Site-specific N-linked glycan profiling on the fusion protein aflibercept using a novel fragmentation technique

Featuring the ZenoTOF 7600 system from SCIEX with electron activated dissociation (EAD) and Biologics Explorer software

Xuezhi Bi[1] and Zoe Zhang[2]
*[1]Bioprocessing Technology Institute, A*STAR Research Entities, Singapore; [2]SCIEX, USA*

In this work, a comprehensive glycopeptide analysis of the Fc fusion protein aflibercept is presented using novel electron activated dissociation (EAD)[1,2] as part of an automated data-dependent acquisition (DDA) workflow. Data reduction was accomplished with Biologics Explorer software, providing extensive glycan libraries and highly accurate results, optimized for raw data files acquired on SCIEX accurate mass instruments.

Aflibercept is an Fc fusion protein that consists of 5 different N-linked glycosylation sites with extensive glycan heterogeneity exhibiting additional levels of complexity in terms of characterization. Glycan structures and glycan occupancy both play a vital role in the stability and efficacy of a therapeutic. Therefore, the glycan profile is often considered a product quality attribute (PQA) or critical quality attribute (CQA) for these molecules, which requires comprehensive characterization. A bottom-up peptide mapping workflow is generally used for glycopeptide characterization, as it provides information about both protein sequence and glycan distribution simultaneously. However, traditional fragmentation technology often fails to accurately confirm the sequence of the peptide and the localization of the modifications due to primarily fragmenting the sidechain glycans. Current electron-based dissociation (ExD) techniques, such as electron-transfer dissociation (ETD) and electron-capture dissociation (ECD) are also limited in their ability to easily and reproducibly provide confident identification for PQAs and CQAs to ensure safety and efficacy. Here, the ability of EAD for peptide mapping MS/MS analyses is examined to address these limitations.

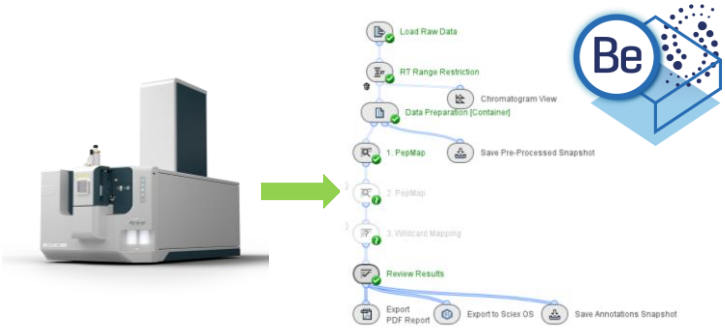

Figure 1. A streamlined characterization workflow for glycoproteins. Data are acquired on the ZenoTOF 7600 system from SCIEX and analyzed with Biologics Explorer software.

EAD is a newly developed fragmentation technique that is unique to the ZenoTOF 7600 system from SCIEX (Figure 1). EAD provides optimized peptide fragmentation with a single platform method.[1,2] This allows for accurate localization of glycans and confident identification of the peptide through high fragment coverage of the peptide backbone.[3,4] Additionally, Zeno EAD enables fast and sensitive data-dependent acquisition (DDA) enhancing sensitivity with the Zeno trap enabled. This approach overcomes long-existing challenges of alternative fragmentation, such as long reaction times, limited charge state fragmentation and low sensitivity. EAD allowed for analysis of a wide range of peptide types, derived from digested aflibercept, in a single data-dependent analysis, while Biologics Explorer software offers an accurate peptide identifications from the acquired EAD data, providing a streamlined characterization workflow.

Key features of the ZenoTOF 7600 system

- **New depths of glycopeptide mapping analysis:** EAD with a fast DDA platform method enables accurate and comprehensive glycopeptide characterization 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 confidence in identity of glycopeptides and in the localization of the modifications
- **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 Biologics Explorer software simplify the entire user experience

Methods

Sample preparation: Aflibercept containing 5 N-linked glycosylation sites was denaturated with 7.2 M guanidine hydrochloride in 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: Ten 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) platform method using the ZenoTOF 7600 system with EAD as fragmentation mode. General method parameters are shown in Table 2.

