Native mass spectrometry analysis of biotherapeutics and aggregates with enhanced sensitivity

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Native mass spectrometry (MS) enables accurate mass measurement and rapid impurity assessment of intact biotherapeutics under native conditions. It was demonstrated that the ZenoTOF 8600 system provided a >3X MS sensitivity improvement for intact mass analysis compared to the previous platform.[1] This technical note focuses on native MS analysis of monoclonal antibodies (mAbs), a cysteine (Cys)-linked antibody-drug conjugate (ADC), and mAb aggregates with enhanced sensitivity (Figure 1), which reduces the sample consumption required to generate high-quality data under native conditions.

The limitations of native MS analysis currently include the need for high sample amounts and the inherent fragile nature of non-covalent complexes.[2] The ZenoTOF 8600 system addresses these challenges with hardware improvements that allow sensitive native MS analysis under soft source condition, enabling the detection of non-covalently bound assemblies, such as Cys-linked ADCs and low-abundance mAb aggregates.

Key features of enhanced native MS analysis for biotherapeutics characterization

- **High sensitivity:** Highly sensitive native MS analysis of intact biotherapeutics from sample amounts as low as 100 ng was achieved using the ZenoTOF 8600 system.
- **Enhanced analysis of Cys-linked ADCs:** The soft source condition preserves non-covalent interactions in the Cys-linked ADCs, enabling direct drug-to-antibody ratio (DAR) measurements with minimal in-source fragmentation.
- **Aggregates profiling:** The enhanced MS sensitivity and the preservation of non-covalent interactions enabled rapid analysis of mAb aggregates under native conditions.
- **Streamlined native MS workflow:** The enhanced native MS workflow is streamlined from data acquisition using SCIEX OS software to automatic data analysis using intuitive Biologics Explorer software.

