Confirmation of disulfide linkages in a multispecific monoclonal antibody (mAb) using electron activated dissociation (EAD)

Featuring the ZenoTOF 7600 system with EAD from SCIEX and Protein Metrics Inc. software

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The data presented in this technical note show the confirmation of disulfide-linked peptides for a multispecific monoclonal antibody (mAb). The analysis of disulfide-linked peptides was performed using data dependent acquisition (DDA) of a non-reduced digestion of the multispecific mAb with the ZenoTOF 7600 system (Figure 1). Automated data interpretation with Protein Metrics Inc. software was incorporated as part of a disulfide mapping solution. With this workflow, standard and advanced characterization leveraging a novel fragmentation technique based on electron activated dissociation (EAD)[1,2] is achievable in 1 injection, enabling a streamlined characterization accessible to every user level.

The role of disulfide bonds is essential for maintaining tertiary and/or quaternary structures in proteins. Since the overall structure of proteins is related to their function, ensuring disulfide bond arrangements are as expected is critical for the safety and efficacy of biotherapeutics. Characterization of disulfide bond arrangements using bottom-up workflows has become increasingly popular due to advances in modern high-resolution mass spectrometry, novel fragmentation technologies and automated data processing software.[3,4] However, disulfide bonds prevent effective fragmentation around linked cysteine residues when using collision induced dissociation (CID), which limits the information available for identifying disulfide-bound peptides as well as disulfide patterns. Although alternative fragmentation mechanisms exist, these techniques can suffer from long reaction times, low sensitivity and lack of reproducibility.

Figure 1. The ZenoTOF 7600 system from SCIEX.

In this technical note, a new fragmentation type based on EAD[1,2] is used to confirm the disulfide linkages of a multispecific mAb in which 2 different heavy chains and 2 identical light chains are linked. One of the heavy chains was designed with an additional c-terminal single-chain variable fragment (scFv) through a $(GGGS)_2$ linker (Figure 2). The scFv domain also contained multiple intrachain disulfide bonds. The data were acquired using a 10 Hz DDA method and interpreted with Byos software (Protein Metrics Inc.). This workflow demonstrates a routine approach for the streamlined characterization of disulfide-linked peptides with a new level of data quality.

Key features of the ZenoTOF 7600 system

- **New depths of peptide mapping analysis:** EAD with fast DDA enables alternative fragmentation for routine, in-depth analysis of next-generation protein therapeutics and standard mAbs.
- **Higher levels of structural information:** Changing the mechanism of fragmentation by tuning the electron energy may provide a higher level of structural information, particularly for disulfide bond identification.
- **Higher MS/MS sensitivity:** Increased detection of fragments (by 5- to 10-fold) using the Zeno trap enables higher confidence in data assignment.
- **High reproducibility:** Reproducible fragmentation with EAD for singly, doubly and multiply charged ions enables analysis of more precursors than other alternative and low-reproducibility fragmentation techniques.
- **Streamlined and easy to use:** Fully automated data acquisition in DDA mode using EAD with SCIEX OS software and automated data interpretation with Byos software (Protein Metrics Inc.) simplifies the entire user experience.

Methods

Sample preparation: The multispecific mAb sample (Figure 2) was denaturated with 7.2 M guanidine hydrochloride (HCl) in a buffer of 50 mM Tris-HCl, pH 7.0, and free cysteines were capped with 5 mM iodoacetamide. Digestion was performed with either trypsin/Lys-C or trypsin/Lys-C/Asp-N at 30°C for 16 h. The reaction was stopped with 1% formic acid.

Chromatography: A total of 3 µL (4 µg) of the trypsin/Lys-C digest were separated with a Waters ACQUITY CSH C18 column (2.1 × 100 mm, 1.7 µm, 130 Å) using an ExionLC AD system. The mobile phase (A) consisted of water with 0.1% formic acid, while the organic phase (B) was acetonitrile with 0.1% formic acid. A gradient profile was used at a flow rate of 350 µL/min (Table 1). The column temperature was maintained at 50°C.

Table 1. Chromatography for peptide mapping analysis.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
Initial	*98*	*2.0*
1.00	*98*	*2.0*
86.00	*55*	*45*
86.50	*20*	*80*
87.00	*2.0*	*98*
87.50	*2.0*	*98*
87.51	*98*	*2.0*
90.00	*98*	*2.0*

Mass spectrometry: Data were acquired using DDA with the ZenoTOF 7600 system. The electron energy for the alternative fragmentation in the EAD cell was set to a value of 7 eV. Detailed method parameters are summarized in Table 2.

Table 2. Mass spectrometry parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*IDA dependent*
Polarity	*positive*	
Gas 1	*50 psi*	
Gas 2	*50 psi*	
Curtain gas	*35 psi*	
Source temperature	*550°C*	
Ion spray voltage	*5500 V*	
Declustering potential	*80 V*	
Collision energy	*12 V*	
CAD gas	*7*	
Maximum candidate ion	*10*	
Intensity threshold	*125 cps*	
Charge states	*2 to 10*	
Exclusion time	*6 s after 2 occurrences*	
Start mass	*200 m/z*	*100 m/z*
Stop mass	*2,000 m/z*	*3,000 m/z*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*4750 nA*
ETC	*NA*	*Dynamic*
Zeno trap	*NA*	*ON*
Accumulation time	*0.25 s*	*0.10 s*
Time bins to sum	*8*	*12*

Data processing: Data were processed using Byos software (Protein Metrics Inc.).

Results and discussion

Disulfide bonds are a common post-translational modification (PTM) found in globular proteins that have been comprehensively studied since the 1980s.[5] Because they are tightly related to protein folding structure, they can affect both the efficacy and safety of a biotherapeutic. With the advancement of modern mass spectrometry, bottom-up approaches have become the method of choice for the characterization of multiple product quality attributes, enabling the simultaneous identification and localization of modifications, including disulfide bonds.[4,6] The most commonly used approach, CID, typically leads to peptide backbone fragments while leaving the disulfide bond intact. This can result in a complicated yet incomplete MS/MS spectrum that is difficult to interpret. In addition, CID struggles to obtain high fragmentation coverage for peptides bound via a disulfide bond. While alternative fragmentation techniques have been used for this type of identification in the past, their adoption by the biopharmaceutical industry has been limited by their overall low sensitivity and lack of automated DDA workflows.[7-9]

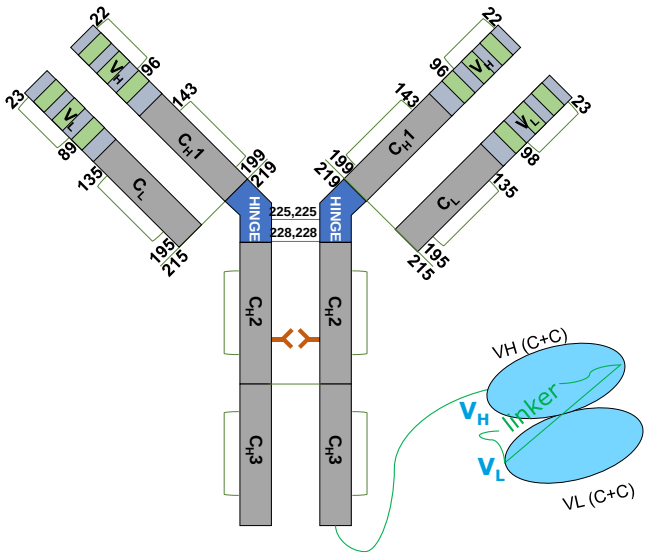

Figure 2. Schematic of the multispecific mAb with inter- and intra-disulfide bonds. The mAb contains 2 identical light chains and 2 different heavy chains. One of the heavy chains was designed with an additional C-terminal single-chain variable fragment (scFv) through a linker.

The study presented here focused on detailed disulfide linkage characterization of a multispecific mAb (Figure 2) using the ZenoTOF 7600 system. A DDA approach in combination with Zeno EAD was used for a routine non-reduced peptide mapping analysis. EAD enables advanced characterization during DDA acquisition while the Zeno trap enhances the detection of fragment ions, allowing for the correct identification of large disulfide peptides, especially for fragments derived from very low abundant precursors. The multispecific mAb contains 21 disulfide bonds (Figure 2) that are critical for maintaining its 3-dimensional structure. A total of 3 interchain disulfides between 2 heavy chains, 2 interchain disulfides between heavy and light chains and 12 intrachain disulfides on the main frame of the antibody were all confirmed in the trypsin/Lys-C digested samples. Further, Asp-N was employed to facilitate digestion at the extended scFv region, breaking the long chain into peptides with an LC-MS-friendly size. As such, 2 additional linkages in the scFv region could be confirmed (Table 3). The 2 disulfide-bonded peptides from the extension of the second heavy chain (H2+cpp**C** and **C**ppc+H2) were out of scope for the sample preparations chosen, as their yielded sizes were very large. These peptides can be targeted using a different enzymatic digestion approach. No scrambled disulfide bonds were detected, indicating the neutral pH used was very efficient at keeping the disulfide linkage intact during the sample preparation of the multispecific mAb.

Table 3. List of expected and identified disulfide bonds.

Chains	Subunit	Cysteine locations		Identified (Y/N)
L1+L1(L2+L2)	V_L	23	98	Y
L1+L1(L2+L2)	C_L	135	195	Y
L1+H1 (L2+H2)	C_L+C_H1	215	219	Y
HC1+HC2	Hinge	225	225	Y
HC1+HC2	Hinge	228	228	Y
H1+H1 (H2+H2)	V_H	22	96	Y
H1+H1 (H2+H2)	C_H1	143	199	Y
H1+H1 (H2+H2)	C_H2	260	320	Y
H1+H2	C_H2	348	353	Y
H1+H1 (H2+H2)	C_H3	366	424	Y
H2+H2	scFv	483	557	Y
H2+cpp**C**	scFv	504	587	N
Cppc+H2	scFv	590	694	N
H2+H2	scFv	617	682	Y

Figure 3 shows an example of a typical MS and EAD MS/MS spectrum for a disulfide-linked peptide. Of the dominant fragments observed in the spectrum, 4 are the +1 and +2 charge states of the 2 corresponding peptides (SGTASVVCLLNNFYPR and VYACEVTHQGLSSPVTK) after the cleavage of the disulfide bond with EAD (green labeled peaks in Figure 2). The accurate mass data therefore provide clear information on which 2

peptides are connected by a disulfide bond. In addition, traditional disulfide-bonded peptides often suffered from low MS/MS coverage for 1 or both of the corresponding peptides when using CID, since the disulfide bond limits the comprehensive fragmentation. Here, great MS/MS fragment coverage of both peptides was achieved with EAD (93.7% for SGTASVVCLLNNFYPR and 87.5 % for VYACEVTHQGLSSPVTK), further enhancing confidence in the assignment.

Additional examples of the EAD MS/MS spectrum for disulfide-linked peptide are shown in Figure 4 (intrachain disulfide for the heavy chain Cys22-Cys96), Figure 5 (interchain disulfide between the 2 heavy chains H1 Cys 348-H2 Cys 353) and Figure 6 (intrachain disulfide for the scFv region Cys483-Cys557). Each example represents a unique disulfide linkage

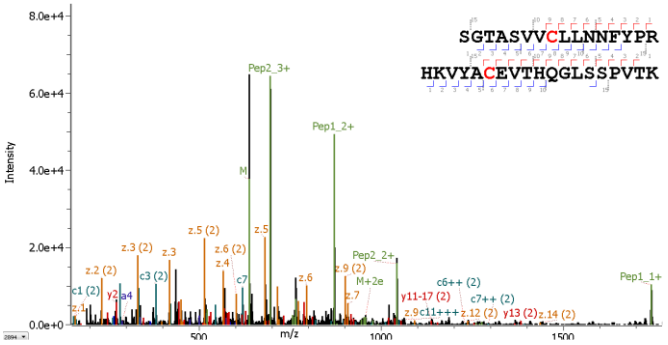

Figure 3. MS (top) and EAD MS/MS (bottom) spectrum for the disulfide-linked peptides SGTASVVCLLNNFYPR and VYACEVTHQGLSSPVTK. The peptides shown are derived from the light chain showing an intrachain disulfide bond (Cys135-Cys195).

category. All of the examples clearly show the fragment ions for the corresponding peptides and high fragment coverage (>85%) for each of the individual peptides.

