Applications of electron activated dissociation for next-generation biotherapeutic characterization

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INTRODUCTION

Glycoproteins have been used as an essential therapeutic modality in vaccine development, with many approved clinical applications. Glycosylation of these proteins, including glycan structures and glycan occupancy, plays a vital role in stability and efficacy. Therefore, the glycan profile is often considered a product or critical quality attribute (PQA/CQA) for these molecules. A bottom-up peptide mapping workflow is often used as part of glycopeptide characterization as it provides both protein sequence information and glycan profiling, simultaneously. However, traditional fragmentation technology often fragments side-chain glycans, failing to provide an accurate confirmation of the peptide backbone or localization of the glycosylation.

Disulfide bonds are another CQA to be monitored in all protein therapeutics. 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.[1,2] However, disulfide bonds prevent effective fragmentation around linked cysteine residues when using collision-induced dissociation (CID). This 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.

EAD, a newly developed fragmentation technique unique to the ZenoTOF 7600 system (SCIEX), allows optimized peptide fragmentation in a premade platform method.[3,4] This allows for accurate localization of the linked glycans alongside confident identification of the peptide through high MS/MS sequence coverage of the peptide backbone.[5,6] Also Zeno EAD enables fast and sensitive information-dependent acquisition (IDA). This approach overcomes challenges of alternative fragmentation, such as long reaction times, limited charge state fragmentation, and low sensitivity. Two different next generation biologics were employed to demonstrate the application of this new technology. Glycoprotein X, a vaccine containing six different N-glycan sites was used as an example to show how this method can be applied for the streamlined characterization of glycopeptides and in relation to the understanding of biotherapeutics. In addition, a multispecific mAb in which 2 different heavy chains and 2 identical light chains are linked, was used to demonstrate confirmation of disulfide linkages. One of the heavy chains was designed with an additional c-terminal single-chain variable fragment (scFv) through a linker. The scFv domain also contained multiple intrachain disulfide bonds.

MATERIALS AND METHODS

Sample preparation:
A sample of glycoprotein vaccine was denaturated with 7.2 M guanidine hydrochloride (Gua-HCl), 100 mM Tris buffer pH 7.2, followed by reduction with 10 mM DL-dithiothreitol (DTT) and alkylation with 30 mM iodoacetamide (IAM). Digestion was performed with trypsin/Lys-C enzyme at 37ºC for 16 h.

The multispecific mAb sample was denaturated with 7.2 M Gua-HCl in a buffer of 50 mM Tris-HCl, pH 7.0, and free cysteines were capped with 5 mM IAM. Digestion was performed with either trypsin/Lys-C or trypsin/Lys-C/Asp-N at 30ºC for 16 h. The reaction was stopped with 1% formic acid.

HPLC conditions:
The digests were separated with a CSH C18 column (2.1 × 100 mm, 1.7 µm particle size, 130 Å, Waters) using an ExionLC system (SCIEX). 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 300 µL/min. The column temperature was maintained at 50ºC.

MS and MS/MS conditions:
Data were acquired with an DDA method either using CID or EAD for MS/MS with the ZenoTOF 7600 system (SCIEX).

Format 6b: FAFbSASB-Tetrapod HC-scFv+LC x HC-scFv+LC

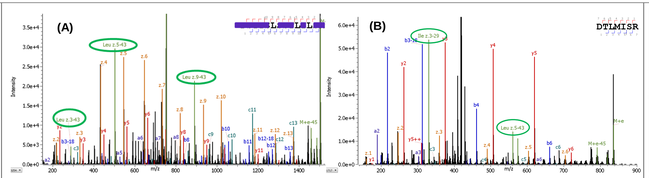

Figure 1. EAD MS/MS spectra to differentiate Leu/Ile isomers from the multispecific mAb sample. (A) MS/MS spectra of a peptide with three Leu, each of which is confirmed by *z*-43 fragment. (B) MS/MS spectra of a peptide with both Leu and Ile. *Z*-43 and *z*-29 provide the confirmation of identity.

RESULTS

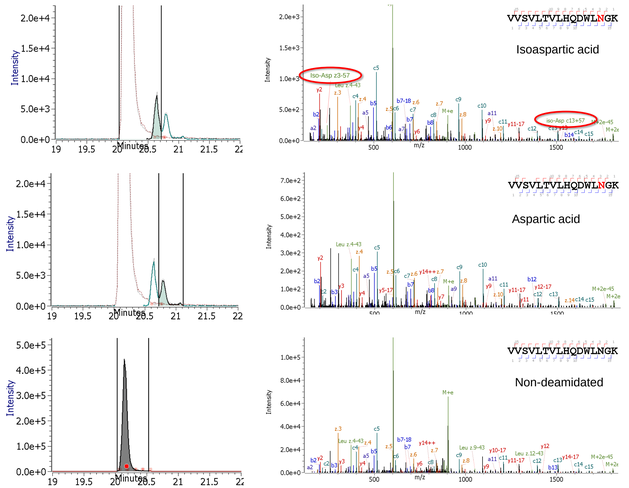

Figure 2. Data of peptide with deamidation-based modification from the multispecific mAb sample. Extracted ion chromatograms with red dots indicating the time point for MS/MS data acquisition on the left and associated EAD spectra on the right for (A) iso-aspartic acid with diagnostic ions highlighted by circles (B) aspartic acid (C) non-deamidated main form. For (A) diagnostic fragment ions *z*3-57 and *c*13+57 were observed.

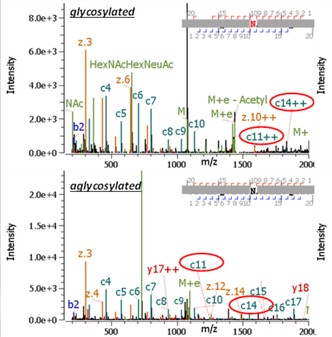

Figure 3. Comparison of glycosylated and aglycosylated peptide fragmented by EAD. MS/MS spectra are shown for a glycopeptide from glycoprotein X carrying HexNAc(5)Hex(5)NeuAc(1) at N38. The red circles highlight the *c*-ions that differ between the two peptides. Blue and red hash marks depict fragment ion coverage.

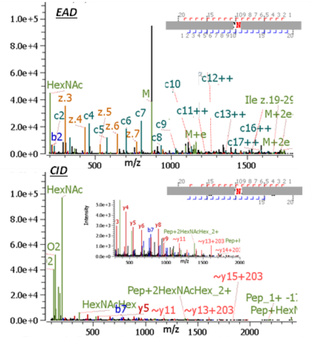

Figure 4. Comparison of glycopeptide fragmented using EAD and CID. MS/MS spectra are shown for a glycopeptide from glycoprotein X carrying G0 at N38. EAD provided not only higher fragment coverage, but localization of the glycosylation, whereas fragments lost the glycan in the case of CID.

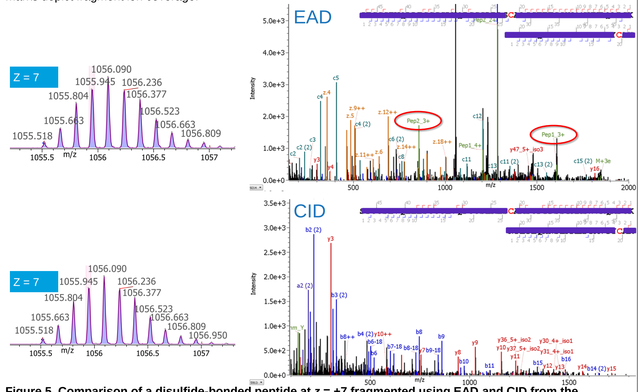

Figure 5. Comparison of a disulfide-bonded peptide at *z* = +7 fragmented using EAD and CID from the multispecific mAb sample. EAD provides ions for individual peptides (encircled fragments) and achieved an overall higher fragment coverage compared to CID.

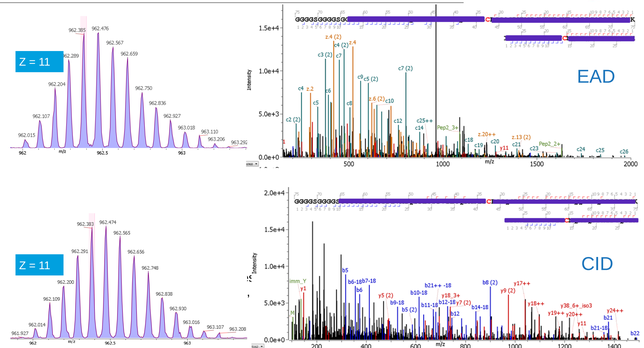

Figure 6. Comparison of disulfide peptide containing linker at *z* = +11 using EAD and CID for fragmentation from the multispecific mAb sample. EAD provides ions for individual peptides (green fragments) and achieved an overall higher fragment coverage compared to CID.

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. State-of-the art processing software from Protein Metrics Inc. is directly compatible with EAD raw data files.

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TRADEMARKS/LICENSING