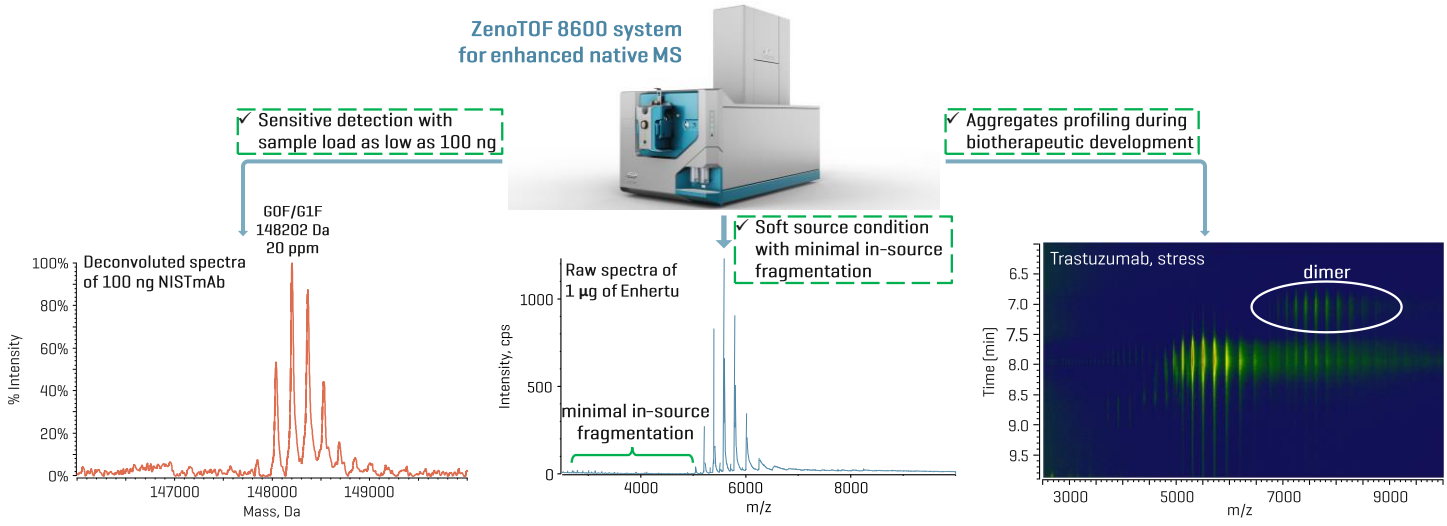

Figure 1. Sensitive native MS analysis using the ZenoTOF 8600 system. The ZenoTOF 8600 system enables highly sensitive native MS analysis of biotherapeutics with a high mass accuracy (~20 ppm) and low sample load of 100 ng (left panel). The soft source condition of the ZenoTOF 8600 system introduces minimal in-source fragmentation, preserving non-covalent interactions under native conditions, enabling accurate mass measurement and DAR determination for Cys-linked ADCs, such as trastuzumab-deruxtecan (Enhertu) (middle panel). The MS sensitivity improvement provided by the ZenoTOF 8600 system also enabled sensitive detection of a low-abundant dimer in the stressed trastuzumab (right panel).

Introduction

Native MS is a powerful analytical method to characterize the structural heterogeneity of biotherapeutic molecules. It preserves the original conformation and the non-covalent interactions, enabling the analysis of intact species and higher-order structures.[3] However, traditional native size exclusion chromatography (SEC)-MS workflows suffer from low MS sensitivity and high sample consumption.

Native MS is particularly valuable for the analysis of Cys-linked ADCs, which are generated by reducing the interchain disulfide bonds, followed by the payload conjugation to the cysteine residues. As a result, these ADCs have the intact structure held by non-covalent interactions. Maintaining structural integrity during native MS analysis is essential for these molecules, as unintended fragmentation can impact impurity assessment and DAR quantitation.[4]

Another important aspect of biotherapeutic characterization is detecting and monitoring protein aggregates, which can reduce drug efficacy and increase the risk of immunogenicity.[5] Forced degradation studies are usually performed to assess the tendency of biotherapeutics to form aggregates and identify degradation pathways. A thorough understanding and control of aggregation are essential to ensure the consistency, safety, and effectiveness of biotherapeutics.

Methods

Sample preparation: The dilution series of NISTmAb (RM8671, NIST) and Enhertu were prepared by diluting the 10 µg/µL stock solutions to concentrations ranging from 10 ng/µL to 1 µg/µL prior to native SEC-MS analysis.

The stressed trastuzumab sample for aggregate analysis was prepared by incubating 1 µg/µL of the mAb in ammonium acetate (pH 3.6) at 50°C with shaking at 700 rpm for 20 hours. The resulting solution was directly injected for native SEC-MS analysis.

Size-exclusion chromatography (SEC): Native SEC separation was conducted using an ACQUITY UPLC Protein BEH SEC column (4.6 × 150 mm, 1.7 µm, 200 Å; Waters) with an isocratic gradient. The mobile phase consists of 20 mM ammonium acetate. The total run time was 15 minutes with a flow rate of 0.15 mL/min.

Mass spectrometry: The native SEC-MS data were acquired using the ZenoTOF 8600 system (SCIEX). The key source and TOF MS settings are shown in Table 1.

Table 1: Source and TOF MS parameters for native SEC-MS analysis.

Parameter	SEC-MS
Workflow	Intact proteins
Start mass	2,500 Da
Stop mass	10,000 Da
Spray voltage	3,500 V
Curtain gas	40 psi
CAD gas	9
Ion source gas 1	60 psi
Ion source gas 2	60 psi
Source temp	250°C-300°C
QJet DP	120 V
Collision energy	12 V
Accumulation time	0.25 s
Time bins to sum	120

Data analysis: The 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 were specified for NISTmAb and trastuzumab. For Enhertu, the conjugation is defined as a gain of molecular formula $C_{52}H_{56}FN_9O_{13}$, with a maximum of 8 conjugates.

Sensitive detection with low sample consumption

The ZenoTOF 8600 system provides a >3X MS sensitivity improvement for intact mass analysis, enhancing the detection of low-abundant species with reduced sample consumption.[1] In this work, a dilution series of NISTmAb was prepared to test different sample loads ranging from 100 ng to 5 µg with native SEC-MS analysis.

Figure 2 shows the representative native SEC-MS data of NISTmAb from 1 µg and 100 ng sample injections. The ZenoTOF 8600 system produced an excellent signal of different glycoforms and consistent charge state distributions for both sample loads (Figures 2A and 2B). By comparison, intact NISTmAb was barely or not detected with 1 µg or 100 ng sample injections, respectively, using native SEC-MS on the previous platform (data not shown). The background-subtracted spectra revealed a low valley-to-peak ratio of ~10% between the G0F/G0F and G0F/G1F glycoforms for both sample loads, indicating sufficient desolvation of the intact molecule. Both spectra were successfully deconvoluted, generating nearly identical deconvoluted spectra with clear identification of major glycoforms. The most abundant glycoform G0F/G1F showed good mass accuracy, measured at 148,202 Da with a mass error of ~20 ppm (Figure 2C).

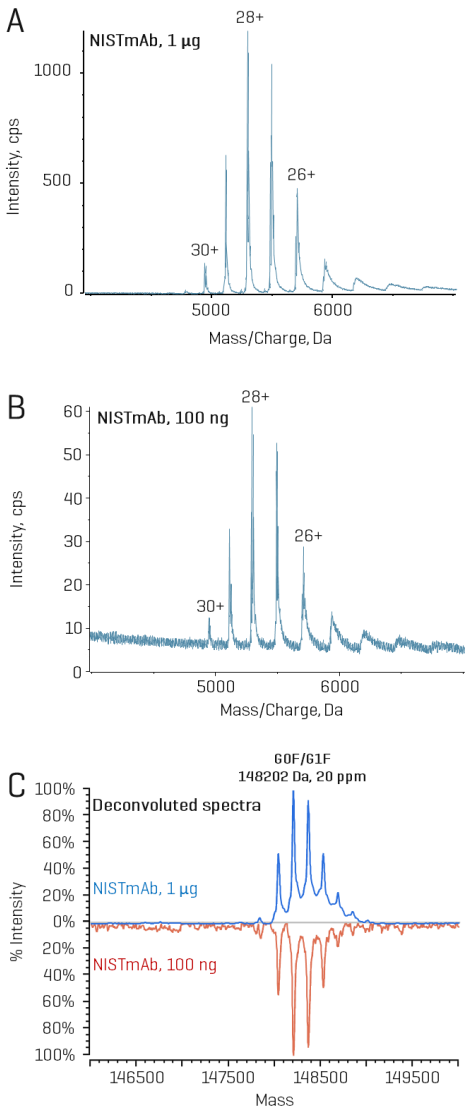

Figure 2. Sensitive native SEC-MS analysis of NISTmAb. Native SEC-MS analysis of NISTmAb was performed using different sample loading amounts. The mass spectra from 1 µg (A) and 100 ng (B) sample injections were shown as representative examples. Similar charge state distributions and valley-to-peak ratios (~10%) between the G0F/G0F and G0F/G1F glycoforms were observed in the 2 spectra (A and B). The deconvoluted mass spectra (C) are also highly similar between the two conditions, with a good mass accuracy of ~20 ppm obtained for the most abundant glycoform, demonstrating the sensitivity and reliability of native MS analysis at low sample loads.

Enhanced native SEC-MS analysis of a Cys-linked ADC

Cys-linked ADCs, such as Enhertu, consist of an antibody whose Cys residues involved in inter-chain disulfide bonds are conjugated with cytotoxic payloads. The light and heavy chains (LC and HC) of Cys-linked ADCs are held together by non-covalent interactions, which can be disrupted under the denaturing conditions employed for RPLC separation. By comparison, the intact form of a Cys-linked ADC is preserved under the native SEC-MS condition. However, traditional native SEC-MS approaches suffer from low sensitivity and require a large amount of samples (>10 µg) for the detection of intact Cys-linked ADCs.[6] Here, the enhanced MS sensitivity provided by the ZenoTOF 8600 system was leveraged to analyze Enhertu with a significant reduction in sample consumption. Figure 3 shows the native SEC-MS data of Enhertu with 1 µg and 100 ng loading amounts. The 1 µg data acquired at a source temperature of 250°C shows minimal in-source fragmentation