Table 2. 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*	*0.09 s*
Time bins to sum	*8*	*12*
Zeno trap	*ON*	
Electron KE	*7 eV*	
Electron beam current	*5500 nA*	
ETC	*Dynamic*	

Data processing: Data were processed in Biologics Explorer software using the peptide mapping extended workflow template, offering a quick start optimized for SCIEX raw data files.

Results and discussion

Since glycosylations of a biotherapeutic can largely affect its stability and immunogenicity, they are frequently classified as CQAs and therefore closely monitored with different analytical assays. Compared to many mAb products, the glycosylation of an Fc fusion protein can be more complex in terms of the diversity of the glycan structure and the number of glycosylation sites. In the case of aflibercept, a part of the human vascular endothelial growth factor receptor was combined with the Fc portion of human IgG.[3] Both parts contain several glycosylation sites, which leads to convoluted glycan structures as well as a variety of distribution on each site. 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 both an identification of the peptide sequence and

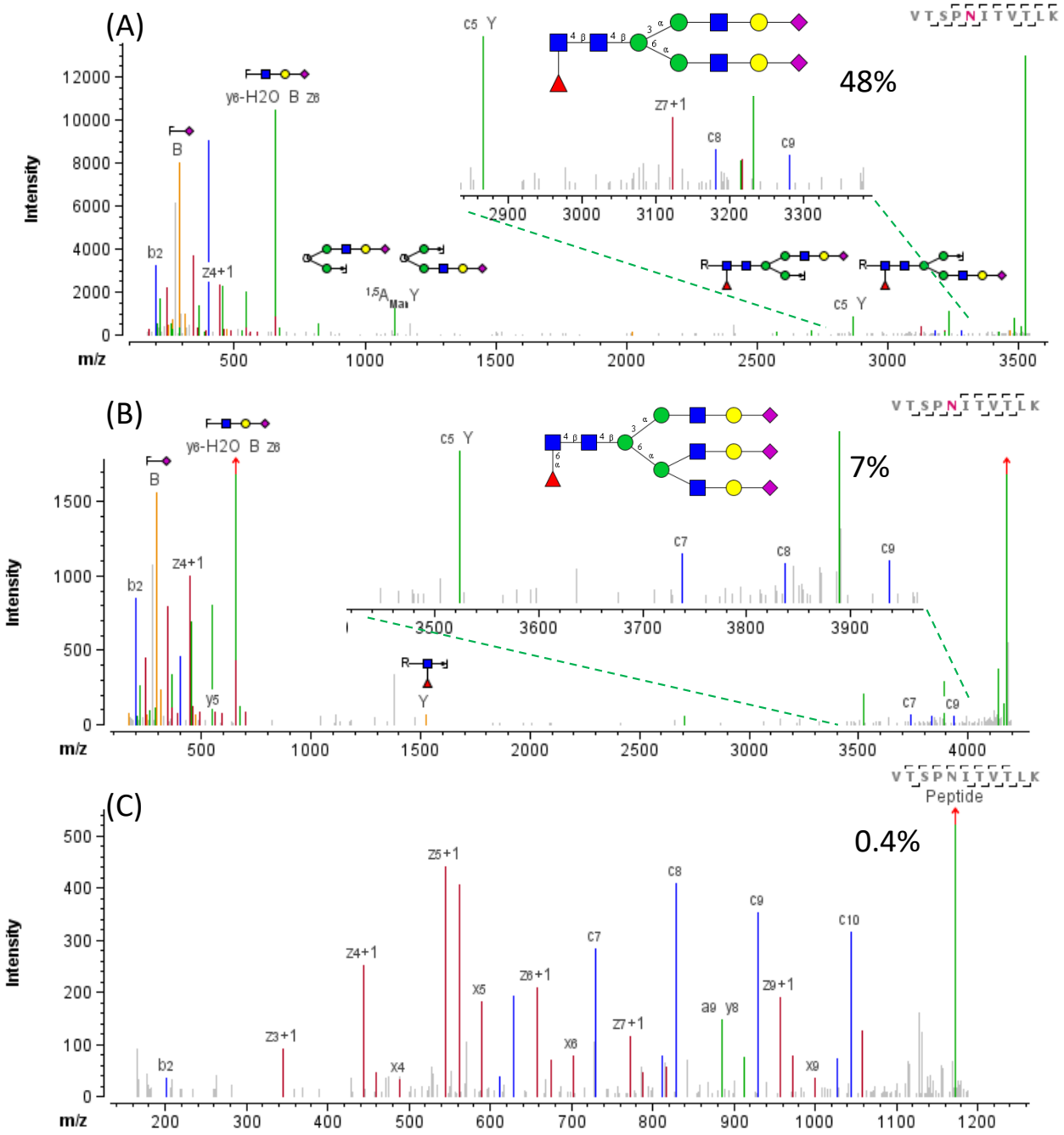

Figure 2. EAD MS/MS spectra of the peptide, VTSPNITVTLK, which contains an N-linked glycosylation site at N36. (A) The EAD MS/MS spectrum for the most abundant glycopeptide (48%) carrying G2FS2 at N36. (B) The EAD MS/MS spectrum of trace glycopeptide (7%) carrying G3S3F at N36. (C) EAD MS/MS spectrum of aglycosylated peptide (0.4%) at N36. EAD provided unambiguous information on the localization of the glycosylation through comprehensive ion series with intact glycosylation (e.g. *z7, c5*).

information about the exact position, occupancy and nature of the glycan.[4]

