Comprehensive characterization of a cysteine-linked antibody-drug conjugate using electron-activated dissociation (EAD)

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Electron-activated dissociation (EAD) is a fast and sensitive MS/MS approach for biotherapeutic sequence characterization, payload and post-translation modification (PTM) localization, disulfide bond mapping and amino acid isomer differentiation. In this work, the reduced and non-reduced EAD-based peptide mapping workflows were leveraged to comprehensively characterize a Cys-linked antibody-drug conjugate (ADC). The high-quality EAD data provided high sequence coverage, accurate drug-antibody ratio (DAR) determination, precise payload and PTM localizations, confident disulfide bond mapping and clear differentiation of amino acid isomers.

Comprehensive characterization of antibody sequence, drug payload and PTMs is essential to ensuring the quality, safety and efficacy of ADC products.[1,2] Peptide mapping is a common approach for the comprehensive characterization of antibody-based therapeutics in addition to intact mass analysis.[3-10] Collision-based MS/MS techniques employed by traditional peptide mapping approaches cause extensive fragmentation of the payload and cleavage of labile PTMs, leading to the loss of site-specific information about these important moieties. By comparison, EAD can preserve labile modifications for their accurate localization.[3-5] EAD can also generate signature fragments for unambiguous differentiation of amino acid isomers, such as Asp *vs.* isoAsp and 3- *vs.* 4-hydroxyproline.[6-8] Additionally, EAD provides superior results for disulfide bond mapping compared to collision-based MS/MS approaches.[9]

Key features of EAD for comprehensive characterization of ADCs

- **High sensitivity and sequence coverage:** Zeno EAD is highly sensitive and can be used as a single-injection DDA method to obtain complete protein sequence coverage.
- **Accurate localization:** EAD preserves labile PTMs and ADC payloads for their accurate localizations.
- **Disulfide bond mapping:** EAD cleaves the peptide backbone and S-S bond in disulfide-linked peptides, leading to extensive sequence fragmentation for increase confidence in identification.
- **Amino acid isomer differentiation:** EAD generates signature fragments for clear differentiation of amino acid isomers, such as as Asp *vs.* isoAsp and Leu *vs.* Ile.
- **Minimal optimization**: EAD methods require minimal development and optimization and are easy to implement by users with different MS experience.

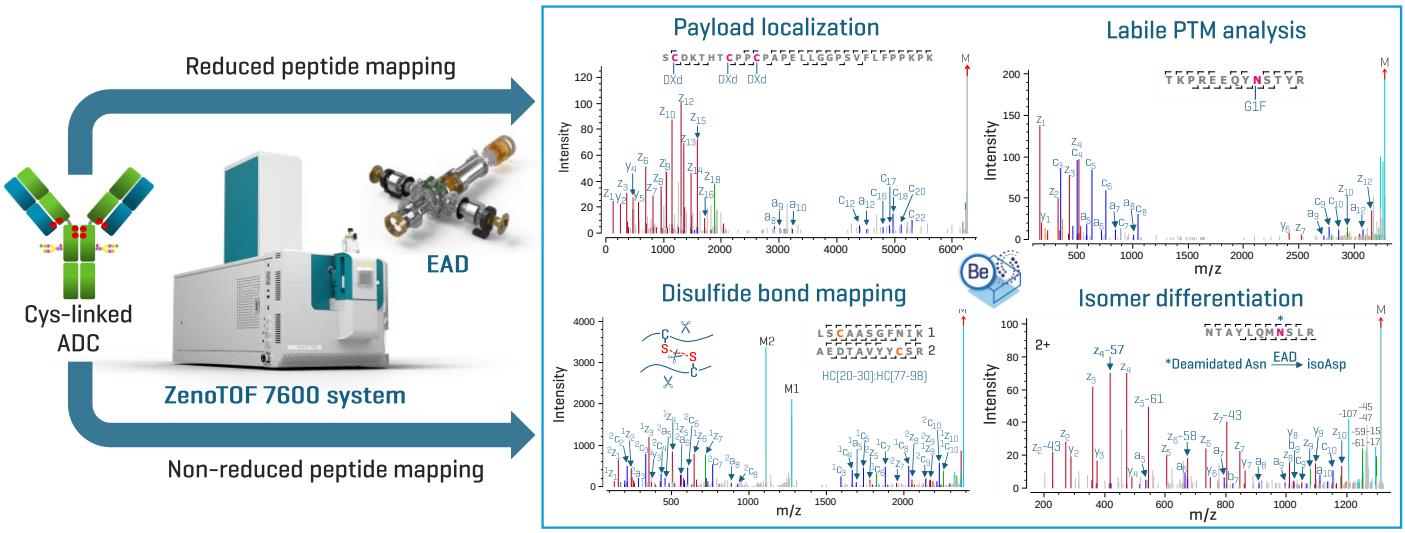

Figure 1. Comprehensive characterization of Cys-linked ADCs using the ZenoTOF 7600 system equipped with EAD. EAD-based reduced and non-reduced peptide mapping workflows enable comprehensive characterization of Cys-linked ADCs such as trastuzumab deruxtecan (Enhertu, T-DXd). These powerful approaches provide high sequence coverage, DAR determination, accurate PTM and payload localizations, confident disulfide bond mapping and clear isomer differentiation.

Introduction

ADCs are highly complex biotherapeutics consisting of mAbs conjugated with cytotoxic payloads through chemical linkers. New-generation ADCs mostly employ site-specific conjugation chemistry. The 4 pairs of cysteine residues involved in inter-chain disulfide bond linkages are often the target of site-specific conjugation, producing ADCs with a fixed drug-antibody ratio (DAR) of 8, such as trastuzumab deruxtecan (Enhertu, T-DXd)[11] characterized in this work.

Comprehensive ADC characterization can involve antibody sequence analysis, DAR determination, payload localization, disulfide bond mapping, isomer differentiation and PTM analysis. The characterization of these product quality attributes (PQAs) poses an analytical challenge to the traditional peptide mapping approach using collision-based MS/MS. EAD provides a viable solution to address all these analytical challenges. In this work, an EAD-based peptide mapping workflow was employed to characterize T-DXd digests prepared under reduced or non-reduced conditions.

Methods

Sample preparation: Lyophilized T-DXd powder was dissolved in a 50mM Tris buffer, followed by denaturation using 7.6M guanidine hydrochloride in 50 mM Tris. The denatured solution was treated with or without dithiothreitol (DTT) for the reduced or non-reduced peptide mapping experiment. The reduced and non-reduced samples were diluted prior to enzymatic digestion overnight using the trypsin/Lys-C mix (Promega). 5-10 µg of the final digests was injected for LC-MS analyses.

Liquid chromatography: Tryptic peptides were separated with the gradient displayed in Table 1 using an ACQUITY BEH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters). A flow rate of 0.25 mL/min was used for the chromatographic separation. The column was kept at 60°C in the column oven of an ExionLC AD system (SCIEX). Mobile phase A was 0.1% formic acid (FA) in water and mobile phase B was 0.1% FA in acetonitrile.

Mass spectrometry: Reduced and non-reduced peptide mapping data were acquired using a data-dependent acquisition (DDA) method with CID or EAD on the ZenoTOF 7600 system (SCIEX). The key DDA parameters are shown in Table 2.

Data analysis: LC-MS data were interpreted using a peptide mapping template within Biologics Explorer software (SCIEX). A custom modification of DXd (+1033 Da, $C_{52}H_{56}F_1N_9O_{13}$) was created for the Cys residue in Modification Editor and selected as a variable modification during peptide mapping analysis. For the analysis of the non-reduced data, 4 and 2 intra-chain disulfide linkages were defined for heavy chain (HC) and light chain (LC), respectively.

Table 1. LC gradient for peptide separation.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
Initial	98	2
2	98	2
62	60	35
65	50	50
67	10	90
70	10	90
71	98	2
75	98	2

Table 2. EAD and CID DDA parameters.

