Comprehensive characterization of advanced glycation end products (AGEs) 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 demonstrates the power of electron activated dissociation (EAD) to confidently identify and characterize advanced glycation end products (AGEs) in protein therapeutics. EAD resulted in excellent fragmentation and complete coverage of AGEs, leading to high confidence in sequence identification and localization of AGE moieties.

Protein therapeutics with glycation can undergo further degradation to produce AGEs *in vivo*.[1-2] AGEs might cause discoloration of therapeutic products and induce adverse immune responses.[1-4] Despite their significance to product quality, AGEs are poorly characterized because they have low abundances and are difficult to fragment using traditional collision-based MS/MS approaches.[2-4] In comparison, EAD can provide excellent fragmentation of glycated peptides,[4] allowing the accurate localization of the glycation moiety to differentiate between positional isomers.

In this technical note, EAD was employed to characterize the AGEs produced from forced glycation of NISTmAb (Figure 1). EAD enabled the precise localization of the AGE moieties, even in the presence of glycation and/or glycosylation. To our knowledge, this is the first report on the characterization of AGEs using an alternative fragmentation approach.

Key features of EAD for the comprehensive characterization of glycation and AGEs

- **Confident identification**: EAD enables excellent fragmentation of glycated peptides and AGEs, leading to high confidence in sequence identification
- **Accurate localization**: The glycation or AGE moieties are retained in EAD, allowing unambiguous localization of these labile modifications and differentiation between isomeric Lys- and Arg-associated AGEs
- **High quality**: The Zeno trap provides a 5–10-fold increase in the detection of MS/MS fragments, improving EAD data quality for glycated peptides or AGEs at varied concentrations
- **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)

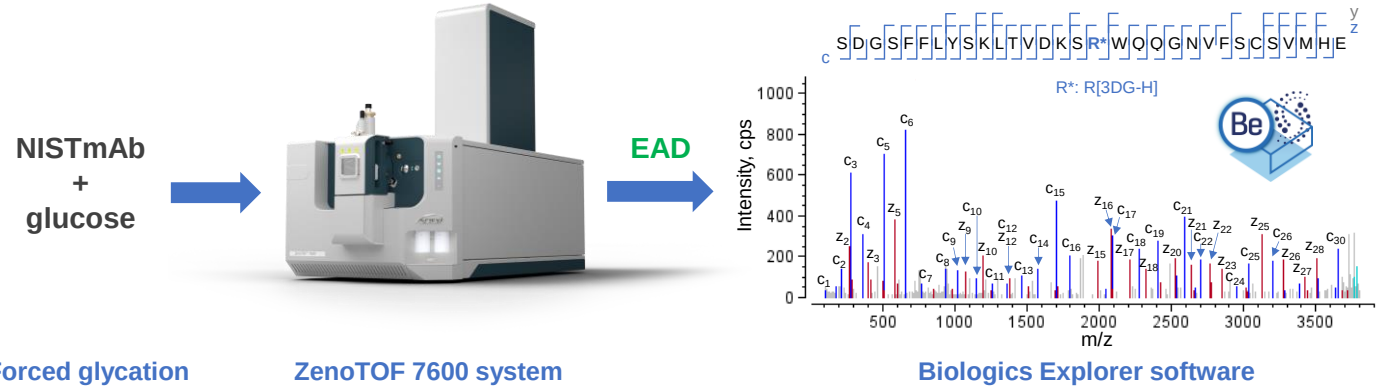

Figure 1. Comprehensive characterization of AGEs using the SCIEX ZenoTOF 7600 system and Biologics Explorer software. EAD provides excellent fragmentation of AGEs for confident identification and accurate localization of the AGE moieties. Illustrated on the right is a deisotoped EAD spectrum of an AGE in the forced glycation sample of NISTmAb. EAD generated a complete series of sequence ions while retaining the 3-deoxyglucosone hydroimidazolone (3DG-H) moiety for this AGE, enabling unambiguous localization of 3DG-H to the Arg residue.

Methods

Sample preparation: Three vials of 10 µg/µL NISTmAb (RM 8671, NIST) were mixed with 500mM glucose (Sigma-Aldrich) and incubated at 60ºC for 4 days. The solution turned yellow-brown following forced glycation. The stressed samples were denatured in guanidine hydrochloride, reduced with dithiothreitol and alkylated with iodoacetamide. Buffer exchange was then performed using Bio-Spin 6 columns (Bio-Rad Laboratories). Three vials of sample were separately digested (at 37ºC for 3 hours) using 3 different enzymes, including trypsin/Lys-C mix (Promega), chymotrypsin (Promega) and Glu-C (Promega). The final digests were injected in 10 µL aliquots (~10 µg) for EAD analysis.

