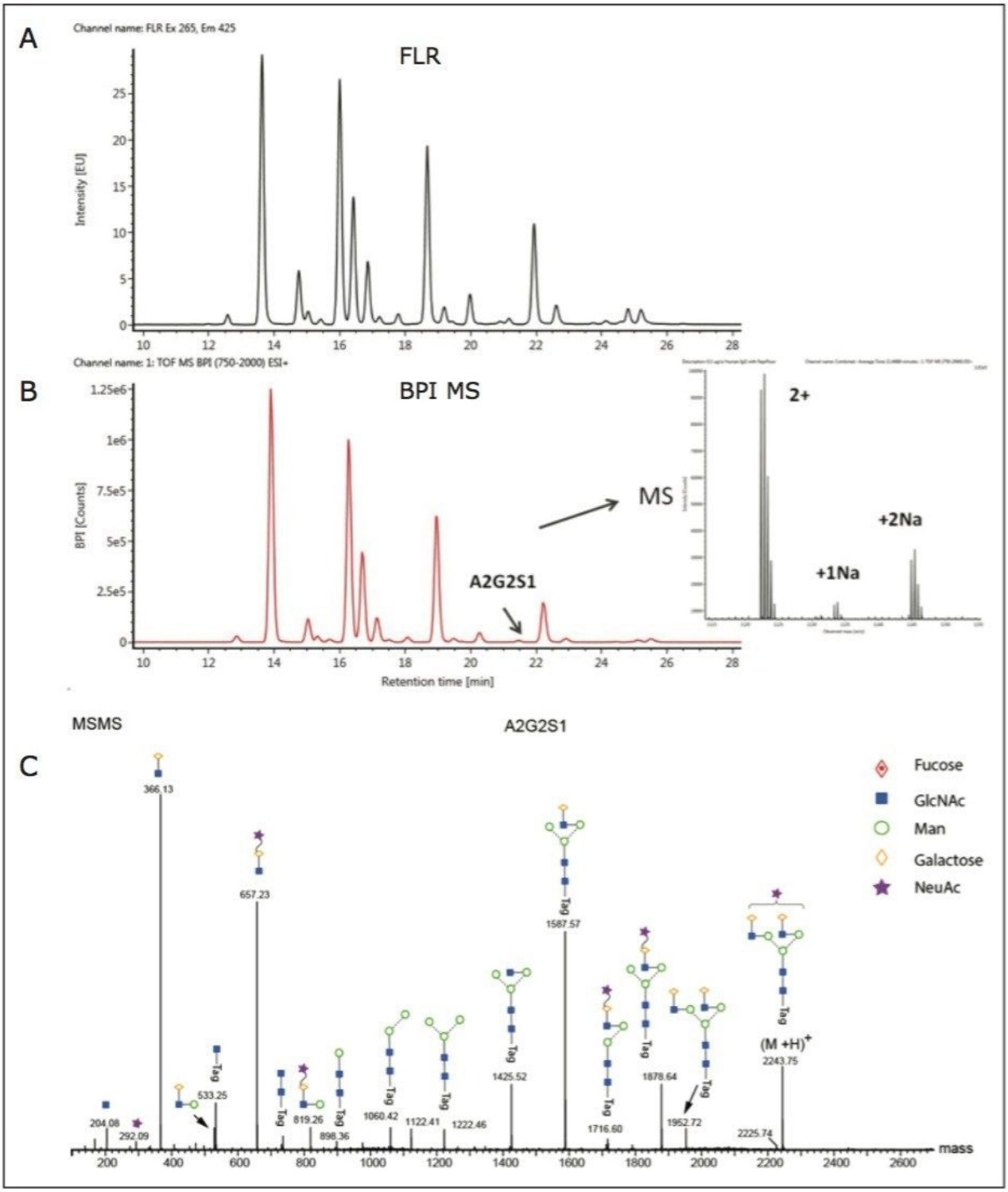

Figure 2. UPLC-FLR/MSMS analysis of Rapi Fluor labeled human IgG N-glycans. A) FLR data channel. B) BPI MS

data channel. The MS spectrum of a low intensity ion was inserted (A2G2S1). The dominant ions were doubly charged with minor sodium adduct ions. C) Deconvoluted MS/MS spectrum of A2G2S1 was displayed.

The MS and MS/MS fragmentation spectra were also shown as an example in Figure 2 for a minor glycoform, A2G2S1, which is present at 0.1% level. The MS spectrum shows doubly charged ions with minor sodium adduct ions in the raw MS spectrum.

We observed a similar fragmentation pathway for the *Rapi*Fluor-MS labeled glycans compared to the 2-AB labeled glycans. The MS/MS fragmentation of A2G2S1 showed that glycosidic bond cleavage from both reducing and non-reducing end was the dominant fragmentation pathway. The observed sequential neutral losses from the non-reducing end stops at the first GlcNAc residue at the reducing end with the *Rapi*Fluor-MS label attached. Also, the counter fragment ions from the non-reducing end, oxonium ions, were readily observed.

In addition to human IgG, we also tested the *Rapi*Fluor-MS labeled glycans released from a mouse IgG1 sample. It is well known that N-glycolyneuraminic acid and alpha (1-3) galactose containing N-glycans on mAbs generated from murine cell lines are glycans with immunogenic epitopes. These glycans present analytical challenges, due to 1) their low abundance in the glycan mixture, and 2) difficulty to characterize them structurally due to poor MS and MS/MS signals from using the conventional labels.

