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Note d'application

Characterization of Released N-linked Glycans in Biosimilar mAb Drug Products Using the Xevo™ G3 QTof

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Abstract

As an increasing number of biosimilar monoclonal antibodies (mAb) are being developed, there is a need for streamlined characterization to ensure products meet the appropriate requirements prior to release into the market. Despite having the same amino acid sequence, biosimilar mAbs often have differences in modifications such as glycosylation. Characterizing the glycans is important because glycosylation can affect immunogenicity and other biological activity. However, it can be challenging to analyze due to the low abundance and complex branched and isomeric structures of glycans present. This study shows how the Xevo G3 QTof Mass Spectrometer can be used with GlycoWorks™ *Rapi*Fluor-MS™ labeling, HILIC chromatographic separation, and waters_connect™ informatics tools to characterize the population of released N-linked glycans. The glycosylation profiles of Remicade™ (originator) and Renflexis™ (biosimilar) infliximab drug products were compared using low and elevated energy MS data coupled with fluorescence detection. The results show how the sensitivity and structural information offered by this workflow can assist in the characterization of N-glycans.

Benefits

- N-linked glycan characterization benefiting from the high sensitivity and specificity of Xevo G3 QTof Mass

Spectrometer

- MS^E acquisition (DIA) enables in-depth comparability assessment of biosimilars and confirmation of glycan structure assignments based on the elevated energy fragment ion spectra
- Streamlined workflow in waters_connect for in-depth interrogation of biosimilar mAb product differences

Introduction

With the increasing pipeline of biosimilar monoclonal antibody (mAb) drug products in development, there is a need for streamlined analysis of product attributes to ensure they meet the appropriate requirements for analytical comparability to the innovator. Biosimilars must be demonstrated to be highly similar to the originator product, and any differences must be carefully evaluated to establish that no negative impacts to product safety and efficacy will arise. Glycosylation is an important modification that can affect immunogenicity and other biological activities.[1] It is also a modification that can vary notably between mAb products, despite having the same protein amino acid sequence, due to differences in manufacturing processes and cell lines from which the drug products originate.[2] Full characterization of glycans in a mAb product provides information on the monosaccharide composition and linkages, enabling these evaluations to take place.

While characterizing glycosylation is a critical step in ensuring a biosimilar meets the required criteria, it can also be challenging. This challenge is due to the low abundance of many glycoforms, their lack of a chromophore for optical detection, and their complex branched and isomeric structures. This study shows how the Xevo G3 QTof Mass Spectrometer can be used to characterize released N-glycans in mAb drug products with sufficient sensitivity for a comparability assessment, using the wealth of structural information provided by the collision induced fragmentation, in addition to accurate mass measurement of the glycan precursor, and relative quantitative profiling using an inline fluorescence detector.

In this study, GlycoWorks *Rapi*Fluor-MS was used to enable effective glycoform resolution by HILIC chromatography, sensitive fluorescence detection, and high MS ionization efficiency.[3] Released glycan samples, using the GlycoWorks *Rapi*Fluor-MS Kit for Automation, were prepared using an Andrew+™ Pipetting Robot, followed by HILIC UPLC separation on an ACQUITY™ Premier LC System coupled to inline ACQUITY FLR and Xevo G3 QTof detectors. Released N-glycan profiles were generated for infliximab innovator (Remicade) and an approved biosimilar (Renflexis). The analysis confirmed several biosimilar-innovator differences in glycovariant

patterns and was able to assign differences to specific structures, an important step towards establishing cogent biosimilarity arguments.

Experimental

Sample Description

Enzymatically released glycan samples from innovator infliximab (Remicade) and an approved biosimilar (Renflexis) were prepared using the Andrew+ Pipetting Robot following the GlycoWorks *Rapi*Fluor-MS (RFMS) automation protocol.[4] For each sample, 2 µL of labeled glycans (from ~0.5 µg mAb) was injected on column.