Chromatography: The peptides were separated with the gradient displayed in Table 1 using an ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters). A flow rate of 0.25 mL/min was used for the peptide separation. The column was kept at 60ºC 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.

Mass spectrometry: 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.

Data processing: EAD data were analyzed using the peptide mapping workflow templates within the Biologics Explorer software. A maximum of 4 hexose modifications at Lys or Arg residues per peptide sequence was allowed in peptide mapping. The AGE modifications on Lys residues that were considered for data analysis included Hex-1H_2O (+144.04 Da), Hex-2H_2O (+126.03 Da), carboxymethyl (CML, +58.01 Da), carboxyethyl (CEL, + 72.02 Da), furosine or pyrraline (FRS/PRL, +108.02 Da). The AGE modifications on Arg residues that were considered for data analysis included 3DG-H (+144.04 Da), dihydropyrimidine (DHPM, +126.03 Da), glyoxal hydroxyimidazolone (G-OH, +37.98 Da) and glycoal hydroimidazolone (G-H, +39.99 Da). Oxidations at Met, His and Trp residues were set as variable modifications.

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*

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 EAD.

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

Glycation and AGEs of mAbs

Glycation of a recombinant monoclonal antibody (mAb) occurs when the proteins are exposed to reducing sugars, such as glucose, during fermentation or storage.[1-2] Glycated mAbs can undergo further degradation to form AGEs *in vivo* or under stress conditions.[1-2] While glycation does not change the color of mAbs, AGEs are linked to the discoloration of the product.[3-4] Additionally, AGEs might trigger the expression of AGE-specific receptors and induce adverse immune responses.[1-2] Given these safety concerns, glycation and AGEs must be fully characterized and closely monitored during the lifecycle of a protein therapeutic.

Detailed characterization of AGEs by LC-MS has been scarce[3] due to the low abundances of glycation and AGEs and the poor fragmentation of these species with traditional collision-based MS/MS approaches. In this work, NISTmAb was thermally stressed in the presence of glucose for 4 days to promote the formation of AGEs, which were then characterized using EAD. The results described in this technical note enhance our understanding of AGEs and highlight the power of EAD to comprehensively characterize these challenging modifications.

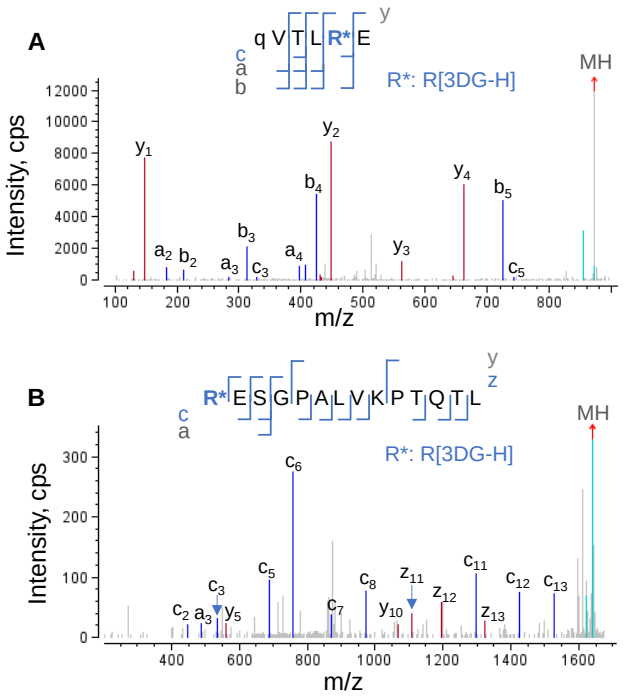

Figure 2. EAD MS/MS spectra of 2 Arg[5]-modified AGEs from Glu-C and chymotrypsin digestions of NISTmAb. The heavy chain (HC) peptides HC[1-6] (A) and HC[5-18] (B) from Glu-C and chymotrypsin digestions of NISTmAb, respectively, contained the same AGE moiety (3DG-H) at the same amino acid residue (Arg[5]). Excellent fragmentation of 2 peptides carrying AGEs by EAD led to confident identification and accurate localization of the AGE moiety. In addition, high-quality EAD data excluded the possibility of an isomeric species for HC[5-18] (B), in which the Lys residue is modified with Hex-1H_2O. Note: All the EAD spectra shown in this technical note are deisotoped spectra from the Biologics Explorer software. q = pyro-Glu