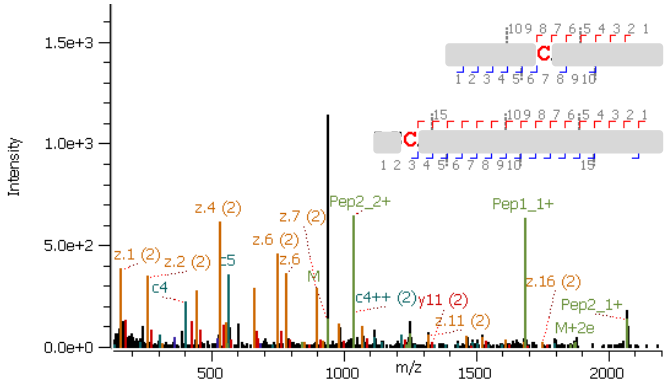

Figure 4. EAD MS/MS spectrum for an intrachain disulfide bonded peptide from the heavy chain Cys22-Cys96

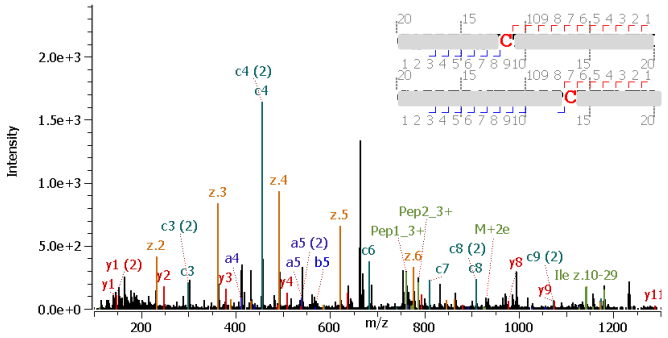

Figure 5. EAD MS/MS spectrum for a disulfide-linked peptide. The peptides shown contain an interchain disulfide bond between 2 heavy chains H1 Cys 348-H2 Cys 353.

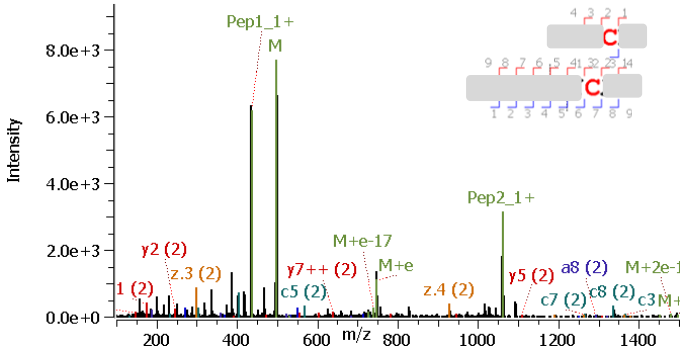

Figure 6. EAD MS/MS spectrum for a peptide with an intrachain disulfide bond in the scFv region Cys483-Cys557.

Conclusions

- Confident sequence and disulfide linkage confirmation was achieved with EAD, a novel fragmentation technique offered with the ZenoTOF 7600 system.
- MS/MS fragment detection can be significantly enhanced compared to traditional accurate mass MS/MS analysis when using the Zeno trap. This enables confident fragment assignment due to excellent data quality, even for low-abundant species.
- The robust, reproducible and easy-to-use alternative fragmentation technique of EAD enables users to directly answer challenging analytical questions with the ZenoTOF 7600 system powered by SCIEX OS software.
- Automatic, state-of-the-art data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner using Byos software from Protein Metrics Inc.

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