Monitoring and characterization of glycation in a biotherapeutics degradation study using icIEF-UV/MS and LC-MS workflows

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This technical note demonstrates the unique capabilities of imaged capillary isoelectric focusing (icIEF)-UV/MS and electron-activated dissociation (EAD)-based LC-MS workflows to separate, identify and characterize glycation in a biotherapeutics degradation study on a single MS platform. Glycation impacts the quality of biotherapeutics by reducing antigen binding, promoting aggregation and increasing heterogeneity.[1,2] Therefore, thorough characterization and monitoring of glycation throughout the drug lifecycle are crucial to maintaining product safety and efficacy. The orthogonal icIEF-UV/MS and EAD workflows (Figure 1) provide multi-level insights into product quality attributes (PQAs) of biotherapeutics, supporting crucial decision-making processes early in the development pipeline. These workflows are valuable tools for comprehensive charge heterogeneity analysis during biotherapeutic discovery and development, ensuring drug quality and safety.

It is challenging to detect and confidently identify the low-abundant glycation species using traditional cIEF or intact LC-MS workflows. Additionally, the accurate localization of labile glycation poses an analytical challenge to conventional collision-based MS/MS approaches.[3,4] The streamlined icIEF-UV/MS and EAD-based LC-MS workflows address these challenges by providing high-resolution separation, sensitive detection, confident identification and comprehensive characterization of biotherapeutic charge variants.[5,6]

Key features of SCIEX's icIEF-UV/MS and LC-MS workflows for charge heterogeneity analysis

- **Accurate peak assignment:** The MS capability offered by icIEF-UV/MS enables a rapid assessment of charge profile changes over the course of forced glycation.
- **Confident identification**: The combined data from icIEF-UV/MS and LC-MS workflows provide confident identification of proteoforms within each charge variant.
- **Sensitive detection and high-resolution separation:** icIEF-UV offers high resolution and high sensitivity for the separation and detection of low-abundant variants or impurities.
- **Unique capabilities of EAD**: EAD-based LC-MS workflow provides high sequence coverage and site-specific information about glycation.
- **Platform assays**: The streamlined icIEF-UV/MS and EAD-based LC-MS workflows involve a single MS platform and can be easily implemented for routine and advanced charge heterogeneity analysis.

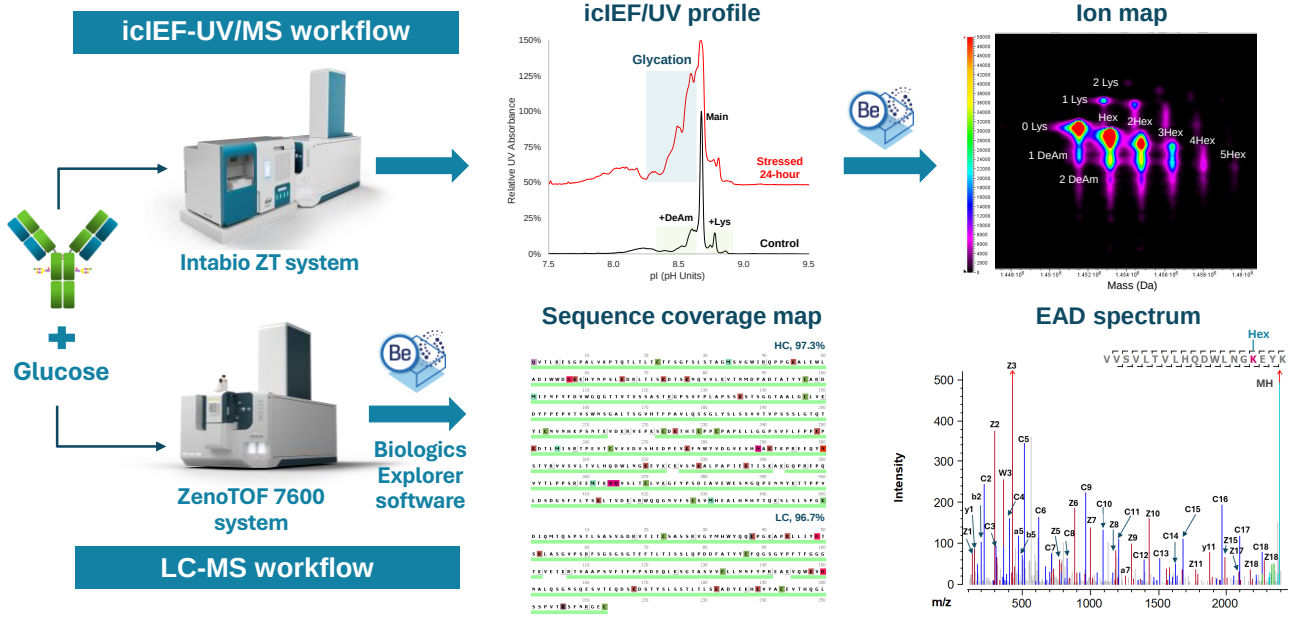

Figure 1. Comprehensive characterization of glycation in a forced degradation study using icIEF-UV/MS and EAD-based LC-MS workflows. The Intabio ZT system–coupled with the ZenoTOF 7600 system–provides high-resolution separation, sensitive detection and comprehensive characterization of glycation in a time-course stress study of NISTmAb in the presence of glucose. The icIEF-UV/MS workflow enables a rapid assessment of charge profile changes over the course of forced glycation, while the EAD-based LC-MS workflow provides site-specific information about glycation. The icIEF-UV/MS and EAD data are processed and visualized using the optimized workflow templates within Biologics Explorer software.

Introduction

Antibody-based therapeutics are highly heterogeneous due to the presence of various glycoforms, degradation products, aggregates and charge variants.[1,2] The charge variants arise mainly from various PTMs, including the C-terminal lysine (Lys), pyroglutamate formation, deamidation (DeAm), glycosylation, glycation (Hex) and oxidation.[7] Glycation contributes to an increased abundance of acidic variants and sample heterogeneity of biotherapeutics.[1,2] Further degradation of glycated species can lead to the formation of advanced glycation end products, which are associated with product discoloration.[2] Glycation is a potential critical quality attribute, therefore, it is crucial to characterize and monitor glycation in biotherapeutic development to ensure the safety and efficacy of drug molecules.

Traditionally, characterizing glycation is challenging due to the low abundance and labile nature of this PTM.[3,4] The icIEF-UV/MS and EAD-based LC-MS workflows offer a viable solution for high-resolution separation, sensitive detection, confident identification and accurate localization of glycation in biotherapeutics. In this work, these 2 powerful approaches were leveraged to characterize glycation species in time-course degradation samples of NISTmAb in the presence of glucose.

Methods

Sample preparation: The non-stressed sample of NISTmAb (RM 8671, NIST) was diluted to 5 µg/µL in water and kept at -40°C for 5 days prior to icIEF-UV/MS and enzymatic digestion. The time-course stressed NISTmAb samples were prepared by incubating 5 µg/µL of NISTmAb in 500mM glucose (Sigma-Aldrich) at 37°C for up to 24 hours. The stressed samples were split into 2 portions for subsequent LC-MS and icIEF-UV/MS analyses.

icIEF-UV/MS: The non-stressed and glycated NISTmAb samples were desalted and subjected to deglycosylation overnight using the PNGase F protease (New England Biolabs). The deglycosylated samples were mixed with the master mix solution containing arginine, pharmalyte 5 to 8 (Cytiva), pharmalyte 8 to 10.5 and 6.0 µg/mL peptide pI markers. The solutions were vortexed and degassed by centrifugation and analyzed using the Intabio ZT cartridges (SCIEX).

LC-MS: The non-stressed and glucose-stressed NISTmAb samples were denatured with 7.2M guanidine hydrochloride in 100mM Tris buffer (pH=7.2), followed by reduction with 10mM DL-dithiothreitol and alkylation with 30mM iodoacetamide. Digestion was performed with the trypsin/Lys-C protease at 37°C for 5 hours. The final digests were injected in 20 µL aliquots (~20 µg) for EAD analysis.

Chromatography: The tryptic peptides were separated using an ACQUITY BEH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters). A 0.25 mL/min flow rate was used for the chromatographic separation. The column was kept at 60°C in the column oven of an ExionLC AD system (SCIEX). Mobile phase A was 0.1% formic acid (FA) in water and mobile phase B was 0.1% FA in acetonitrile. The peptide mapping data was acquired using an EAD data-dependent acquisition (DDA) method described previously.[3] The key EAD DDA parameters are provided in Table 1.

Table 1. Key EAD DDA parameters.

Parameter	Value
Start mass	*100 m/z*
Stop mas	*2000 m/z*
Q1 resolution	*Unit*
Zeno trap	*ON*
Zeno threshold	*100,000 cps*
Accumulation time	*0.1 s*
Electron beam current	*5,000 nA*
Electron KE	*7*
ETC	*Dynamic*
Reaction time	*20 ms*
EAD RF	*100 Da*

Data analysis: The icIEF-UV/MS and LC-MS data were interpreted using Biologics Explorer software (SCIEX). Each peak in the charge profile was integrated by Intabio software to determine its peak area and percentage composition. Intact masses of the main peak and charge variants were determined from the corresponding deconvoluted mass spectra.

Charge heterogeneity analysis of non-stressed and stressed NISTmAb using icIEF-UV/MS

The Intabio ZT system enables simultaneous separation, detection and identification of charge variants in a single assay by integrating charge separation and UV detection offered by icIEF-UV with the accurate mass measurement capability of the ZenoTOF 7600 system. During icIEF-UV/MS analysis, the separation efficiency and resolution of icIEF-UV are maintained after chemical mobilization, ensuring reliable MS identification of proteoforms within each charge variant peak. Previous studies have demonstrated that this streamlined workflow enables reproducible separation and detection of intact biotherapeutics and their charge variants.[8-12]

Figure 2 shows the overlaid icIEF-UV profiles and base peak electropherograms (BPEs) of the non-stressed and 24-hour stressed NISTmAb samples. The samples were treated with the PNGase F protease to remove the N-linked glycosylation, allowing better detection and monitoring of the glycation events during the time-course study. The similarities between the icIEF-UV (Figure 2A) and BPE (Figure 2B) profiles demonstrate that the charge variant separation was maintained after chemical mobilization.

It is evident in Figure 2A that forced glycation led to a drastic change with the icIEF-UV profile. While the main peak is the dominant species in the non-stressed sample (black trace in Figure 2A), the increasing content of acidic variants made it challenging to identify the main species in the stressed sample (red trace in Figure 2A) using icIEF-UV alone. This challenge can be overcome by leveraging the MS capability offered by the icIEF-UV/MS workflow. The MS capability also enables accurate mass measurement of the proteoforms within each variant peak, leading to their confident assignments.

The total percent compositions of the acidic and basic variants detected in the icIEF-UV profiles are provided in the table in Figure 2A. In the non-stressed sample, the percent compositions of the main, acidic and basic species were calculated to be 49.2%, 40.4%, and 10.4%, respectively. The major acidic species detected in the non-stressed sample carry 1 to 2 deamidation, while the basic peaks are composed of the C-terminal Lys variants (black trace in Figure 2A). These results agree with the data described in the previous study,[10] highlighting the reproducibility of the icIEF-UV/MS workflow.

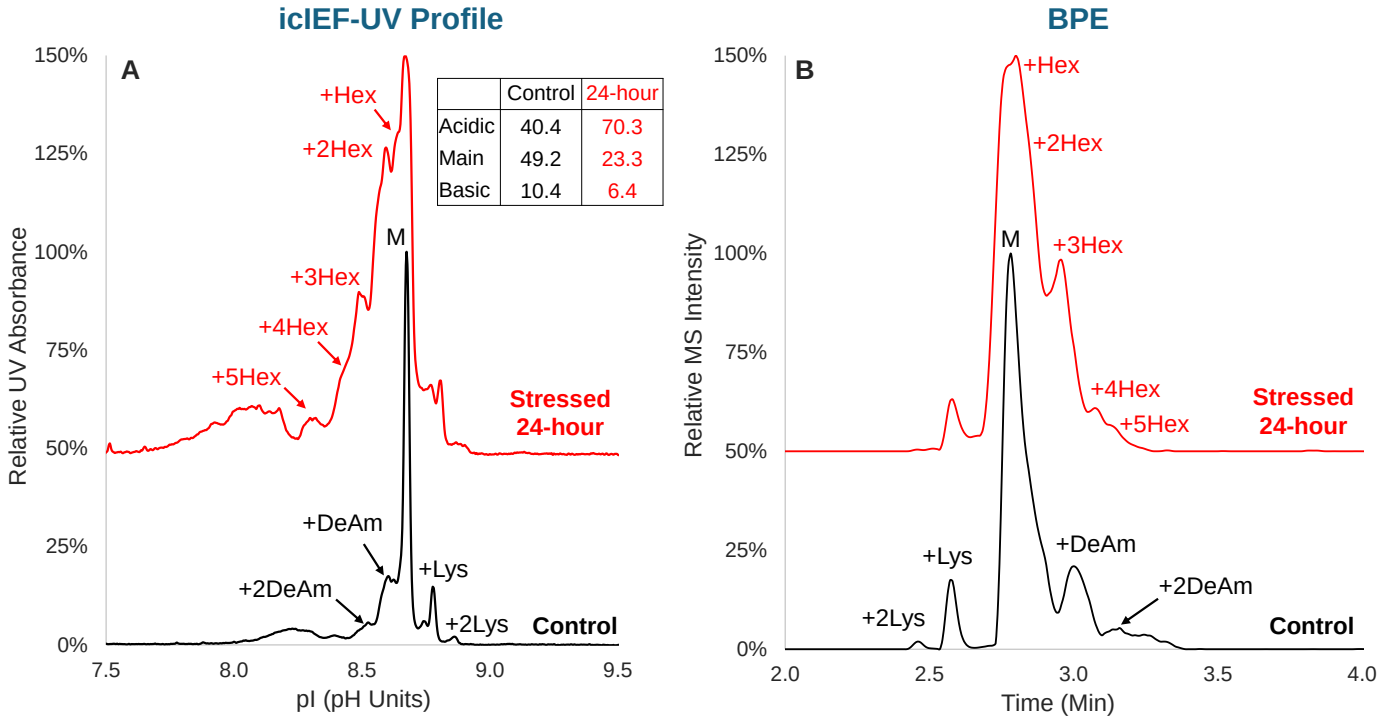

Figure 2. icIEF-UV/MS analysis of the non-stressed and stressed NISTmAb using the Intabio ZT system. The icIEF-UV profiles and base peak electropherograms (BPEs) of the non-stressed (black traces in A and B) and 24-hour stressed (red traces in A and B) NISTmAb samples following deglycosylation demonstrate the high-resolution separation of the main species, basic and acidic charge variants before and after chemical mobilization. The proteoforms under each charge variant peak were confidently assigned based on the accurate mass measurement. The table in A shows the percent compositions of the main, acidic and basic species in the non-stressed and 24-hour stressed samples. The forced glycation of NISTmAb led to a significant increase in the acidic variant content. The acidic variants are composed primarily of the glycated species carrying up to 5 hexose moieties (red traces in A and B). M: deglycosylated NISTmAb. Hex: glycation. Lys: C-terminal lysine. DeAm: deamidation.

Substantial changes in charge variant content were observed in the icIEF-UV and BPE profiles of the glycated NISTmAb sample (red traces in Figure 2) compared to the non-stressed sample (black traces in Figure 2). The total percent composition of the acidic variants in the 24-hour stressed sample was calculated to be 70.3% (table in Figure 2A). This value is much higher than that measured for the non-stressed sample (40.4%). The deconvolution results revealed the modification of NISTmAb with up to 5 Hex moieties in the 24-hour stressed sample (red traces in Figure 2). This explains the dramatic increase in the acidic content in the stressed sample.

The analysis of icIEF-UV/MS data is streamlined using the optimized intact MS analysis workflow offered by Biologics Explorer software. This intuitive software also provides powerful tools to facilitate automated data interpretation and results visualization. Figure 3 shows the annotated ion map for the non-stressed and 24-hour stressed NISTmAb samples. The ion map offered by Biologics Explorer software provides excellent visualization of the charge profile changes associated with forced degradation. Multiple glycation events can be clearly observed in the ion map of the 24-hour stressed sample (Figure 3B).

The results described above demonstrate the ability of the icIEF-UV/MS workflow to provide high-resolution separation, sensitive detection and confident identification of charge variants, enabling a detailed understanding of the charge profile changes caused by forced degradation. Compared to traditional icIEF-UV workflows, the added MS capability of icIEF-UV/MS allows confident peak assignment and detailed analysis of multiple proteoforms within each variant peak, eliminating the need for labor-intensive and time-consuming fraction collection followed by MS analysis. The icIEF-UV/MS workflow can be a valuable tool for the rapid monitoring and characterization of biotherapeutic charge variants, accelerating product development and quality assessment.

LC-MS analysis of glycated NISTmAb using EAD

In addition to offering the unique icIEF-UV/MS capability described above, the Intabio ZT system consists of the ZenoTOF 7600 system that provides a full suite of LC-MS workflows for comprehensive biotherapeutic characterization. Previous studies have demonstrated the exceptional capability and versatility of the ZenoTOF 7600 system to deliver unparalleled EAD-based LC-MS results for confident sequence confirmation, PTM analysis and isomer differentiation.[13] In this work, the EAD-based LC-MS workflow was

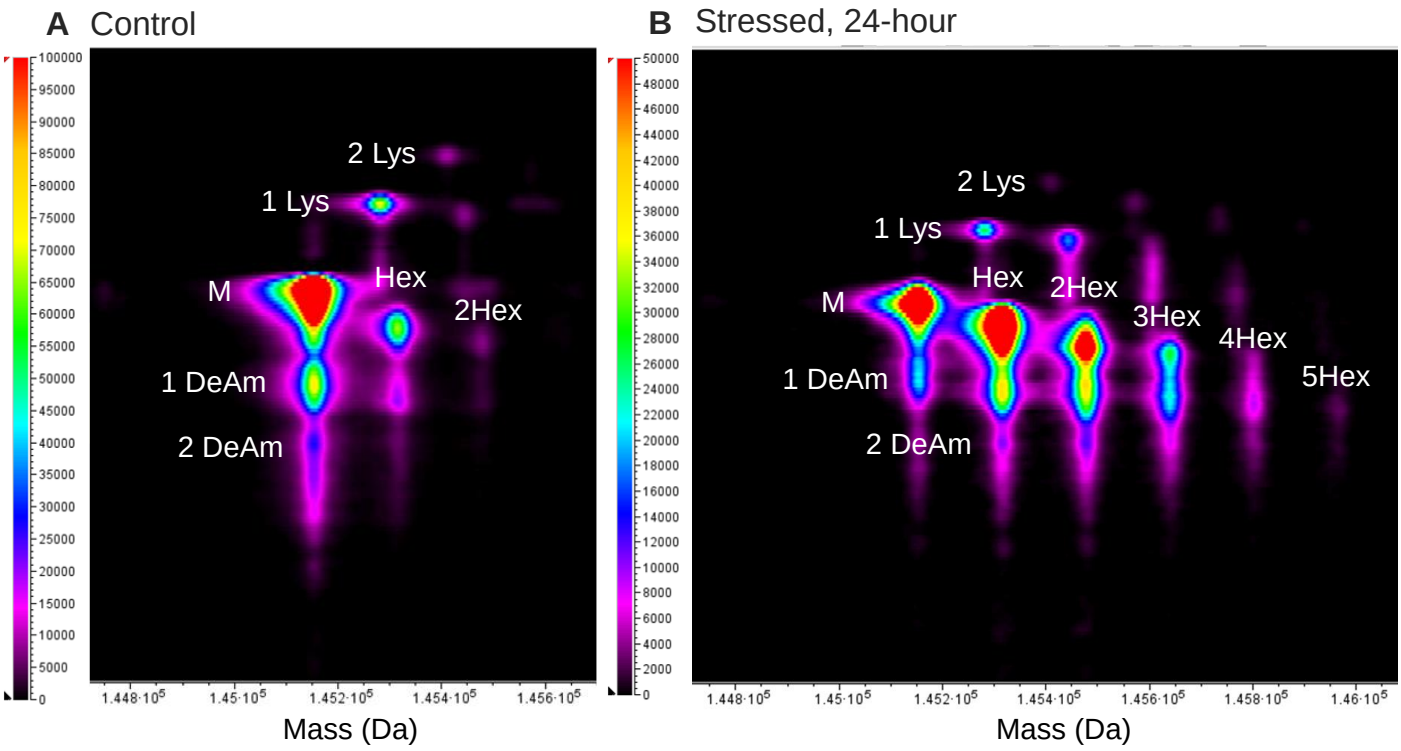

Figure 3. Visualization of the icIEF-UV/MS result using the ion map within Biologics Explorer software. The ion map from Biologics Explorer software provides excellent visualization of the charge variants identified from icIEF-UV/MS analysis of the non-stressed (A) and 24-hour stressed (B) NISTmAb sample. The color scheme of the ion map indicates the relative abundances of the main species and charge variants. A significant increase in the glycation content (addition of up to 5 Hex moieties) was detected in the 24-hour stressed sample (B). The charge variants identified from the stressed sample were further characterized by the EAD-based LC-MS workflow. Not all species were annotated for figure clarity. M: deglycosylated NISTmAb. Hex: glycation. Lys: C-terminal lysine. DeAm: deamidation.

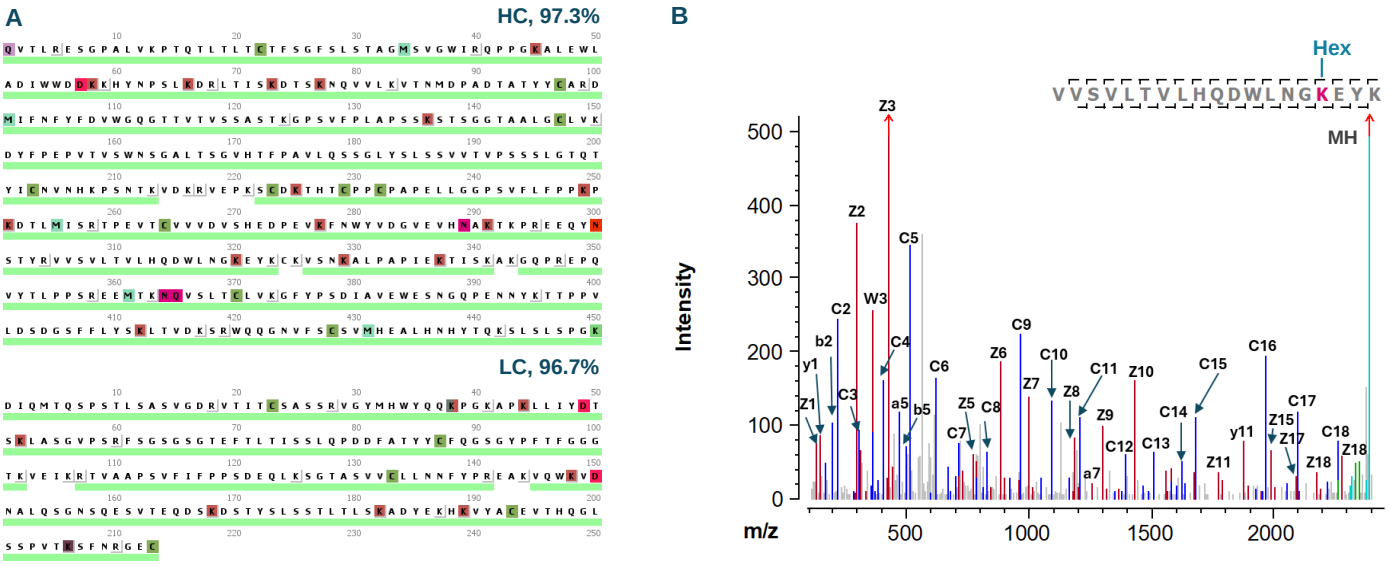

Figure 4. Confident identification and localization of glycation in the time-course NISTmAb samples using an EAD-based LC-MS workflow. The EAD-based LC-MS workflow provided a high sequence coverage (>96%) of the heavy (HC) and light chain (LC) in a single injection (A). The deisotoped EAD spectrum of a selected glycated peptide identified in the glucose-stressed samples shows that EAD led to extensive fragmentation of the peptide backbone while preserving the labile glycation moiety, leading to confident sequence confirmation and accurate localization of the modification (B).

employed to identify and localize glycation in NISTmAb tryptic digests. The combination of the icIEF-UV/MS and LC-MS results leads to a comprehensive charge heterogeneity analysis of the non-stressed and glycated NISTmAb samples.

The EAD-based LC-MS workflow led to high sequence coverage of LC and HC (>96%) in a single injection (Figure 4A), demonstrating the capability of this powerful workflow for routine biotherapeutic characterization. It was reported previously that EAD provides a clear advantage over traditional collision-based MS/MS approaches for fragmentation and localization of glycation due to its ability to preserve labile modifications.[3,4] Figure 4B shows the deisotoped EAD spectrum of a glycated peptide identified using Biologics Explorer software. EAD led to extensive fragmentation of this glycated peptide for its confident identification. The ability of EAD to preserve the intact glycation moiety in the MS/MS fragments enabled unambiguous localization of this modification in the peptide sequence. Additionally, the EAD-based LC-MS workflow provides relative quantitation of each glycated species across time-course samples (data not shown), as described previously.[4]

In summary, the orthogonal, streamlined icIEF-UV/MS and EAD-based LC-MS workflows offered by the Intabio ZT and ZenoTOF 7600 system provide comprehensive charge heterogeneity analysis of glycated NISTmAb in a time-source study. The icIEF-UV/MS workflow provides high-resolution separation, sensitive detection and confident identification of glycated species, enabling rapid screening and monitoring of glycation from early discovery through late development. The EAD-based LC-MS workflow is a powerful tool for comprehensive and routine characterization of biotherapeutics. The ability of EAD to preserve labile PTMs offers site-specific information about glycation in the peptide sequences. The combined benefits of these 2 orthogonal workflows can be leveraged for comprehensive charge heterogeneity analysis of routine and stability samples, facilitating informed decision-making throughout the development pipeline.

Conclusions

- The orthogonal icIEF-UV/MS and LC-MS workflows, offered by a single MS platform, are powerful tools for comprehensive charge heterogeneity analysis of routine and stability samples from the biopharmaceutical pipeline
- The icIEF-UV/MS workflow provides high-resolution separation, sensitive detection, rapid quantitation and confident identification of intact glycated species in a time-course degradation study of NISTmAb
- The EAD-based LC-MS workflow leads to confident identification of glycated peptides and accurate localization of glycation in the peptide sequence
- While the icIEF-UV/MS workflow can be leveraged for rapid charge heterogeneity analysis and impurity assessment, the EAD-based LC-MS workflow provides in-depth characterization of proteoforms or impurities
- The streamlined icIEF-UV/MS and EAD-based LC-MS workflows, coupled with intuitive Biologics Explorer software, can be easily implemented by analytical teams in different biopharmaceutical departments

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