Aflibercept is a 48 kDa glycoprotein with 5 N-linked glycosylation sites used to treat wet macular degeneration and metastatic colorectal cancers. The LC-MS analysis consisted of a platform peptide mapping workflow utilizing DDA. In the study, a standard kinetic energy of 7 eV was utilized to demonstrate the streamlined implementation of this platform assay. No method optimization was performed for this analysis, as 7eV has been proved capable of providing comprehensive information for characterization in a single injection.[5]

EAD on the ZenoTOF 7600 system produces rich fragmentation, providing information about the peptide backbone and glycans in a single spectrum. The data were acquired in a non-targeted manner, allowing for identification of all detectable peptides in a given sample. Figure 2 shows 3 MS/MS spectra of different glycopeptides identified at site N36 in varying abundances. Excellent fragment coverages of ~91% were obtained for all 3 peptides, despite their differences in abundance. Figure 2A shows the EAD spectrum from the most abundant glycan species, G2F (48.7%) at site N36. The spectrum provides confident sequence confirmation and localization information by the presence of *c* and *z*-ions, such as *c*5 and *z*7. Meanwhile, Figure 2B demonstrates an EAD spectrum of a less abundant species, G3S3F. For low abundance glycopeptides, it is more challenging to obtain descriptive fragmentation information. The unique capability of the Zeno trap in combination with EAD provides a solution for such cases, as it enhances the overall MS/MS sensitivity and spectral quality by significantly increasing S/N, as shown previously.[6] Despite the low abundance of G3S3F (7%), its localization can be confirmed confidently because the sensitivity afforded by the Zeno trap enables the confident detection of *c*-ions, such as *c*5, *c*7, *c*8 and *c*9. In addition to a vast glycan distribution, low-level aglycosylated peptides (0.4%) can often be present in heavily glycosylated fusion proteins, as shown in Figure 2C. As expected, the *z*-ions, such as *z3–z6,* are identical for all three peptides. However, the *c*-ions, *c5–c10,* show drastic differences, confirming that glycan modifications are present in Figures 2A and 2B, but not in 2C.

