Comprehensive glycopeptide analysis of a protein-based vaccine

Featuring the SCIEX ZenoTOF 7600 system with electron activated dissociation (EAD) and Protein Metrics, Inc. software

Andrew Mahan[1], Hirsh Nanda[1], Zhengwei Chen[2], Zoe Zhang[2], Fang Wang[2], Kerstin Pohl[2]
[1]Janssen, Spring House, PA, USA; [2]SCIEX, USA

In this work, a comprehensive glycopeptide analysis of a glycoprotein vaccine is presented, using novel electron activation dissociation (EAD)[1, 2] as part of an automated data-dependent acquisition (DDA) workflow. Data reduction was accomplished with Protein Metrics, Inc. software, providing extensive glycosylation libraries and highly accurate results.

Glycoproteins have been used as an essential therapeutic modality in vaccine development, with many approved applications to date. The glycosylation of these protein-based vaccines, including glycan structures and glycan occupancy, play a vital role in stability and efficacy. Therefore, the glycan profile is usually considered a product or critical quality attribute (PQA/CQA) for these molecules. A peptide mapping workflow is generally used for glycopeptide characterization as it provides both protein sequence information and glycan profiling, simultaneously. However, CID technology often fragments the glycan structures, failing to provide an accurate confirmation of the peptide backbone and localization information. Especially with complex glycoproteins, such as vaccines containing multiple partially occupied glycosylation sites with no consensus sequence, achieving accurate and comprehensive information is becoming increasingly challenging. In comparison, EAD, a novel fragmentation technique available on the ZenoTOF 7600 system (Figure 1), allows for optimized peptide fragmentation.[1,2] It enables accurate localization of the linked glycans and confident identification of the peptides through high sequence coverage.[3,4] In addition, high-quality MS/MS information can be obtained for even low-abundant attributes by leveraging the Zeno trap in an automatic fashion during information-dependent acquisition (IDA). This approach overcomes challenges of alternative fragmentation such as long reaction times, limited charge state fragmentation, and low MS/MS sensitivity.

Figure 1. The SCIEX ZenoTOF 7600 system.

Here, a vaccine in development, containing six different N-glycan sites, was used as an example to showcase the streamlined characterization of glycopeptides and the gains EAD can provide in the understanding of complex 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, especially suited for glycopeptides
- **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 glycoprotein-based vaccine, expressed in a CHO cell line and of 25 kDa in size, containing 6 N-linked glycosylation sites, was used. It 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.

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: Data were acquired with an information dependent acquisition (IDA) method using the ZenoTOF 7600 system with either CID or EAD as fragmentation mode. General method parameters were kept the same between the two methods (Table 2). Parameters specific for EAD or CID can be found in Table 3.

Data processing: Data were processed in Byos software (Protein Metrics, Inc.). The standard PTM workflow was easily modified to create a multi Byonic node workflow containing two Byonic processing nodes, one for CID data processing, one for EAD data processing. This allowed for a simple side-by-side comparison of the two fragmentation types used. Peptide identification and fragment mass tolerance were set at 6 ppm and 20 ppm, respectively.

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 ions	*15*	
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*
Accumulation time	*0.25 s*	*
Time bins to sum	*8*	*12*

*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*

Results and discussion

Since the glycosylation of a biotherapeutic can affect its stability and immunogenicity, it is frequently classified as a CQA and therefore closely monitored. Compared to many mAb-based products, the glycosylation of a protein-based vaccine is even more complex in terms of the diversity of the glycan structure and the number of glycan sites, which can be contributed to by the expression system. Peptide mapping using liquid chromatography coupled to mass spectrometry (LC-MS) is a versatile tool for the characterization of protein glycosylation, as it can provide an ID of the protein through amino acid sequence information and the opportunity to understand the exact attachment site and nature of the glycans.[3] In addition, relative composition of each glycan and occupancy level at the site of attachment need to be examined.

