Comprehensive characterization of an antibody-drug conjugate (ADC) using electron activated dissociation (EAD)

Featuring an EAD-based peptide mapping workflow using the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

Zoe Zhang[1], Haichuan Liu[1], Takashi Baba[2], Pavel Ryumin[2], Bill Loyd[2], Jason Causon[2] and Kerstin Pohl[1]
[1]SCIEX, USA; [2]SCIEX, Canada

This technical note describes an in-depth characterization of a lysine-linked ADC using a streamlined, EAD-based peptide mapping workflow in a single injection. This workflow combines the powerful capabilities of EAD for peptide mapping with automated data interpretation offered by Biologics Explorer software. The workflow provides confident ADC sequence confirmation and accurate localization of the payload. The EAD-based peptide mapping workflow involves minimal method development and optimization, allowing its easy implementation by different levels of users.

Traditional collision-based tandem mass spectrometry (MS/MS) approaches lead to fragmentation of the cytotoxic payload in the ADC. Often CID does not provide site-specific information critical to biotherapeutic development. Although conventional electron-based MS/MS approaches can provide site information about the payload, these techniques suffer from slow scan rates, long reaction times, low sensitivity and low reproducibility. By comparison, EAD can be implemented as a data-dependent acquisition (DDA) platform method for comprehensive biotherapeutic characterization with high sensitivity and high reproducibility.[1-3]

In this technical note, the EAD-based peptide mapping workflow (Figure 1) was leveraged to provide complete sequence coverage and accurate localization of the payload in ado-trastuzumab emtansine (T-DM1) in a single injection.

Key features of the ZenoTOF 7600 system for ADC characterization

- **New depth in peptide mapping analysis:** The EAD DDA platform method enables comprehensive analysis of monoclonal antibodies (mAbs) and ADCs in a single injection
- **Confident payload localization and characterization:** EAD provides information for accurate localization and detailed structural characterization of the payload
- **High MS/MS sensitivity:** The Zeno trap improves the detection of fragments by 5- to 10-fold, enabling higher confidence in fragment assignment and peptide identification
- **High quality and reproducibility:** Excellent fragmentation reproducibly with EAD improves confidence in the results
- **Streamlined and easy-to-use:** Automated interpretation of EAD DDA data using Biologics Explorer software allows for easy method implementation and improved user experience

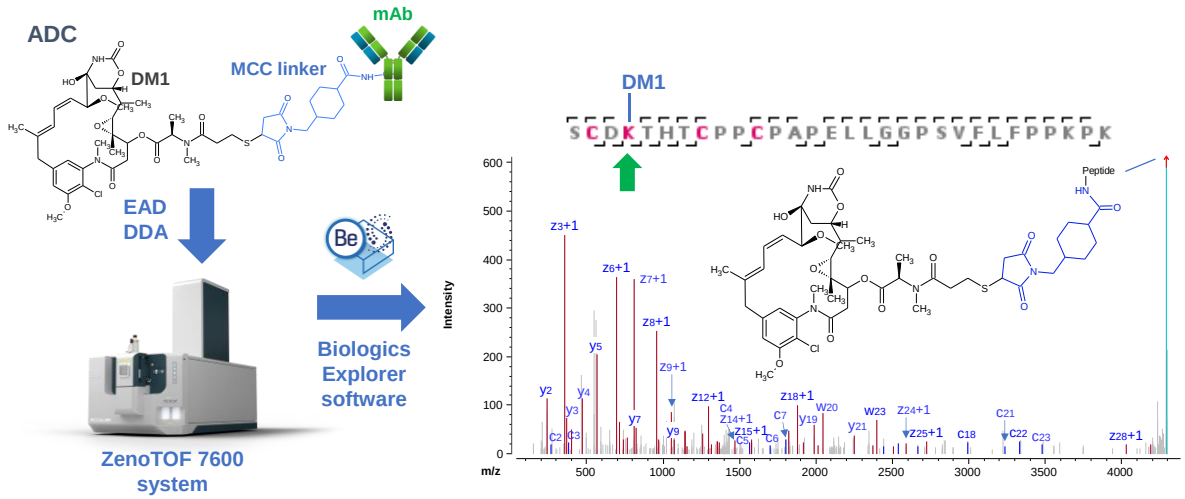

Figure 1. The EAD-based peptide mapping workflow offered by the ZenoTOF 7600 system enables confident sequence confirmation and accurate determination of the payload conjugate sites in T-DM1. The cytotoxic payload, DM1, was retained in the EAD fragments, leading to its accurate localization in the ADC using Biologics Explorer software. MCC: maleimidomethyl cyclohexane-1-carboxylate.