Protein	Location	Modification	EAD_1 [%]	EAD_2 [%]	EAD_3 [%]
Protein	N36	A2G2F1S2	48.7	48.9	49.0
Protein	N36	A2G2F1S1	30.1	29.9	30.7
Protein	N36	A3F1S3	7.3	6.9	6.3
Protein	N36	A3F1S2	6.3	6.2	6.0
Protein	N36	A2G2F1	5.4	5.8	6.0
Protein	N36	A2G1F1S1	0.7	0.7	0.7
Protein	N36	A4F1S3	0.4	0.4	0.4
Protein	N36	A4F1S4	0.2	0.3	0.0
Protein	N36	A3F1	0.2	0.2	0.2
Protein	N36	M4A2G2S1	0.2	0.2	0.2
Protein	N36	Deamidated	0.1	0.1	0.1

Figure 3. Glycans identified at site N36 of aflibercept digest. Three replicates were performed to demonstrate the reproducibility of the identification. Only modifications above 0.1%Vol are shown in the table.

A summary of all identified glycans (≥ 0.1%Vol) at N36 was generated, including relative quantitative results based on the volume, using Biologics Explorer software (Figure 3). More than 10 different glycans were identified by MS/MS. Not only bi-antennary glycans were detected, but also tri- and tetra-antennary glycans were successfully identified, including those with multiple sialic acids, such as A4FS3 and A4FS4. Three replicate injections were examined and the relative quantification shows high reproducibility of the experimental setup and narrow CV across the replicates. The obtained results are consistent with previously published data.[7] In addition to glycan modifications, a trace level (0.1%) of deamidation was detected at N36.

The remaining 4 N-linked sites were also investigated in detail. Glycan profiles varied across the sites. A much higher level of aglycosylated species (70%) was detected for the peptide containing the N68 site (Figure 4A). This peptide was observed predominantly in its singly charged form. The analysis of singly charged peptides has been a long-standing challenge for traditional ExD approaches, limiting their adoption as a general

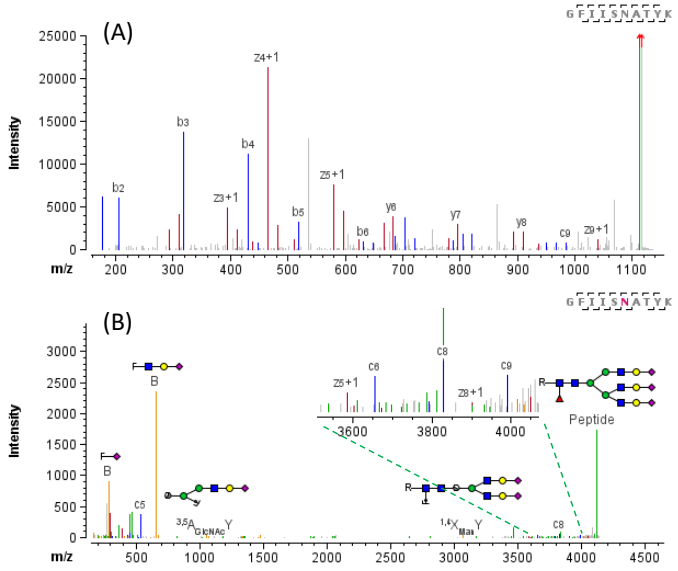

Figure 4. Zeno EAD MS/MS spectra of the peptide, GFIISNATYK, which contains an N-linked glycosylation site at N68. (A) The EAD MS/MS spectrum is shown for the +1 charged state aglycosylated peptide containing site N68. Since the peptide was found in its singly charged state, most of the fragments obtained with EAD were type *b*- and *y*-ions. (B) The MS/MS spectrum is shown for a glycopeptide carrying 0.5% G3S3F at N68. Fragments at *m/z* 204 and *m/z* 656 represent oxonium ions derived from the glycan, providing additional confirmation that the spectrum is from a glycopeptide. Furthermore, *c*-ions, such as *c*6–*c*9, provide the localization of the glycan modification.