Comprehensive characterization of AGEs from NISTmAb with forced glycation

As described in a previous technical note, 3 enzymes with complementary cleavage patterns, including trypsin/Lys-C, chymotrypsin and Glu-C, were employed to facilitate the complete characterization of glycated peptides that resulted from the forced glycation of NISTmAb.[5] Here, digestion of glycated

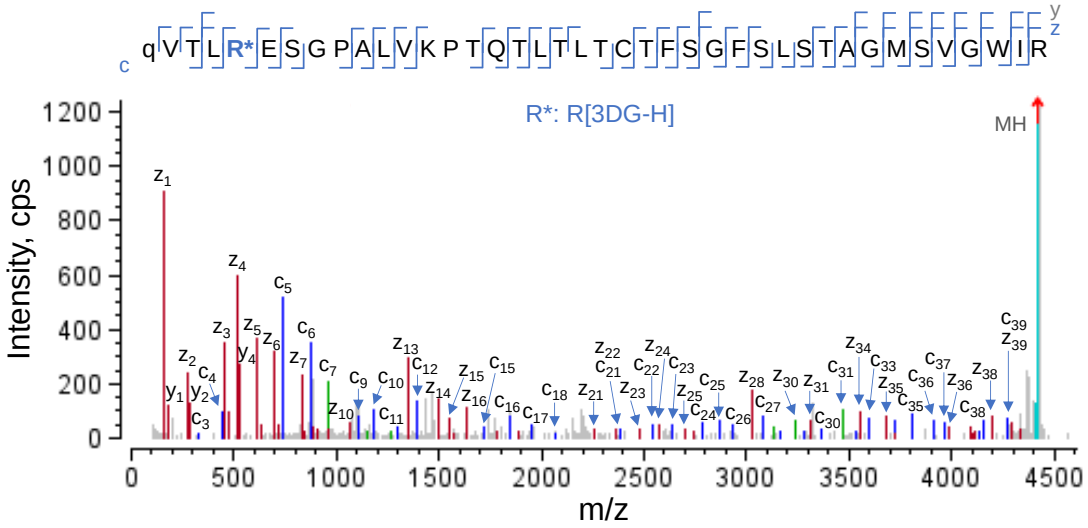

Figure 3. EAD MS/MS spectrum of an Arg[5]-modified AGE from the trypsin/Lys-C digestion of NISTmAb. Trypsin/Lys-C digestion of NISTmAb produced a long peptide HC[1-40] containing 1 3DG-H modification. EAD of this AGE led to the formation of a nearly complete series of *c*/*z* ions containing 3DG-H which enabled precise localization of the modification to the Arg[5] residue. q = pyro-Glu

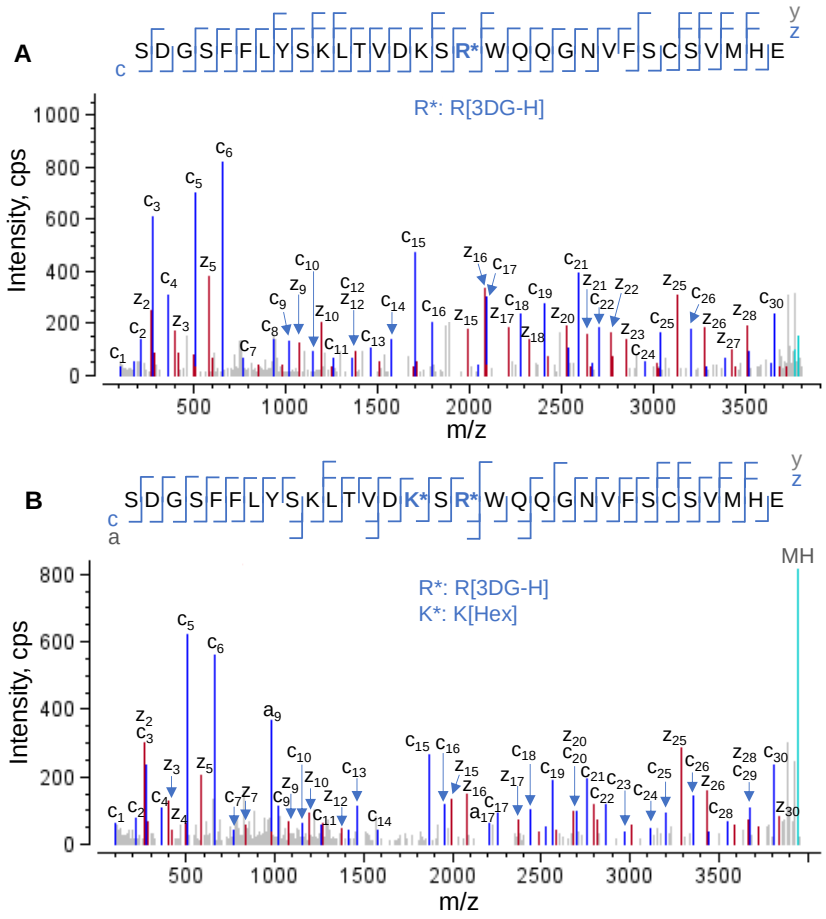

Figure 4. EAD MS/MS spectra of the peptide HC[403-433] carrying 1 or 2 modifications from the Glu-C digestion. The excellent fragmentation coverage provided by EAD enabled accurate localization of the 3DG-H modification in HC[403-433] containing 3DG-H only (A) or 1 3DG-H and 1 glycation moiety (B).

NISTmAb using these enzymes produced different length peptides that carried the same AGE modification on the same site (Lys or Arg). High-quality EAD data of these peptides, regardless of the length, provided additional confidence in identifying the AGEs.

EAD MS/MS spectra of 3 N-terminal HC peptides that contained the same AGE modification (3DG-H at Arg^5) are displayed in Figures 2 and 3. Glu-C digestion of glycated NISTmAb produced an N-terminal peptide from HC[1-6], with 6 amino acid residues (Figure 2A). In contrast, the chymotrypsin and trypsin/Lys-C digestions produced the longer peptides HC[5-18] and HC[1-40], respectively. Despite a drastic difference in peptide length, EAD resulted in complete or nearly complete fragmentation of these peptides with AGE modifications, enabling confident peptide identification and accurate localization of the 3DG-H moiety to Arg^5 (Figures 2 and 3).

One of the challenges faced when characterizing glycation or AGE modifications is the differentiation between positional isomers that carry modifications with identical Δmass values on different amino acid residues. For example, the differentiation between isomeric AGEs containing a Hex-$1H_2O$ modification on a Lys residue or a 3DG-H on an Arg residue ($+C_6H_8O_4$ or +144.04Da for both AGE modifications), is challenging. EAD can address this challenge because it yields excellent fragmentation of peptides with AGEs while retaining the labile modifications. The accurate localization of 3DG-H to Arg^5 in the AGEs described here (Figures 2 and 3) eliminated the possibility of a positional isomer in which a Lys residue was modified with Hex-$1H_2O$.

Excellent fragmentation of AGEs using EAD was also achieved for those containing multiple modifications. Figure 4 displays EAD MS/MS spectra of the peptide HC[403-433] modified with a 3DG-H moiety (Figure 4A) and the same peptide carrying both a 3DG-H and a glycation moiety (Figure 4B). The detection of a nearly complete series of *c*/*z* fragments for 2 AGEs allowed accurate localization of 3DG-H individually (Figure 4A) or simultaneously with the glycation moiety (Figure 4B).

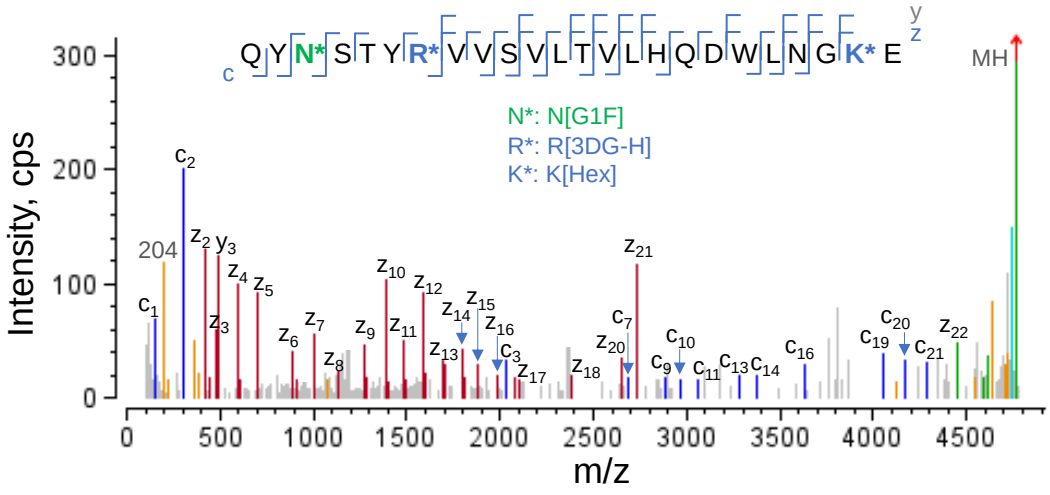

Figure 5. EAD MS/MS spectrum of the peptide HC[298-321] carrying 1 glycan (G1F), 1 glycation and 1 AGE (3DG-H) moiety from the Glu-C digestion. The ability of EAD to provide extensive fragmentation of the peptide backbone while preserving labile modifications allowed for simultaneous localizations of glycosylation, glycation and AGE moieties in the same sequence.

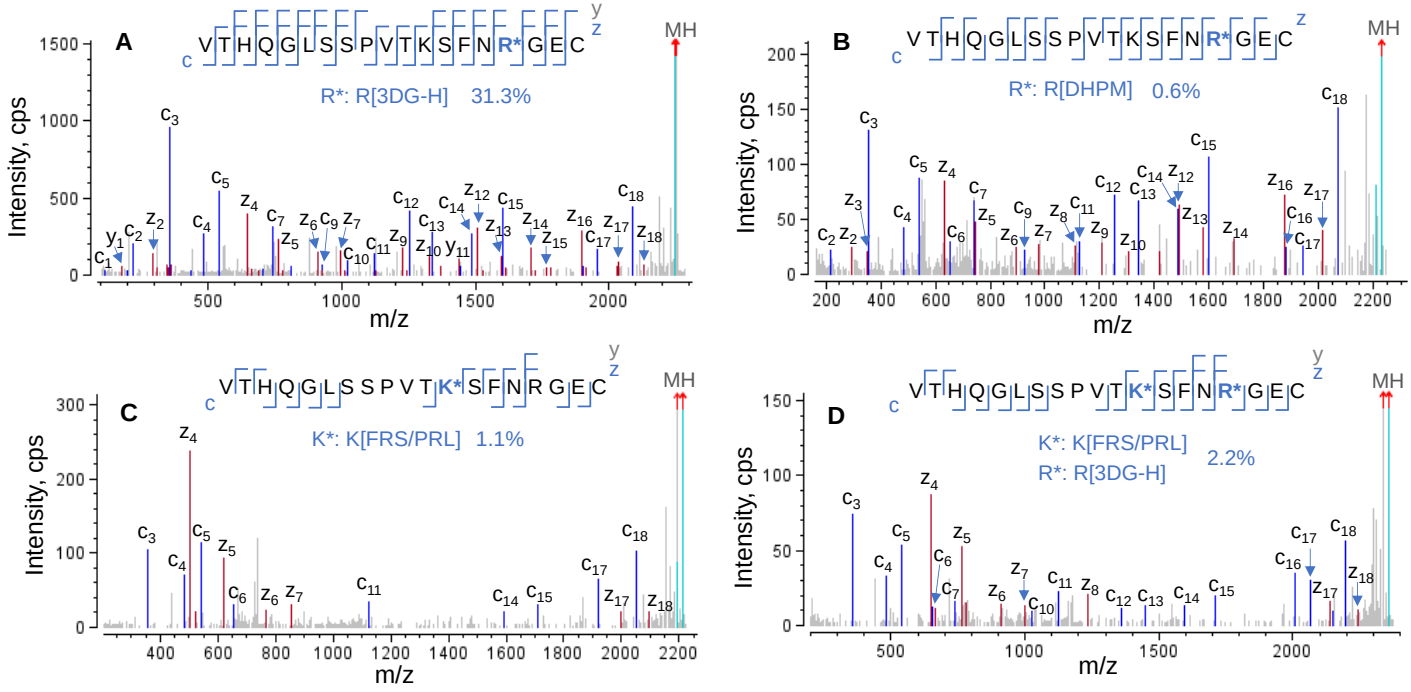

Figure 6. EAD MS/MS spectra of selected AGEs for the peptide LC[195-213] from the Glu-C digestion. The AGEs of LC[195-213] from the Glu-C digestion were present at different levels, including relative abundances of >30% for 3DG-H (A), ~1-2% for 2 FRS/PRL-containing species (C and D) and as low as 0.6% for DHPM (B). Despite such differences in relative abundance, high-quality EAD data were obtained for all AGEs, leading to confident sequence identification and accurate localization of the AGE moieties. The relative abundances of AGEs were calculated by considering the native form and all glycation/AGE species identified for LC[195-213]. The detailed quantification result of this peptide is displayed in Figure 8.

Figure 5 displays the EAD spectrum of an HC peptide carrying 3 different labile modifications, including 1 glycation residue (Hex), 1 AGE moiety (3DG-H) and 1 glycan (G1F). Despite the complex modification pattern of this peptide, EAD led to nearly complete coverage of the sequence containing the 3 labile moieties, allowing precise localization of each modification. For example, the detection of 2 complementary pairs of c_2/z_{22} and c_3/z_{21}

fragments confirmed the G1F glycosylation on Asn[3] and the detection of c_7/z_{18} ions localized 3DG-H to Arg[7].

Excellent EAD data enabled the confident identification of the same sequence modified with different AGE moieties. Figure 6 shows the EAD spectra of LC[195-213] modified with different AGE moieties, including 3DG-H (Figure 6A), DHPM (Figure 6B), FRS/PRL (Figure 6C) and the combination of 3DG-H and FRS/PRL (Figure 6D). These 4 AGEs were present at different levels, ranging from a relative abundance of >30% for 3DG-H (Figure 6A) to as low as 0.6% for DHPM (Figure 6B). Regardless of the type and relative abundance of these AGEs, EAD produced excellent MS/MS fragmentation to confidently identify and localize the AGE moieties.

Additionally, EAD DDA is a highly sensitive approach because the use of the Zeno trap provides a 5–10-fold increase in the detection of MS/MS fragments. Two examples that highlight the ability of EAD to characterize AGEs at low levels are displayed in Figure 7. The CML on peptide HC[372-384] (Figure 7A) and CEL on peptide LC[149-161] (Figure 7B) were present at ~0.1% relative abundance, whereas the corresponding glycated species were present at 85-95% relative abundance (data not shown). Despite the low abundances of CML and CEL, EAD DDA

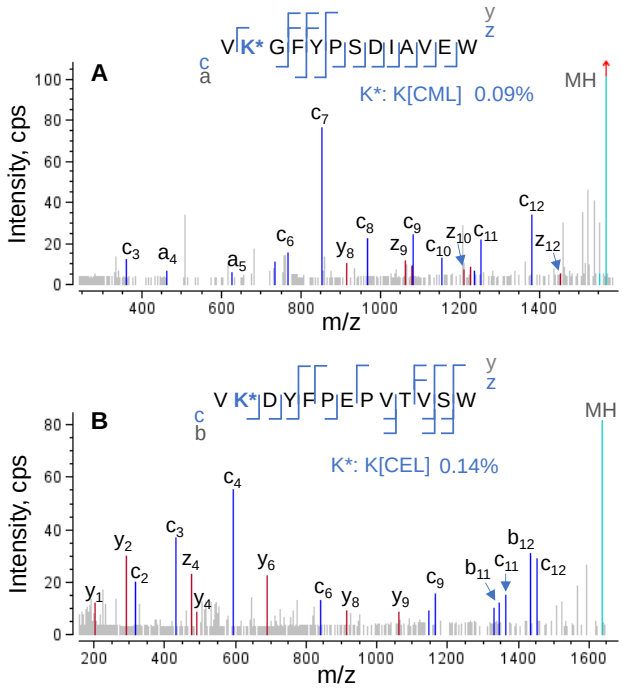

Figure 7. EAD MS/MS spectra of 2 low abundant AGEs from the chymotrypsin digestion. Despite the low abundance (~0.1%) of peptides HC[372-384] carrying CML (A) and LC[149-161] carrying CEL (B), EAD generated enough sequence ions for confident identification of these 2 AGEs and localization of the AGE moiety (CML or CEL).

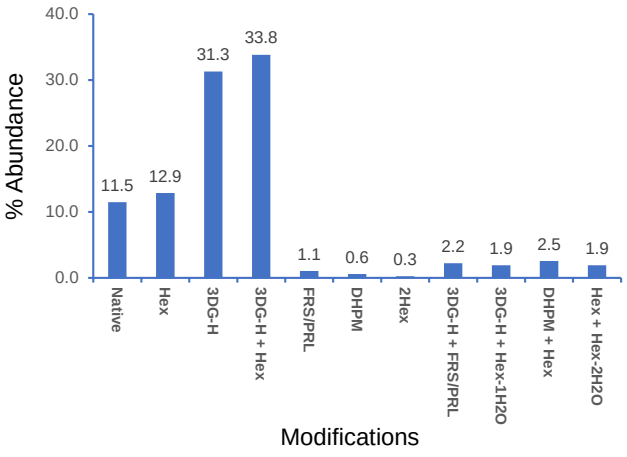

Figure 8. Relative abundances of modifications identified for peptide LC[195-213] (VTHQGLSSPVTKSFNRGEC) from the chymotrypsin digestion. The majority (88.5%) of peptide LC[195-213] was modified with 1 or 2 glycation and/or AGE moieties. The 2 dominant AGEs (31-34%) identified for this peptide contained 1 3DG-H moiety with or without a glycation residue (Hex).

Table 4. List of AGEs identified in NISTmAb forced glycation samples that were digested using 3 different enzymes.

Chain	Arg AGEs		Lys AGEs		
	3DG-H	DHPM	Hex-1H_2O/2H_2O	FRS/PRL	CML/CEL
HC	*R5, R40, R68, R99, R258, R295, R304, R347, R358, R419*	*R5, R68, R258, R419*	*K13, K45, K59, K66, K73, K77, K150, K277, K323, K325, K337, K341, K412, K417*	*K13, K45, K73, K77, K83, K124, K249, K277, K323, K325, K343, K363, K373, K395, K417*	*K77, K83, K150, K277, K363, K373, K395, K417*
LC	*R18, R28, R60 R107, R141, R210*	*R141, R210*	*K41, K44, K52, K106 K144, K168, K206*	*K38, K41, K44, K52 K102, K106, K125 K144, K187, K206*	*K41, K52, K148, K182*

produced enough sequence ions to confidently identify the 2 species and localize the AGE moieties.

Summary of AGEs identified by EAD

Forced glycation of NISTmAb resulted in the formation of numerous glycated species and AGEs. The previous technical note described selected examples of glycated peptides identified by EAD.[5] Table 4 summarizes the major AGEs and their modification sites identified from glycated NISTmAb using EAD in this work. The dominant AGE modification identified in the forced glycation samples was 3DG-H on the Arg residues (Table 4). Other common AGE modifications observed included Hex-$1H_2O/2H_2O$ and FRS/PRL at Lys and DHPM at Arg. The AGEs with CML or CEL were only present at low levels.

Figure 8 shows the relative abundances of the glycated species and AGEs identified for the peptide LC[195-213] from the chymotrypsin digestion. Forced glycation of NISTmAb resulted in extensive modification of this LC peptide, with 88.5% of the peptide modified with 1 or 2 glycations and/or AGE moieties. The dominant AGEs identified contained 1 3DG-H moiety with or without 1 glycation residue (Hex). The relative abundances of these 2 AGEs (31-34%) were much higher than those of other species, including the glycated peak (12.9%), indicating the prevalence of the 3DG-H modification. The relative quantification of glycation and AGE modifications will be described in another technical note.

In summary, EAD is powerful for the comprehensive characterization of glycation and AGEs, which are challenging modifications to characterize using traditional collision-based MS/MS approaches. EAD resulted in excellent fragmentation of AGEs to confidently identify peptide sequences. Additionally, this method preserved AGE moieties to accurately localize these labile modifications and enable differentiation between positional isomers.

Conclusions

- EAD is a powerful tool for in-depth characterization of glycation and AGEs, which are important for the quality of protein therapeutics but challenging to characterize using traditional collision-based MS/MS approaches
- EAD led to nearly complete fragmentation of AGEs while retaining the AGE moieties for confident sequence identification, accurate localization of labile modifications and differentiation between positional isomers
- EAD enabled the confident characterization of AGEs of different lengths and present at abundances as low as ~0.1%
- The major AGE modifications identified from forced glycation samples of NISTmAb included Arg-derived 3DG-H and DHPM and Lys-derived Hex-$1H_2O/2H_2O$, FRS/PRL and CML/CEL, among which 3DG-H was the dominant moiety.

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