LC Conditions

LC system:	ACQUITY Premier BSM UPLC
Detection:	ACQUITY Premier FLR Detector ($\lambda_{excitation}$=265 nm, $\lambda_{emission}$=425 nm, 2 Hz)
Vials:	QuanRecovery™ with MaxPeak HPS vials (p/n: 186009186)
Column(s):	ACQUITY Premier Glycan BEH™ Amide Column (1.7 µm, 130 Å, 2.1 × 150 mm) (p/n: 186009524)
Column temperature:	60 °C
Sample temperature:	8 °C
Injection volume:	2 µL
Mobile phase A:	50 mM ammonium formate (pH 4.4) prepared from Waters™ Ammonium Formate Solution – Glycan

Analysis (p/n: 186007081)

Mobile phase B: Acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.400	25	75	6
35.00	0.400	46	54	6
36.50	0.200	80	20	6
39.50	0.200	80	20	6
43.10	0.200	25	75	6
47.60	0.400	25	75	6
55.00	0.400	25	75	6

MS Conditions

MS system: Xevo G3 QTof

Ionization mode: ESI, positive

Acquisition range: 100–2000 *m/z*

Capillary voltage: 3.0 V

Collision energy: Low energy: 6 V

Elevated energy ramp: 15–40 V

Cone voltage: 40 V

Source temperature: 120 °C

Desolvation temperature:	300 °C
Cone gas:	35 L/hr
Desolvation gas:	800 L/hr
Intelligent data capture (IDC) setting:	Low (5)

Data Management

Data were acquired and processed with the waters_connect informatics platform with the integrated UNIFI™ App (version 3.1.0.16). The Glycan FLR with MS confirmation workflow was used to identify glycans in each sample. The same data were then reprocessed with the accurate mass screening workflow using the compiled list of identified glycans to facilitate comparison between innovator and biosimilar.

Results and Discussion

When biosimilarity is being evaluated, any differences detected in glycovariant profiles generate the need for rapid identification of any additional species detected in the candidate biosimilar. Obtaining confirmation that abundance differences in those profiles are due to the same species in both samples is also important to establish, effectively restarting an exercise in product characterization. Characterization of released glycans in a mAb sample requires sufficient sensitivity to detect lower abundance N-linked glycoforms in complex labeled released glycan mixtures to unambiguously assign the monosaccharide composition and linkages of each structure. When glycans are labeled with GlycoWorks *Rapi*Fluor-MS, fluorescence detection provides high sensitivity optical detection for comprehensive glycan profile quantification, while precursor and fragment ion accurate mass data provide information about the monosaccharide composition and structure of the glycan.

Figure 1 shows the wealth of information obtained from the two detectors, including chromatograms obtained from the fluorescence detector, low, and elevated collision energy MS channels, that enable generation of the precursor and fragment ions' mass spectra (DIA mode) for the indicated peak. Such data can be used for routine profiling and accurate confirmation of glycan profiles, using a glucose unit based calibrated retention value and MS1 mass confirmation searched against a comprehensive glycan library. The FLR + Mass Confirmation

workflow in UNIFI is often used for this purpose, in combination with the Waters RFMS Glycan GU Library containing 177 N-glycan structures encompassing those typically identified in mAb samples.[5] Using this information, glycan structures can be confidently assigned to measure the glycan profiles of mAb drug products. This workflow is discussed in greater detail in an earlier application note where innovator and biosimilar Infliximab were compared.[6]