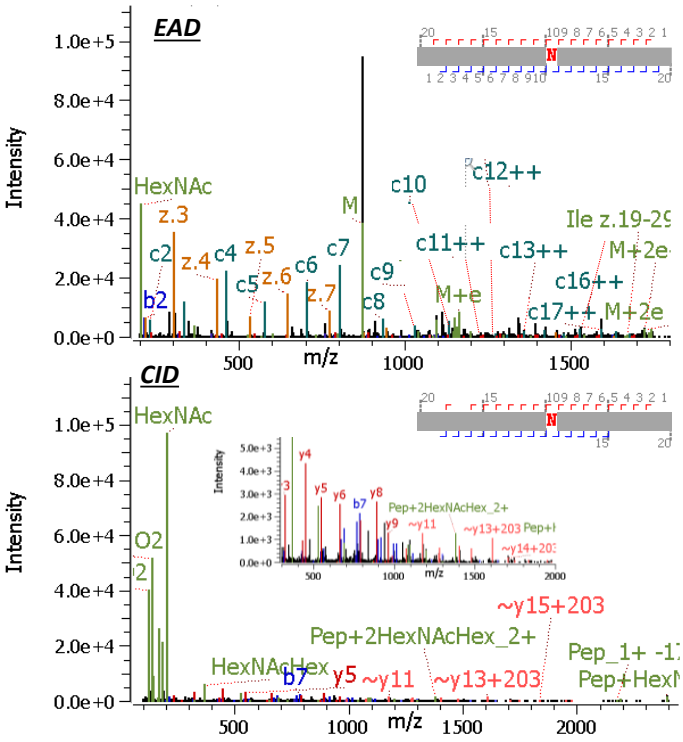

Figure 2. Comparison of MS/MS spectra of a glycopeptide using EAD and CID. MS/MS spectra are shown for a glycopeptide carrying G0 at position N38. Blue and red hash marks depict fragment ion coverage. EAD provided unambigious information on the localization of the glycosylation through comprehensive ion series with intact glycosylation (e.g. *c*11++). CID resulted in high-abundant oxonium ions and the loss of the fragile modification, limiting confident localization.

The vaccine digest used in this study was a 25 kDa glycoprotein with 6 N-linked glycosylation sites. The LC-MS analysis consisted of a platform peptide mapping workflow utilizing DDA. In the case of alternative fragmentation with EAD, a standard energy of 7 eV was used. In the case of CID, rolling collision energy was used. In both cases, standard fragmentation energies (rolling collision energy for CID and 7 eV for EAD) were used.

Sequence coverages of ~80% were obtained for both MS/MS acquisition strategies (data not shown). However, significant differences between CID and EAD in terms of the information obtained were observed (Figure 2). In addition to higher fragment coverage of the peptide backbone, EAD preserved the glycosylation on the fragments allowing for exact and unambiguous localization (*c*-ion series in Figure 2, top). In the case of CID, the labile glycans dissociated from all backbone fragments (Figure 2, bottom). Traditional CID approaches can either provide fragment information of the glycans when applying low collision energies or of the peptide backbone when higher energies are employed. The high energies used for CID usually result in the dissociation of the glycan structures from the peptide backbone. Therefore, the identification and exact localization of glycans are challenging using CID, especially if a peptide contains multiple potential modification sites. Conversely, EAD in the ZenoTOF 7600 system produces rich fragmentation providing information for peptide backbone and glycans in a single spectrum. The data was acquired in a non-targeted manner potentially allowing for ID of all detectable peptides in a given sample.

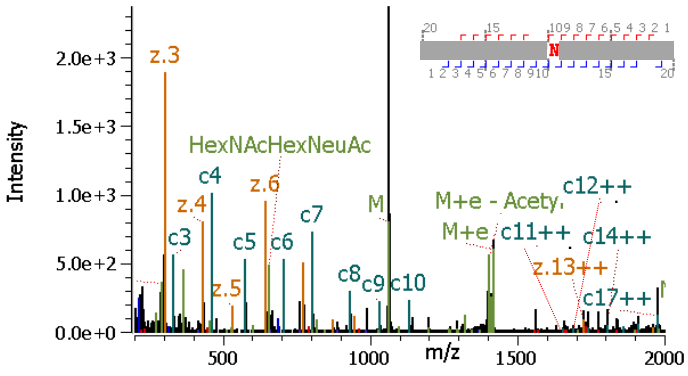

Figure 3. A low-abundant glycopeptide fragmented using Zeno EAD. MS/MS of a low-abundant (estimate ~ 0.4%) glycopeptide carrying G2FS1 at position N38. Blue and red hash marks depict fragment ion coverage with all N-terminal fragment ions in blue and the C-terminal ions in red. EAD provided unambigious information on the localization of the glycosylation through comprehensive ion series with intact glycosylation.

