Comprehensive site-specific glycan profiling of a protein-based flu vaccine using electron activated dissociation (EAD)

Featuring alternative fragmentation and streamlined data analysis software

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ABSTRACT

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.

Introduction

Glycoproteins are an essential modality in vaccine development, with many approved applications to date. Glycosylation, including glycan structures and occupancy, plays a vital role in the stability and efficacy of protein-based vaccines. A peptide mapping workflow is generally used for glycopeptide characterization, as it provides protein sequence information and glycan profiling, simultaneously. Traditional collision-induced dissociation (CID) preferentially dissociates glycans and often fails to provide accurate confirmation of peptide sequences and localization information. Alternative fragmentation can provide more descriptive fragmentation, however, long reaction times and the need for peptide-based tuning has limited its usage as a routine characterization method. Here, the ability of latest ECD technology to characterize a vaccine protein with multiple glycosylation sites was examined.

MATERIALS AND METHOD

Sample preparation: A sample of glycoprotein-based vaccine, expressed in a CHO cell line of 25 kDa containing 6 N-linked glycosylation sites 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: A total of 3 µL (4 µg) of the 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 (SCIEX). The mobile phase consisted of water with 0.1% formic acid, while the organic phase was acetonitrile with 0.1% formic acid. A 60 min gradient profile was used at a flow rate of 250 µL/min. The column temperature was maintained at 50°C.

Mass spectrometry: Data were acquired using DDA with the ZenoTOF 7600 system (Figure 1). The electron energy for the alternative fragmentation in the EAD cell was set to a value of 7 eV.

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 identification using the Byonic processing nodes, one for CID data processing, one for EAD data processing. Peptide identification and fragment mass tolerance were set at 6 ppm and 20 ppm, respectively.

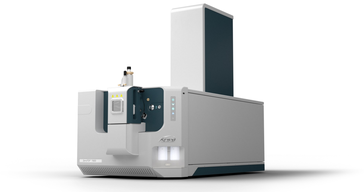

Figure 1. The ZenoTOF 7600 system from SCIEX.

RESULTS & DISCUSSION

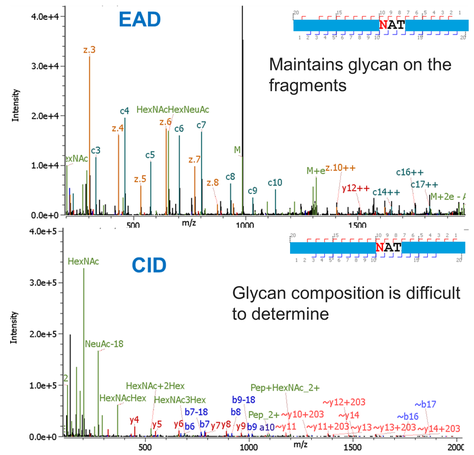

Figure 2. Comparison of MS/MS spectra of a glycopeptide using Zeno EAD and Zeno CID. MS/MS spectra are shown for a glycopeptide carrying G0 at position N38. Blue and red hash marks depict fragment ion coverage. In addition to higher fragment coverage with better signal-to-noise ratio (S/N), EAD provided unambiguous information on the localization of the glycosylation through comprehensive ion series with intact glycosylation (such as, *c*11++ onwards). CID resulted in high-abundance oxonium ions and the loss of the fragile modification, limiting confident localization of the modification.

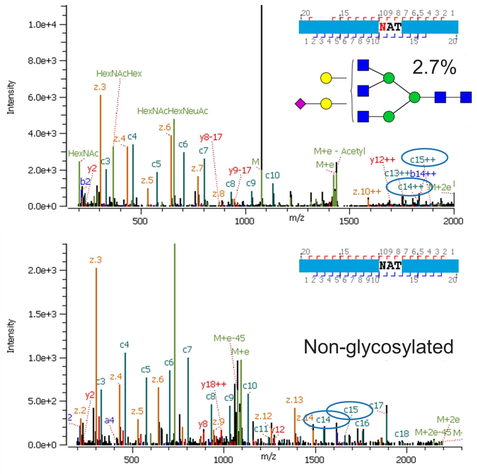

Figure 3. Comparison of MS/MS spectra of glycosylated and aglycosylated peptide using Zeno EAD. MS/MS spectra are shown for a glycopeptide carrying HexNAc(5)Hex(5)NeuAc(1) and its aglycosylated counterpart at position N38. The red circle highlight exemplary *c*-ions that differ between the two peptides based on the presence or absence of the modification. Blue and red hash marks depict fragment ion coverage with all N-terminal fragment ions in blue and the C-terminal ions in red.

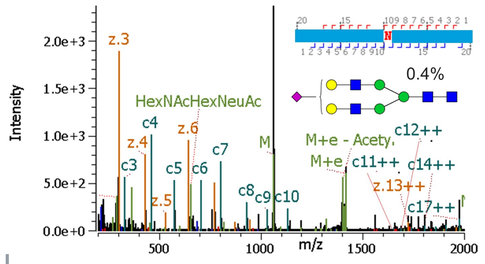

Figure 4. MS/MS spectra of low-abundance glycopeptide using Zeno EAD. MS/MS of a low-abundance (estimate ~ 0.4%) glycopeptide carrying G2FS1 at position N38. Blue and red hash marks depict fragment ion coverage with all N-terminal fragment ions in blue and the C-terminal ions in red. EAD provided unambiguous information on the localization of the glycosylation through a comprehensive ion series with intact glycosylation (*c*11++ onwards). The unique capability of the Zeno trap in combination with EAD can be a solution for analytes with very low abundance as it enhances the MS/MS sensitivity and spectral quality in terms of S/N, significantly. Here, the MS/MS spectrum of the glycopeptide showed an excellent S/N of fragments with 90% fragment coverage. In addition, the diagnostic ions for localization of the glycosylation were identified confidently.

Glycans	Glycan Short Name	MS Aliaz name ←	2021 ELR3 CID (%)	2021 ELR3 EAD (%) ↓
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
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

Table 1. Identification of N-linked glycosylation at position N38. The table summarizes 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). The results show consistent relative abundances between CID and EAD, proofing that EAD is capable of identifying peptides confidently and reproducibly allowing relative quantification.

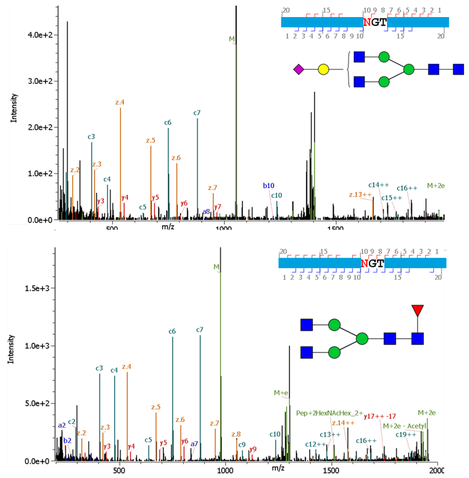

Figure 5. MS/MS spectra of glycopeptides using Zeno EAD. MS/MS spectrum for glycopeptide carrying (A) HexNAc(4)Hex(4)SA and (B) HexNAc(4)Hex(3)Fuc(1) (G0F) at N223 are shown. Blue and red hash marks depict fragment ion coverage with all N-terminal fragment ions in blue and the C-terminal ions in red. EAD provided unambiguous information on the localization of the glycosylation through comprehensive ion series with intact glycosylation (*c*12++ onwards).

Glycans	Glycan Short Name	MS Aliaz name ←	2021 ELR3 CID (%) ↓	2021 ELR3 EAD (%)
HexNAc(3)Hex(3)	G0-GlcNAc		10.9	9.5
HexNAc(4)Hex(3)	G0		10.1	9.2
HexNAc(4)Hex(5)NeuAc(1)	G2S		7.9	7.5
HexNAc(4)Hex(4)NeuAc(1)	G1S		7.5	7.1
HexNAc(3)Hex(3)Fuc(1)	G0F-GlcNAc		7.4	8.2
HexNAc(2)Hex(3)	Man3		7.2	8.1
HexNAc(4)Hex(3)Fuc(1)	G0F		6.6	6.4
HexNAc(4)Hex(4)	G1		6.2	7.6
HexNAc(4)Hex(4)Fuc(1)	G1F		5.0	5.3
HexNAc(4)Hex(5)	G2		4.1	3.8
HexNAc(3)Hex(4)	G1-GlcNAc		4.0	3.6
HexNAc(4)Hex(5)Fuc(1)NeuAc(1)	G2FS1		3.9	3.7
HexNAc(2)Hex(5)	Man5		3.4	3.8
HexNAc(5)Hex(6)Fuc(1)NeuAc(2)	FA3G3S2		2.9	2.6
HexNAc(6)Hex(7)Fuc(1)	FA4G4		2.7	3.0
HexNAc(4)Hex(5)Fuc(1)	G2F		1.7	1.5
HexNAc(3)Hex(4)Fuc(1)	G1F-GlcNAc		1.4	1.3
HexNAc(4)Hex(5)NeuAc(2)	G2S2		1.1	1.5
HexNAc(4)Hex(5)Fuc(1)NeuAc(2)	G2FS2		1.1	1.3
HexNAc(2)Hex(3)Fuc(1)	Man3F		1.0	0.9
HexNAc(5)Hex(4)Fuc(1)	G1F+GlcNAc		0.8	0.6
HexNAc(5)Hex(6)Fuc(1)NeuAc(1)	FA3G3S		0.5	0.5
HexNAc(5)Hex(6)NeuAc(1)	A3G3S		0.5	0.5
HexNAc(5)Hex(6)Fuc(1)	FA3G3		0.5	0.5
HexNAc(5)Hex(5)Fuc(1)	G2F+GlcNAc		0.4	0.5
HexNAc(5)Hex(5)NeuAc(2)	A3G2S2		0.4	0.6
HexNAc(5)Hex(6)NeuAc(2)	A3G3S2		0.4	0.4
HexNAc(6)Hex(6)Fuc(1)NeuAc(2)	FA4G3S2		0.2	0.2
HexNAc(5)Hex(6)	A3G3		0.2	0.2

Table 2. Identification of N-linked glycosylations at position N223. The table summarizes 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).

From the six glycosylation sites, three were confidently identified with reversed-phase LC-MS/MS. Additionally, HILIC separation confirmed another glycosylation site on a short glycopeptide, which was not retained by the reversed phase column here (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 peptides, a different enzymatic digestion approach separating these sites is recommended for obtaining most accurate information.

CONCLUSION

- **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 analyses 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

REFERENCES

1. Baba T *et al.* (2015) Electron capture dissociation in a branched radio-frequency ion trap, *Anal Chem*, **87**, 785−792.

2.Baba T *et al.* (2020) Dissociation of biomolecules by an intense low-energy electron beam in a high sensitivity time-of-flight mass spectrometer. *Am. Soc. Mass Spectrom.* 2021, 32, 8, 1964−1975.

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