Parameter	CID	EAD
Start mass	100 m/z	
Stop mass	2000 m/z	
Q1 resolution	Unit	
Zeno trap	ON	
Zeno threshold	100,000 cps	
Maximum candidate ions	12	8
Charge state	1-8	2-10
Accumulation time	0.06 s	0.1 s
CE	Dynamic	10 V
Electron beam current	-	5500 nA
Electron KE	-	7 eV
ETC	-	Dynamic
EAD RF	-	120 Da
Reaction time	-	20 ms

Reduced and non-reduced peptide mapping

Previous work has demonstrated the high MS/MS sensitivity, fragmentation reproducibility and versatile capabilities of the EAD-based peptide mapping workflow for comprehensive biotherapeutic characterization.[3-9]

Figure 2 shows high sequence coverage of HC and LC (≥99%) from the reduced T-DXd digest obtained using the EAD-based peptide mapping workflow in a single injection. Similar results were also obtained for the T-DXd digest prepared under the non-reduced condition (data not shown). In addition to obtaining high sequence coverage of T-DXd, the EAD DDA method identified peptides carrying the DXd payload, intra-chain disulfide linkage or various PTMs, such as glycosylation, glycation, oxidation and deamidation, for in-depth characterization of these PQAs. Fast and sensitive EAD DDA can be employed as a single-injection method for comprehensive biotherapeutic characterization[3-9] or combined with CID DDA in a single method[10] to leverage the complementary capabilities of these 2 MS/MS approaches.

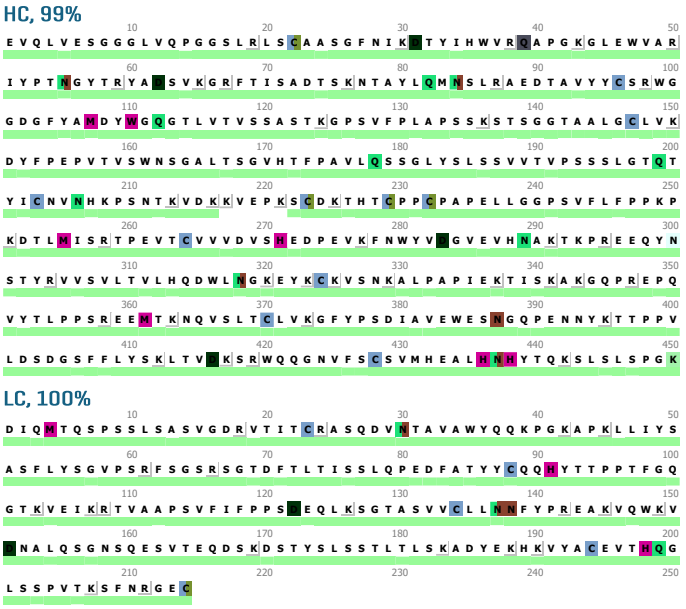

Figure 2. High sequence coverage of T-DXd using the EAD-based peptide mapping workflow. A nearly complete sequence coverage (≥99%) of HC and LC was obtained for T-DXd using the EAD DDA method in a single injection.

