Comprehensive characterization of glycation in protein therapeutics using electron activated dissociation (EAD)

Featuring the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

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This technical note highlights the power of electron-activated dissociation (EAD) for the comprehensive characterization of glycation in protein therapeutics Figure 1. Collision-induced dissociation (CID) provided insufficient fragmentation of glycated peptides. In comparison, EAD resulted in excellent fragmentation and coverage of glycated peptides, significantly increasing confidence in the identification and the accuracy of the localization of glycation.

Glycation is a common non-enzymatic modification of monoclonal antibodies (mAbs). Glycation increases the heterogeneity of mAbs and may also lead to protein aggregation and discoloration of the products.[1-2] Therefore, it is critical to fully characterize and closely monitor glycation in different phases of the drug lifecycle to ensure product quality. Characterizing glycated peptides using traditional collision-based MS/MS approaches, such as CID, is challenging due to the preferential cleavage of water from the hexose moiety and the low yield of sequence ions. By comparison, electron-based MS/MS approaches can provide complete sequence information about glycated peptides for accurate localization of glycation in the sequence.[1–2]

In this technical note, CID and EAD were employed to characterize glycated peptides produced from forced glycation of NISTmAb. The advantages of EAD over CID for identification and localization of glycation are highlighted.

Key features of EAD for comprehensive characterization of glycated peptides

- **Confident identification**: EAD provides an excellent fragmentation of glycated peptides, leading to high confidence in sequence identification
- **Accurate localization**: The glycation moiety remains intact in EAD fragments, allowing unambiguous localization of the modification
- **High quality**: The Zeno trap of the ZenoTOF 7600 system provides 5–10-fold increases in detection of MS/MS fragments, leading to excellent EAD data for glycated peptides
- **Fast and flexible**: EAD can be operated in data-dependent acquisition (DDA) or MRM^{HR} mode with a fast scanning rate (~20 Hz in DDA mode) and with the ability to tune electron kinetic energy (KE)

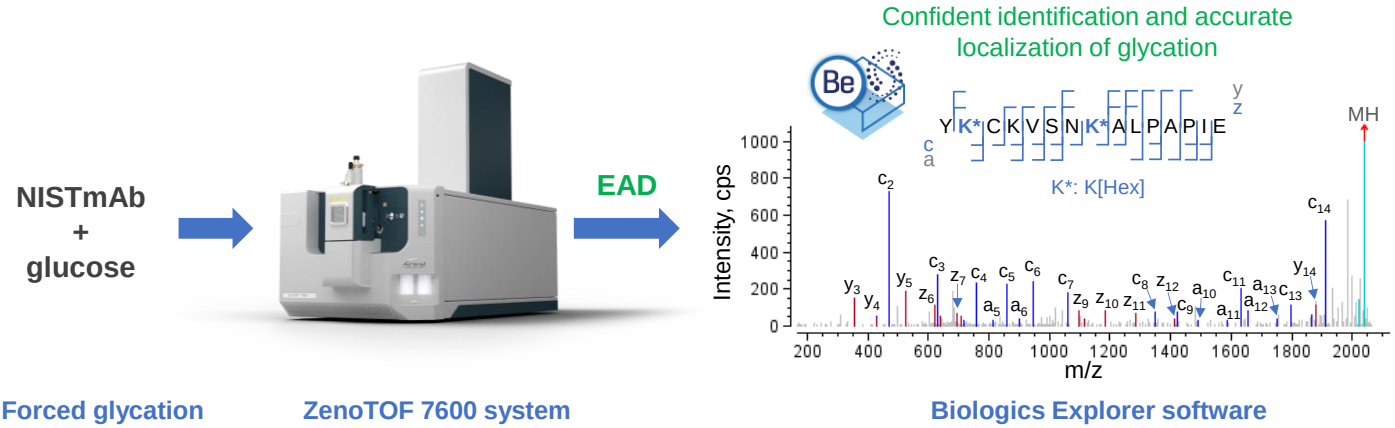

Figure 1. Comprehensive characterization of glycation using the SCIEX ZenoTOF 7600 system. EAD provides an excellent fragmentation of glycated peptides for confident identification and accurate localization of glycation. Illustrated on the right is a deisotoped EAD spectrum of a doubly glycated peptide from the Biologics Explorer software. The positions of 2 glycation moieties among 3 potential glycation sites (Lys residues) were determined unambiguously given the detection of a complete series of sequence ions.