For low-abundant glycopeptides, it is even more challenging to obtain descriptive fragmentation information. The unique capability of the Zeno trap in combination with EAD can be a solution for such cases as it enhances the MS/MS sensitivity and spectral quality (in terms of S/N) significantly as shown

previously.[5] In Figure 3 an example of a low-abundant glycopeptide containing G2FS1 is shown, which only makes up ~0.4% of the total glycan species on that specific site. The spectrum showed an excellent signal-to-noise ratio for fragments with 90% fragment coverage. In addition, the diagnostic ions for localization of the glycosylation were identified (Figure 3).

The existence of aglycosylated peptide species is common in heavily glycosylated proteins such as vaccines (Figure 4). As expected, the series of *c*-ions from *c*3 to *c*10 are identical for both peptides. However, from *c*11-c17 (where the glycan is attached at the asparagine), the *c*-ion series differed from each other drastically. This example nicely illustrates how EAD can provide both peptide ID and the location of the glycan.

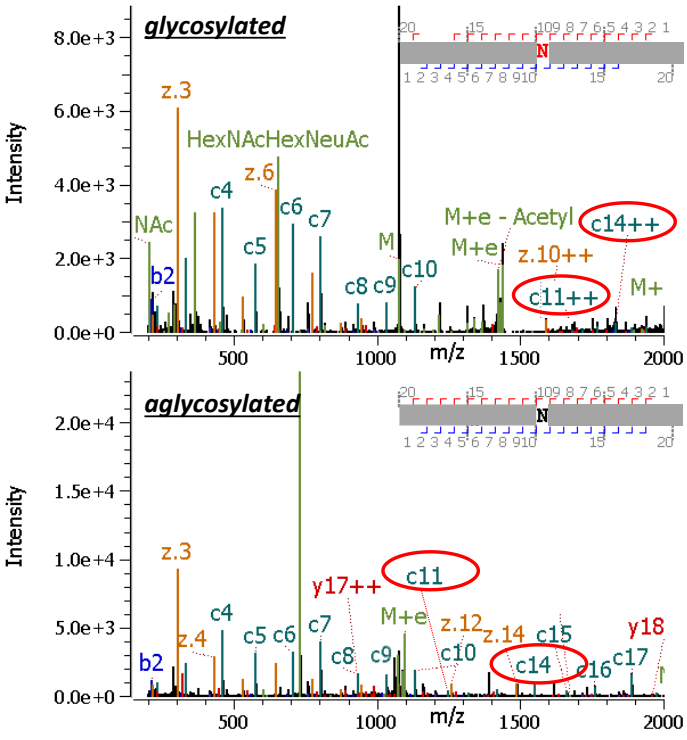

Figure 4. Comparison of MS/MS spectra of glycosylated and aglycosylated peptides using Zeno EAD. MS/MS spectra are shown for a glycopeptide carrying HexNAc(5)Hex(5)NeuAc(1) and its aglycosylated counterpart at position N38. The red circles highlight exemplary *c*-ions that differ between the two peptides. Blue and red hash marks depict fragment ion coverage with all N-terminal fragment ions in blue and the C-terminal ions in red.

Another example of glycopeptide identified from a different site, N223, is shown in Figure 5. Also at this position, the high confidence in glycan identification and localization including significant fragment coverage were obtained using Zeno EAD.

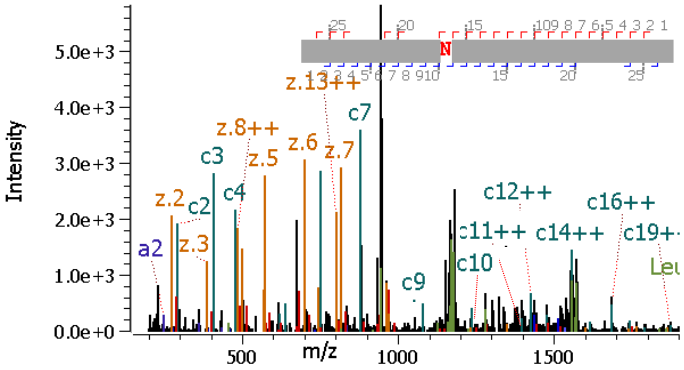

Figure 5. Glycopeptide fragmented using Zeno EAD. MS/MS spectrum for glycopeptide carrying HexNAc(4)Hex(3)Fuc(1) (G0F) at N223 is shown. Blue and red hash marks depict fragment ion coverage with all N-terminal fragment ions in blue and the C-terminal ions in red. EAD provided unambigious information on the localization of the glycosylation through comprehensive ion series with intact glycosylation (e.g. *c*11++ onwards).

A summary of all identified glycans (≥ 0.1%) at N38 and N223 was generated based on the extracted ion chromatograms using Byos (example for N38 in Figure 6). The results show consistent relative abundances between CID and EAD, providing EAD is not only capable for ID of such peptides but also for reproducible relative quantification.

From the six glycosylation sites, three were confidently identified with reverse-phase LC-MS/MS. Additionally, HILIC separation confirmed another glycosylation site on a short glycopeptide, which was not retained by the reverse-phase column (data not shown). The remaining two glycosylation sites were located on the same peptide when using a trypsin/Lys-C digest. Although Zeno EAD can provide information on such a peptide, a different enzymatic digestion approach separating these sites is recommended for obtaining the most accurate information.

			MS Alias name ←	2021 ELR3 CID (%)	2021 ELR3 EAD (%) ↓
Var. Pos. Protein	Glycans	Glycan Short Name			
	HexNAc(4)Hex(3)	G0		19.5	18.9
	HexNAc(2)Hex(3)	Man3		15.6	15.7
	HexNAc(4)Hex(4)NeuAc(1)	G1S		14.0	13.5
	HexNAc(4)Hex(4)	G1		10.0	10.0
	HexNAc(3)Hex(3)	G0-GlcNAc		7.4	9.0
	HexNAc(4)Hex(5)NeuAc(1)	G2S		7.5	7.6
	HexNAc(2)Hex(5)	Man5		4.4	4.2
	HexNAc(4)Hex(5)	G2		3.8	3.4
	HexNAc(5)Hex(5)NeuAc(1)	G2S+GlcNAc		2.7	2.7
	HexNAc(3)Hex(4)	G1-GlcNAc		2.2	2.0
	HexNAc(5)Hex(5)	A3G2		1.9	1.9
	HexNAc(4)Hex(3)Fuc(1)	G0F		2.1	1.9
	HexNAc(2)Hex(4)	Man4		1.3	1.2
	HexNAc(3)Hex(4)NeuAc(1)	G1S-GlcNAc		1.1	1.2
38	HexNAc(5)Hex(6)NeuAc(1)	A3G3S		1.1	1.2
	HexNAc(5)Hex(6)	A3G3		1.0	1.0
	HexNAc(5)Hex(5)NeuAc(2)	A3G2S2		0.7	0.7
	HexNAc(4)Hex(5)NeuAc(2)	G2S2		0.6	0.6
	HexNAc(3)Hex(3)Fuc(1)	G0F-GlcNAc		0.5	0.5
	HexNAc(4)Hex(4)Fuc(1)	G1F		0.5	0.5
	HexNAc(5)Hex(6)NeuAc(2)	A3G3S2		0.5	0.4
	HexNAc(6)Hex(4)	A4G1		0.4	0.4
	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	G2FS1		0.4	0.4
	HexNAc(4)Hex(5)Fuc(1)	G2F		0.3	0.3
	HexNAc(5)Hex(3)Fuc(1)	G0F+GlcNAc		0.2	0.2
	HexNAc(5)Hex(5)Fuc(1)	G2F+GlcNAc		0.2	0.2
	HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	FA3G3S		0.1	0.1
	HexNAc(5)Hex(6)Fuc(1)	FA3G3		0.1	0.1
	HexNAc(3)Hex(4)Fuc(1)	G1F-GlcNAc		0.2	0.1

Figure 6. Identification of N-linked glycosylations at position N38. The table summarize the relative abundances of identified glycan species based on the XIC of the MS1. The color coding indicates the abundances from high abundance (dark blue) to low abundance (pastel).

Conclusions

- Identification, localization, and relative quantification with highly descriptive data was shown for challenging N-linked glycopeptides as part of a standard DDA peptide mapping method using Zeno EAD.
- Low-abundant glycoforms were confidently identified with high-quality MS/MS spectra leveraging the automatic signal enhancement enabled by the Zeno trap in combination with EAD, reducing the need to optimize and or run targeted methods.
- Automated and highly accurate data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner using Protein Metrics Inc. software.

References

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