Introduction

With advancements in protein engineering, antibodies and ADCs have become an important class of biotherapeutics.[3] ADCs are typically composed of a ~150 kDa mAb covalently coupled with a cytotoxic payload through synthetic linkers.[4] ADCs have more complex structures and higher sample heterogeneity compared to unconjugated mAbs because the variants carry different numbers of the payload at different conjugation sites.[5] As a result, it is critical to fully characterize ADCs during their development to ensure drug quality, safety and efficacy. Comprehensive characterization of ADCs typically involves confirmation of the mAb sequence, identification and localization of post-translational modifications (PTMs) on the mAb, measurement of the drug-to-antibody ratio (DAR) and determination of payload conjugation sites. While intact mass analysis is commonly utilized to determine the DAR, sequence confirmation and localization of the payload are typically achieved using a bottom-up approach.

Methods

Sample preparation: T-DM1 was denatured with 7.2M guanidine hydrochloride (HCl) in 100mM Tris-HCl buffer (pH = 7.2), followed by reduction with 10mM DL-dithiothreitol and alkylation using 30mM iodoacetamide. Digestion was performed with the trypsin/Lys-C protease at 37°C for 16 h.

Chromatography: A 4 µL (4 µg) sample of the trypsin/Lys-C digest was separated with a CSH C18 column (2.1×100 mm, 1.7 µm, 130 Å, Waters) using an ExionLC AD system. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Table 1 shows the LC gradient used for peptide separation at a 300 µL/min flow rate. The column temperature was maintained at 50°C in the column compartment.

Table 1. Chromatography for peptide mapping analysis.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
Initial	*98*	*2*
5	*98*	*2*
6	*90*	*10*
40	*55*	*45*
44	*10*	*90*
46	*10*	*90*
47	*98*	*2*
50	*98*	*2*
51	*10*	*90*
54	*10*	*90*
55	*98*	*2*
60	*98*	*2*

Mass spectrometry: EAD DDA data were acquired using SCIEX OS software on the ZenoTOF 7600 system (SCIEX). Detailed TOF MS and EAD DDA method parameters are summarized in Table 2.

Table 2. MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*DDA*
Polarity	*Positive*	
Gas 1	*40 psi*	
Gas 2	*40 psi*	
Curtain gas	*30 psi*	
Source temperature	*350°C*	
Ion spray voltage	*5,200 V*	
Declustering potential	*20 V*	
Collision energy	*8 V*	
CAD gas	*7*	
Maximum candidate ion	*5*	
Intensity threshold	*100 cps*	
Charge states	*2 to 10*	
Exclusion time	*6 s after 2 occurrences*	
Start mass	*100 m/z*	*150 m/z*
Stop mass	*1,800 m/z*	*2,500 m/z*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*4,750 nA*
ETC	*NA*	*100*
Zeno trap	*NA*	*ON*
Accumulation time	*0.25 s*	*0.20 s*
Time bins to sum	*4*	*4*

Data processing: EAD DDA data were processed using the peptide mapping workflow template within Biologics Explorer software. The combination of DM1 and the maleimidomethyl cyclohexane-1-carboxylate (MCC) linker was defined as a variable modification for the protein N-terminus and lysine (K) residue.

Peptide mapping analysis of T-DM1

T-DM1 was approved in 2013 by the U.S. Food and Drug Administration (FDA) for the treatment of human epidermal growth factor receptor 2 (HER2)-positive, metastatic breast cancer.[5,6] T-DM1 is composed of trastuzumab covalently conjugated to the cytotoxin DM1 via a non-cleavable MCC linker, which is connected to the primary amine of the protein N-terminal or lysine residue (Figure 2). Previous intact mass studies showed that the average DAR for T-DM1 is ~3.5.[5,6] Considering that trastuzumab contains 88 lysine and 4 N-terminal residues, the addition of DM1 to these potential conjugation sites may result in >4.5 million possible variants of the T-DM1.[5] Since the structure and conjugation site of the payload can impact drug safety and efficacy, it is important to confidently identify the payload-containing peptides and accurately determine the sites of payload conjugation.

Peptide mapping is the method of choice for providing simultaneous identification of payload-containing peptides and localization of the payload. Traditional collision-induced dissociation (CID) of DM1-containing peptides resulted in the preferable fragmentation of the payload, leading to the formation of small fragments such as m/z 547.2, 485.2 and 453.2.[5] This fragmentation pattern increases the spectral complexity and ambiguity of the payload localization. This work employed the EAD-based peptide mapping workflow to obtain confident sequence confirmation and precise localization of the DM1 payload in T-DM1. This powerful workflow leverages the unique capabilities of EAD for the preservation of labile PTMs and automated data analysis using Biologics Explorer software, leading to comprehensive ADC characterization in a single injection.