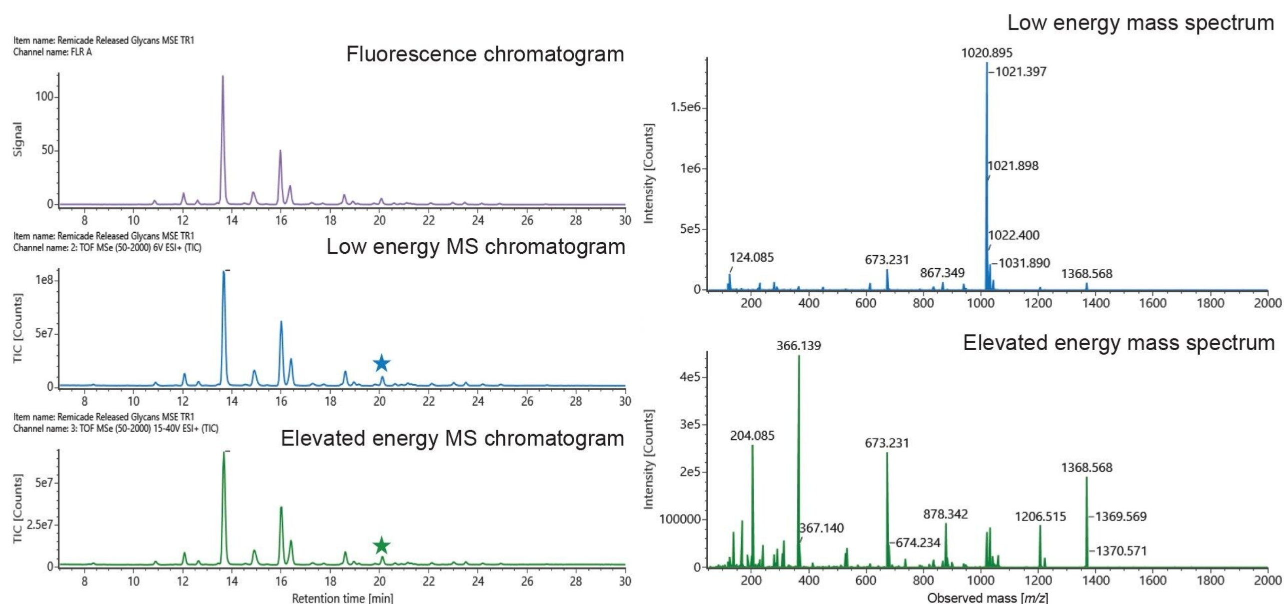

Figure 1. Released N-glycan data, including (left) fluorescence, low energy MS, and elevated energy MS chromatograms, and (right) low energy and elevated energy mass spectra for the peak indicated by the star, the sialylated glycan FA1G1NeuGc1.

Figure 2 shows an overlay of TIC chromatograms of the originator (Remicade, blue trace) and a biosimilar (Renflexis, red trace). As is evident by the overlaid chromatograms, there are numerous differences between the two mAb products. Some notable glycans that are detected in one mAb or present at varying abundances are labeled in the chromatogram with their GU + Mass assigned structures. Interestingly, many of the differences observed were in the sialylated glycans, which elute later in the HILIC separation. There were additional differences present for non-sialylated structures, such as the presence and abundance of high mannose glycans (M5 and M6), as well as differences in abundance of the mono-antennary (A1) and bi-antennary (A2) α-galactosylated glycans.