characterization tool in assay and process development. In this study, a high-quality MS/MS EAD spectrum with dominant *b*- and *y*-ions was obtained for this singly charged peptide (Figure 4). The most abundant glycan species observed at site N68 was G2FS2, with nearly 17% relative abundance. A trace level of G3FS3 was also detected at 0.5% (Figure 4B). Oxonium ions were detected in this spectrum at *m/z* 204 and *m/z* 656, confirming the existence of the glycan modifications. The presence of *c6–c9* fragments confirmed the location and identity of the glycan.

Aflibercept is known for having hybrid glycan occupancy, as shown previously.[6] In this study, multiple hybrid glycans were detected at sites N123 and N196, such as M4A1G1S1 and M5A1G1S1. One example of hybrid glycan detection at site N123 is shown in Figure 5. The glycan was identified and localized to this position with high confidence using Zeno EAD. The high-abundance glycan oxonium ion was detected at *m/z* 656 from HexHexNAcSA, confirming the spectrum was derived from a glycopeptide (Figure 5). Multiple *c*- and *z*-ions, such as *c*5 and *z*6, confirm that this hybrid glycan is located at position N123. A second example of a hybrid glycopeptide identified at another site, N196, is shown in Figure 6. This 5 amino acid long peptide contains an N-terminal glycan modification. A series of *z*-ions, *z*2–*z*5, in the low *m/z* range provide unambiguous sequence confirmation (Figure 6 bottom), while a series of *c*-ions confirm glycan localization and identification (Figure 6 top).

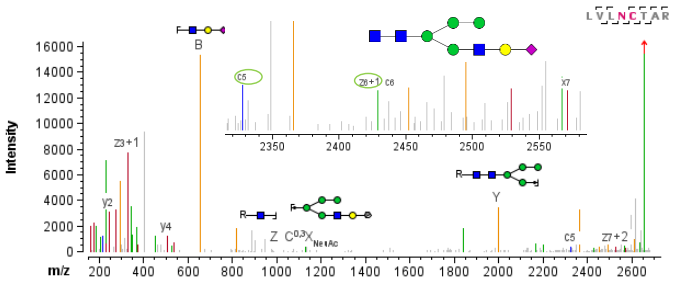

Figure 5. Glycopeptide with modification at N123 fragmented using Zeno EAD at N123. The MS/MS spectrum for a glycopeptide carrying M4A1G1S1 at N123 is shown. Zeno EAD provided unambiguous information about the localization of the glycosylation by the presence of characteristic ions, such as *c*5 and z6.

A summary of all identified glycans (≥ 0.1%) at N196 and their relative abundances based on volume was generated using Biologics Explorer software (Figure 7). The glycosylation site N196 showed a more complex glycan distribution compared to N36 (Figure 7 vs. Figure 3). Similarly, the results show consistent relative abundances across different replicates, of that DDA leveraging EAD is capable of comprehensive identification of singly and multiply charged peptides, and can allows for reproducible relative quantification at the same time. Besides detection of multiple complex and hybrid glycans, a few

