


Probing the cause of antibody discoloration via relative quantification of glycation and associated species using an EAD-based multi-attribute methodology (MAM)

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ABSTRACT

This poster highlights a streamlined solution that combines the power of capillary isoelectric focusing (cIEF) to monitor antibody discoloration and an electron-activated dissociation (EAD)-based MAM workflow to determine the cause of color change. The cIEF profiles of the time-course forced glycation samples of NISTmAb revealed a significant increase in acidic variants in the discolored samples. EAD-based MAM was employed to identify and quantify the acidic species to assess their role in the discoloration of protein therapeutics.

INTRODUCTION

Color is a potential critical quality attribute of protein therapeutics.[1,2] Undesirable color changes can lead to increased product heterogeneity and concerns about the safety and efficacy of a drug. Previous studies showed that advanced glycation end products (AGEs) are partially responsible for the discoloration of recombinant antibodies.[1,2] Glycated and AGE species are often challenging to characterize using collision-based MS/MS approaches because they are ineffective for fragmenting these species.[3-6] In contrast, EAD-based MAM enabled confident identification and relative quantitation of the glycated species and AGEs, providing molecular insights into the role of AGEs in the discoloration of protein therapeutics.[3-6] Here, the cIEF and EAD-based MAM workflows were leveraged together (Figure 1) to provide a streamlined solution from product quality tracking to cause investigation.

MATERIALS AND METHODS

Sample:
The non-stressed control sample of NISTmAb (RM 8671, NIST) was kept at -40°C for 10 days prior to enzymatic digestion. The stressed samples were prepared by incubating NISTmAb at 60°C for 7-10 days or in 500mM glucose (Sigma-Aldrich) at 50°C or 60°C for up to 10 days. The time-course glycation samples were split into 2 portions for subsequent LC-MS and cIEF analyses. The samples for LC-MS analysis were denatured in guanidine hydrochloride, reduced with dithiothreitol and alkylated with iodoacetamide. The samples were then digested using trypsin, chymotrypsin or Glu-C (Promega).

HPLC:
The peptides were separated 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 AD system (SCIEX). For cIEF separation, buffers and reagents were prepared following the instructions in the BioPhase 8800 Capillary Isoelectric Focusing (cIEF) kit. The cIEF data were acquired using the BioPhase 8800 system (SCIEX). The UV detection wavelength was 280 nm.

MS/MS:
EAD data-dependent acquisition (DDA) experiments were performed using the ZenoTOF 7600 system (SCIEX) and SCIEX OS software (SCIEX). EAD data were analyzed using the peptide mapping workflow templates in the Biologics Explorer software, as described in previous technical notes.[3-6]

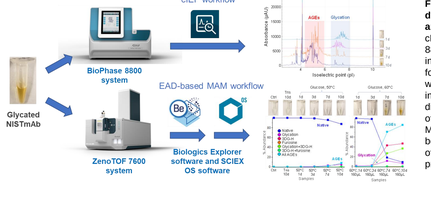

Figure 1. Characterization of the discolored NISTmAb using the cIEF and EAD-based MAM workflows. The cIEF results obtained from the BioPhase 8800 system revealed a gradual increase in acidic variants in the time-course forced degradation samples of NISTmAb without color change and a dramatic increase in acidic species in the discolored samples. Relative quantitation of glycation and AGEs by the EAD-based MAM workflow established a correlation between the color change and the level of AGEs but not glycated species present, consistent with the cIEF data.

RESULTS

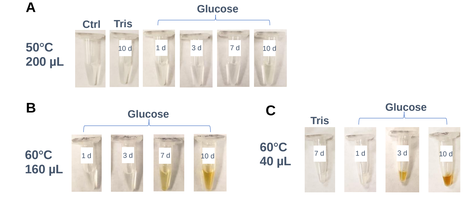

Figure 2. Time-course forced glycation of NISTmAb. The control (Ctrl in A) and NISTmAb samples that were thermally stressed in the presence of Tris-HCl only (Tris in A and C) did not display color change. No discoloration was observed for the NISTmAb samples that were incubated with glucose at 50°C (samples 1d-10d in A). By comparison, a visible color change was detected in the samples that were incubated at 60°C for an extended period of time (7 days in B and 3 days in C). The color change occurred more quickly in samples that had a smaller incubation volume (40 µL in C vs. 160 µL in B).

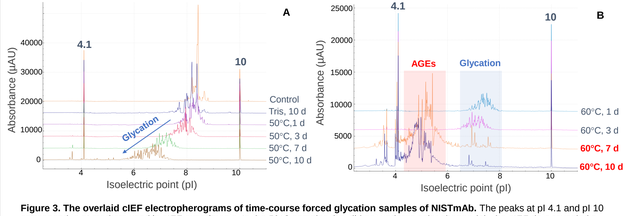

Figure 3. The overlaid cIEF electropherograms of time-course forced glycation samples of NISTmAb. The peaks at pI 4.1 and pI 10 correspond to pI markers used in cIEF experiments. In the 50°C samples that did not undergo color change (A), the cIEF data revealed a gradual shift of the charge profile of NISTmAb to the acidic region (lower pI) with increasing incubation duration. This gradual pI shift can be attributed to the increasing level of the glycated species. A dramatic shift of the charge profile was observed in the 2 discolored samples that were incubated at 60°C (highlighted in red in B), in which nearly all the acidic species were detected at pI <6, compared to pI 6-9 in the samples without color change. LC-MS analyses identified the AGEs as the species that were likely responsible for the significant pI shift and discoloration.

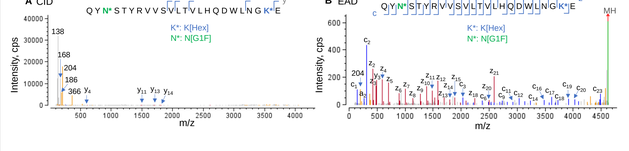

Figure 4. CID and EAD spectra of the HC peptide occurring at residues 298-321 and carrying 1 glycosylation (G1F) and 1 glycation. The CID spectrum (A) of this glycated glycopeptide was dominated by the oxonium ions generated from the glycan (G1F). Only a few y ions at very low abundance were produced by CID. By comparison, EAD led to complete coverage of the peptide, facilitating accurate localization of both glycosylation and glycation in the same sequence.

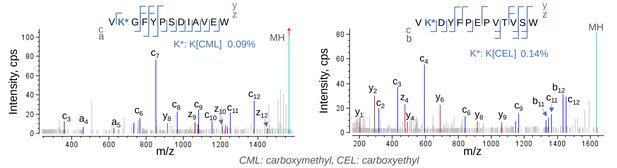

Figure 5. EAD MS/MS spectra of 2 low-abundant AGEs from the chymotrypsin digestion. Despite the low abundance (~0.1%) of peptides HC[372-384] carrying CML (A) and LC[149-161] carrying CEL (B), EAD generated enough sequence ions for confident identification of these 2 AGEs and localization of the AGE moiety (CML or CEL).

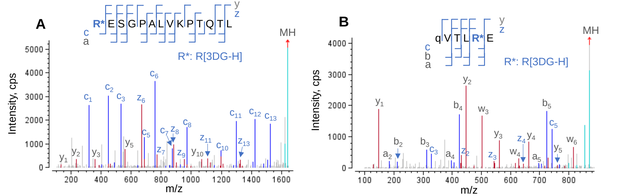

Figure 6. Deisotoped EAD MS/MS spectra of 2 AGEs derived from the same Arg residue. EAD led to excellent fragmentation of 2 AGEs containing the 3DG-H modification on the same Arg residue in 2 peptides from the trypsin/Lys-C (A) and Glu-C (B) digestions, respectively. q = pyroglutamic acid.

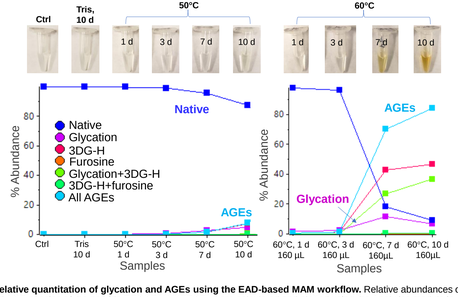

Figure 7. Relative quantitation of glycation and AGEs using the EAD-based MAM workflow. Relative abundances of the native, glycated and AGE species of the peptide RESGPALVKPTQTL were calculated and plotted using the Analytics module in SCIEX OS software. The color of each sample is shown at the top of the plots to facilitate comparison. The AGEs line corresponds to the sum of the relative abundances of all AGE species identified. These data revealed a correlation between discoloration and the relative abundance of AGEs but not the relative abundance of glycation.

*Samples	*%Native ...	*%Hex_RESG	*%3DGH_RESG	*%Furosine ...	*%Hex_3DGH...	*%3DGH_Furosine ...	*%AGEs
Control	99.94	0.06	0.00	0.00	0.00	0.00	0.000
Tris-HCl, 10d	99.97	0.03	0.00	0.00	0.00	0.00	0.000
Glucose, 50c, 1d	99.73	0.22	0.05	0.00	0.00	0.00	0.054
Glucose, 50c, 3d	99.17	0.61	0.22	0.00	0.00	0.00	0.216
Glucose, 50c, 7d	95.64	2.65	1.71	0.00	0.00	0.00	1.711
Glucose, 50c, 10d	87.52	4.88	7.26	0.00	0.34	0.00	7.605
Glucose, 60c, 1d	97.71	1.67	0.62	0.00	0.00	0.00	0.619
Glucose, 60c, 3d	96.24	2.44	1.29	0.00	0.03	0.00	1.320
Glucose, 60c, 7d	18.21	11.67	42.81	0.19	26.84	0.28	70.122
Glucose, 60c, 10d	9.10	6.75	46.63	0.17	36.79	0.55	84.146

Figure 8. 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 day 3 glucose stress samples. The level of glycation increased in the first 7 days of incubation with glucose but then decreased by day 10. In comparison, the relative abundances of AGEs containing 3DG-H increased significantly by day 7 and remained the dominant species in the day 10 sample.

CONCLUSIONS

- The streamlined cIEF and EAD-based MAM workflows offer a useful solution to monitor the changes in charge profile associated with antibody discoloration and determine the cause of color change.
- The color change of NISTmAb was affected by factors including the absence or presence of glucose, temperature, duration of incubation and sample volume.
- The cIEF data revealed a shift of the charge profile to pI<6 in the samples that displayed the distinct yellow or brown color.
- While CID led to incomplete fragmentation of glycated peptides or AGEs, particularly for the long or multiply modified species, EAD provided excellent fragmentation of these challenging molecules in different lengths, carrying a different number of glycation or AGE moieties or containing other labile modifications, such as glycosylation.
- EAD enabled the confident characterization of AGEs of different lengths and present at abundances as low as ~0.1%.
- EAD-based MAM identified AGEs as the species that are likely responsible for the discoloration of antibody-based therapeutics.

REFERENCES

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