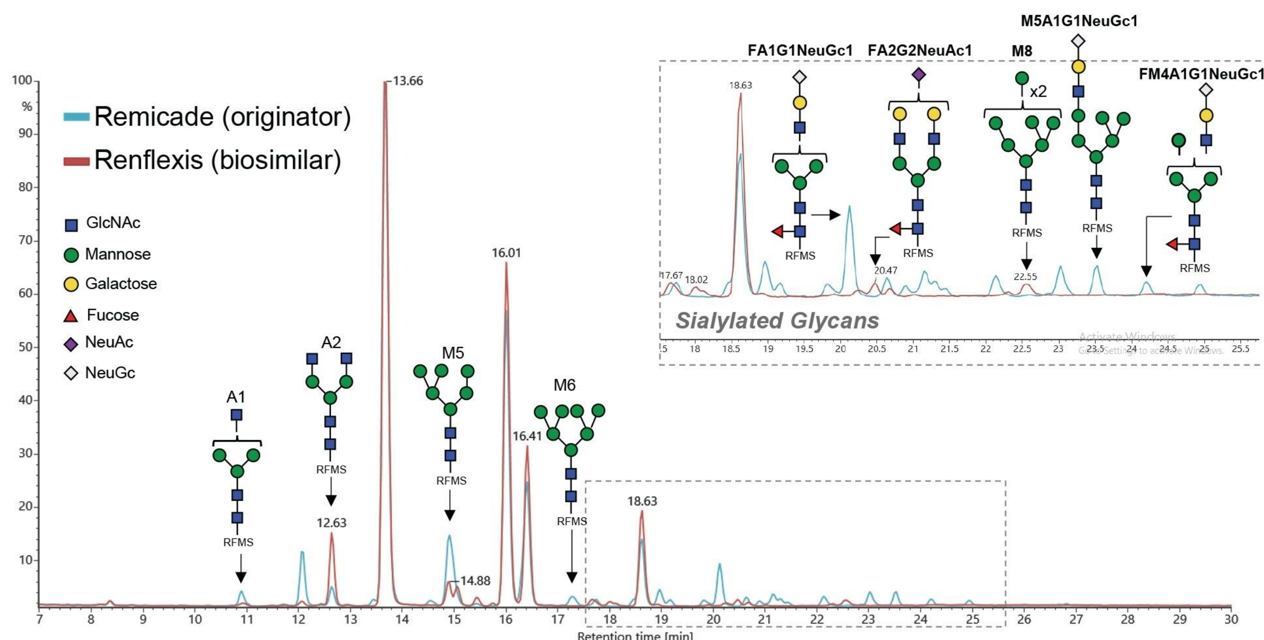

Figure 2. Overlaid TIC chromatograms of an infliximab originator (Remicade) and a biosimilar (Renflexis) released N-glycan analysis. Some of the glycans differing between the two samples, either in presence or relative abundance, are indicated in the chromatogram with their structures.

In addition to observing differences in the chromatograms based on the MS1 TIC chromatogram, useful information can also be obtained from elevated energy mass spectra obtained using data independent acquisition (DIA, MS^E). With MS^E, MS spectra are alternatingly acquired at low and elevated collision energy, capturing both precursor and fragment ions from all components in a sample. This acquisition method enables users to gain information from diagnostic fragment ions in the elevated energy mass spectra. For example, two of the major forms of Sialic acid, NeuAc and NeuGc, have distinct masses of 291.095 and 307.090, respectively.

Generating extracted ion chromatograms of these fragment ions in the elevated energy channel readily reveals the profile of sialylated glycans in each sample. Figure 3 shows the extracted ion chromatograms of Remicade and Renflexis for NeuGc (left) and NeuAc (right) characteristic oxonium ions. Here, it becomes clear that Remicade contains more NeuGc glycans, while Renflexis contains more NeuAc glycans. This difference is to be expected because these mAbs originate from different cell lines, *i.e.*, Remicade from murine origin cells and Renflexis from Chinese hamster ovary. However, confirming these differences is important, as they may impact the immunogenicity, PK, and receptor interactions of the drug product.