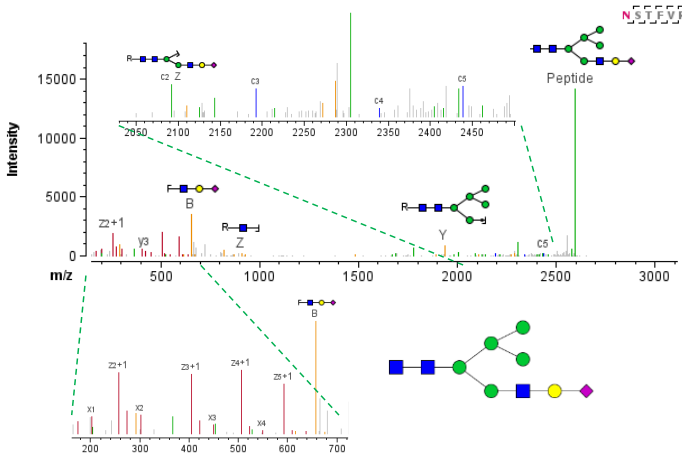

Figure 6. Zeno EAD spectrum of glycopeptide at N196. The MS/MS spectrum for a glycopeptide carrying M5A1G1S1 at N196 is shown. EAD provided unambiguous information about the localization of the glycosylation by the presence of characteristic ions, such as *c*2, *c*3, *c*4, and *c*5, top inset). (Bottom inset) A series of *z*-ions at low *m/z* range confirm the sequence of this short peptide.

high-mannose species were detected, such as Man5. Site-specific glycan profiling of each of the 5 sites (N36, N68, N123, N196 and N282) was performed, though only data from sites N36 and N196 are shown as examples in this technical note. The glycan distribution observed were consistent with previous published data.[7]

Protein	Location	Modification	EAD_1 [%]	EAD_2 [%]	EAD_3 [%]
Protein	N196	A2G2S1	37.8	39.1	39.5
Protein	N196	A2G2	21.7	18.0	19.5
Protein	N196	A2G2S2	15.8	16.2	16.2
Protein	N196	M5	8.3	10.6	8.8
Protein	N196	A2G1	3.0	2.5	2.7
Protein	N196	M4A1G1	2.4	1.6	1.5
Protein	N196	M4A1G1S1	2.2	2.4	2.3
Protein	N196	A2G1S1	2.1	2.4	2.5
Protein	N196	A2G2F1	1.9	1.8	2.0
Protein	N196	M5A1G1S1	1.8	1.9	1.9
Protein	N196	M5A1G1	1.4	1.8	1.5
Protein	N196	M4	0.6	0.4	0.3
Protein	N196	A2G2F1S2	0.3	0.3	0.3
Protein	N196	M4A2G2S1	0.3	0.3	0.3
Protein	N196	M4A2G2	0.3	0.2	0.2
Protein	N196	A2G2Sg1	0.1	0.1	0.1
Protein	N196	A2G1B	0.1	0.1	0.0
Protein	N196	A1	0.1	0.1	0.0
Protein	N196	A2G1F1	0.0	0.1	0.1
Protein	N196	A2G2B	0.0	0.2	0.2

Figure 7. Glycans identified at site N36. Three technical replicates were performed to demonstrate the reproducibility of the identification. Only modifications above 0.1%Vol are shown in the table.

Conclusions

- Identification, localization and relative quantification with highly descriptive data were achieved for challenging N-linked glycopeptides as part of a standard DDA peptide mapping method using Zeno EAD and Biologics Explorer software
- Low abundance glycoforms were confidently identified with high-quality MS/MS spectra, leveraging the automatic signal enhancement enabled by the Zeno trap in combination with EAD, thus 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 Biologics Explorer software

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.
3. Hermosilla, Jesus (2020) Comprehensive biophysical and functional study of ziv-aflibercept:characterization and forced degradation. *Scientific Reports* 10, Article number 2675.
4. Comprehensive peptide mapping of biopharmaceuticals utilizing electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12639-B.
5. An evaluation of a single injection platform method for advanced characterization of protein therapeutics using electron activation dissociation (EAD). SCIEX technical note, RUO-MKT-02-13965-A.
6. ZenoTOF 7600 system BE EXTRAORDINARY. SCIEX Brochure, RUO-MKT-07-13304-A.
7. Shen Z et al (2021) Analytical comparability assessment on glycosylation of ziv-aflibercept and the biosimilar candidate. *Int J Biol Macromol*. 180:494-509.

SCIEX

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

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices