A new electron activated dissociation (EAD) approach for comprehensive glycopeptide analysis of therapeutic proteins

Featuring the SCIEX ZenoTOF 7600 system using Zeno EAD and Protein Metrics Inc. software

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The data presented here demonstrate the advantage of the novel electron activated dissociation (EAD)[1,2] over traditionally used collision induced dissociation (CID) with regards to glycopeptide characterization and localization in a peptide mapping workflow. In addition, streamlined, advanced characterization in one injection is being offered through high speed, highly reproducible, alternative fragmentation.[3-6] This solution takes peptide mapping experiments to a new level.

As biotherapeutics are becoming more complex, challenges for in-depth characterization increase simultaneously. Their characterization involves a myriad of analytical methods which include, but are not limited to, amino acid sequence confirmation and identification and localization of post-translational modifications (PTMs).[3,6] Glycosylations in particular are PTMs frequently considered critical quality attributes, as their composition and levels can affect the effector functions and the in vivo half-life of a biotherapeutic product. Glycosylations tend to be present in a highly heterogenous manner in terms of structure and abundancies, increasing the complexity of their analysis.[7-8] A robust glycopeptide mapping solution can assign the glycosylation sites, determine the compositions of the attached glycans and estimate their relative abundances.

Figure 1. The SCIEX ZenoTOF 7600 system.

EAD, a newly developed dissociation approach which is unique to the SCIEX ZenoTOF 7600 system, allows for a tunable electron energy that produces varied fragmentation patterns for a wide range of peptides in a peptide mapping workflow.[1,2] The resulting MS/MS fragment ions from glycopeptides showed peptide backbone fragment ions with glycosylation remaining intact. This allows for accurate localization of the linked glycans along with confident identification of the peptide through high MS/MS sequence coverage of the peptide backbone. Zeno EAD (Figure 1) enables fast and sensitive data dependent acquisition (DDA). This approach overcomes challenges of alternative fragmentation such as long reaction times and low sensitivity. Trastuzumab was used as an example to show how users of all levels can apply this technology for their streamlined characterization of glycopeptides, improving efficiency and understanding of their biotherapeutics.

Key features of the SCIEX 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 well-suited for glycopeptide characterization
- **Higher MS/MS sensitivity:** Increased detection of fragments (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

Sample preparation: A sample of trastuzumab was denaturated with 7.2 M guanidine hydrochloride, 100 mM Tris buffer pH 7.2, followed by reduction with 10 mM DL-dithiothreitol and alkylation with 30 mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 37 °C for 16 h.

Chromatography: 10 µl (4 µg) of the trypsin/Lys-C digest were separated with a CSH C18 column (1.7 µm particle size, 130 Å, 2.1×100 mm, Waters) using an ExionLC system. The mobile phase A consisted of water with 0.1% formic acid, while the organic phase B was acetonitrile 0.1% formic acid. A gradient profile was used at a flow rate of 300 µL/min (Table 1). The column temperature was maintained at 50°C.

Mass spectrometry: Data were acquired with an information dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system. General method parameters were kept the same and are summarized in Table 2. Parameters specific for EAD or CID can be found in Table 3.

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*

Data processing: Data were processed in Byos software (Protein Metrics Inc.). To achieve side by side comparison, the standard PTM workflow was modified to include two MS/MS Id Byonic processing nodes, one for CID data processing, one for EAD data processing. All other processing parameters were kept the same. Peptide identification and fragments mass tolerance were set 6 ppm and 20 ppm, respectively. The processed results were filtered to eliminate results with MS/MS scores lower than 100.

Table 2. General MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*IDA dependent*
Gas 1	*50 psi*	
Gas 2	*50 psi*	
Curtain gas	*35 psi*	
Source temperature	*450 °C*	
Ion spray voltage	*5500 V*	
Declustering potential	*80 V*	
Collision energy	*12 V*	*
CAD gas	*7*	
Maximum candidate ion	*15*	
Intensity threshold	*125 cps*	
Charge states	*1 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*
Accumulation time	*0.25 s*	*
Time bins to sum	*8*	*10*

*specific for EAD/CID.

Table 3. MS parameters for CID and EAD.

Parameter	CID	EAD
Collision energy	*rolling*	*12 V*
Electron KE	*NA*	*7 eV*
Electron beam current	*NA*	*5500 nA*
ETC	*NA*	*100*
Zeno trap	*ON*	*ON*
Accumulation time	*0.05 s*	*0.09 s*

Glycopeptide ID

In biotherapeutics characterization, glycosylations are usually being classified as a critical quality attribute and therefore closely monitored. Liquid chromatography mass spectrometry (LC-MS) based peptide mapping is considered to be a versatile tool for characterization of protein glycosylation, since it eliminates the need to remove the glycan from the protein, while providing very comprehensive information about the molecule sequence and other PTMs.[3,6] However, traditional CID approaches can either provide fragment information of the fragile glycans when applying low collision energies or of the peptide backbone when higher collision energies are used. Achieving both at the same time and at high quality, along with a general peptide mapping approach, remains a challenge with CID. In addition, the high energies used for CID usually result in the dissociation of the glycan structures from the peptide backbone. Therefore, identification of the peptide and exact localization of the glycan is limited, especially in the case of multiple potential modification sites in a given peptide. On the other hand, in addition to diagnostic oxonium ions of the glycans, the tunable electron energy in the SCIEX ZenoTOF 7600 system produces rich peptide backbone fragment ions and fragments with the intact glycan attached, simultaneously. These data allow for confidence in the correct identification of peptides and the accurate localization and identification of the attached glycans. Using the Zeno trap in combination with EAD allows for accurate and detailed identification of even low abundant glycopeptides due to a boost in the sensitivity of the fragments.

The most intense glycopeptides for each glycan type found in the trastuzumab digest contained one miscleavage site after R304 due to the steric hindrance introduced by glycans at N300 (Figures 2, 3 and 5). Figure 2 shows an example of a glycopeptide carrying G0F. The precursor ion and fragment ion spectra from both CID and EAD were compared side-by-side. MS data were matched with a tolerance of maximal 6 ppm. Subsequent data interpretation of MS/MS spectra included the identification of peptide fragments, oxonium ions and peptide-glycan fragments (Figure 2, top right). As seen in the spectrum, the dominant ions were oxonium ions, in the case of CID, and low abundant *b*- and *y*-ions. It should be noted that the default parameters for the rolling collision energy (CID) can be adjusted to increase the coverage of the peptide backbone as shown previously[9], however this approach usually also limits the overall MS/MS sequence coverage for other peptides. Furthermore no peptide fragments with intact glycans were detected in the case of CID. On the contrary, EAD did not only provide very comprehensive fragmentation of the peptide backbone with 100% MS/MS sequence coverage being superior to CID, but the

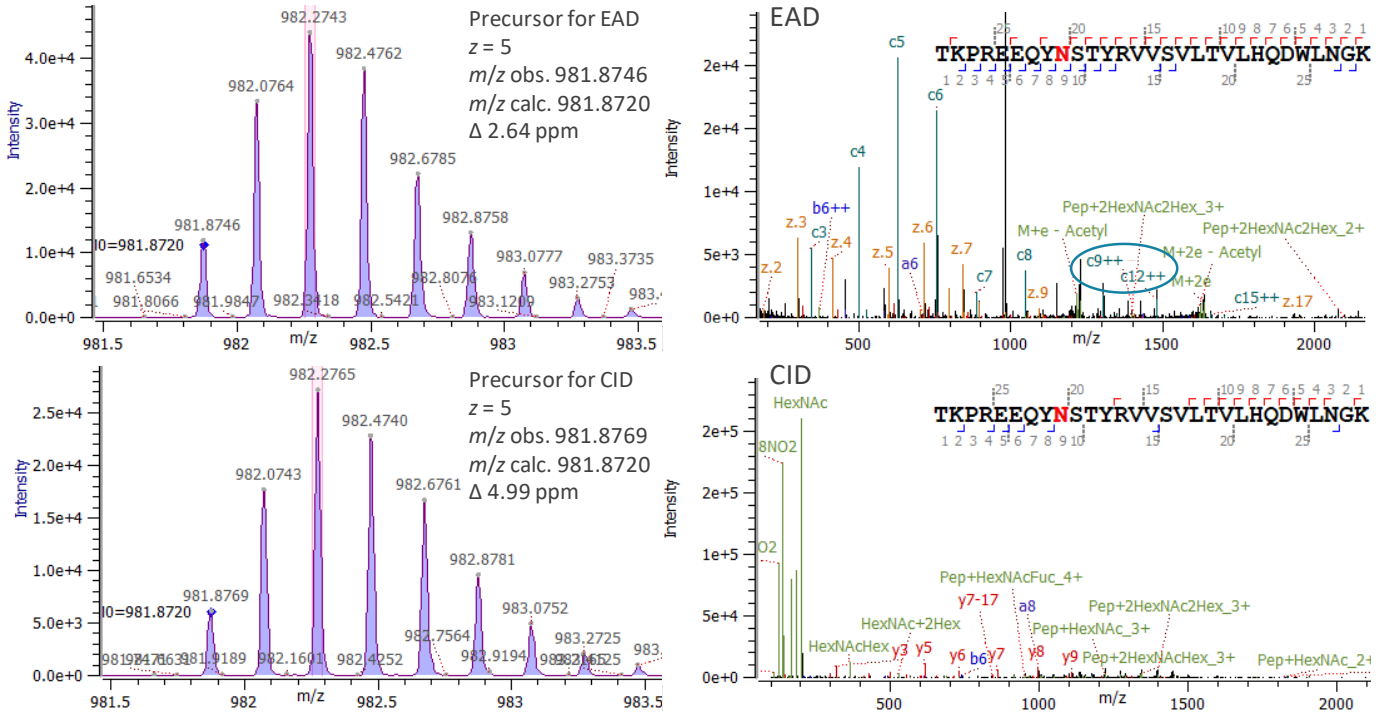

Figure 2. Side-by-side comparison of a glycopeptide fragmented using EAD and CID. Precursor ion spectra (left panel) and respective MS/MS spectra (right panel) are shown for a glycopeptide from trastuzumab carrying G0F. Blue and red hash marks depict fragment ion coverage. EAD resulted in a higher fragment coverage and better S/N for peptide backbone fragments than CID. In addition, diagnostic fragment ions confirm the localization of the glycosylation (encircled ions) in the case of EAD; whereas CID does not provide this information.

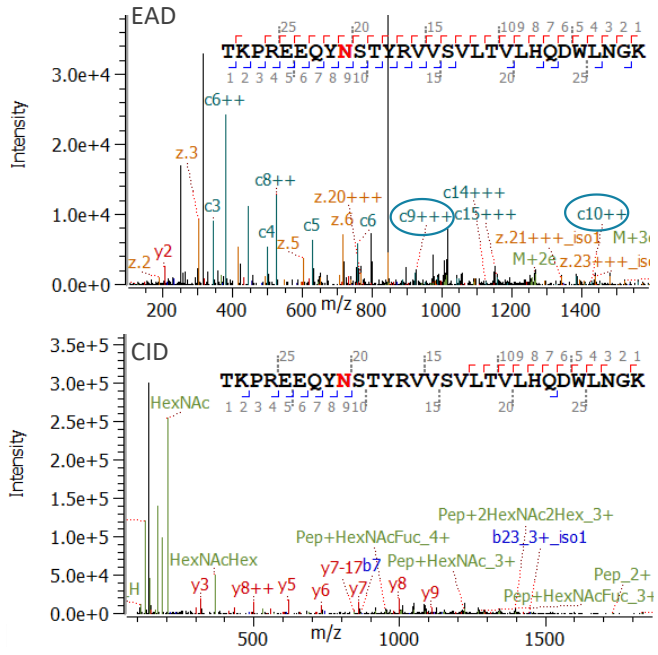

Figure 3. Comparison of glycopeptide, fragmented using EAD and CID. MS/MS spectra are shown for a glycopeptide from trastuzumab carrying G1F (precursor *z* = +6). Blue and red hash marks depict fragment ion coverage. EAD resulted in a higher fragment coverage and better S/N for peptide backbone fragments than CID. In addition, diagnostic fragment ions confirm the localization of the glycosylation (encircled ions) in the case of EAD; whereas CID does not provide this information.

doubly charged *c*9 and *c*12 ion with intact glycan also provided accurate localization of the site of modification (encircled ions in Figure 2, top right).

For the peptide with a G1F modification, a similar behavior was observed (Figure 3). Comprehensive fragmentation coverage was achieved with EAD compared to CID and ions proving the localization of the fragile modification could be detected (*c*9+++ and *c*10++ etc.). Apart from the high abundant glycosylation forms of G0F and G1F, lower abundant forms were reproducibly identified (Figure 4). One example is a high mannose species (Man5 at ~ 3%) in Figure 5. Despite its low abundance (more than 10x lower in relative abundance than the G0F-containing peptide, see Figure 4), high-quality fragment ion spectra were achieved, demonstrating the high sensitivity of Zeno EAD and Zeno CID. In addition, a full series of *z*1- *z*21 ions together with a series of *c* ions allowed for 96-100% fragment coverage for EAD, while CID only achieved 61-68% fragment coverage depending on the glycopeptide (Figures 2, 3 and 5).

For an easy review of the data, a glycan profiling report was generated. The template was formatted to sum and report the glycol forms detected in different peptide sequences (including tryptic cleaved peptides and missed cleavages) and filtered to show peptides with N-linked glycan as a single modification. The automated color coding heat map facilitates a quick understanding of which glycoforms are present in relative high, medium or low abundance, ranging from 44% to 0.2%. All N-linked glycosylations found to be present were in alignment with those previously reported (Figure 4).[10] The results demonstrate great repeatability of the Zeno EAD technology for glycopeptide analysis across different abundancies.

				MS Alias name ←	Trastuzumab EAD r(1) (%)	Trastuzumab EAD r(2) (%)	Trastuzumab EAD r(3) (%)
Protein name ↑	NGlycan ↑	Glycans ↑	Glycan Short Name ↑				
Anti-HER2 Heavy chain (1 and 2)	300	HexNAc(2)Hex(5)	Man5		3.5	3.3	3.1
		HexNAc(2)Hex(6)	Man6		0.4	0.4	0.4
		HexNAc(3)Hex(3)	G0-GlcNAc		1.4	1.4	1.6
		HexNAc(3)Hex(3)Fuc(1)	G0F-GlcNAc		6.9	6.5	7.4
		HexNAc(3)Hex(4)Fuc(1)	G1F-GlcNAc		1.5	1.3	1.7
		HexNAc(4)Hex(3)	G0		3.8	3.8	3.8
		HexNAc(4)Hex(3)Fuc(1)	G0F		44.3	44.1	42.8
		HexNAc(4)Hex(4)			1.2	1.2	1.2
		HexNAc(4)Hex(4)Fuc(1)	G1F		32.7	33.4	33.6
		HexNAc(4)Hex(5)Fuc(1)	G2F		4.0	4.1	4.2
		HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	G2FS2		0.3	0.4	0.2

Figure 4. Identification of N-linked glycosylations in trastuzumab at N300. The table summarizes the identified glycan species based on MS/MS with EAD and the relative abundance based on the XIC of the MS1 for three replicate injections. The color coding indicates the abundances from high abundant (dark red) to low abundant (pastel).

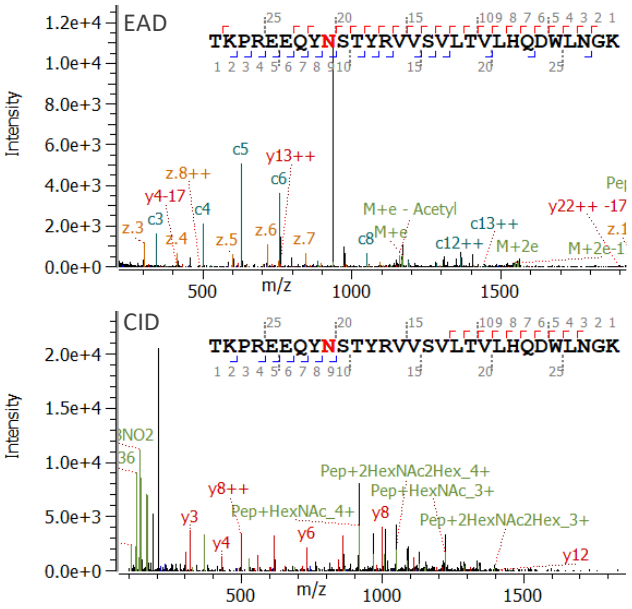

Figure 5. Comparison of glycopeptide, fragmented using EAD and CID. MS/MS spectra are shown for a glycopeptide from trastuzumab carrying Man5 (precursor *z* = +5). EAD resulted in a higher fragment coverage and better S/N for peptide backbone fragments than CID. In addition, diagnostic fragment ions confirm the localization of the glycosylation in the case of EAD; whereas CID does not provide this information.

Conclusions

- The robust, reproducible and easy-to-use alternative fragmentation mechanism EAD enables users to identify, fully characterize, and relatively quantify glycopeptides along with a general peptide mapping analysis in one single injection
- Excellent fragment coverage and localization of fragile modifications can be achieved with Zeno EAD with very high reproducibility, allowing for full confidence in peptide ID
- MS/MS fragment detection was significantly enhanced compared to traditional MS/MS analyses, enabling great data quality for confident fragment assignment even for precursors with medium or very low intensities such as modified peptides utilizing Zeno EAD
- Automatic data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner using Protein Metrics Inc. software

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