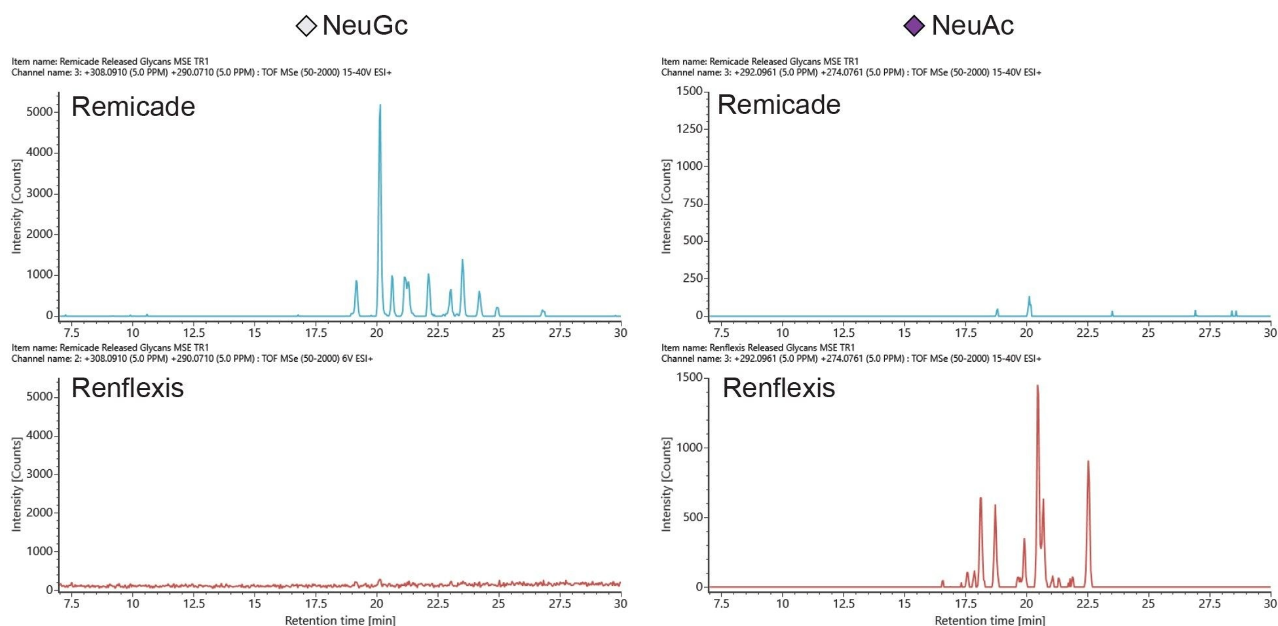

Figure 3. Extracted ion chromatograms of elevated energy MS channel (MS^E) data for diagnostic fragment ions for NeuGc (308.091 m/z) and NeuAc (292.096 m/z) from both infliximab samples.

Elevated energy fragment ion spectra can also be used to further confirm the identity of assigned glycans in a sample. While additional techniques such as ion mobility or NMR are needed to assign stereochemistry, this platform enables verification of the presence of monosaccharide units and glycosidic structural connectivity. To obtain informative fragment ion information from the elevated energy spectra, the collision energy should be optimized such that fragmentation of monosaccharide units is observed but over-fragmentation does not complicate the spectrum and obscure important information. Here, we found the optimum collision energy ramping to be from 15 to 40 V.

It should be noted that the significantly enhanced ionization due to the RFMS glycan tag produces a fragmentation pattern preferentially extending from the RFMS-containing termini, simplifying interpretation of the structure. An example MS^E elevated energy spectrum is shown in the top of Figure 4 for the N-glycan M5A1G1NeuGc1, a hybrid sialylated N-glycan structure, which is present in the originator but not in the biosimilar product. Despite this N-glycan's low relative abundance in the TIC chromatogram (~1.4%), the elevated energy spectrum shows sufficient fragment ions to establish the glycan structure. Several key fragment ions are labeled with their matching structures, showing fragmentation denoting monosaccharide connectivity.

For more complex samples with chromatograms indicating multiple coeluting species, data dependent acquisition (DDA) may provide cleaner fragmentation spectra, as the individual precursor ions are isolated for fragmentation. An example DDA spectrum is shown in the bottom of Figure 4 for a complex sialylated N-glycan structure, FA2G2NeuAc, which was detected at lower abundance (~1%) and only in the biosimilar Renflexis. This fragmentation data proves useful to confirm assignments in the case of ambiguous structures, as isomeric glycans can have vastly different impacts on activity and safety of biotherapeutic products.

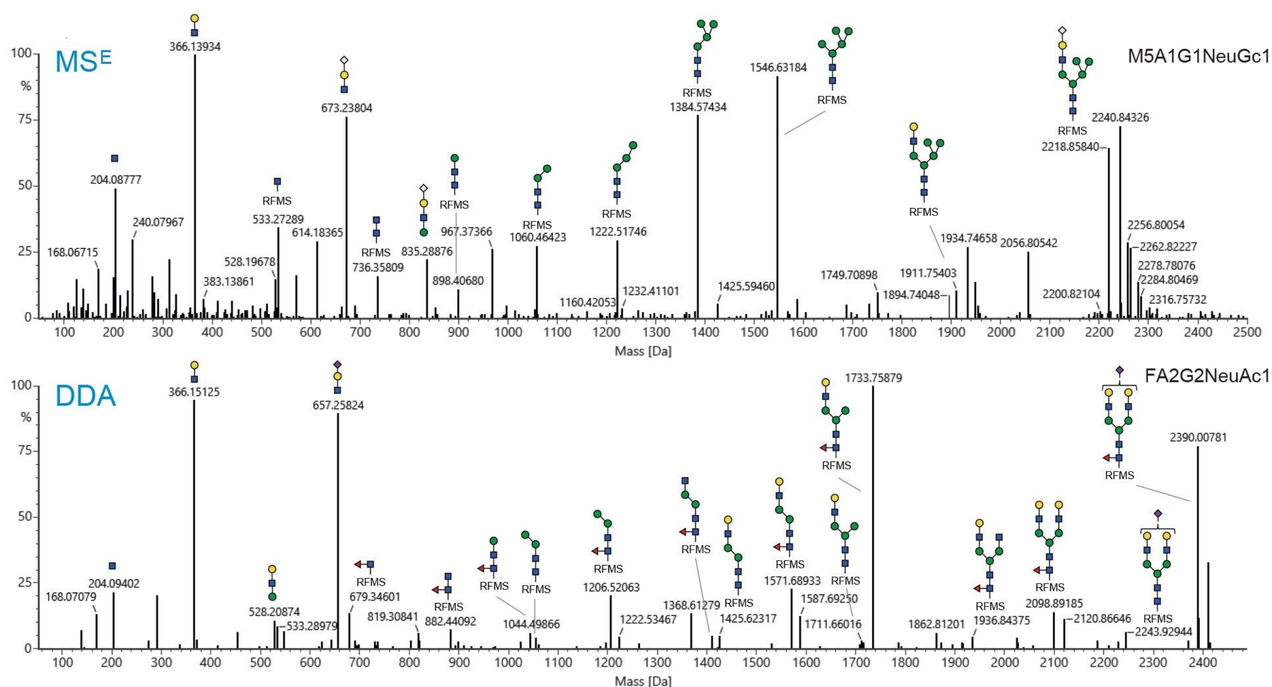

Figure 4. Elevated energy deconvoluted MS spectra showing the fragment ions of two RFMS labeled sialylated glycans, M5A1G1NeuGc1 in Remicade (top) and FA2G2NeuAc1 in Renflexis (bottom). The top spectrum was acquired with MS^E fragmentation enabled, and the bottom was acquired with a DDA fragmentation method. Fragment ions showing structural linkage information are labeled with the corresponding structure.

Conclusion

When differences in glycovariation profiles are seen for batches of a molecule or between an innovator and

biosimilar, some degree of extended product characterization is required for assessment of the potential impacts of those differences. Characterizing glycosylation of mAbs is important to ensure biosimilarity of mAb products to an originator product, but this process can be challenging due to the structural diversity of glycans. The quantitative profile and RFMS-labeled glycan structural information obtainable from a system comprised of an ACQUITY Premier UPLC, inline ACQUITY FLR, and Xevo QTof detectors facilitates the characterization of glycans released from a mAb product, enabling the thorough comparison of originator, and biosimilar products. The additional information offered by fragment ion spectra generated by elevated energy MS^E acquisition or targeted MS/MS provides users with greater detail to confirm these glycan compositional and structural assignments. The sensitivity and specificity offered by this workflow with the Xevo QTof MS enables characterization of mAb glycosylation profiles, even for glycovariants detected at lower abundance.

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720008084, October 2023

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