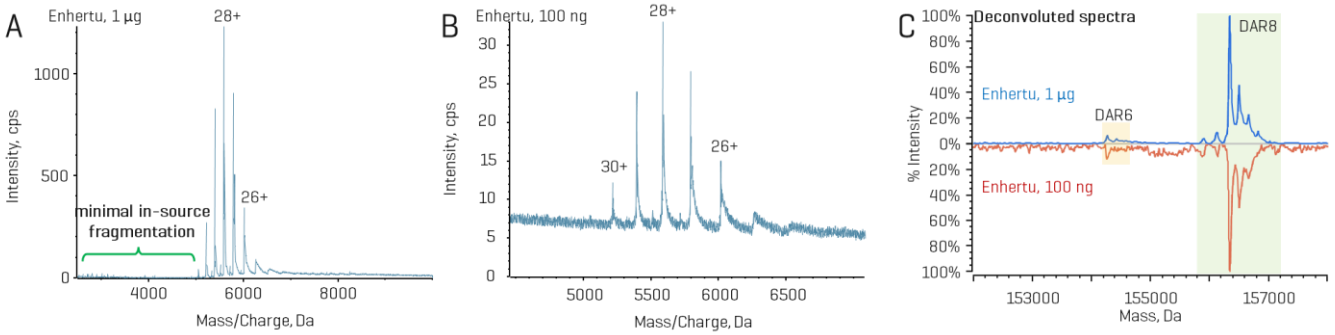

Figure 3. Sensitive native SEC-MS analysis of Enhertu–a Cys-linked ADC. Native SEC-MS analysis of 1 µg Enhertu at a source temperature of 250°C shows minimal in-source fragmentation in the low mass range (A). The MS sensitivity improvement provided by the ZenoTOF 8600 system led to the detection of intact Enhertu at a low sample load of 100 ng (B). Similar results were observed between the 1 µg and 100 ng sample loads, as revealed in the mass spectra (A and B) and the deconvoluted results (C), with consistent detection of the DAR8 and low-abundant DAR6 species.

due to the soft source condition of the ZenoTOF 8600 system (Figure 3A). It should be noted that the source parameters, such as QJet declustering potential and source temperature, can be fine-tuned to minimize ion-source fragmentation depending on the stability of the analyte. The MS sensitivity improvement provided by the ZenoTOF 8600 system led to the detection of intact Enhertu at sample loads as low as 100 ng (Figure 3B). The deconvoluted spectra for both sample loads confirmed that the major Enhertu proteoforms carry a DAR of 8 (Figure 3C). The improvement in MS sensitivity also enabled the detection of a low-abundant DAR6 species (Figure 3C).

Aggregate analysis using native SEC-MS

As described above, the ZenoTOF 8600 system provides MS sensitivity improvement, compared to the previous platform, for enhanced native SEC-MS analysis of mAbs and ADCs with reduced sample consumption. This increased sensitivity also enables the detection of low-abundance impurities in biotherapeutics. In this study, the forced degradation sample of trastuzumab was prepared under low pH and elevated temperature conditions and evaluated for the stress-induced aggregation using the enhanced native SEC-MS workflow.

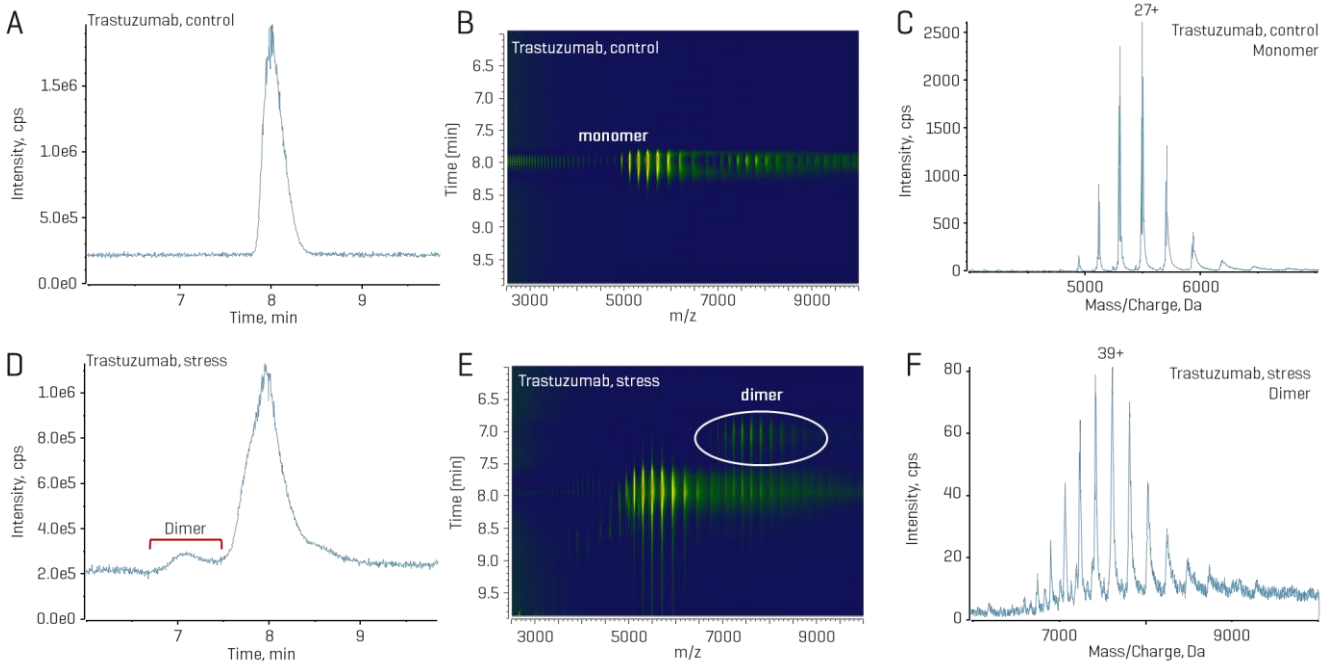

Figure 4. Aggregate analysis of the control and stressed trastuzumab samples using native SEC-MS. The TIC of the control sample exhibits a single peak corresponding to monomeric trastuzumab (A), while the stressed sample shows an additional peak at an earlier retention time that corresponds to the dimer species (B). The native monomer and dimer detected in the control and stressed samples, respectively, were confirmed in the corresponding mass spectra (C and F). The ion map of the Biologics Explorer software provides an excellent visualization of different species in the control and stressed samples (B and E). The dimer was observed in the stressed sample but not in the control sample, which was clearly visualized in the ion map (sold circle in E).

Figure 4 presents the native SEC-MS results of the control and stressed trastuzumab samples analyzed using the ZenoTOF 8600 system. The total ion chromatograms (TICs) clearly show a single, distinct peak in the control sample (Figure 4A), indicating the presence of monomers only. In contrast, the TIC of the stressed sample displays an additional peak eluting earlier than the main peak that corresponds to the dimer species (Figure 4D). The native trastuzumab monomer and dimer detected in the control and stressed samples, respectively, were confirmed in the corresponding mass spectra (Figures 4C and 4F). The ion map of the Biologics Explorer software enables excellent data visualization for rapid aggregate analysis and impurity assessment (Figures 4B and 4E).

Conclusions

- The ZenoTOF 8600 system demonstrates excellent MS sensitivity for enhanced native SEC-MS analysis of biotherapeutics with reduced sample consumption.
- The soft source condition enables the detection of intact Cys-linked ADCs with minimal in-source fragmentation, leading to accurate DAR measurement.
- The enhanced MS sensitivity enables rapid aggregate analysis and impurity assessment using native SEC-MS.
- The ZenoTOF 8600 system, coupled with SCIEX OS software and Biologics Explorer software, provides a streamlined native SEC-MS workflow from data acquisition to result visualization.

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