Methods

Sample preparation: Three vials of 10 µg/µL NISTmAb (RM 8671, NIST) were mixed with 500mM glucose (Sigma-Aldrich) and incubated at 60°C for 4 days. The forced glycation samples were denatured in guanidine hydrochloride, reduced with dithiothreitol and alkylated using iodoacetamide, followed by buffer exchange using Bio-Spin 6 columns (Bio-Rad Laboratories). The 3 vials of samples were separately digested (at 37°C for 3 hours) using 3 different enzymes, including trypsin/Lys-C mix (Promega), chymotrypsin (Promega), and Glu-C (Promega). 5 µL and 10 µL of the final digests (~5-10 µg) were injected for CID and EAD analysis, respectively.

Chromatography: The peptides were separated on an ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters) using the gradient shown in Table 1. A flow rate of 0.25 mL/min was used for the separation. The column was kept at 60°C in the column oven of an ExionLC system (SCIEX). Mobile phase A was 0.1% formic acid (FA) in water and mobile phase B was 0.1% FA in acetonitrile.

Table 1. LC gradient for peptide separation.

Time (min)	A (%)	B (%)
Initial	*98*	*2*
2	*98*	*2*
62	*65*	*35*
65	*50*	*50*
67	*10*	*90*
70	*10*	*90*
71	*98*	*2*
75	*98*	*2*

Mass spectrometry: CID and EAD DDA data were acquired in SCIEX OS software using the ZenoTOF 7600 system. The key TOF MS and MS/MS settings are listed in Tables 2 and 3, respectively.

Table 2. TOF MS parameters.

Parameter	Value
Spray voltage	*5500 V*
TOF start mass	*400 m/z*
TOF stop mass	*1800 m/z*
Accumulation time	*0.1 s*
Source temperature	*400ºC*
Declustering potential	*80 V*
Collision energy	*10 V*
Time bins to sum	*8*

Table 3. MS/MS parameters using CID or EAD.

Parameter	CID	EAD
IDA criteria	*Peptide*	
Maximum candidate ions	*10*	
Charge state	*1-10*	*2-10*
Isotope to select	*Most intense*	
Exclude time	*4 s after 2 occurrences*	
Dynamic CE for MS/MS	*True*	*False*
Dynamic ETC for MS/MS	*False*	*True*
Start mass	*100 m/z*	
Stop mass	*2,000 m/z*	
Zeno trap	*ON*	
Zeno threshold	*100,000 cps*	
Accumulation time	*0.08 s*	*0.1 s*
Declustering potential	*80 V*	
Time bins to sum	*10*	
Electron beam current	*N/A*	*5,500 nA*
Electron KE	*N/A*	*7 eV*
Reaction time	*N/A*	*20 ms*

Data processing: CID and EAD data were analyzed using the Biologics Explorer software. A maximum of 4 hexose modifications at Lys or Arg residues per peptide sequence was allowed in peptide mapping.

Glycation of mAbs

Glycation of a recombinant mAb occurs when the proteins are exposed to reducing sugars, such as glucose, during fermentation or storage.[1-2] Glycation may cause several negative consequences to the mAbs, including 1) reduced antigen binding, 2) increasing heterogeneity, 3) higher likelihood for protein aggregation, and 4) the possibility of further degradation to generate advanced glycation products (AGEs), which are linked to discoloration of the product.[1-2] Given these negative impacts on product quality, glycation is a potential critical quality attribute that must be fully characterized and closely monitored.

Typically, the level of glycation in mAbs is low under the optimized fermentation and storage conditions. There is no consensus sequence for glycation, so the modification may occur at any accessible residues (Lys or Arg), further limiting the abundance of glycated peptides. In this work, forced glycation of NISTmAb in the presence of glucose was performed to increase the level of glycation for better characterization and enhanced understanding of this important modification.

