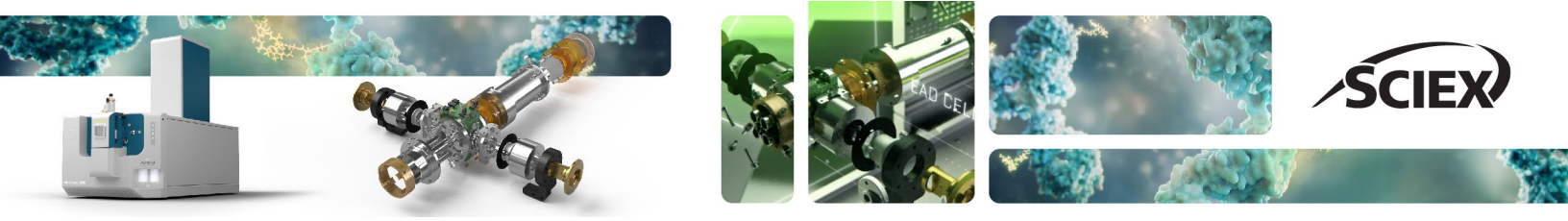

In-depth characterization of monoclonal antibodies and antibody-drug conjugates using native and middle-down mass spectrometry

Haichuan Liu and Zoe Zhang

SCIEX, USA

This technical note highlights the combined power of native mass spectrometry (MS) and electron activated dissociation (EAD)-based middle-down workflows for rapid and comprehensive characterization of monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) using a single MS platform.

Native MS offers 2 main advantages over reverse-phase liquid chromatography (RPLC)-MS for mAb and ADC analysis.[1-3] First, non-covalent interactions are preserved during native MS analysis, allowing intact mass measurement of Cys-linked ADCs that may dissociate in the denaturing condition. Second, native MS leads to the formation of high m/z ions with low charge states, increasing the spectral space between different species,[1] which facilitates the characterization of biotherapeutics with high complexity. As an orthogonal approach to native MS, an EAD-based middle-down workflow has proven highly effective for biotherapeutic sequence analysis, post-translation modification (PTM) localization and intrachain disulfide bond mapping.[4-8]

In this work, the size exclusion chromatography (SEC) based native MS and EAD-based middle-down workflows were leveraged to characterize mAbs and ADCs using the ZenoTOF 7600 system. For trastuzumab deruxtecan (T-DXd)—a Cys-linked ADC—native MS enables rapid detection of intact molecule and determination of the drug antibody ratio (DAR), while an EAD-based middle-down workflow provided confident localization of the payload conjugation sites.

Key features of native MS and EAD-based middle-down workflows

- **Simple sample preparation:** Native MS and middle-down workflows involve simpler sample preparation than traditional peptide mapping.
- **Preservation of non-covalent interactions in native MS:** Non-covalent interactions in the Cys-linked ADCs are preserved in the native MS condition, enabling direct DAR measurement of these challenging molecules.
- **Power of EAD**: EAD-based middle-down workflow leads to high sequence coverage, accurate localization of labile PTMs and rapid mapping of intrachain disulfide bonds.
- **Platform assays**: Native MS and EAD-based peptide mapping workflows are streamlined and can be implemented for routine biotherapeutic characterization by different levels of LC-MS users.