Figure 3 shows an example of a UPLC-FLR/QTof MS analysis of the mouse IgG1 glycans that contain these immunogenic epitopes. Structural informative fragments (with asterisks) are observed for a low abundant immunogenic glycan, FA2Gal1Sg1, which is present at about 0.1% level. The fragment ion at *m/z* of 528.2 suggests this glycan contains alpha-gal when this ion was the most dominant fragment ion in the entire spectrum; also another diagnostic ion at *m/z* of 2260.8 was generated from losing one NeuGc from the precursor ion. This glycan was also observed in FLR chromatogram of 2-AB labeled glycans without sufficient MS signals to obtain good quality CID fragmentation (data not shown). With *Rapi*Fluor-MS labeling chemistry, sufficient amount of precursor ions were obtained for subsequent MS/MS fragmentation.

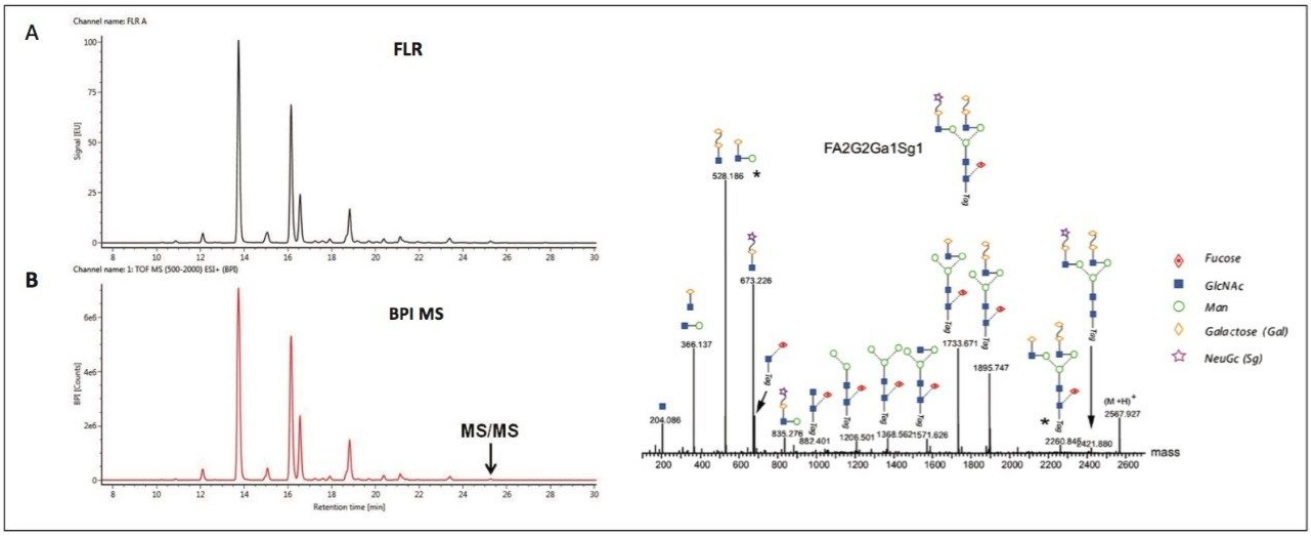

Figure 3. UPLC-FLR/MSMS analysis of Rapi Fluor-MS labeled mouse IgG1 N-glycans. A) FLR data channel. B) BPI MS data channel. One of the last eluting glycans were selected for MS/MS fragmentation. The deconvoluted fragmentation data from FA2Ga1Sg1 was displayed in C); "Ga" stands for galatose and "Sg" stands for NeuGc. Structurally informative fragments (with asterisks) are observed for this low abundant ion (< 0.1% relative abundance). Fragment ion at m/z of 528.2 suggests this glycan contains alpha-gal when this ion was the most dominant fragment ion in the entire spectrum; also another diagnostic ion at m/z of 2260.8 was generated from the loss of one NeuGc from the precursor ion.

Overall, we demonstrated that *Rapi*Fluor-MS labeling chemistry enhances MS and MS/MS sensitivity to obtain high quality precursor and fragmentation ion spectra. Therefore, rich structural information for low abundant glycan species are achieved using this approach.

Conclusion

LC/FLR analysis of N-glycans released from protein therapeutics is performed routinely in analytical laboratories around the world. For scientists who want to add MS characterization capability to their glycan analysis, they often struggle with low MS signals and poor quality MS/MS fragmentation for mass confirmation and structure elucidation using conventional FLR labels such as 2-AB and 2-AA. To address these challenges, Waters offers

enabling technologies that include the novel *Rapi*Fluor-MS labeling chemistry for rapid glycan sample preparation, and a UPLC-FLR/QTof MS system controlled by UNIFI Scientific Information System. The improved FLR and MS sensitivity from the *Rapi*Fluor-MS label and the QTof MS with StepWave Technology allow confident identification and characterization of minor but critical glycoforms from mAbs.

References

1. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent. Waters and New England Biolabs application note (p/n 720005275en).
2. GlycoWorks *Rapi*Fluor-MS Kit Care and Use Manual (p/n 715004793en.)

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