Peptide mapping of glycated NISTmAb

Since glycation occurs at Lys or Arg residues, which renders these sites inactive for proteolytic cleavages, trypsin is not the ideal enzyme for generating glycated peptides. For this reason, 3 enzymes with complementary cleavage patterns, including trypsin/Lys-C, chymotrypsin, and Glu-C, were employed to obtain a complete peptide map of NISTmAb. EAD data for individual digests provided varying degrees of coverage for NISTmAb with forced glycation (60%-83% for heavy chain (HC) and 75%-99% for light chain (LC)), the combined peptide map from all EAD data led to an excellent sequence coverage of NISTmAb (~95% for HC and 100% LC, Figure 2). This result was comparable to that obtained using CID (~93% for HC and 100% LC). However, as will be described below, EAD provided much better fragmentation of glycated peptides than CID, resulting in higher confidence in identification and more accurate localization of glycation.

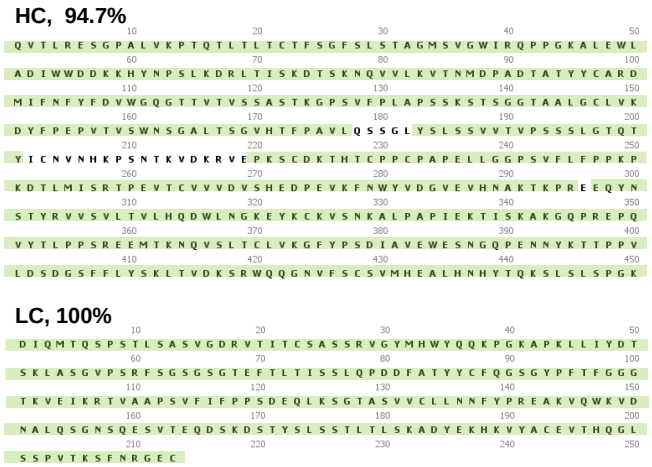

Figure 2. Sequence coverage of NISTmAb from EAD data from forced glycation samples. Sequence coverages of ~95% and 100% were obtained for the HC and LC of NISTmAb, respectively based on EAD data of 3 forced glycation samples prepared using trypsin/Lys, chymotrypsin, or Glu-C.

The HC and LC of NISTmAb contain 49 Lys and 17 Arg residues in total. The combined EAD data from 3 digests led to the identification of glycation for nearly all of these sites (46 Lys and 17 Arg), indicating the prevalence of this modification under the forced degradation condition. The detailed results of peptide mapping using CID and EAD will be presented elsewhere. The comparisons of CID vs. EAD fragmentation of representative glycated peptides from the Glu-C digest will be highlighted in the following section.

Identification and localization of glycation

CID is not the ideal MS/MS approach for characterization of glycated peptides due to the preferential cleavage of the hexose moiety of glycation and generally insufficient fragmentation of the

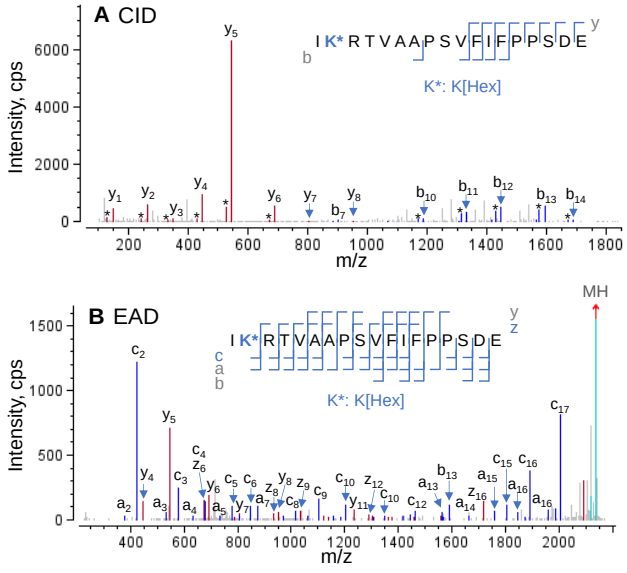

Figure 3. CID *vs.* EAD of the singly glycated peptide 105-122 from LC. This glycated peptide contains two potential glycation sites (1 Lys and 1 Arg residue). Although CID (A) produced enough fragments for confident identification, it cannot pinpoint the glycation site due to incomplete fragmentation of the peptide. By comparison, a nearly complete coverage of the peptide was achieved by EAD (B), leading to unambiguous localization of the glycation moiety to the Lys residue in this peptide. Note: not all the fragments were labeled for spectral clarity. All the CID/EAD spectra shown in this technical note are deisotoped spectra from the Biologics Explorer software. * Water loss products of the adjacent *b/y* ions.

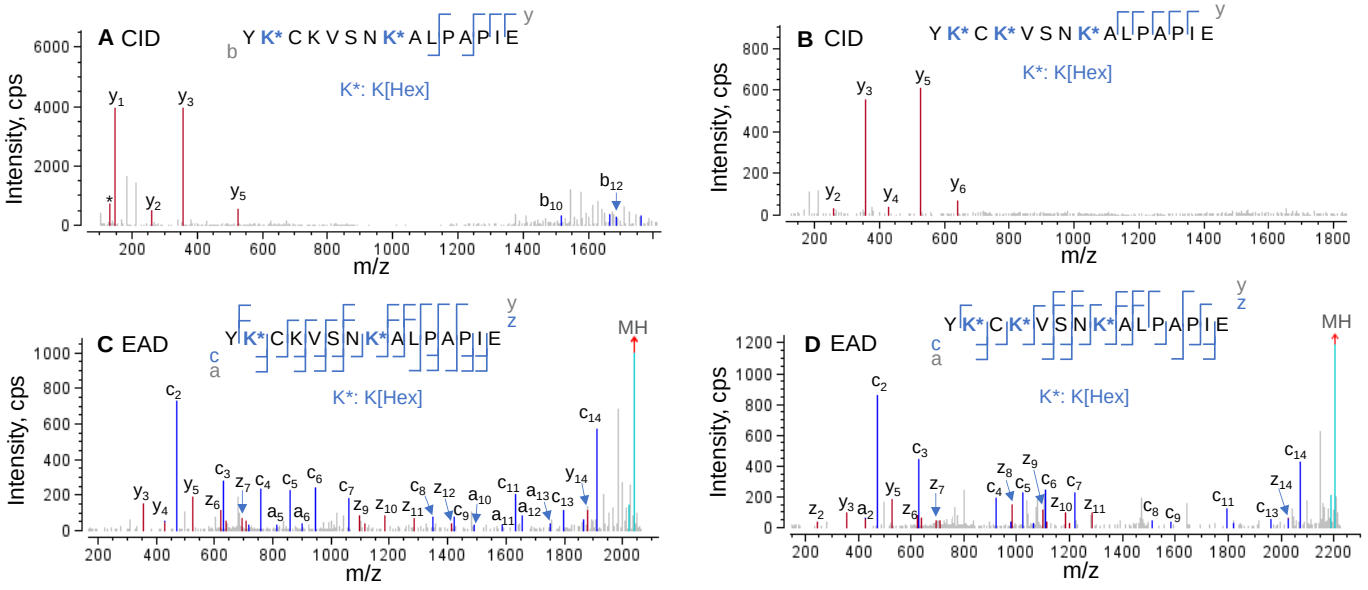

Figure 4. Localization of glycation in the doubly and triply glycated peptide 322-336 from HC. CID led to poor fragmentation of the doubly and triply glycated peptide 322-336 (A and B), making it challenging to pinpoint the glycation sites. By comparison, the EAD spectra of these 2 glycated peptides (C and D) were rich in sequence ions (*c*/*z*/*a*/*y*), resulting in higher confidence in peptide identification and no ambiguity in localization of the glycation moieties. Specifically, 2 glycation sites (out of three Lys residues) in the doubly glycated peptide cannot be confidently determined with the CID data (A), whereas unambiguous localization of these 2 glycation moieties can be achieved using EAD (C).

backbone of a glycated peptide.[1-2] It was demonstrated previously that EAD is superior to CID in the characterization of labile modifications such as glycosylation and sulfation.[3-5] The peptide mapping data from this work showed that EAD also provided significant benefits for the characterization of glycated peptides, particularly for the long or multiply glycated peptides.