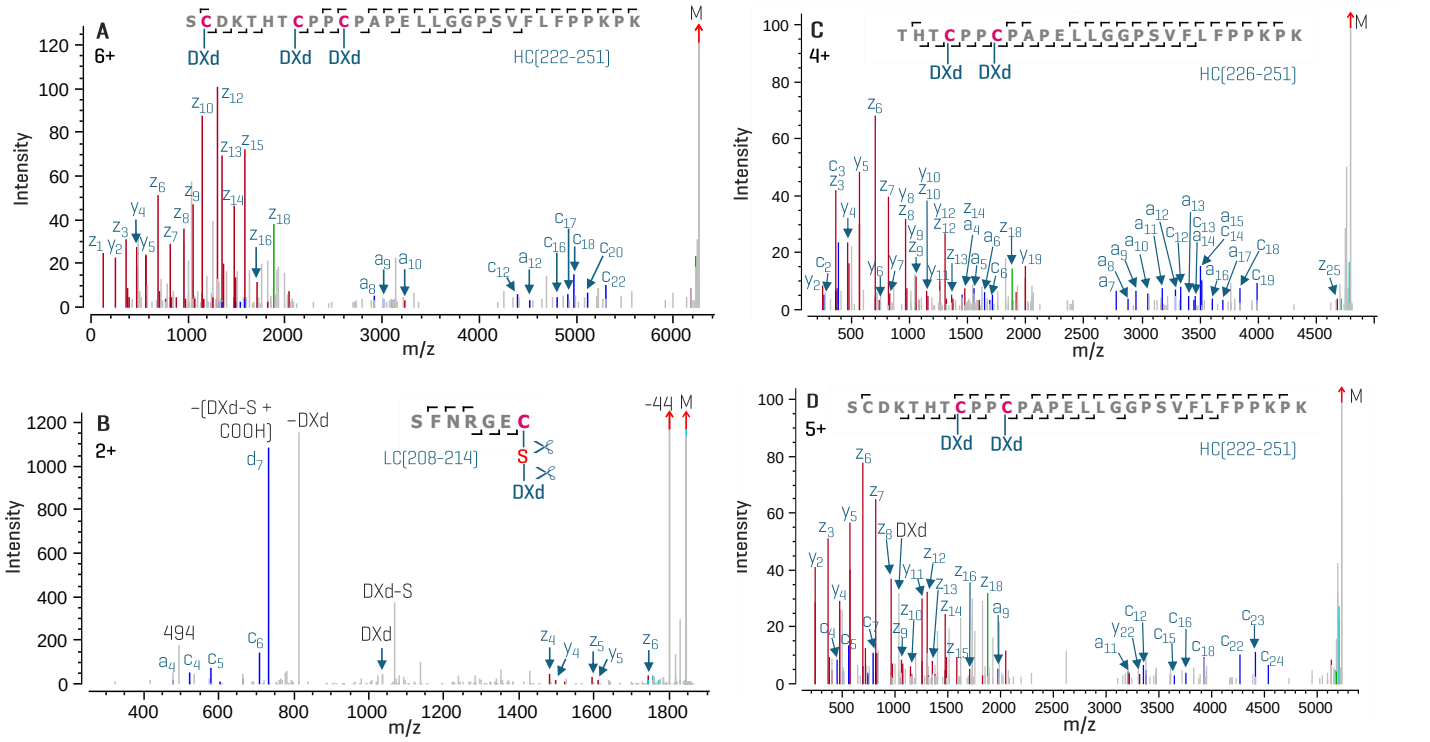

Figure 3. Deisotoped EAD MS/MS spectra of payload (DXd)-containing peptides from T-DXd. EAD provided extensive fragmentation of DXd-containing peptides while preserving the payload in the sequence fragments (*a*, *c* or *z*), leading to confident peptide identification and accurate payload localization. The high-quality EAD data confirmed Cys conjugations of 3 DXd on the peptide HC[222-251] (A) and 1 DXd at the C-terminus of LC (B). EAD of the doubly charged LC[208-214] led to an interesting fragmentation pattern where both sides of the -S- moiety were cleaved, producing the DXd or DXd-S peak (B). 2 of 3 DXd conjugation sites on the HC were further confirmed by excellent EAD data of the peptide HC[226-251] (C). In addition, EAD identified low-abundant T-DXd peptides without or with partial Cys conjugation, such as the peptide HC[222-251] containing 2 instead of 3 DXd (D). Detailed results of these interesting species will be described separately. Not all fragments were labeled for spectral clarity.

DAR determination and payload localization

DAR determination is critical to ensuring the product quality of ADCs.[2] T-DXd consists of 3 and 1 DXd payloads on each HC and LC, respectively, leading to a fixed DAR of 8. The identification of DXd-containing peptides will allow the accurate DAR determination of T-DXd. Figure 3 shows representative EAD spectra of the DXd-containing HC and LC peptides. EAD led to extensive fragmentation of these peptides for confident sequence confirmation while preserving the payloads in the fragments for their accurate localization. The identification of the peptide HC[222-251] carrying 3 DXd (Figure 3A) and LC[208-214] with 1 DXd (Figure 3B) confirmed a DAR of 8 for the T-DXd. The ability of EAD to preserve intact payloads in the fragments of these 2 peptides enabled accurate localization of all 4 DXd in HC and LC. The identification of shorter HC[222-225] (data not shown) and HC[226-251] (Figure 3C) peptides carrying 1 and 2 DXd, respectively, further confirmed the 3 payload sites on the HC. EAD spectrum of the doubly charged LC[208-214] revealed an interesting fragmentation pattern where both sides of the -S- moiety were cleaved to produce the payload-specific peaks (DXd or DXd-S, Figure 3B). Further work will be performed to study the mechanism of this fragmentation pattern.

Compared to other electron-base MS/MS fragmentation techniques, EAD is highly sensitive due to the use of the Zeno trap, which increases the detection of MS/MS fragments by 5-fold.[3] The high sensitivity of EAD allowed the confident identification of low-abundant T-DXd peptides with partial or incomplete conjugation. Figure 3D shows the EAD spectrum of the peptide HC[222-251] conjugated with 2 DXd.