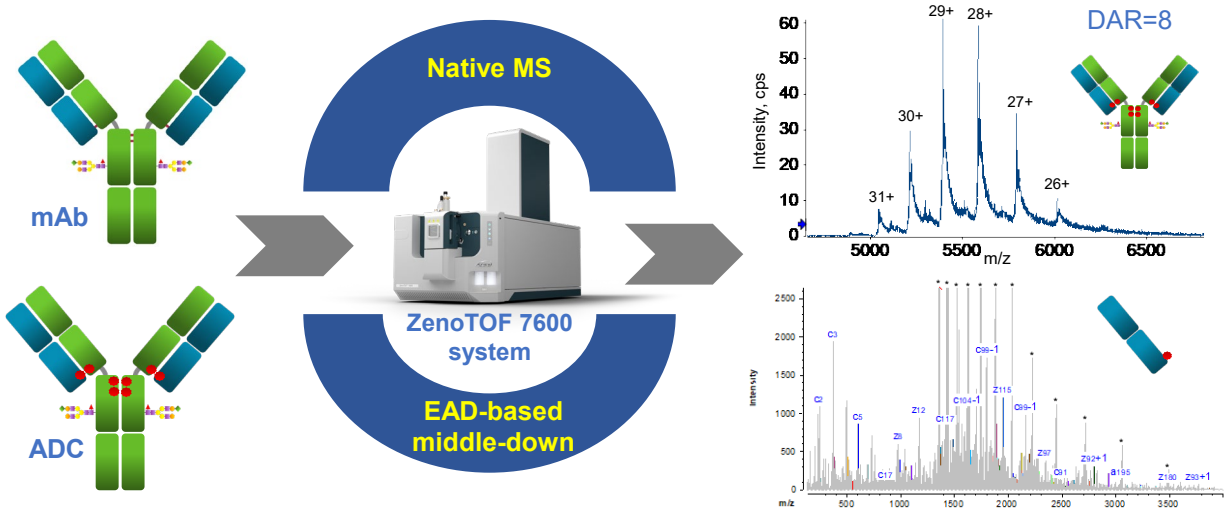

Figure 1. Rapid confirmation of antibody sequences and determination of payload conjugation sites by the combined power of native MS and EAD-based middle-down workflows on the ZenoTOF 7600 system. The native SEC-MS workflow provides an accurate mass measurement of mAbs and ADCs under the native condition, leading to direct DAR determination of Cys-linked ADCs that may dissociate under the denaturing condition, such as T-DXd. The EAD-based middle-down workflow enables rapid sequence confirmation, PTM and payload localization and disulfide bond mapping. * represents the payloads in an ADC.

Methods

Sample preparation: 25-50 µg of 5 µg/µL mAbs (NISTmAb and infliximab) and ADCs [trastuzumab emtansine (T-DM1) and T-DXd] were injected for SEC separation followed by native MS analysis. 10 µg of T-DXd was injected for RPLC-MS analysis and middle-down fragmentation using EAD.

Chromatography: RPLC separation of T-DXd was performed at a flow rate of 0.3 mL/min using an ExionLC AD system (SCIEX) installed with an ACQUITY UPLC Protein BEH C4 column (2.1 × 50 mm, 1.7 µm, 300 Å, Waters). Table 1 shows the gradient used for RP separation. 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 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.

Table 1. LC gradient for RP separation of T-DXd.

Time (min)	A (%)	B (%)
Initial	80	20
2	80	20
14	60	40
15	10	90
17	10	90
17.5	80	20
20	80	20

Data acquisition:

Native, RPLC and middle-down MS data were all acquired using the ZenoTOF 7600 system (SCIEX). Table 2 shows source and TOF MS settings for native and RPLC MS analyses. The LC subunit of T-DXd generated during RPLC separation was targeted for EAD fragmentation in an MRM^{HR} experiment. A kinetic energy of 1 eV and a reaction time of 10 ms were applied to the 18+ charge state of the LC subunit containing 2 intra-chain disulfide bonds. Table 3 shows key MRM^{HR} parameters used for middle-down analysis.

Table 2. TOF MS parameters for native and RPLC MS LC MS analyses.

Parameter	RPLC	Native
Spray voltage	5500 V	
Curtain gas	35	30
CAD gas	7	12-15
Ion source gas 1	50 psi	70 psi
Ion source gas 2	50 psi	70 psi
Source temperature	400°C	250-400°C*
TOF start mass	500 m/z	1000 m/z
TOF stop mass	3,000 m/z	15,000 m/z
Accumulation time	0.1 s	0.5 s
Declustering potential	80 V	290 V
Collision energy	10 V	12 V
Time bins to sum	8	80

* Varied during method optimization. Final settings are 300°C for NISTmAb and infliximab and 250°C for T-DXd.

Table 3. MRM^{HR} parameters using EAD.