The CID and EAD spectra of a singly glycated peptide 105-122 from LC were compared in Figure 3. This peptide contains 2 potential glycation sites (1 Lys and 1 Arg residue) near the N-terminus. Although CID generated enough *b*/*y* ions for confident identification of this glycated peptide, it did not provide clear evidence for accurate localization of the glycation moiety (Figure 3A). By comparison, EAD produced a nearly complete series of *c*/*z* fragments and additional *a*/*b*/*y* ions for improved confidence in the identification and precise location of glycation (Figure 3B). The location of the glycation moiety in this peptide can be pinpointed to the Lys residue based on a complementary pair of c_2/z_{16} fragments detected by EAD.

The advantage of EAD over CID was more significant for the characterization of long or multiply glycated peptides. Figures 4A and 4B show that the CID of a doubly and a triply glycated peptide 322-336 from HC generated a limited number of *b*/*y* fragments, leading to low confidence in sequence identification. Additionally, the exact locations of the glycation moieties in these 2 multiply glycated peptides cannot be accurately determined from the CID data (Figure 4A and 4B). In contrast, EAD resulted in an excellent fragmentation of 2 glycated peptides (Figure 4C and 4D), leading to a complete sequence coverage and unambiguous localization of the glycation moieties. Specifically, the 2 glycation sites were determined to be Lys^{323} and Lys^{329} in the doubly glycated peptide occurring at residues 322-336 (Figure 4C).

The CID and EAD spectra of a long peptide (7-48 from HC) carrying 1 or 2 glycation moieties are displayed in Figures 5 and 6, respectively. Similar to the examples described above (Figure 4A and 4B), few fragments were detected in the CID data from this long singly or doubly glycated peptide (Figure 5), resulting in an ambiguity in localization of glycation among 3 potential sites (2 Lys and 1 Arg residues). In stark contrast with the CID data, the excellent EAD spectra of 2 long glycated peptides (Figure 6) led to the highly confident identification and unambiguous localization of the glycation moieties. EAD data revealed that the Lys^{45} near the C-terminus was glycated in the singly glycated peptide 7-48 (Figure 6A), while the other Lys residue but not Arg residue was also glycated in the doubly glycated peptide (Figure 6B).

Another excellent example showcasing the power of EAD for the characterization of glycation is displayed in Figure 7. Glu-C digestion of the forced glycation sample led to the formation of an HC peptide at residues 298-321 that carries 1 N-linked glycosylation and 2 potential glycation sites. Since glycation is a hexose modification (+162 Da), the peptide modified with 1 G1F and 1 glycation moieties has the identical mass as that carrying 1 G2F. Differentiation between these 2 isomeric species is not possible at the MS1 level. The CID spectrum of the peptide occurring at residues 298-321 that was modified with 1 G1F and 1 glycation moieties was dominated by the oxonium ions produced from the glycan (Figure 7A). Only several *y*-type sequence ions in very low abundance were detected in CID. Hence, the CID data were insufficient for confident identification of this glycated glycopeptide, nor for accurate localization of the 2 modifications or differentiate between this peptide and its G2F isomer. In comparison, the EAD MS/MS of this glycated glycopeptide produced rich sequence ions for highly confident identification (Figure 7B). In addition, the unique ability of EAD to retain labile modifications[3-5] in the fragments enabled accurate localization of 1 G1F and 1 glycation, instead of 1 G2F, in this peptide. For example, the detection of a c_3 ion containing the

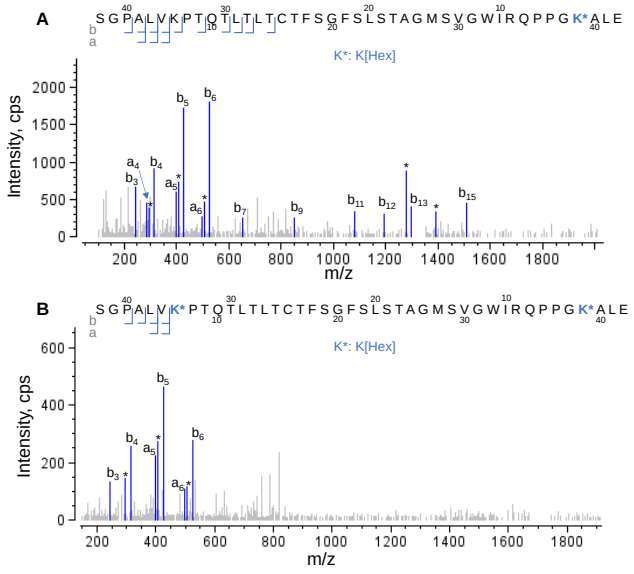

Figure 5. CID spectra of a long glycated peptide occurring at residues 7-48 of the HC. * Water loss products of *b* ions. CID fragmentation of the singly (A) and doubly (B) glycated peptide 7-48 was far from desirable for high confident identification and localization of glycation.

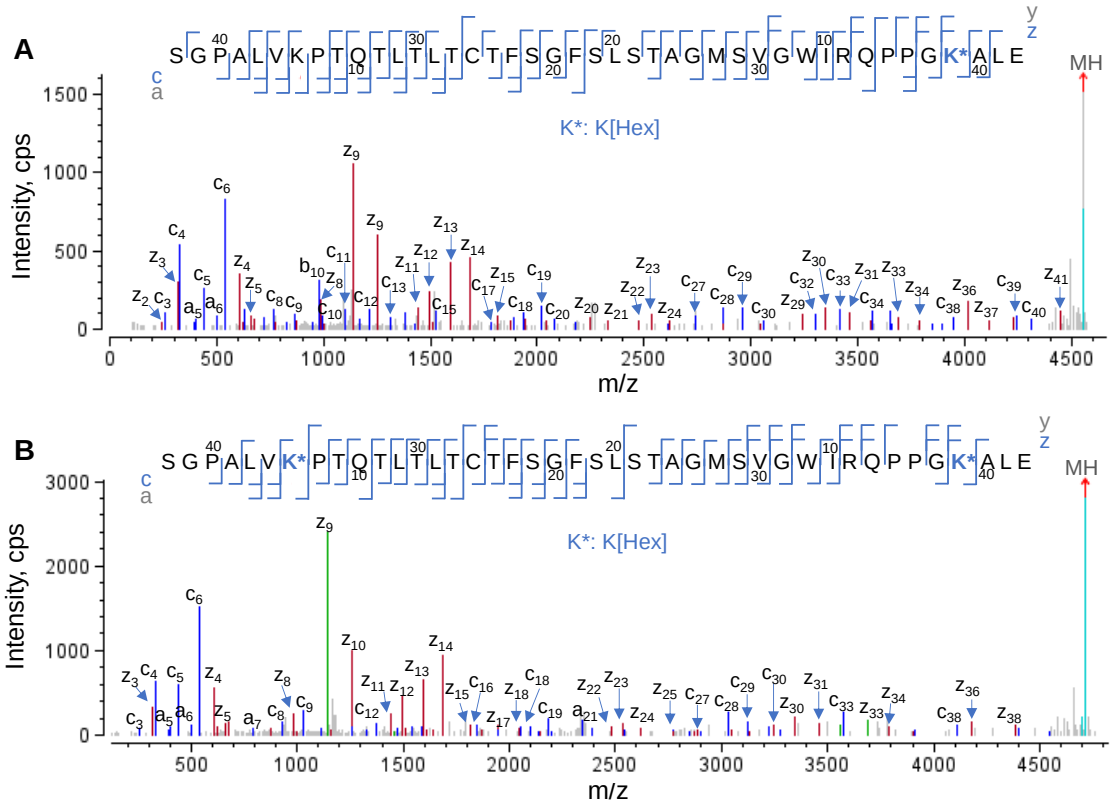

Figure 6. EAD spectra of a long glycated peptide occurring at residues 7-48 of the HC. EAD resulted in extensive fragmentation of this long peptide that was modified with 1 (A) or 2 (B) glycation, leading to confident peptide identification and accurate localization of glycation.