The EAD-based peptide mapping workflow benefited from the excellent fragmentation and high sensitivity of Zeno EAD to provide 100% sequence coverages of the light chain (LC) and heavy chain (HC) subunits of trastuzumab from T-DM1 in a single injection (Figure 3). This result allowed confident sequence confirmation of trastuzumab and in-depth characterization of DM1-containing peptides.

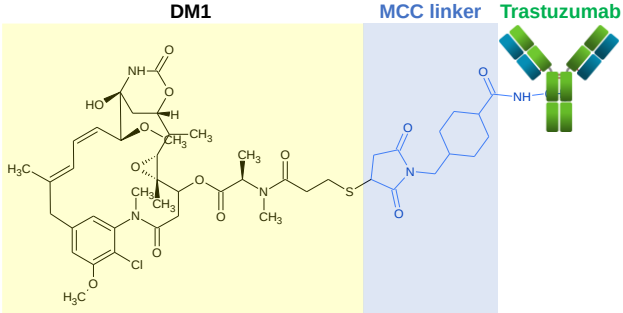

Figure 2. Structure of the T-DM1 ADC. T-DM1 consists of the cytotoxin DM1 (in yellow background) conjugated to trastuzumab via the MCC linker (in blue background), which is covalently bound to the primary amines on the mAb.

Figure 3. Complete sequence coverage of the LC and HC subunits of trastuzumab from T-DM1. EAD-based peptide mapping led to 100% sequence coverage of LC and HC of trastuzumab in a single injection.

Characterization of DM1-containing peptides

The unique ability of EAD to retain intact labile modifications in the fragments was leveraged here to provide accurate localization of the payload in DM1-containing peptides.

Figure 4 shows the deisotoped EAD spectrum of the DM1-containing HC peptide (HC222-251) from trastuzumab. EAD led to the generation of extensive *c*/*y*/*z* fragments with or without DM1, enabling the confident identification of the peptide sequence and precise localization of the payload. Specifically, the detection of an unmodified c_3 and DM1-containing c_4 fragments pinpointed the position of DM1 to the Lys^{225} residue among 3 potential conjugation sites (Lys^{225}, Lys^{249} and Lys^{251}) in the peptide HC222-251. The Lys^{249} residue was also identified as a DM1 conjugation site from EAD of the peptide HC229-232 (data not shown). In addition to generating dominant sequence-related fragments, EAD of HC222-251 produced a diagnostic fragment of DM1 at m/z 547.2 (Figure 4), facilitating the confirmation of the payload structure.

Figure 5 displays the EAD spectra of the peptide LC25-45 with or without the DM1 payload. EAD provided excellent fragmentation