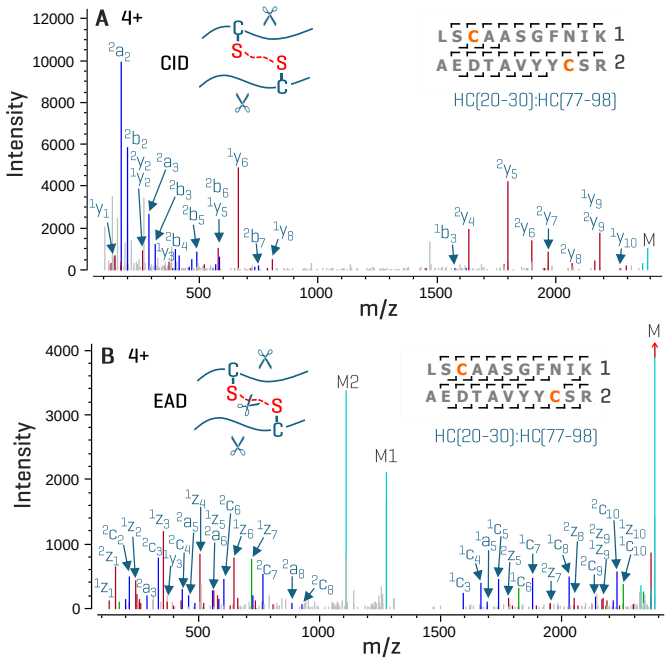

Figure 4. Disulfide bond mapping using CID and EAD. CID (A) and EAD (B) provide complementary results for a disulfide-linked peptide from the HC of T-DXd. Compared to CID (A), EAD led to more extensive fragmentation of 2 peptides due to its ability to cleave the disulfide bond for increased confidence in sequence identification (B). Additionally, the detection of 2 full-length peptides (M1 and M2) in the EAD spectrum (B) provided accurate mass information for sequence confirmation. The upper left numbers in fragment annotations correspond to the peptide number given by the sequence. The scissors icons indicate the peptide backbone or S-S bond cleavages. Not all fragments were labeled for spectral clarity.

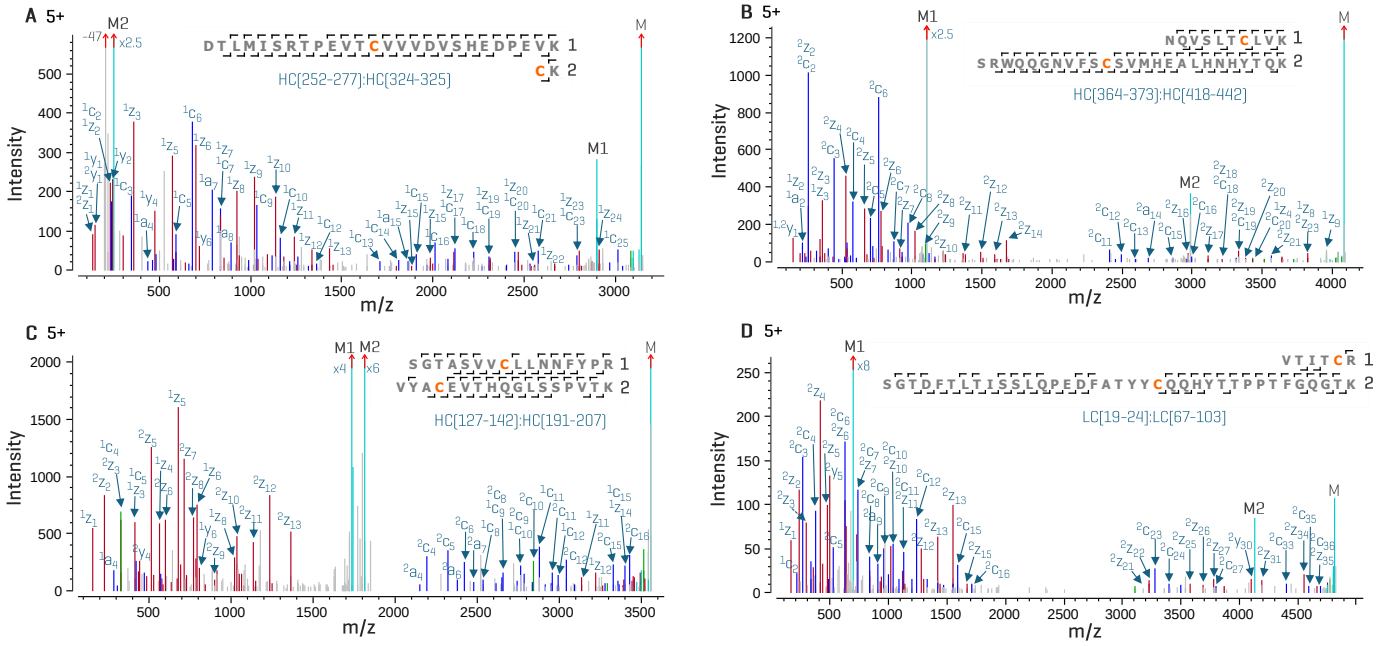

Figure 5. Deisotoped EAD MS/MS spectra of disulfide-linked peptides from T-DXd. The ability of EAD to cleave both peptide backbone and disulfide bond led to confident confirmation of all 6 intra-chain disulfide linkages (4 on HC and 2 on LC) in T-DXd. In addition to the example shown in Figure 4B, 3 disulfide-linked peptides from HC (A-C) and 1 from LC (D) are shown in this figure.

High-quality EAD data led to the localization of 2 DXd to Cys[229] and Cys[232]. The peptide HC[222-251] conjugated with 1 DXd and LC[208-214] without DXd were also identified (data not shown). A detailed study of these ADC impurities will be described separately.

Disulfide bond mapping

EAD provides complementary and superior fragmentation to CID for disulfide bond mapping. EAD can simultaneously cleave the peptide backbone and the disulfide bond, leading to more extensive fragmentation and increased confidence in sequence identification.[9] In this work, CID and EAD DDA were employed to analyze the T-DXd digest prepared under the non-reduced condition.

Figure 4 shows the CID and EAD spectra of a disulfide-linked peptide from the HC. While both MS/MS techniques produced high-quality data for confident identification of this peptide, EAD generated more sequence fragments for higher peptide coverage by cleaving the disulfide bond (Figure 4B). The cleavage of the disulfide bond also led to the detection of two intact peptides (M1 and M2 in Figure 4B), providing additional mass confirmation of 2 disulfide-linked peptides.

High-quality CID and EAD data allowed the confirmation of all the 6 intra-chain disulfide linkages on the T-DXd (4 on HC and 2 on LC). Figure 5 shows EAD spectra of 3 disulfide-linked peptides from HC and 1 from LC. Similar to the example described above (Figure 4B), excellent EAD fragmentation allowed confident identification of these disulfide-linked peptides with various lengths.

PTM analysis and isomer differentiation

In addition to payload analysis and disulfide bond mapping, EAD offers unique capabilities for the localization of labile PTMs and differentiation of amino acid isomers.[3-8]

Figure 6 shows an example of glycopeptide characterization and isomer differentiation using EAD. The ability of EAD to preserve labile PTMs in the fragments led to precise localization of the glycan G1F in the glycopeptide TKPREEQYNSTYR (Figure 6A) and the glycation moiety (Hex) in the glycated peptide LSCAASGFNIKDTYIHWVR (Figure 6B).

The high-sensitive EAD DDA method provided excellent fragmentation of deamidated peptide NTAYLQMNSLR despite its low abundance (~0.6%, Figure 6B). The detection of a diagnostic z_4 – 57 fragment allowed the confident assignment of the deamidated Asn as an isoAsp residue instead of an Asp. EAD also generated signature z – 29 and z – 43 fragments for the confirmation of Ile and Leu residues, respectively (Figures 6B and 6C). Additionally, EAD cleaved the side chains of the z fragments to produce characteristic neutral-loss peaks for certain amino acid residues. For example, the z_5 – 61 and z_6 – 58 fragments are indicative of Met and Gln residues, respectively (Figure 6C).

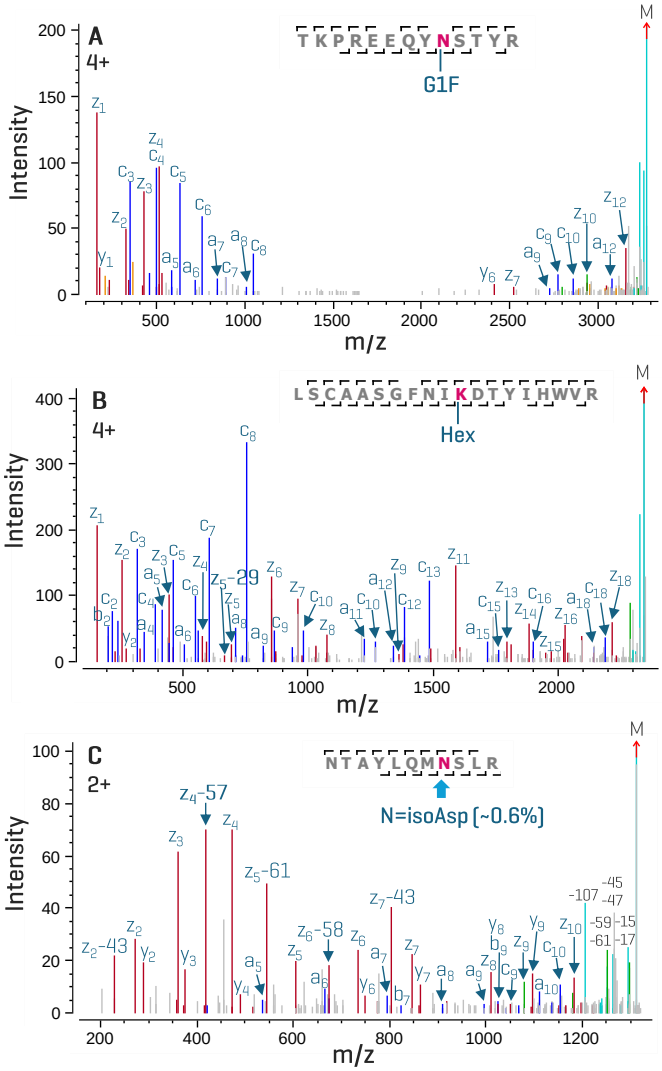

Figure 6. PTM localization and isomer differentiation using EAD. EAD is powerful for accurate localization of labile PTMs, such as glycosylation and glycation (Hex). The detection of G1F-containing *a*/*c*/*z* fragments (e.g. c_9) from the peptide HC[292-304] pinpointed the location of this glycan to N300 (A). Extensive fragmentation of a glycated peptide HC[20-38] (~1%) allowed its confident identification and accurate localization of glycation (B). Highly sensitive EAD offers a viable solution for clear differentiation of amino acid isomers. EAD generated a signature z_4 – 57 fragment for the deamidated peptide HC[77-87] despite its low abundance (~0.6%), confirming the conversion of Asn to isoAsp (C). EAD also led to the detection of diagnostic z_2 – 43 and z_7 – 43 fragments for the confirmation of 2 Leu residues in the sequence. In addition, EAD resulted in side-chain losses of z fragments diagnostic to certain amino acid residues, such as the loss of 61 for Met (z_5 – 61 in C) and 58 for Gln (z_5 – 58 in C).

In summary, the high-quality data presented in this technical note demonstrate the powerful capabilities of the EAD-based peptide mapping workflow for comprehensive ADC characterization. This workflow offers high sequence coverage of the antibody, confident identification of payload-containing peptides for accurate DAR determination, precise localization of the payloads and labile PTMs,

high-confidence disulfide bond mapping and clear differentiation of amino acid isomers. The high sensitivity offered by the EAD-based peptide mapping workflow also enabled the detection and identification of low abundant impurities in ADCs, such as the partially conjugated species.

Conclusions

- The EAD-based peptide mapping workflow provides a comprehensive characterization of the T-DXd, a new-generation Cys-conjugated ADC
- EAD DDA led to high sequence coverage of the reduced or non-reduced T-DXd in a single injection
- EAD provided sensitive detection and confident identification of T-DXd peptides with partial or complete payload conjugation for accurate DAR determination and impurity assessment
- EAD offered high-quality data for confident determination of all intra-chain disulfide linkages in T-DXd
- EAD preserves the payloads and labile PTMs in the fragments for their accurate localization and generates diagnostic fragments for clear isomer differentiation

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