Parameter	Value
Start mass	100 m/z
Stop mass	4000 m/z
Q1 resolution	Low
Zeno trap	ON
Zeno threshold	100,000 cps
Accumulation time	0.2 s
Declustering potential	80 V
CE	12 V
Time bins to sum	8
Electron beam current	5,000 nA
Electron KE	1 eV
ETC	100%
Reaction time	10 ms
EAD RF	150 Da

Data analysis: Intact and subunit mass analyses were conducted using an intact workflow template in Biologics Explorer software. EAD MRM^{HR} data were interpreted using the middle-down workflow template.

Method optimization of native MS

Native MS provides a rapid mass measurement of intact biotherapeutics under native conditions. The formation of lower charge states under the native vs. denaturing condition leads to more m/z separation and better detection of different species in the mass spectrum.[1] Additionally, non-covalent interactions are preserved during native MS analysis,[1-3] allowing intact mass measurement of Cys-conjugated ADCs that may dissociate in the denaturing condition. In this work, an SEC-based native MS workflow was employed to characterize 2 mAbs (NISTmAb and infliximab) and 2 ADCs (Lys-conjugated T-DM1 and Cys-conjugated T-DXd).

SCIEX OS software provides an intuitive user interface for optimizing the MS and source conditions of the ZenoTOF 7600 system to achieve optimal native MS results. The key parameters to optimize include source gas settings and source temperature (Tables 2 and 3). Compared to RPLC analysis, native MS required more source gas flows and lower source temperature (Table 2). Figure 2 shows the effect of source temperature on the native MS signal of NISTmAb. The increase of the source temperature from 300°C to 500°C led to more extensive formation of the LC subunit and unfolded NISTmAb at m/z below ~5,000 (Figure 2). The ion map in Biologics Explorer software offers an excellent visualization of the changes with the intact profiles (insets of Figure 2). It was determined that a source temperature of 250-300°C provided sufficient desolvation of mAbs and ADCs while keeping gas-phase denaturation to the minimum. The optimized native MS method was employed to characterize 2 mAbs and 2 ADCs, as will be described in the following sections.

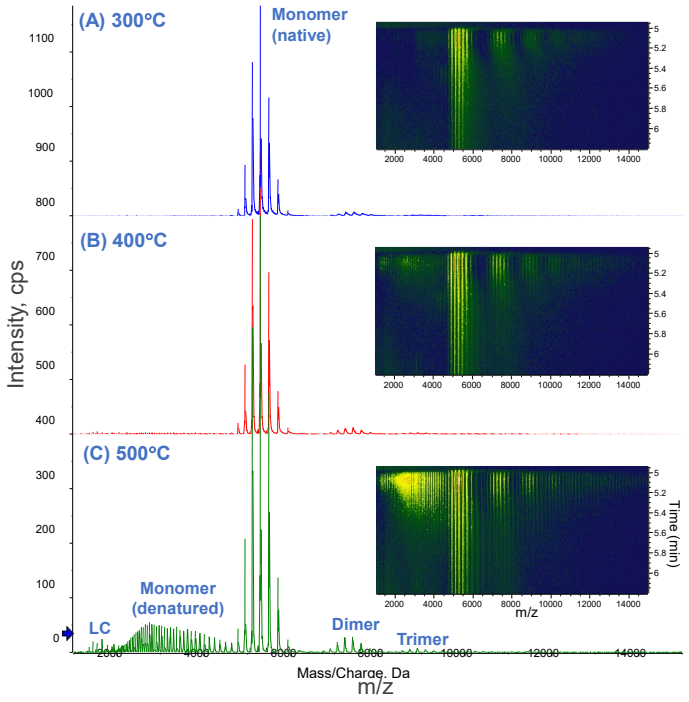

Figure 2. Effect of source temperature on native MS analysis of NISTmAb. The major charge states of native NISTmAb were detected in the m/z range of ~5,000-6,000 (A-C). No LC and denatured species were detected at a source temperature of 300°C or below (A). Gas-phase denaturation was boosted with increasing source temperatures (B and C). Insets are the ion maps from Biologics Explorer software. The ion map provides an excellent visualization of the changes in biotherapeutics.

Native MS analysis of mAbs

Figure 3 shows the deconvoluted spectra of NISTmAb and infliximab from native MS analyses. The major glycoforms and C-terminal Lys variants are annotated in the deconvoluted spectra together with the measured mass values and errors. While G0F/G0F, G0F/G1F and

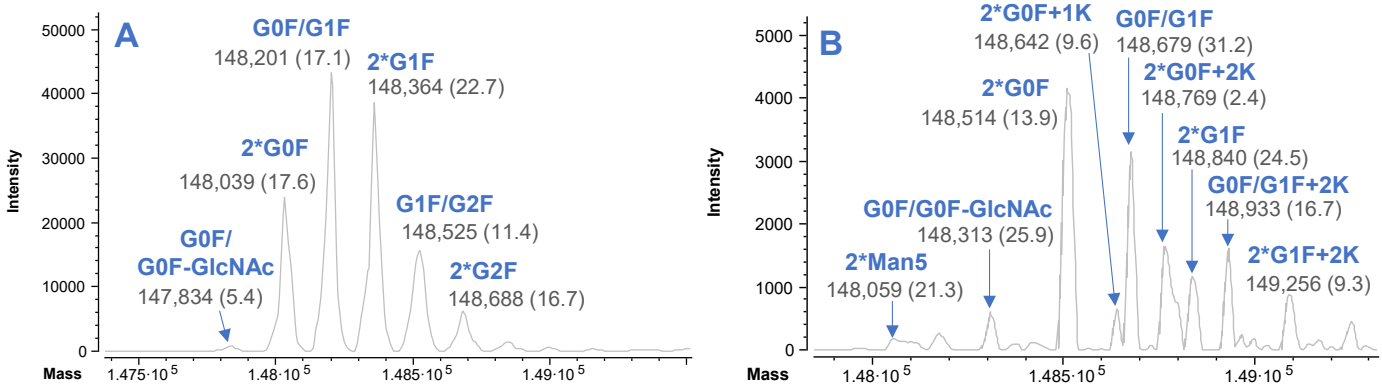

Figure 3. Deconvoluted results of NISTmAb and infliximab from native MS analyses. Native MS analyses performed using the ZenoTOF 7600 system provided rapid detection of intact glycoforms and C-terminal Lys (K) variants of NISTmAb (A) and infliximab (B) with high mass accuracy. A much higher level of C-terminal Lys variants was observed for infliximab (B) compared to NISTmAb (A). Mass errors are provided in parentheses.

G1F/G1F were the dominant glycoforms detected in NISTmAb and infliximab, these species were present in different ratios in 2 mAbs (Figure 3). Native MS results also provided rapid detection of abundant C-terminal Lys variants in infliximab (Figure 3B). Detailed characterization of infliximab and its charge variants using orthogonal peptide mapping, middle-down and icIEF-UV/MS workflows will be described in a separate technical note.

Native MS analysis of T-DM1

ADCs are highly complex biotherapeutics consisting of mAbs conjugated with cytotoxic payloads through chemical linkers. One of the challenges with ADC characterization is high sample heterogeneity. Traditional Lys-linked ADCs, such as T-DM1, show a highly complex MS profile due to the presence of various glycoforms and a broad DAR distribution (DAR as high as 8 for T-DM1).[9] Native MS offers advantages over RPLC-MS approaches for analyzing Lys-linked ADCs with heterogenous DAR by generating lower charge states in the higher m/z region,[1] enabling direct analysis of these ADCs without the deglycosylation treatment.

Figure 4 shows the native MS result of Lys-linked T-DM1. Despite high sample heterogeneity, native MS analysis led to the detection of multiple glycoforms carrying up to 8 payloads (DM1). The deconvolution of the native MS spectrum resulted in an average DAR of 3.5 for T-DM1, in good agreement with the values reported previously.[9]

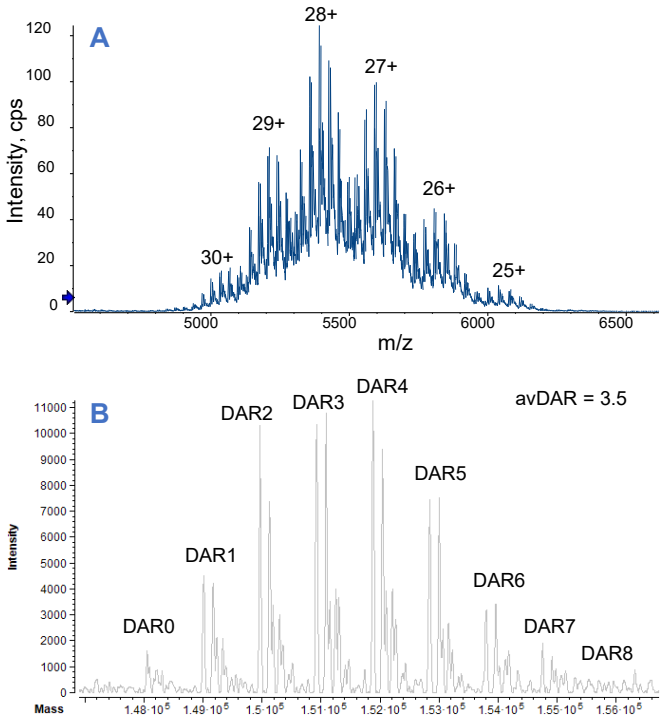

Figure 4. Native MS analysis of Lys-conjugated T-DM1. Native MS led to the detection of intact T-DM1 carrying up to 8 payloads in the m/z range of ~4,500-6,500 (A). An average DAR (avDAR) of 3.5 was determined from the deconvoluted result of T-DM1 using Biologics Explorer software (B). This DAR value is in consistent with those reported previously.[9]

Characterization of T-DXd by RPLC and native MS workflows

Another challenge with ADC characterization using RPLC-MS is that Cys-linked ADCs, such as T-DXd, can fall apart under the denaturing condition. T-DXd is composed of trastuzumab where Cys residues involved in the formation of inter-chain disulfide bonds are reduced and conjugated with cytotoxic payloads (DXd), resulting in a homogenous DAR of 8.[3] The LC and HC in T-DXd are held together non-covalently due to the lack of inter-chain disulfide bonds. This non-covalent interaction can be disrupted under the denaturing condition employed for RPLC separation, leading to the dissociation of T-DXd into LC and HC subunits. By comparison, the intact form of T-DXd can be preserved under the native MS condition.

Figure 5 shows the RPLC-MS result of T-DXd. Under the condition used for RPLC separation, T-DXd was denatured and dissociated into LC and HC subunits (Figure 5A). Accurate mass measurement of these 2 subunits revealed the presence of 1 and 3 DXd in LC and HC, respectively (Figure 5B-5E). Based on this result, a DAR of 8 can be calculated for T-DXd, in agreement with the previous report.[3]

Figure 6 shows the native MS spectrum and deconvoluted result of T-DXd. While T-DXd fell apart during RPLC separation (Figure 5), its intact form was preserved under the native condition (Figure 6A), allowing direct mass measurement and DAR calculation for this ADC. The deconvolution of the native MS spectrum led to a rapid determination

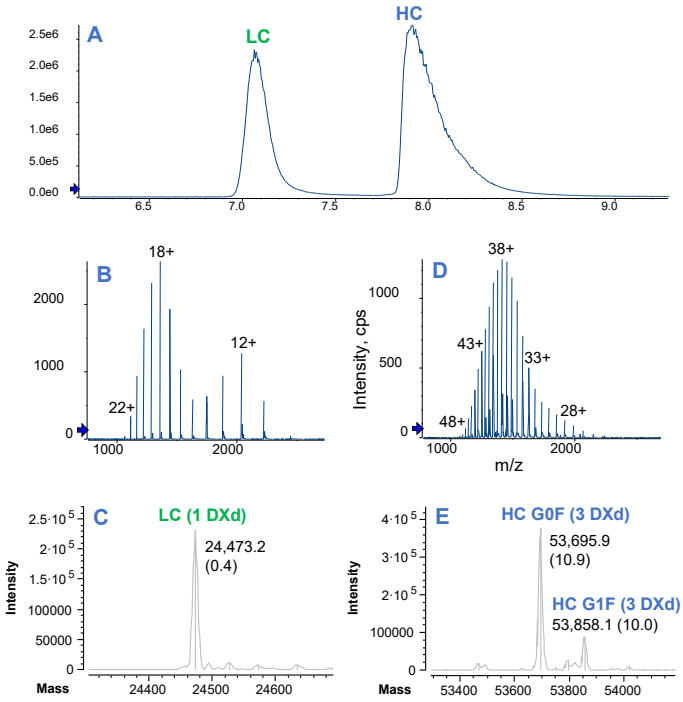

Figure 5. RPLC-MS analysis of Cys-linked T-DXd. Under the denaturing condition employed for RPLC separation, T-DXd was dissociated to produce LC and HC (A). Accurate mass measurement of these 2 subunits (B-E) confirmed the presence of 1 and 3 DXd payloads in LC and HC, respectively. Mass errors are provided in parentheses.

of DAR=8 for T-DXd (Figure 6B). This result is consistent with those obtained from RPLC-MS analysis (Figure 5) and reported in the literature.[3]

Characterization of T-DXd subunits by EAD-based middle-down MS

The capabilities of the EAD-based middle-down workflow for sequence confirmation, PTM localization and disulfide bond mapping have been well documented in the previous technical notes.[4-8] This powerful EAD approach was leveraged to confirm the conjugation sites of the payload in T-DXd. Figure 7 shows the EAD spectrum of the disulfide-linked LC subunit of T-DXd detected in RPLC-MS analysis (Figure 5). The detection of *z*-series fragments, such as z_8 shown in the inset of Figure 7, led to the confident determination of the payload conjugation with the C-terminal Cys residue. In addition to payload localization, the EAD-based middle-down workflow provides confident sequence and disulfide linkage confirmation for T-DXd subunits, as will be described in detail in a separate technical note.

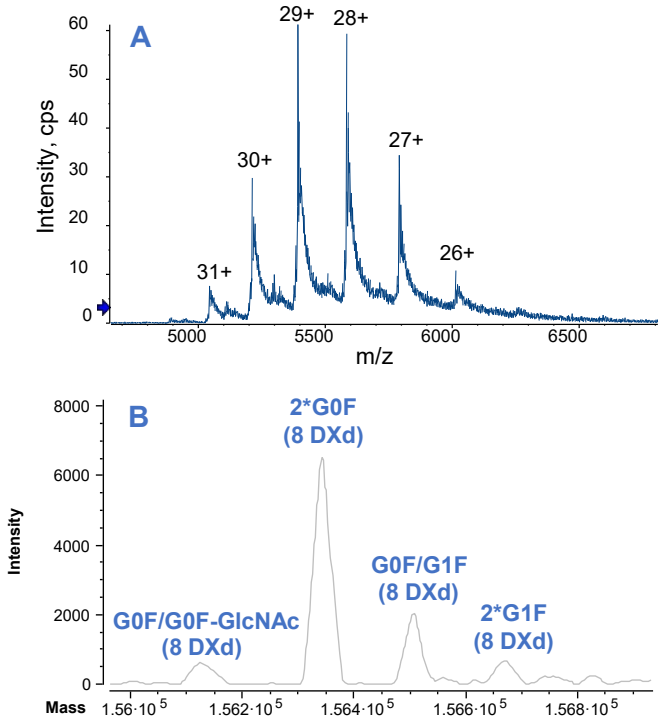

Figure 6. Native MS analysis of T-DXd. The intact form of T-DXd was preserved under the native condition during native MS analysis (A). The deconvoluted result confirmed the presence of 8 DXd in T-DXd (B). Mass errors are provided in parentheses.

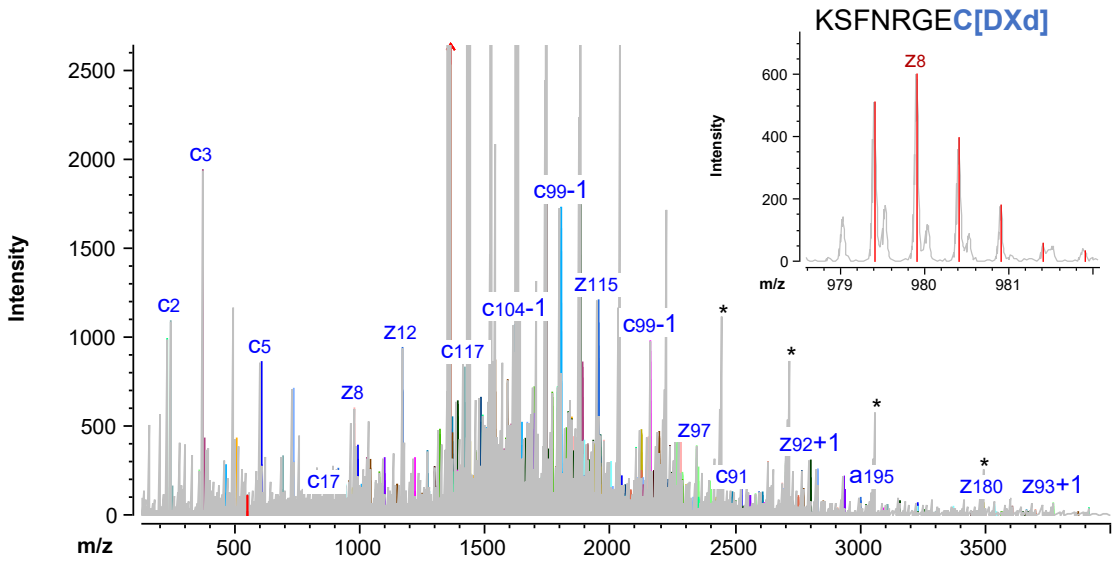

Figure 7. Middle-down analysis of the disulfide-linked LC subunit of T-DXd using EAD. EAD resulted in extensive fragmentation of the disulfide-linked LC subunit of T-DXd. The detection of *z*-series fragments, such as z_8 shown in the inset, confirmed the payload conjugation of the C-terminal Cys residue. A detailed description of the middle-down results of T-DXd subunits will be published in a separate technical note. The peaks labeled with * are the precursor or charge-reduced species.

Conclusions

- The ZenoTOF 7600 system provides a single-instrument solution to obtain high-quality native MS and middle-down data for rapid characterization of mAbs and ADCs with confidence
- Native MS offered accurate mass measurement of biotherapeutics and rapid DAR measurement of ADCs with minimal sample preparation
- The Cys-linked T-DXd was dissociated into LC and HC under the denaturing condition during RPLC-MS analysis, whereas noncovalent interactions in this ADC were preserved in native MS, leading to direct DAR determination
- EAD-based middle-down workflow offered high-quality fragmentation data for sequence confirmation and payload localization
- Native MS and EAD-based middle-down workflows are streamlined and can be rapidly adopted to meet different needs for biotherapeutic characterization, including intact mass measurement, DAR analysis for ADC, sequence confirmation, PTM and payload localization and disulfide bond mapping

References

1. Iain Campuzano and Wendy Sandoval. (2021) Denaturing and native mass spectrometric analytics for biotherapeutic drug discovery research: historical, current, and future personal perspectives. *J. Am. Soc. Mass Spectrom.* **32**(8): 1861-1885.
2. Amber Rolland and James Prell. (2022) Approaches to heterogeneity in native mass spectrometry. *Chem Rev.* **122**(8): 7909-7951.
3. Desligniere E *et al.* (2022) A combination of native LC-MS approaches for the comprehensive characterization of the antibody-drug conjugate trastuzumab deruxtecan. *Front. Biosci. (Landmark Ed)* **27**(10):2905.
4. An enhanced single-injection middle-down workflow to achieve high sequence coverage and disulfide mapping of antibody subunits. SCIEX technical note, MKT-30575-A.
5. A streamlined single-injection middle-down workflow using electron activated dissociation (EAD) for biotherapeutics characterization. SCIEX technical note, MKT-26997-A.
6. Obtaining high sequence coverage and confident post-translational modification (PTM) analysis of biotherapeutics using an electron activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27223-A.
7. Confident sequence analysis of a trispecific antibody using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-27784-A.
8. Achieving ultrahigh sequence coverage and high-confidence disulfide bond mapping of biotherapeutics using an electron-activated dissociation (EAD)-based middle-down workflow. SCIEX technical note, MKT-28565-A.
9. Charge variant analysis of antibody-drug conjugates using an icIEF-UV/MS workflow. SCIEX technical note, MKT-29837-A.

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