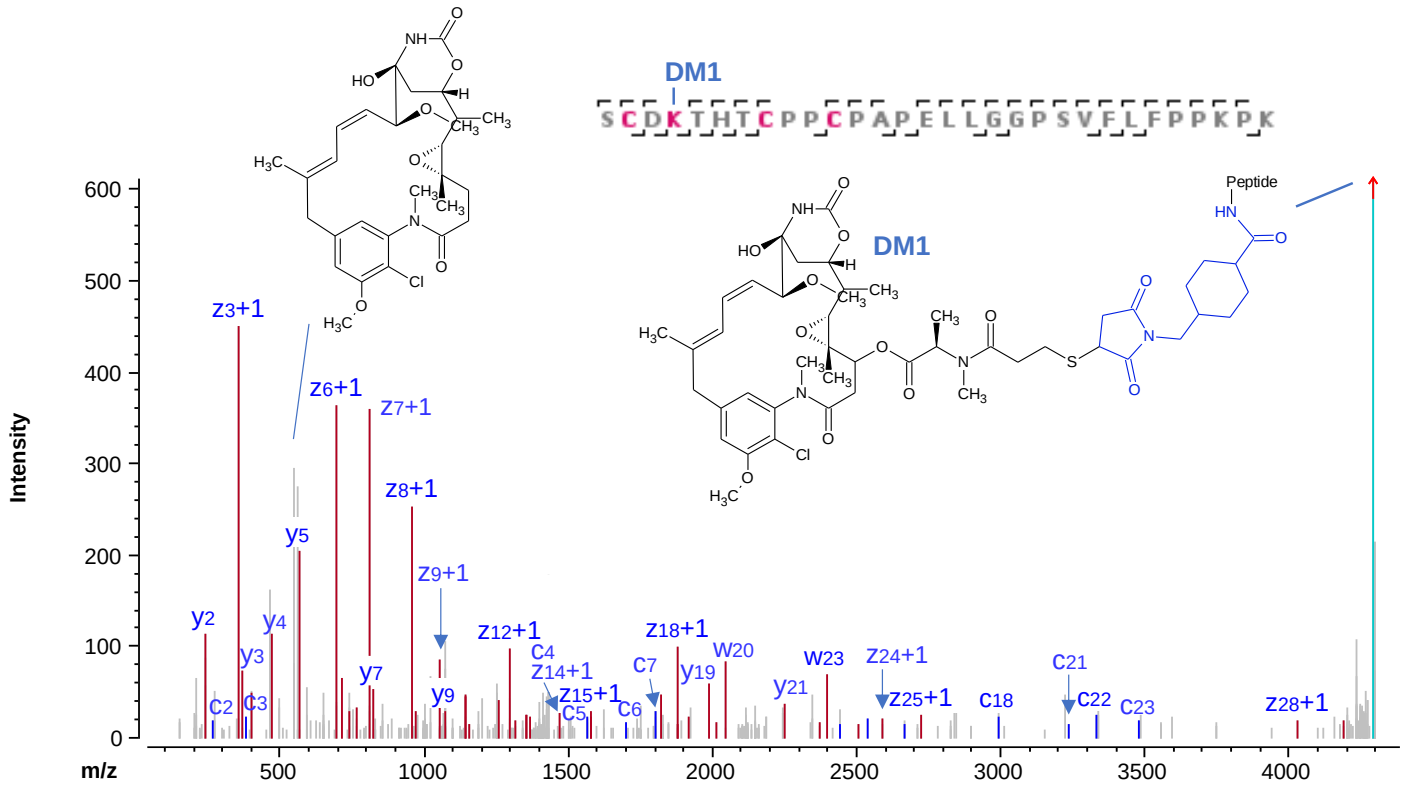

Figure 4. Deisotoped EAD spectrum of the DM1-containing peptide HC222-251 (4+) from Biologics Explorer software. The detection of rich *c*/*y*/*z* fragments allowed the accurate localization of DM1 to the Lys[225] residue in this peptide. The peak at m/z 547.2 corresponds to a characteristic fragment from the DM1 payload.

of 2 peptides for confident sequence confirmation. Similar to the peptide HC222-251 described above, the sequence of LC25-45 contains 3 potential sites of DM1 conjugation (Lys[39], Lys[42] and Lys[45]). The detection of the fragment at m/z 547.2 indicated the presence of the DM1 payload in this LC peptide. The Lys[42] residue was determined to be the DM1 conjugation site in this LC peptide based on the detection of the unmodified z_3+1 and DM1-containing z_4+1 ions (Figure 5B).

The streamlined, EAD-based peptide mapping workflow is a powerful tool for comprehensive ADC characterization in a single injection. The unique capabilities of EAD offered by the ZenoTOF 7600 system coupled with automated data interpretation using Biologics Explorer software enabled simultaneous sequence confirmation and payload localization for ADCs with high confidence.

