

Effortless icIEF-UV/MS to LC-MS switching for comprehensive biotherapeutic characterization

Scott Mack, Haichuan Liu and Zoe Zhang

SCIEX USA

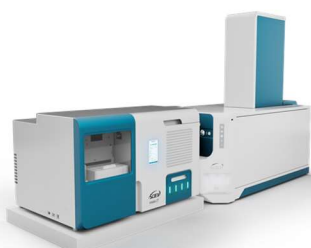
The combined capabilities of imaged capillary isoelectric focusing [icIEF]-UV/MS and electron-activated dissociation [EAD]-based LC-MS workflows on a single MS system offer a rapid and flexible solution to multi-level characterization of biotherapeutics and charge variants.¹⁻³ This technical note describes a streamlined process of alternating between icIEF-UV/MS and LC-MS instrument configurations to achieve rapid charge heterogeneity and peptide mapping analyses. The icIEF-UV/MS and LC-MS results obtained before and after the configuration change are highly consistent, instilling confidence in leveraging these 2 orthogonal separation approaches for comprehensive biotherapeutic characterization [Figure 1].

Comprehensive characterization of biotherapeutics and charge variants is vital to ensuring the quality, safety and efficacy of these drug molecules throughout their development cycles.⁴ Traditionally, achieving in-depth charge heterogeneity analysis and biotherapeutic characterization requires investment in separate icIEF and LC-MS instrumentation and personnel with different skill sets. However, elucidating complex charge profiles and identifying the proteoforms associated with each charge variant peak using conventional icIEF approaches or ambiguous fraction collection methods is challenging. icIEF fractionation followed by off-line MS analysis is labor-intensive, often taking weeks to complete. Furthermore, LC-MS instruments equipped only with collision-based MS/MS techniques struggle with the localization of labile post-translational modifications [PTMs] and differentiation of amino acid isomers.

Key features of SCIEX's icIEF-UV/MS and LC-MS workflows for biotherapeutic characterization

- **Operation simplicity:** Conversion between the icIEF-UV/MS and LC-MS configurations can be achieved in 10 minutes by a single user.
- **Rapid charge heterogeneity analysis:** icIEF-UV/MS offers high-resolution separation, sensitive detection, confident identification and rapid monitoring of biotherapeutic charge variants without the need for off-line fractionation, reducing the time to obtain answers from weeks to hours.
- **Comprehensive biotherapeutic characterization:** EAD-based LC-MS workflows provide high sequence coverage, accurate PTM localization, confident disulfide bond mapping and clear isomer differentiation to achieve comprehensive biotherapeutic characterization.
- **Single-MS solution:** The ease of alternating icIEF-UV/MS and LC-MS analysis on a single MS platform enables quick access to these powerful techniques whenever needed.
- **Platform assays:** The streamlined icIEF-UV/MS and LC-MS workflows can be implemented for routine and advanced characterization of biotherapeutics and charge variants by users with different levels of MS expertise.

Rapid charge heterogeneity analysis and monitoring



Intabio ZT system



Comprehensive biotherapeutic characterization



ZenoTOF 7600 system
ExionLC AD system

Figure 1. The ease of switching between the icIEF-UV/MS and LC-MS configurations on a single MS system allows rapid charge heterogeneity analysis and comprehensive biotherapeutic characterization. The ZenoTOF 7600 system coupled with an Intabio ZT system (left) provides powerful icIEF-UV/MS capabilities for high-resolution separation, sensitive detection, confident identification and rapid monitoring of biotherapeutic charge variants. Coupling the ZenoTOF 7600 system with an ExionLC AD system (right) enables the access to a full suite of LC-MS workflows, such as EAD-based peptide mapping and middle-down MS, offering comprehensive biotherapeutic characterization for sequence confirmation, PTM localization, disulfide bond mapping and isomer differentiation. The icIEF-UV/MS and LC-MS data can be interpreted using a single software package—Biologics Explorer software (middle).

Introduction

Antibody-based biotherapeutics are highly heterogeneous due to various PTMs, degradation products, aggregates and charge variants.¹ Comprehensive characterization of biotherapeutics and their charge variants is important for formulation and stability studies, critical quality attribute (CQA) assessment and quality control of these drug molecules.⁴

Traditional icIEF approaches rapidly separate charge variants, offering a global view of the charge profile. However, without the mass information, these techniques cannot reveal the identity of proteoforms or impurities under each variant peak. The icIEF fractionation followed by off-line MS analysis is time-consuming (days to weeks) and typically involves the efforts from multiple analytical groups. Coupling the ZenoTOF 7600 system with an Intabio ZT or LC system enables quick access to orthogonal icIEF-UV/MS and LC-MS workflows on a single MS system. This allows a single user to perform rapid charge heterogeneity analysis and comprehensive biotherapeutic characterization with operation simplicity and significantly reduces the time to get answers (from days/weeks to hours/days). Additionally, the unique capabilities of EAD offered by the ZenoTOF 7600 system enable accurate localization of labile PTMs and confident isomer differentiation, which are challenging to achieve using traditional collision-based MS/MS approaches.¹⁻³

Methods

Sample preparation: NISTmAb (RM 8671, NIST) was desalted and then 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 solution was vortexed and degassed by centrifugation prior to intact mass analysis using icIEF-UV/MS. For peptide mapping analysis, NISTmAb was denatured using guanidine hydrochloride, reduced with dithiothreitol and alkylated by iodoacetamide, followed by trypsin digestion using the trypsin/Lys-C protease (Promega).

icIEF-UV/MS: NISTmAb sample was separated with an Intabio cartridge (SCIEX) installed on the Intabio ZT system (SCIEX). UV absorbance measurements were collected at 1 Hz during the focusing and mobilization steps. The samples were introduced into the ZenoTOF 7600 system by a metered 3 µL/min flow of chemical mobilizer. The data of 6 replicate injections were acquired.

LC-MS: Upon the completion of icIEF-UV/MS analysis, the Intabio ZT unit was replaced with an ExionLC AD system (SCIEX). In LC-MS experiments, tryptic peptides of NISTmAb were separated using an ACQUITY BEH C18 column [2.1 × 150 mm, 1.7 µm, 130 Å, Waters]. A flow rate of 0.25 mL/min was used for the chromatographic separation. The column was kept at 60°C in the column oven of the ExionLC AD system. 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. 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
Maximum candidate ions	10
Charge state	2 to 10
Electron beam current	5,500 nA
Electron KE	7
ETC	Dynamic
Reaction time	20 ms
EAD RF	120 Da

Data analysis: UV traces and mass spectra from icIEF-UV/MS analysis were interpreted using Biologics Explorer software (SCIEX). Each peak in the charge profile was integrated using the Intabio software to determine its peak area and percentage composition. Intact masses of the main peak and charge variants were determined from the deconvolution of the corresponding mass spectra. EAD DDA data were processed using a peptide mapping workflow template within Biologics Explorer software. Two sets of icIEF-UV/MS and LC-MS data acquired before and after the configuration changes were compared to assess data consistency.

Conversion between icIEF-UV/MS and LC-MS configurations

The Intabio ZT system integrates charge separation and UV detection offered by icIEF-UV with the accurate mass measurement capability of the ZenoTOF 7600 system. icIEF-UV/MS offers simultaneous separation, detection and identification of charge variants in a single assay for rapid charge heterogeneity analysis of biotherapeutics.⁵⁻⁷ When coupled with an LC, the ZenoTOF 7600 system provides a full suite of LC-MS workflows for comprehensive biotherapeutic characterization.⁸⁻¹⁰ Alternating the icIEF-UV/MS and LC-MS configurations allows the user to leverage the unique capabilities of these orthogonal techniques on a single MS platform.

The configuration change from icIEF-UV/MS to LC-MS involves the undocking of the Intabio unit on a wheeled table, removal of a source adapter, installation of an ESI ion source and the connection of an LC system. The reversal of these steps will convert the LC-MS configuration back to icIEF-UV/MS (Figure 2). The process of switching between 2 instrument configurations can be completed in 10 minutes by a single user. The ease and quickness associated with the system conversion enable rapid access to complementary icIEF-UV/MS and

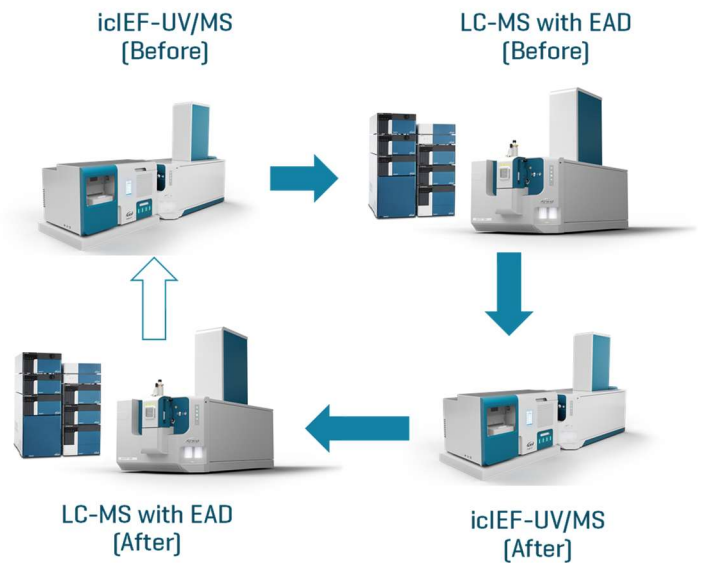


Figure 2. Configuration changes between icIEF-UV/MS and LC-MS. Two sets of icIEF-UV/MS and EAD-based peptide mapping data (Before and After) were acquired by switching between 2 instrument configurations. The unfilled arrow indicates that the system was converted back to the original icIEF-UV/MS configuration at the end of this study.

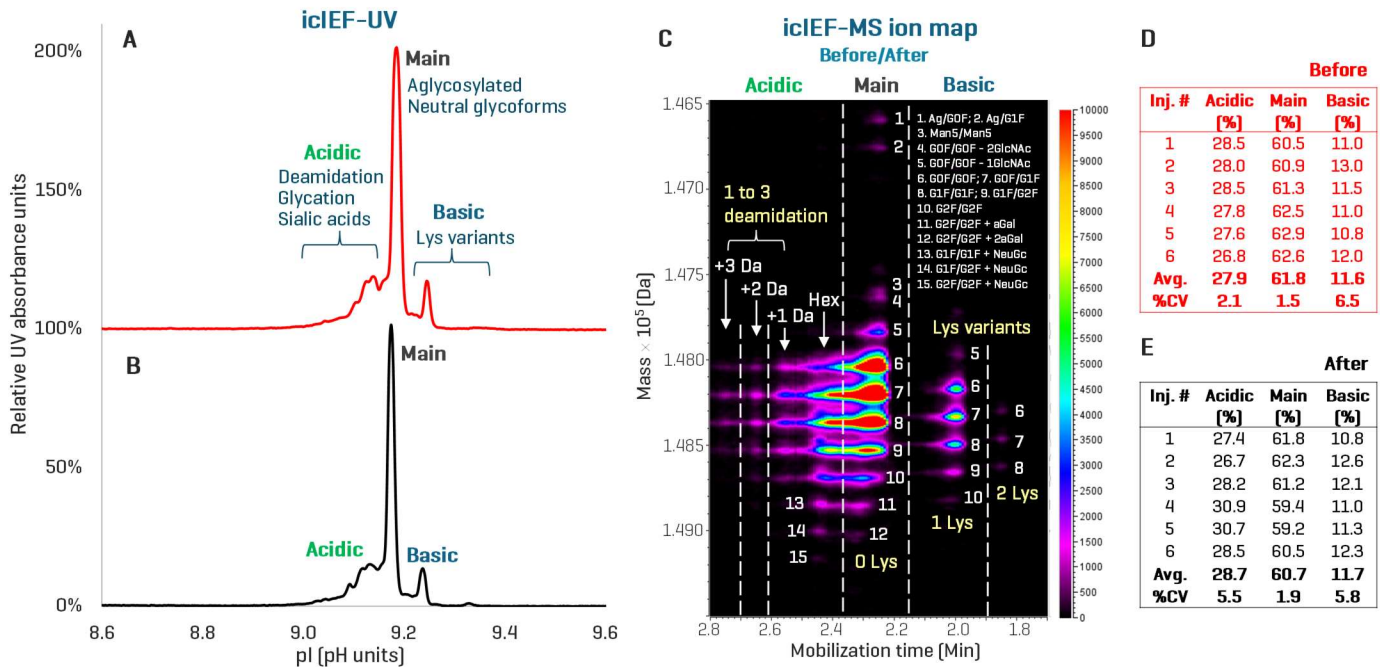


Figure 3. Reproducible separation, relative quantitation and identification of NISTmAb charge variants using the icIEF-UV/MS workflow before and after instrument configuration changes. The icIEF-UV profiles acquired before [A] and after [B] the configuration change show high similarities in charge variant separation and detection, demonstrating the reproducibility of the icIEF-UV/MS workflow. The ion map of the icIEF-MS data from Biologics Explorer software provides excellent visualization of multiple proteoforms, such as various glycoforms (species 1-15), identified from each charge variant peak before and after the configuration change [C]. The tables in D and E display the percentage compositions of the main, acidic and basic species measured for 6 replicate injections of NISTmAb before and after system conversion, respectively. Similar quantitative values were obtained for each species across 12 injections, further demonstrating the minimal impact of instrument configuration change on the data quality and reproducibility. Ag: aglycosylated. Hex: glycation.

LC-MS workflows, depending on the analytical need. The details of configuration changes between icIEF-UV/MS and LC-MS will be described separately. In this work, 2 sets of icIEF-UV/MS and LC-MS data [Before and After] were acquired and compared to assess data consistency before and after the configuration changes [Figure 2].

Charge heterogeneity analysis using the icIEF-UV/MS workflow

Figure 3 shows the icIEF-UV profile, icIEF-MS ion map and relative quantitation results of NISTmAb samples acquired before and after instrument configuration changes. High-resolution separation of acidic and basic variants from the main species was consistently observed in the Before [Figures 3A] and After [Figures 3B] profiles. The MS capability of the icIEF-UV/MS workflow provided consistent identification of various proteoforms, such as various glycoforms [species 1-15], under each charge variant peak following system conversion [Figure 3C]. These results demonstrate the minimal impact of instrument configuration changes on the data quality and reproducibility of the icIEF-UV/MS workflow.

The tables in Figures 3D and 3E show the relative quantitation results obtained for 6 replicate injections of NISTmAb from the Before and After icIEF-UV/MS data, respectively. The average percentage compositions of the main, acidic and basic species measured from the Before data are 61.8%, 27.9% and 11.6% [Figure 3D], respectively, in good agreement with those [60.7%, 28.7% and 11.7%] measured from

the After data [table in Figure 3E]. High reproducibility of the quantitation result [%CV<10%] was also observed across 6 replicate injections before and after instrument configuration changes [Figures 3D and 3E]. These results further demonstrate the reproducibility of charge variant separation, detection and quantitation using icIEF-UV/MS despite instrument configuration changes.

Biotherapeutic characterization using the EAD-based LC-MS workflow

Upon completion of icIEF-UV/MS analysis, the system was converted into the LC-MS configuration following the procedures described above. Two sets of peptide mapping data of NISTmAb [Before and After, Figure 2] were acquired using an EAD DDA method. A similar total ion chromatogram [TIC] intensity of ~7e7 was obtained for the Before and After data [not shown]. The interpretation of 2 data files using the same parameters within Biologics Explorer software led to an identical sequence coverage of HC [98.9%] and LC [97.2%] [Figure 4A]. High-quality EAD spectra of peptides were also obtained consistently from the 2 LC-MS data files. Figures 4B and 4C show the deisotoped EAD spectrum of the glycopeptide TKPREEQYN[GOF]STYR from the Before and After EAD DDA data, respectively. Nearly identical spectral quality and fragment coverage were obtained for this peptide identified in the 2 data files. The detection of a nearly complete series of *c/z* fragments led to accurate localization of labile GOF glycan in both cases. Similar EAD data were also obtained for other glycoforms, such as G1F, G2F and

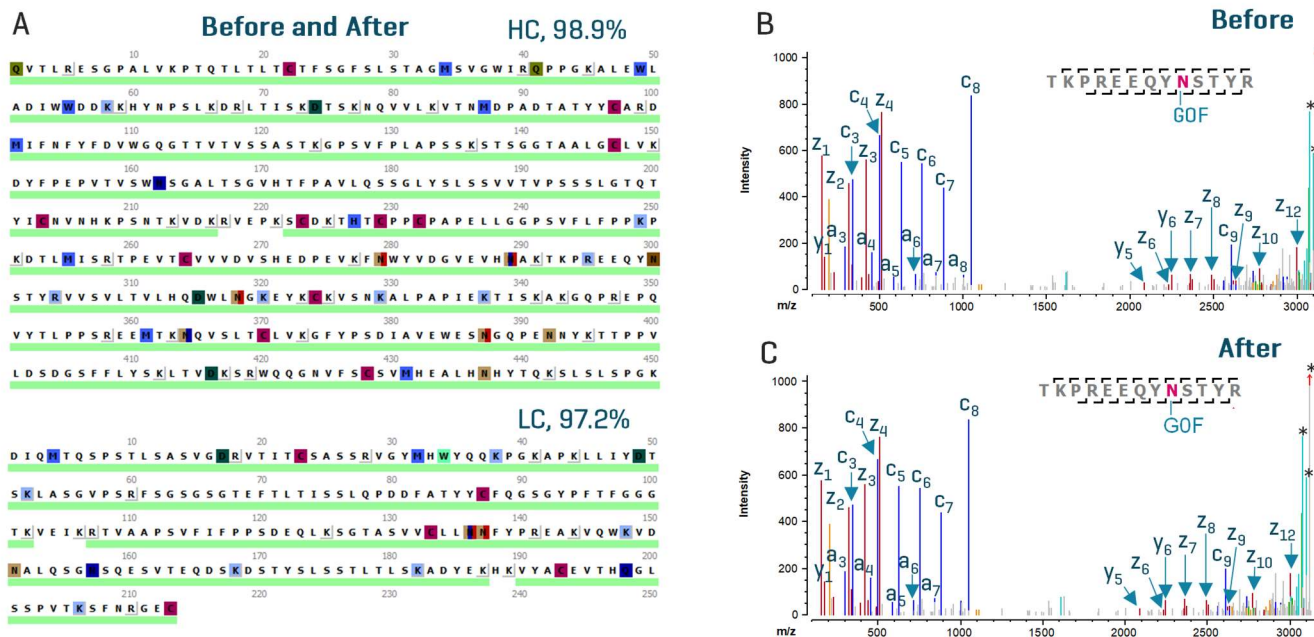


Figure 4. EAD-based peptide mapping results obtained before and after instrument configuration changes. The EAD-based peptide mapping data acquired before and after instrument configuration changes led to an identical sequence coverage of NISTmAb (>97% for HC and LC) [A]. Consistent high-quality EAD data were obtained in the Before and After data. An example demonstrating this similarity is provided in B and C, which show the deisotoped EAD spectra of the glycopeptide TKPREEQYN[GOF]STYR obtained before [B] and after [C] system switching. * Precursor or neutral-loss peaks.

Man5 (data not shown). These LC-MS results confirm the glycoform identification achieved using icIEF-UV/MS (Figure 3C).

In summary, the data presented in this technical note demonstrate the operation simplicity and data reproducibility associated with alternating between icIEF-UV/MS and LC-MS configurations on a single MS system. Highly reproducible icIEF-UV/MS and LC-MS data were obtained before and after the configuration changes, ensuring confidence in leveraging these 2 orthogonal workflows for rapid charge heterogeneity analysis and comprehensive biotherapeutic characterization on a regular basis.

Conclusions

- The ease of switching between icIEF-UV/MS and LC-MS configurations on a single MS platform allows quick access to these powerful workflows on a regular basis
- High-quality icIEF-UV/MS and LC-MS data were obtained reproducibly before and after configuration changes, instilling confidence in leveraging these approaches to meet analytical needs throughout biotherapeutic development
- The combined capabilities of orthogonal icIEF-UV/MS and EAD-based LC-MS workflows provide rapid charge heterogeneity analysis and comprehensive biotherapeutic characterization
- The streamlined icIEF-UV/MS and EAD-based LC-MS workflows, coupled with intuitive Biologics Explorer software, can be easily implemented by users with different levels of MS experience

References

1. Leverage orthogonal technologies: cIEF, icIEF-UV/MS and LC-MS to facilitate biotherapeutics development. [SCIEX technical note, MKT-31116-A](#).
2. In-depth charge heterogeneity analysis of antibody-drug conjugates with a streamlined icIEF-UV/MS and EAD-based peptide mapping workflow. [SCIEX technical note, MKT-32353-A](#).
3. Monitoring and characterization of glycation in a biotherapeutics degradation study using icIEF-UV/MS and LC-MS workflows. [SCIEX technical note, MKT-33706-A](#).
4. Anna Robotham and John Kelly. (2020) LC-MS characterization of antibody-based therapeutics: recent highlights and future prospects. [Approaches to the Purification, Analysis and Characterization of Antibody-Based Therapeutics. Chapter 1: 1-33](#).
5. Direct and rapid multi-attribute monitoring of multiple intact monoclonal antibodies with a wide isoelectric-point range of 7.3 to 9.1. [SCIEX technical note, MKT-26996-A](#).
6. Charge variant analysis of antibody-drug conjugates using an icIEF-UV/MS workflow. [SCIEX technical note, MKT-29837-A](#).
7. In-depth charge heterogeneity analysis of antibody-drug conjugates with a streamlined icIEF-UV/MS and EAD-based peptide mapping workflow. [SCIEX technical note, MKT-32353-A](#).
8. An evaluation of a single injection platform method for advanced characterization of protein therapeutics using electron activation dissociation [EAD]. [SCIEX technical note, RU0-MKT-02-13965-A](#).
9. Leverage orthogonal technologies: cIEF, icIEF-UV/MS and LC-MS to facilitate biotherapeutics development. [SCIEX technical note, MKT-31116-A](#).
10. Achieving ultrahigh sequence coverage and high-confidence disulfide bond mapping of biotherapeutics using an electron-activated dissociation [EAD]-based middle-down workflow. [SCIEX technical note, MKT-28565-A](#).

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