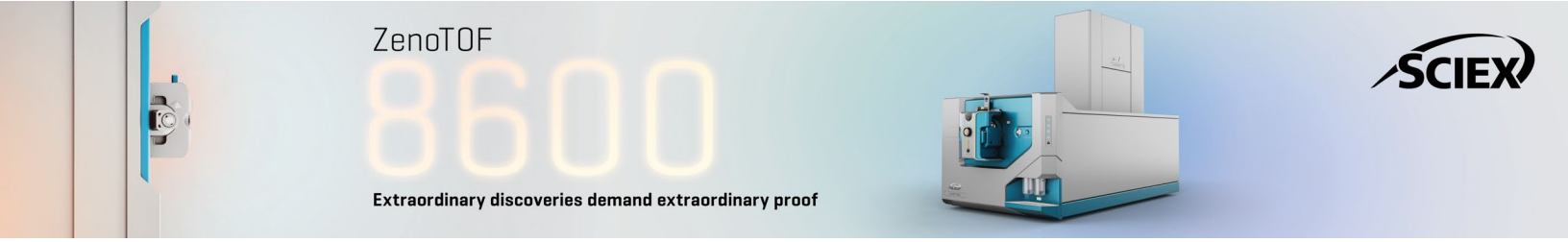

Enhanced sensitivity of intact LC-MS approaches to expand the capability of biotherapeutic analysis

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Sensitive detection and accurate mass measurement of intact biotherapeutics and their impurities are essential to ensure drug's quality, safety and efficacy.[1] This technical note describes enhanced intact mass analysis and impurity assessment of biotherapeutics using intact LC-MS workflows with improved MS sensitivity (Figure 1). The advancements in the ion source, optics and detectors of the novel ZenoTOF 8600 system led to >3X sensitivity increase in the detection of intact biotherapeutics compared to the ZenoTOF 7600 system.

The increasing diversity and complexity of biotherapeutics poses analytical challenges to the analysis of low-abundant proteoforms or impurities in these complex molecules. Intact LC-MS is typically employed for rapid sequence confirmation, impurity assessment and quality control throughout the lifecycle of biotherapeutics. Further improvement in MS sensitivity can enhance the ability of intact LC-MS workflows to analyze low-abundant proteoforms or impurities, benefiting particularly native MS analysis.[2]

Key features of enhanced intact LC-MS workflows for biotherapeutic analysis

- **High sensitivity**: The improved hardware provides >3X gain in MS sensitivity, enabling superior analysis of intact proteoforms or impurities under denaturing or native conditions compared to the previous platforms.
- **Enhanced native MS analysis**: Due to MS sensitivity gain, less sample is needed to generate high-quality data, significantly benefiting native size exclusion chromatography (SEC)-MS analysis.
- **Automatic data interpretation**: Biologics Explorer software provides intuitive templates and tools for automatic data interpretation and results visualization.
- **Streamlined**: Intact LC-MS workflows are streamlined from data acquisition to result analysis and can be easily implemented by scientists from discovery to quality control.

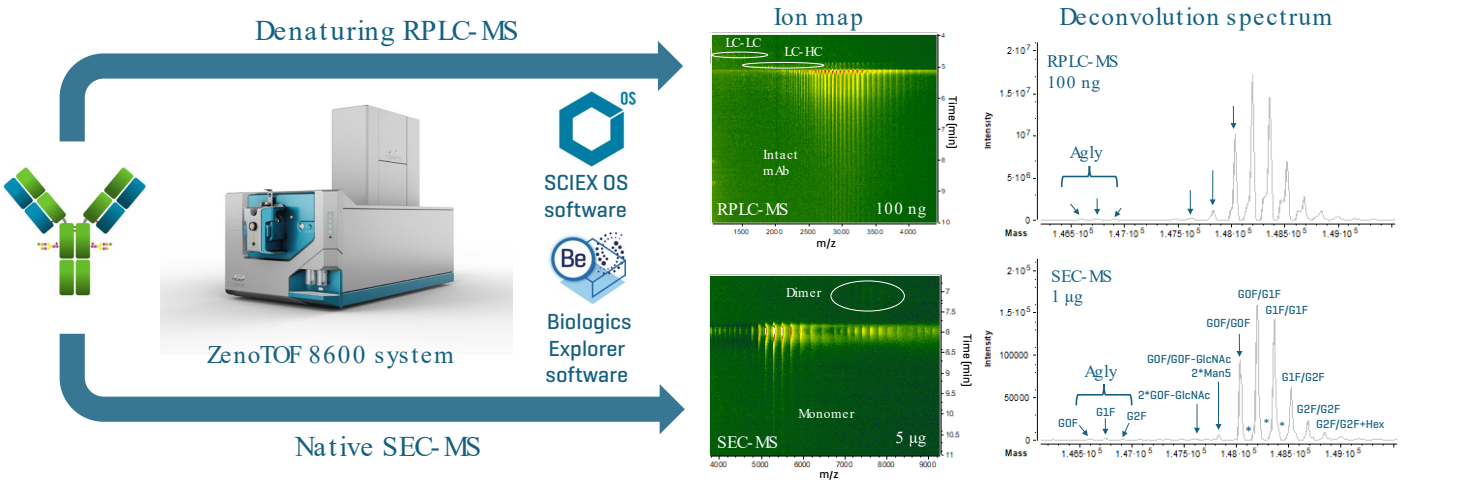

Figure 1. Enhanced intact mass analysis and impurity assessment of biotherapeutics using intact reverse-phase liquid chromatography (RPLC) or native SEC-MS workflows. The novel ZenoTOF 8600 system is equipped with enhanced hardware components—such as the DJet ion guide, QJet ion guide, OptiFlow Pro ion source and TOF optics—that provide >3X MS sensitivity increase for superior intact LC-MS analysis of biotherapeutics compared to the ZenoTOF 7600 system. The MS sensitivity gain led to improved detection of low-abundant proteoforms, such as aglycosylated (Agly) species labeled in the deconvolution spectra (right panel), and product-related impurities detected in the ion maps (middle panel).

Introduction

Antibody-based biotherapeutics, such as monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), are highly heterogeneous due to the coexistence of various proteoforms and product-related impurities.[1] Intact LC-MS analysis of biotherapeutics is essential to confirm the protein sequence and assess sample impurity to ensure product quality, safety and efficacy.

Intact LC-MS analysis is typically performed under denaturing conditions using RPLC or native conditions using SEC. While RPLC-MS offers high sensitivity, this approach does not preserve non-covalent interactions and hence, is not amenable to direct mass determination of Cys-linked ADCs or aggregates. For these molecules, native MS is the method of choice due to its ability to preserve non-covalent interactions.[2,3] However, native MS generally suffers from ion suppression due to the presence of salt in mobile phases. To mitigate this on previous platforms, a strategy has been to use a high sample load.

In this work, RPLC- and SEC-MS analyses of NISTmAb were performed using the ZenoTOF 7600 system and ZenoTOF 8600 system to evaluate the benefits of MS sensitivity improvement provided by the novel QTOF platform.

Methods

Sample preparation: A dilution series of 10 ng/µL to 1 µg/µL NISTmAb (RM8671, NIST) was prepared from the 10 µg/µL stock solution for LC-MS analyses under the denaturing and native MS conditions.

RPLC separation: 10 ng-1 µg of NISTmAb was injected onto an ACQUITY UPLC Protein BEH C4 column (2.1 × 50 mm, 1.7 µm, 300 Å, Waters) installed on an ExionLC AD system (SCIEX). RPLC separation was carried out at a flow rate of 0.25 mL/min using the linear gradient shown in Table 1. The column was kept at 60°C in the column oven. Mobile phase A was 0.1% formic acid (FA) in water and mobile phase B was 0.1% FA in acetonitrile.

SEC separation: Native SEC separation was carried out using an ACQUITY UPLC Protein BEH SEC column (4.6 × 150 mm, 1.7 µm, 200 Å, Waters). An isocratic gradient was employed with the mobile phase containing 20 mM ammonium acetate. The total run time is 15 minutes.

Table 1. LC gradient for RPLC separation.

Time (Min)	Mobile phase A (%)	Mobile Phase B (%)
Initial	90	10
1	90	10
11	20	80
13	20	80
13.1	90	10
15	90	10

Mass spectrometry: Intact RPLC-MS and native SEC-MS data were acquired using the ZenoTOF 7600 system (SCIEX) and ZenoTOF 8600 system (SCIEX). Table 2 shows key source and TOF MS settings for the RPLC-MS and SEC-MS analyses conducted on the ZenoTOF 8600 system.

Table 2. Source and TOF MS parameters for RPLC- and SEC-MS analyses.

Parameter	RPLC-MS	SEC-MS
Workflow	Intact proteins	
Start mass	900 Da	2,500 Da
Stop mass	5,000 Da	10,000 Da
Spray voltage	3,500 V	
Curtain gas	40	
CAD gas	7	9
Ion source gas 1	40 psi	60 psi
Ion source gas 2	40 psi	60 psi
Source temp	400°C	300°C
Declustering potential	120 V	
Collision energy	12 V	
Accumulation time	0.25 s	
Time bins to sum	120	

Data analysis: LC-MS data were interpreted using the Explorer function within SCIEX OS software (SCIEX) and an intact protein analysis template within Biologics Explorer software (SCIEX). An N-linked glycan library and fully connected disulfide connection was specified for intact protein mapping of NISTmAb.

Intact RPLC-MS analysis

The hardware improvements of the ZenoTOF 8600 system provide up to 10X gain in MS sensitivity for different molecules compared to the ZenoTOF 7600 system, significantly improving the detection of low-abundant species in drug products, even with reduced sample load. In this work, the benefits of MS sensitivity gain were evaluated for intact RPLC-MS and SEC-MS analyses of NISTmAb under denaturing and native conditions, respectively.

Figure 2 shows the intact RPLC-MS data of 100 ng NISTmAb acquired using the ZenoTOF 7600 system or the ZenoTOF 8600 system. Similar charge state distributions of NISTmAb were obtained from the 2 QTOF systems (Figures 2A and 2D). However, a close inspection of the 52+ charge state show that the ZenoTOF 8600 system provided a lower "valley-to-peak" ratio (i.e., better peak resolution) of ~11% between the G0F/G0F and G0F/G1F glycoforms (Figure 2E), as compared to ~17% for the previous platform (Figure 2B), indicating better desolvation using the new instrument. To evaluate the sensitivity improvement for intact RPLC-MS analysis using the ZenoTOF 8600 system over the ZenoTOF 7600 system, the S/Ns of the 52+ of the G0F/G1F glycoform were measured from the extracted ion chromatograms (XICs; Figures 2C and 2F). A S/N of ~2,830 was obtained using the ZenoTOF 8600 system (Figure 2F). This value is >3X of the S/N (~890) measured on the ZenoTOF 7600 system. Similar sensitivity increases were also obtained for NISTmAb at other sample loads (10 ng - 500 ng; Table 3). These results demonstrate the enhanced intact RPLC-MS analysis of biotherapeutics using the ZenoTOF 8600 system. As described below, the gain in MS sensitivity offered by the ZenoTOF 8600 system enabled better detection of low-abundant proteoforms or impurities in NISTmAb.

Table 3. S/Ns* of NISTmAb measured at sample loads of 10-500 ng.

Sample load	10 ng	20 ng	50 ng	100 ng	500 ng
ZenoTOF 7600 system	50	120	420	890	2,110
ZenoTOF 8600 system	220	540	1,560	2,830	6,420
Fold change	4.4	4.5	3.7	3.2	3.0

*S/Ns were measured for the XICs of the 52+ of NISTmAb and averaged from 3 replicate injections on a single ZenoTOF 8600 system.