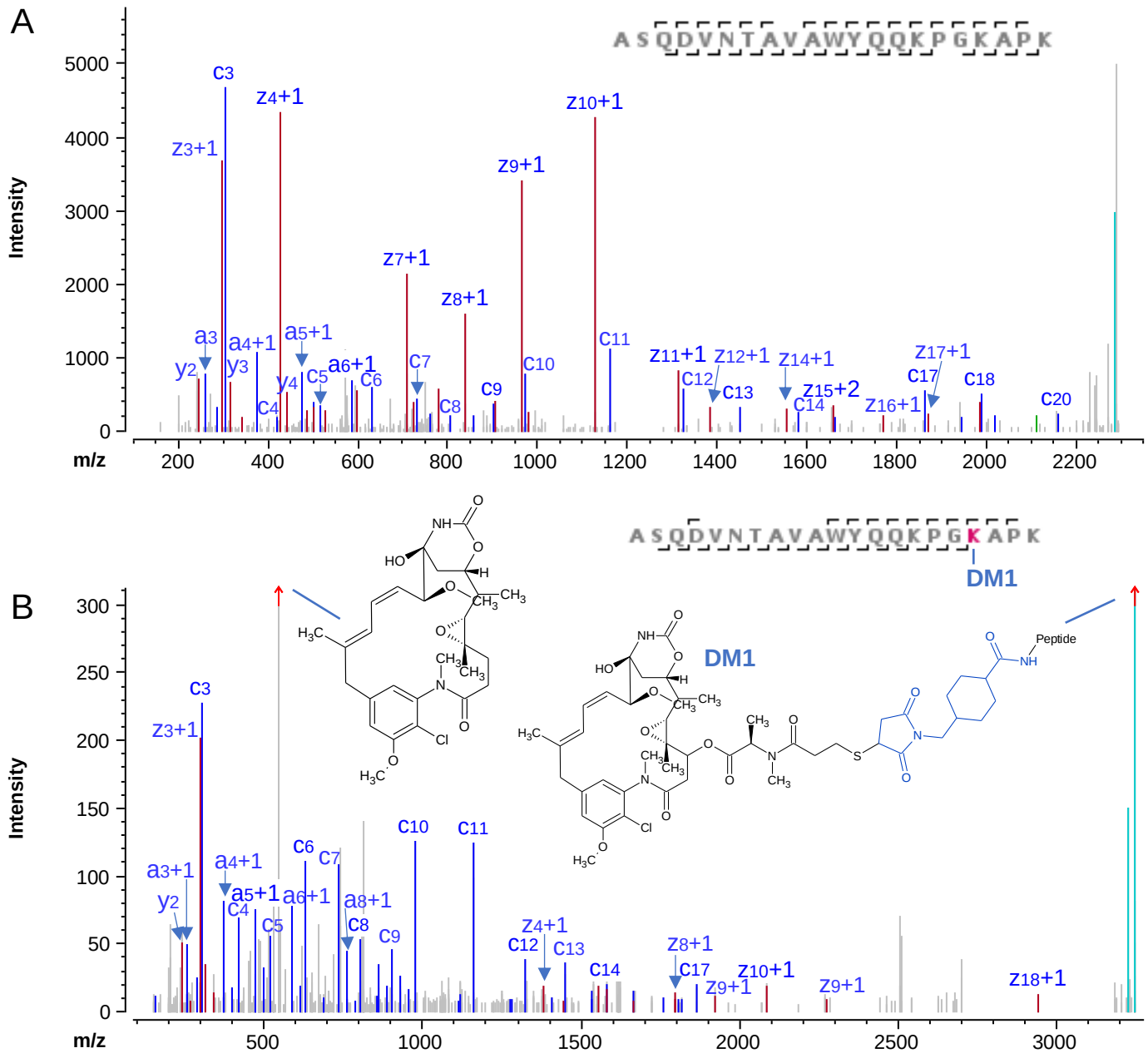

Figure 5. Deisotoped EAD spectra of the peptide LC25-45 (4+) with or without the DM1 from Biologics Explorer software. EAD led to a nearly complete fragment coverage of the peptide LC25-45 (A) without or (B) with the DM1 payload. The peak at m/z 547.2 in B corresponds to a characteristic fragment from the DM1 payload.

Conclusions

- The EAD-based peptide mapping workflow enables comprehensive characterization of complex ADCs in a single injection
- EAD DDA provided a complete sequence coverage of the LC and HC subunits of trastuzumab from T-DM1
- EAD led to extensive fragmentation of DM1-containing peptides for their confident identification while preserving the intact payload for its accurate localization
- EAD DDA coupled with automated data interpretation using Biologics Explorer software offers a streamlined process for biotherapeutic characterization
- The EAD-based peptide mapping workflow requires minimal method development and is easy to implement by different levels of users

References

1. Takashi Baba *et al.* (2015) Electron capture dissociation in a branched radio-frequency ion trap. *Anal Chem*, **87**(1), 785−792.
2. Takashi Baba *et al.* (2021) Dissociation of Biomolecules by an Intense Low-Energy Electron Beam in a High Sensitivity Time-of-Flight Mass Spectrometer. *J Am Soc Mass Spectrom.* **32**(8):1964-1975.
3. An evaluation of a single injection platform method for advanced characterization of protein therapeutics using electron activation dissociation (EAD). SCIEX technical note, RUO-MKT-02-13965-A.
4. Aliain Beck *et al.* (2010) Strategies and challenges for the next generation of therapeutic antibodies. *Nat Rev Immunol* ***10****(5):345-52.*
5. Thomas Botzanowski *et al.* (2017) Insights from native mass spectrometry approaches for top- and middle- level characterization of site-specific antibody-drug conjugates. *Mabs,* ***9****(5):801-811.*
6. Liuxi Chen *et al.* (2016) In-depth structural characterization of Kadcyla (ado-trastuzumab emtansine) and its biosimilar candidate. *Mabs,* ***8****(7): 1210-1223.*

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices