Time-course study of glycation and advanced glycation end products (AGEs) in protein therapeutics using electron activated dissociation (EAD)

Featuring the ZenoTOF 7600 system, Biologics Explorer software and EAD-based multiple attribute methodology (MAM) workflow from SCIEX

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This technical note highlights the power of an electron activated dissociation (EAD) platform method for confident identification and a streamlined multiple attribute methodology (MAM) workflow to monitor the abundance changes of glycation and advanced glycation end products (AGEs) in antibody-based therapeutics. The quantitative information about glycation and AGEs obtained by EAD sheds light on the discoloration phenomenon observed in protein therapeutics.

Previous studies indicated that AGEs might be linked to the discoloration of recombinant antibodies.[1-4] However, the relationship between AGE content and color change was not fully understood due partially to challenges with a comprehensive characterization of AGEs using traditional collision-based MS/MS approaches, which are ineffective for fragmenting AGEs.[1,5,6] By contrast, EAD led to excellent fragmentation of glycated peptides and AGEs, allowing these species to be confidently identified and the glycation and AGE moieties to be accurately localized.[5,6]

In this technical note, an EAD-based MAM workflow[7] was employed to identify and quantify AGEs in time-course forced glycation samples of NISTmAb that exhibit a color change (Figure 1). The results indicated a correlation between the relative abundances of AGEs and the color pattern observed, suggesting that AGEs might play an important role in the discoloration of protein therapeutics.

Key features of EAD for the identification and quantification of glycation and AGEs

- **Confident identification and accurate localization**: EAD results in excellent fragmentation of glycated peptides and AGEs, leading to confident identification and accurate localization of these moieties
- **Quantitatively reproducible**: EAD provides reproducible quantification results of product quality attributes (PQAs)
- **Streamlined workflow**: EAD-based MAM workflow offers a streamlined process from PQA identification and peak integration to relative quantification and results visualization

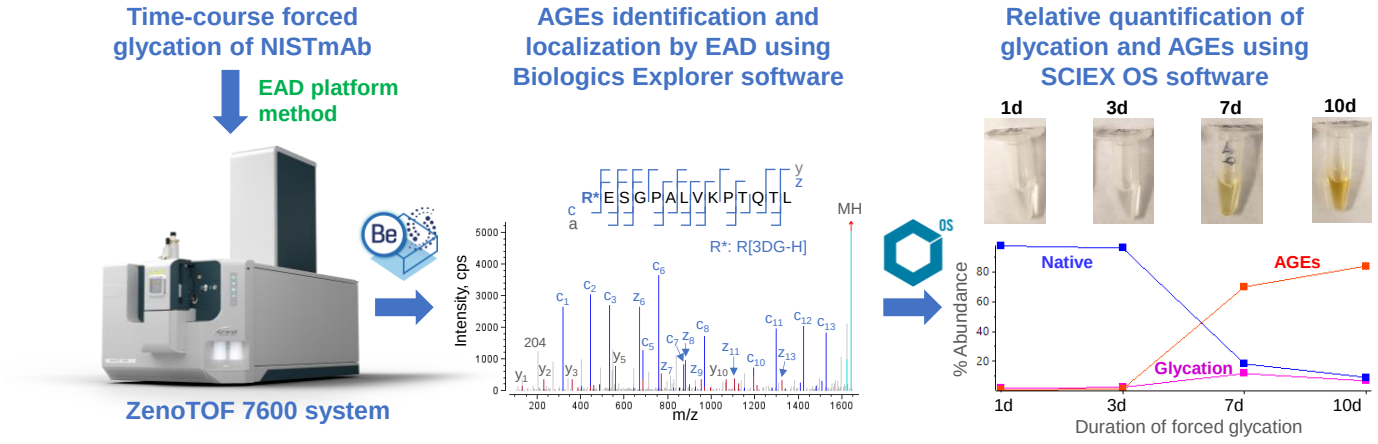

Figure 1. Identification and relative quantification of glycation and AGEs using the ZenoTOF 7600 system, Biologics Explorer software and SCIEX OS software. The time-course forced glycation samples of NISTmAb, in which the color was visible beginning on day 7, were analyzed using the EAD platform method offered by the ZenoTOF 7600 system. EAD led to excellent fragmentation of glycated peptides and AGEs, allowing for their confident identification using Biologics Explorer software. The quantitative results obtained using the EAD-based MAM workflow indicated a correlation between the color pattern and relative abundances of AGEs. 3DG-H: 3-deoxyglucosone hydroimidazolone.

Methods

Sample preparation: The non-stressed control sample of NISTmAb (RM 8671, NIST) was diluted to 5 µg/µL in 25mM Tris-HCl (pH=7.4) buffer and kept at -40°C for 10 days prior to enzymatic digestion. The stressed samples were prepared by incubating 5 µg/µL of NISTmAb in 25mM Tris-HCl (pH=7.4) at 60°C for 10 days or in 500mM glucose (Sigma-Aldrich) at 60°C for 1, 3, 7 and 10 days. All the 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) and the samples were digested using chymotrypsin (Promega). The final digests were injected in 20 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 60°C in the column oven of an ExionLC system (SCIEX). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile.

Mass spectrometry: Data-dependent EAD experiments were performed 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 in the Biologics Explorer software. A maximum of 4 hexose modifications at Lys or Arg residues per peptide sequence was allowed in peptide mapping. A previous technical note provided a list of AGE modifications for peptide mapping.[6] The relative quantification of glycation and AGEs was performed following the EAD-based MAM workflow described previously.[7] Briefly, the AGEs were selected together with the corresponding native (unmodified) and glycated species in the results table and exported to a *.txt file, which was then imported into SCIEX OS software for peak integration and relative quantification. The quantitative results were plotted using the metric plot.

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	*400°C*
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*

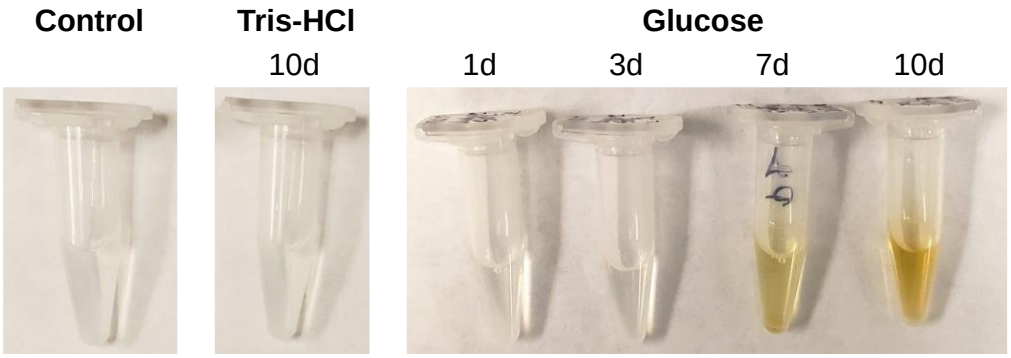

Figure 2. Time-course forced glycation of NISTmAb. The NISTmAb control sample (non-stressed) and the solution stressed in Tris-HCl buffer for 10 days did not show visible discoloration. The NISTmAb solutions incubated in the presence of glucose did not develop color change by day 1 or day 3. Further incubation of the sample to 7 days resulted in a color change to brown/yellow, which grew slightly darker by day 10.

Time-course forced glycation of NISTmAb

Although AGEs might play a role in the discoloration of the recombinant antibodies,[1-4] comprehensive characterization of AGEs by LC-MS is lacking because these species fragment poorly with traditional collision-based MS/MS approaches. It was demonstrated in a previous technical note that EAD provided excellent fragmentation of AGEs, enabling confident sequence identification, accurate localization of the AGE moieties and differentiation of positional isomers.[6] These advantages of EAD further enabled accurate quantification of AGEs in the time-course forced glycation samples of NISTmAb.

In this work, NISTmAb was thermally stressed in the absence or presence of glucose for up to 10 days. The discoloration was not observed for either the NISTmAb control sample or for the solution incubated with Tris-HCl buffer for 10 days (Figure 2). The NISTmAb samples incubated at 60°C in the presence of glucose did not develop discoloration until day 7 when the brown/yellow color became visible and grew slightly darker over the remaining time of incubation (Figure 2). This result demonstrates that the discoloration of protein therapeutics can be reproduced in the lab under harsh stress conditions, therefore enabling investigation of the cause of color change. Here EAD was employed to identify and subsequently quantify glycated peptides and AGEs in the time-course forced glycation samples to determine whether color change correlates with the degree of glycation or concentration of AGEs.

Confident identification of glycated peptides and AGEs

As described in previous technical notes, EAD provided extensive fragmentation of glycated peptides and AGEs, regardless of the length and number of modifications. This approach therefore enabled confident identification of these challenging species and accurate localization of the glycation and AGE moieties.[5,6]

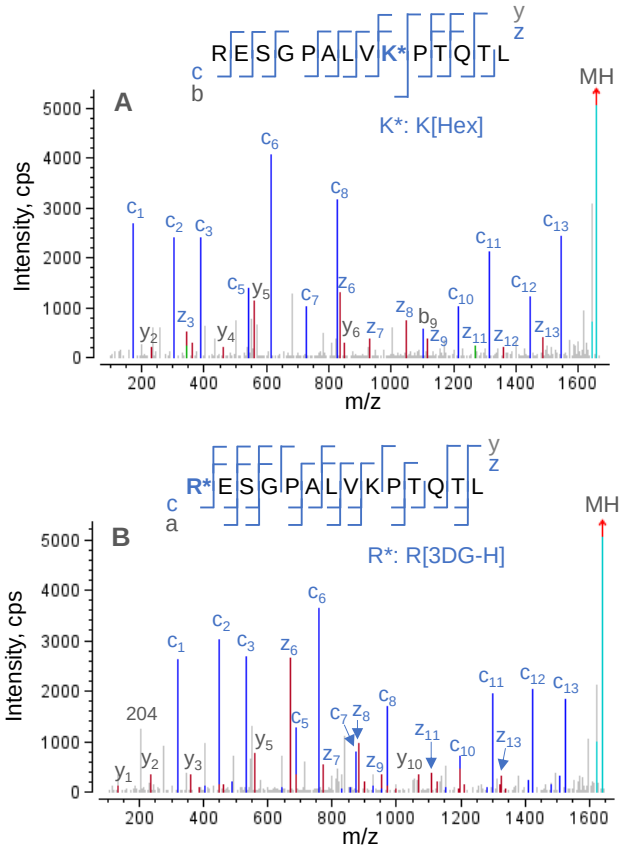

Figure 3. EAD MS/MS spectra of a glycated peptide and an AGE. A complete fragmentation of the peptide backbone by EAD allowed confident identification of a glycated (A) and an AGE (B) species of the peptide RESGPALVKPTQTL. The detection of the sequence ions containing the Hex (glycation) or 3DG-H moiety led to the accurate localization of these moieties. Note: The EAD spectra displayed here are the deisotoped spectra from Biological Explorer software. Not all fragments were labeled for spectral clarity.

This work obtained similar high-quality EAD data for the time-course forced glycation samples. The EAD MS/MS spectra of a glycated and an AGE species identified for the peptide RESGPALVKPTQTL using Biological Explorer software are displayed in Figure 3. The location of the glycation or 3DG-H moiety in the peptide RESGPALVKPTQTL (Figure 3) can be pinpointed based on the detection of a nearly complete series of *c*/*z* fragments. Confident identification of the glycated peptides and AGEs by Biologics Explorer software allowed the relative quantification of these species in the time-course samples using SCIEX OS software.

Relative quantification of glycation and AGE moieties

Relative quantification of glycation and AGE moieties was performed following the EAD-based MAM workflow described previously.[7] Briefly, the native, glycated and AGE species identified from the day 10 discolored sample using Biologics Explorer software were selected in the results table and exported to a *.txt file. This *.txt file was then imported into the processing method created in SCIEX OS software for the subsequent peak integration and relative quantification.

Relative abundances of glycation and AGE species identified for the peptide RESGPALVKPTQTL are summarized in Figure 3. In the control samples, a low level of glycation (<0.1%) was detected for this peptide, while no AGE was identified. The level of glycation increased drastically in the first week of thermal stress in the presence of glucose, from 1.67% on day 1 to 11.67% on day 7. The glycation level then decreased to 6.75% on day 10 due likely to further degradation of glycated species into AGEs. For AGEs, the most significant abundance change

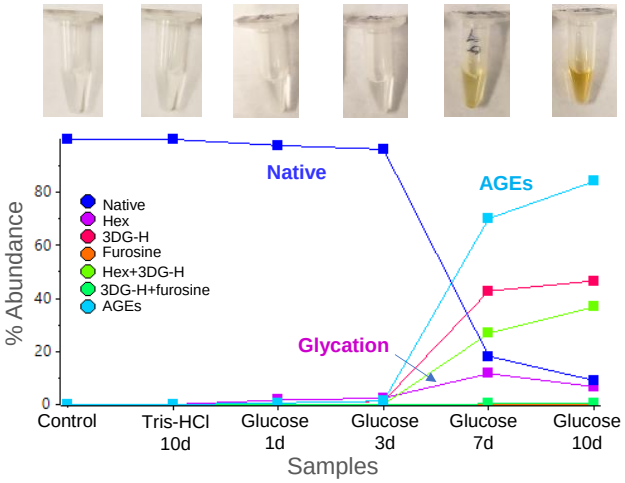

Figure 4. Relative quantification of glycation and AGEs using the EAD-based MAM workflow. Relative abundances of the native, glycated and AGE species of the peptide RESGPALVKPTQTL were calculated and plotted using the Analytics function within SCIEX OS software. The color state of each sample was displayed on the top of the plot to facilitate comparison. The line labeled with "AGEs" corresponds to the sum of the relative abundances of all AGE species identified for this peptide. The data indicated a correlation between color change and relative abundances of AGEs.

was also observed in the day 7 sample. The AGEs containing the 3DG-H and Hex+3DG-H moieties were either absent or identified as the minor components in the day 1 and day 3 samples. However, these 2 AGEs became the dominant species in the day 7 and day 10 samples. Unlike glycation, the relative abundances of the major AGEs continued to increase from day 7 to day 10.

A metric plot that shows the relative abundances of the native, glycated and AGE species of the peptide RESGPALVKPTQTL is displayed in Figure 4 to better visualize the quantification results.

*Samples	*%Native...	*%Hex...	*%3DGH...	*%Furosine...	*%Hex_3DGH...	*%3DGH_Furosine...
Control	99.94	0.06	0.00	0.00	0.00	0.00
Tris-HCl, 10d	99.97	0.03	0.00	0.00	0.00	0.00
Glucose, 1d	97.71	1.67	0.62	0.00	0.00	0.00
Glucose, 3d	96.24	2.44	1.29	0.00	0.03	0.00
Glucose, 7d	18.21	11.67	42.81	0.19	26.84	0.28
Glucose, 10d	9.10	6.75	46.63	0.17	36.79	0.55

Figure 3. The results table from SCIEX OS software that shows the relative abundances of glycation and AGEs identified for the peptide RESGPALVKPTQTL in the control and time-course samples. The native peptide was the dominant species (>96%) detected in the 4 samples that did not develop visible color. These samples included the control, the day 10 Tris-HCl stress sample and the day 1 and 3 glucose stress samples. The level of glycation increased in the first 7 days of incubation with glucose but then decreased on day 10. By comparison, the relative abundances of AGEs containing 3DG-H increased significantly on day 7 and remained the dominant species in the day 10 sample.

The color state of each sample is shown on the top of the plot for comparison. Although the glycation level was elevated in the day 7 and day 10 samples (Figure 4), this trend did not match the color state of the sample, which grew slightly darker from day 7 to day 10. This pattern of results suggests that glycation was not directly responsible for discoloration and is consistent with a previous finding.[2] By comparison, the relative abundance of AGEs shows a strong correlation with the color pattern observed in the samples (Figure 4), suggesting that AGEs are linked to the discoloration of the recombinant antibodies[2]. Compared to the LC-MS/MS approach employed in the previous study,[2] the EAD platform method provides a more complete and more confident identification of AGEs, enabling more accurate relative quantification of these species.

In summary, the data described in this technical note provide insight into the role of AGEs in the discoloration of protein therapeutics. Future work will involve studies of the key factors that affect color change, further investigation of the correlation between discoloration and AGEs and the mapping of the glycation and AGE hot spots in NISTmAb.

Conclusions

- The EAD platform method provided confident identification and accurate quantification of AGEs to help understand the role these species play in the discoloration of protein therapeutics
- A color change was observed in the NISTmAb samples incubated for ≥7 days at an elevated temperature in the presence of glucose
- The EAD data revealed a correlation between color change and the relative abundance of AGEs but not glycation
- The streamlined EAD-based MAM workflow for data analysis using Biologics Explorer software and SCIEX OS software facilitated a comprehensive characterization of glycation and AGEs

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