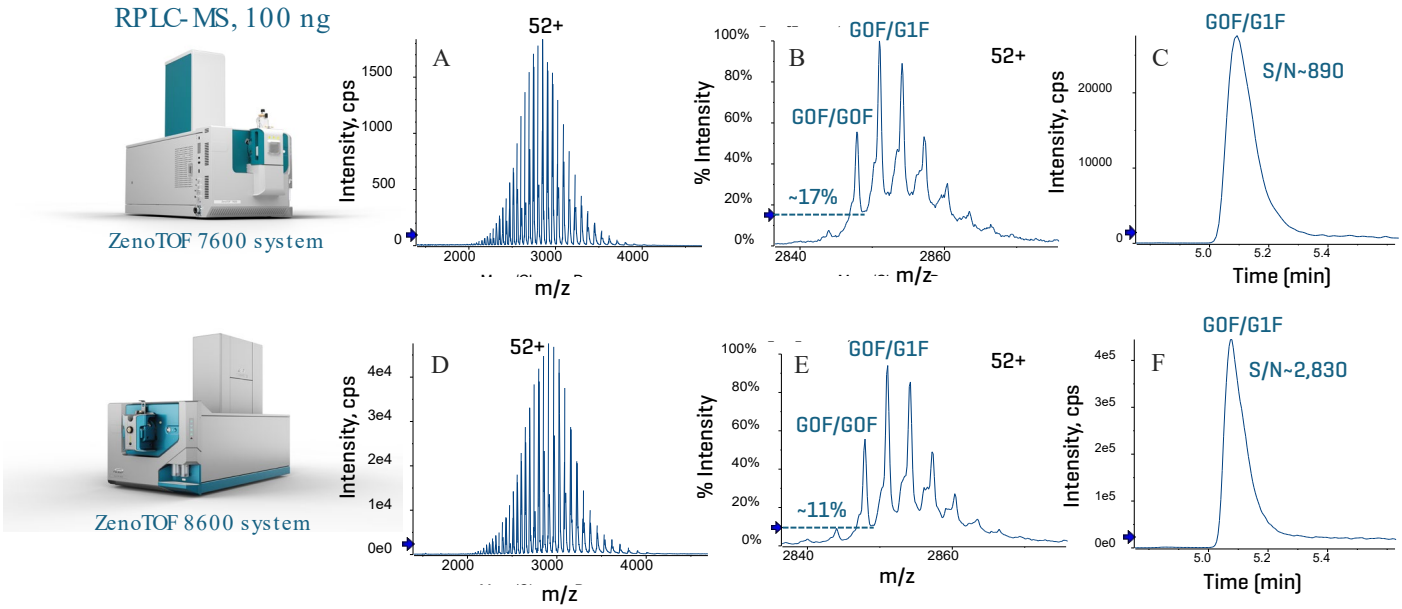

Figure 2. Intact RPLC-MS analysis of 100 ng NISTmAb. Compared to the ZenoTOF 7600 system, the ZenoTOF 8600 system provided a similar charge state distribution (A and D) but a lower "valley-to-peak" ratio (11% vs. 17% for the 52+, see B and E), i.e., better resolution between different glycoforms, as compared to the ZenoTOF 7600 system. The S/Ns measured from the XICs of the 52+ of the G0F/G1F glycoform show that the ZenoTOF 8600 system provided a >3X increase in MS sensitivity compared to the previous platform (C and F).

Native SEC-MS analysis

Compared to RPLC-MS, native MS approaches are advantageous in analyzing large molecules containing non-covalent interactions, such as protein complexes and Cys-linked ADCs,[2,3] which can dissociate under denaturing conditions. The capability of native SEC-MS for the characterization of mAbs and ADCs has been demonstrated previously using the previous QTOF system.[2] The low MS sensitivity caused by ion suppression and high sample consumption were the main challenges with the traditional native SEC-MS analysis. In this work, the benefits of MS sensitivity improvement provided by the ZenoTOF 8600 system for native SEC-MS analysis were evaluated using NISTmAb.

Figure 3 shows native SEC-MS data of 1 µg NISTmAb from the ZenoTOF 7600 system and ZenoTOF 8600 system. Similar to the RPLC-MS results described above (Figure 2), the ZenoTOF 8600 system generated a similar charge state distribution (Figures 3A and 3D) but a better resolution between the G0F/G0F and G0F/G1F glycoforms (Figures 3B and 3E) compared to the data from the previous platform. In addition, the ZenoTOF 8600 system provided a >3X increase in the S/Ns for the G0F/G1F glycoform shown in Figures 3C and 3F. This MS sensitivity improvement enabled the detection of low-abundant impurities and a reduction in sample consumption for native SEC-MS analysis.

Sensitive detection of low-abundant impurities

As described above, the hardware improvements of the ZenoTOF 8600 system led to >3X increase in S/Ns for intact RPLC-MS and native SEC-MS analyses. This sensitivity increase enabled the detection of low-abundant impurities in biotherapeutics.

Figure 4 shows selected low-abundant impurities detected by the enhanced RPLC- and SEC-MS workflows using the ZenoTOF 8600 system. The ion map within Biologics Explorer software offers excellent data visualization for a quick impurity assessment. The ion map of the RPLC-MS data of 100 ng NISTmAb reveals the presence of low-abundant LC-LC and LC-HC impurities (see solid circles in Figure 4A and selected mass spectrum of LC-LC in Figure 4C). These 2 species were only detected with much higher sample loads (≥500 ng) on the previous platform (data not shown). The ion map of the 5 µg SEC-MS data shows a NISTmAb dimer eluting earlier than the native monomer (see solid circle in Figure 4B and mass spectrum in Figure 4D). This aggregate species was not detected even with the maximum sample load (20 µg) tested in this study using the previous platform (data not shown). The oligomers observed at the same retention time as the native monomer were likely formed in the gas phase (see dashed circle in Figure 4B).

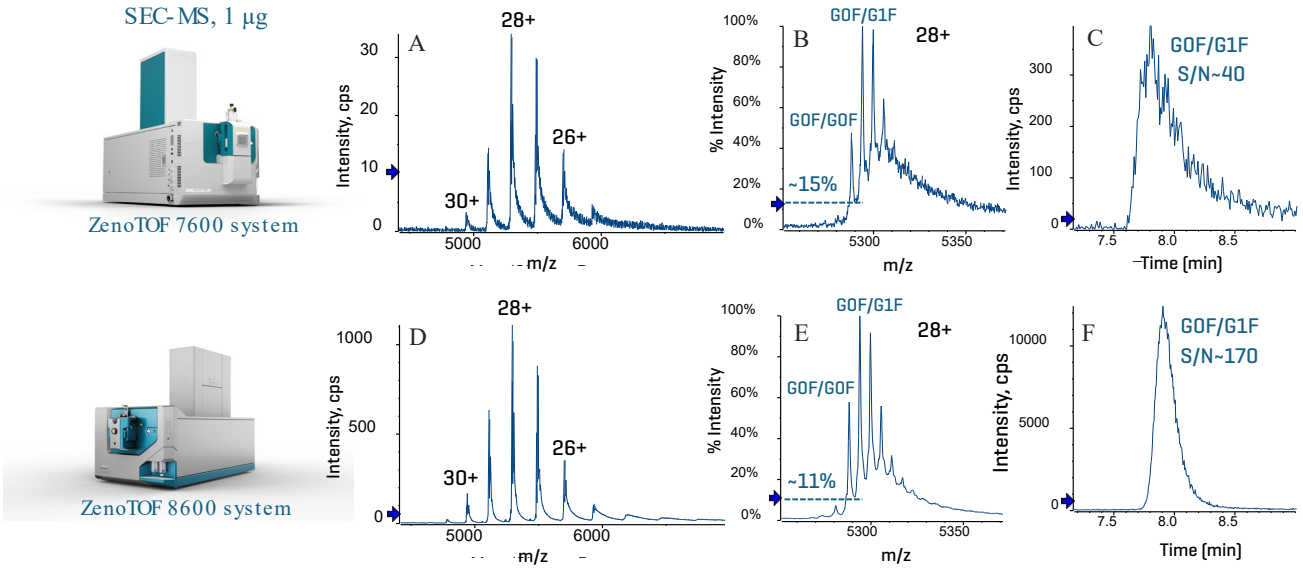

Figure 3. Native SEC-MS analysis of 1 µg NISTmAb. The ZenoTOF 8600 system provided a similar charge state distribution (A and D) but a better resolution between different glycoforms (B and E) compared to the ZenoTOF 7600 system. Additionally, the new QTOF system produced a >3X increase in the S/N of the 28+ of the G0F/G1F glycoform (C and F) compared to the ZenoTOF 7600 system.

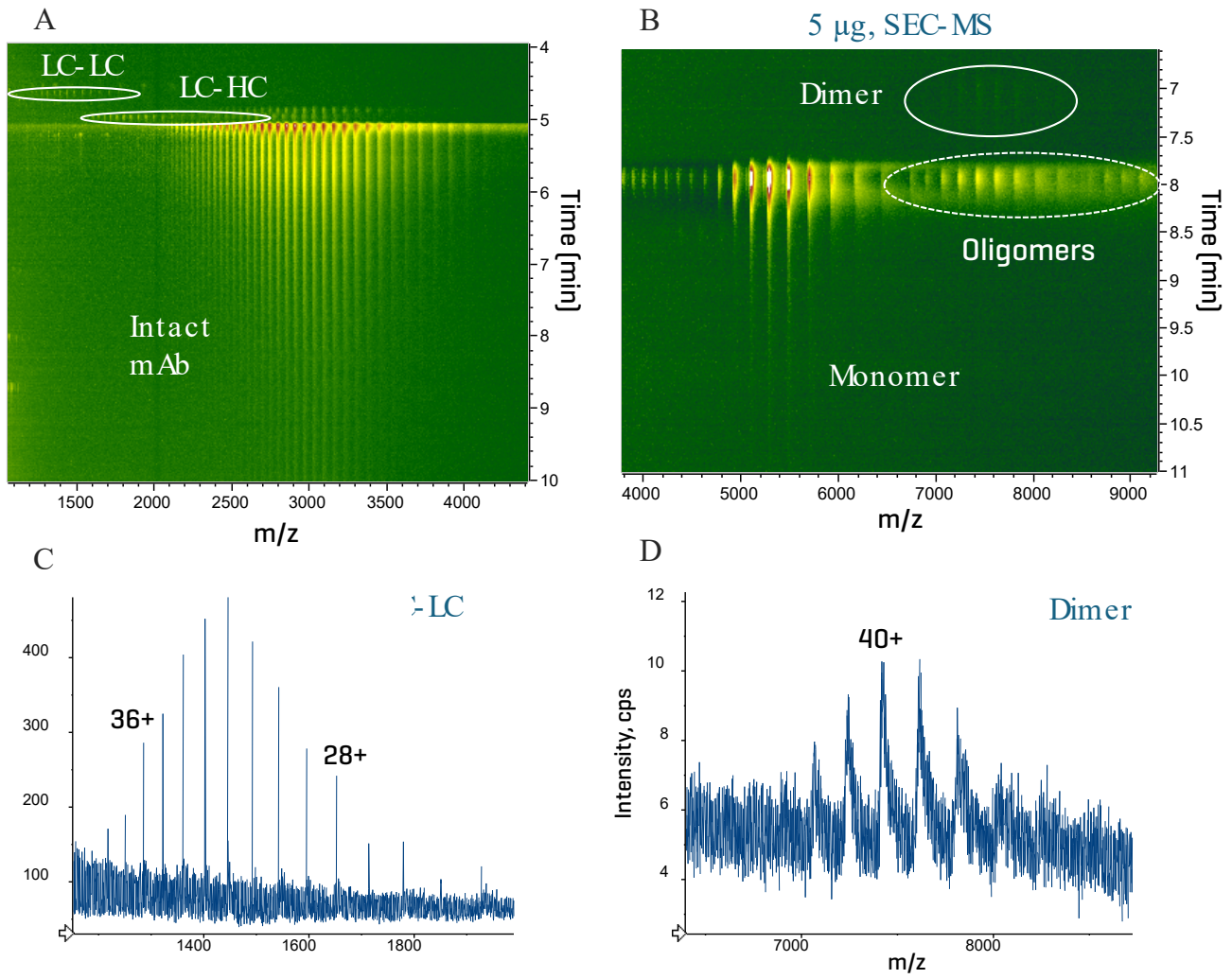

Figure 4. Detection of low-abundant impurities in NISTmAb using the enhanced RPLC-MS and native SEC-MS workflows. Biologics Explorer software provides powerful visualization tools, such as ion map, for data and result reviews. The ion map of the RPLC-MS data from the ZenoTOF 8600 system shows the presence of low-abundant LC-LC and LC-HC impurities with a 100 ng injection of NISTmAb (see solid circles in A). These 2 species were only detected with much higher sample loads (≥500 ng) on the ZenoTOF 7600 system (data not shown). A native dimer of NISTmAb, which was eluting earlier than the monomer, was detected in the 5 µg native SEC-MS data from the ZenoTOF 8600 system (see solid circle in B). This species was not detected with the maximum sample load (20 µg) tested in this study using the ZenoTOF 7600 system (data not shown). The oligomers observed at the same retention time as the native monomer were likely formed in the gas phase (see dashed circle in B). The mass spectra for the LC-LC and dimer detected in the RPLC- and SEC-MS data were shown in C and D, respectively.

Figure 5 shows the deconvolution spectra of the 100 ng RPLC-MS and 1 µg SEC-MS data of NISTmAb from Biologics Explorer software. All the major glycoforms of NISTmAb were detected and confidently annotated based on the accurate mass measurements from RPLC- and SEC-MS analyses. The MS sensitivity improvement offered by the ZenoTOF 8600 system led to the detection of low-abundant species with one aglycosylated (Agly) heavy chain, even with reduced sample loads (Figure 5).

In summary, the improvements in the ion source, optics and detector of the ZenoTOF 8600 system led to >3X sensitivity gain for superior intact RPLC-MS and native SEC-MS analyses of biotherapeutics compared to the previous platform. This sensitivity improvement enabled the detection of low-abundant proteoforms or impurities, facilitating rapid, accurate quality and impurity assessment of biotherapeutics for important decision-making during the discovery and development of biotherapeutics.

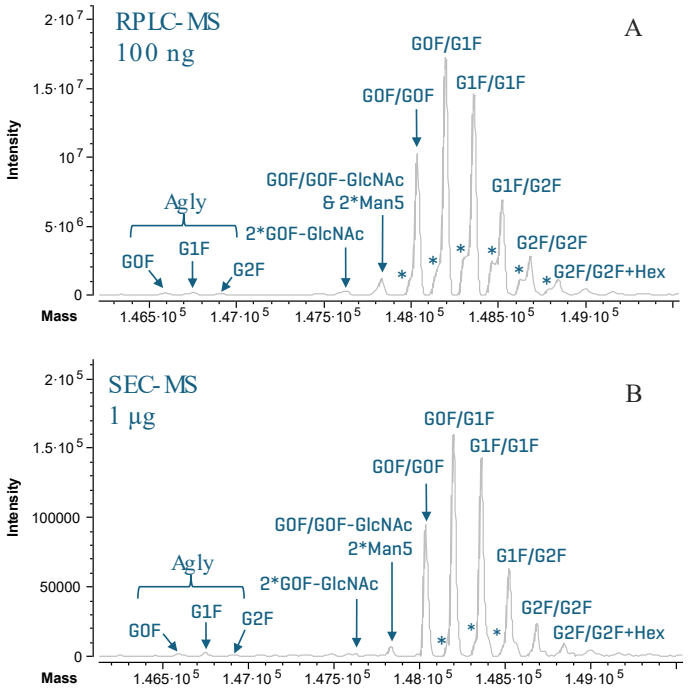

Figure 5. Deconvolution spectra from the 100 ng RPLC-MS (A) and 1 µg SEC-MS data of NISTmAb. All the major glycoforms were detected and confidently assigned for the intact RPLC-MS (A) and SEC-MS (B) data from the ZenoTOF 8600 system. The MS sensitivity gain offered by this novel QTOF system enabled the annotation of low-abundant aglycosylated species (Agly), even with reduced sample loads. The peaks labelled with "*" correspond to a mixture of 1GlcNAc- and 1Lys-loss species.

Conclusions

- The ZenoTOF 8600 system significantly increases MS sensitivity compared to the ZenoTOF 7600 system for enhanced intact mass measurement and impurity assessment of biotherapeutics.
- The MS sensitivity improvement of the ZenoTOF 8600 system compared to the ZenoTOF 7600 system enables the detection of low-abundant species or impurities even with reduced sample load, benefiting intact LC-MS analysis of samples in low concentration and/or with limited quantity.
- The ZenoTOF 8600 system provides a better peak separation between adjacent glycoforms compared to the previous platform.
- Biologics Explorer software provides excellent visualization tools for rapid data review and impurity assessment.

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