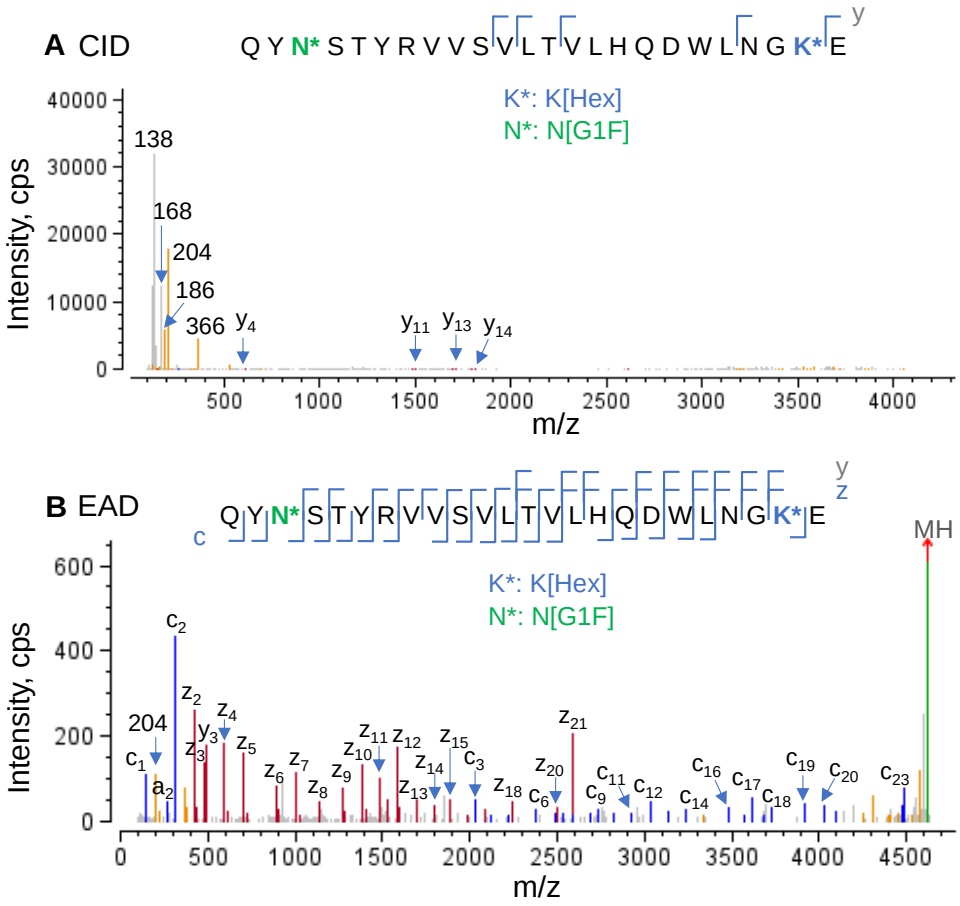

Figure 7. CID and EAD spectra of the HC peptide occurring at residues 298-321 and carrying 1 glycosylation (G1F) and 1 glycation. The CID spectrum (A) of this glycated glycopeptide was dominated by the oxonium ions generated from the glycan (G1F). Only a few y ions at very low abundance were produced by CID. By comparison, EAD led to complete coverage of the peptide, facilitating accurate localization of both glycosylation and glycation in the same sequence.

G1F and z_2/y_2 carrying 1 glycation pinpointed the locations of these two moieties.

In summary, EAD offered significant advantages over CID for the comprehensive characterization of glycation. Compared to CID, EAD resulted in much more extensive fragmentation of glycated peptides, regardless of peptide length and number of glycation moieties, for improved confidence in the identification and localization of glycation.

Conclusions

- EAD is powerful for confident identification and unambiguous localization of glycation — a challenging modification for traditional collision-based approaches
- In general, CID led to incomplete fragmentation of glycated peptides, particularly for the long or multiply glycated species
- EAD provided excellent fragmentation of glycated peptides in different lengths, carrying a different number of glycation moieties or containing other labile modifications (e.g., glycosylation)
- The EAD-based approach described in this technical note can be leveraged to facilitate comprehensive characterization of glycated peptides from protein therapeutics

References

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