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Imaged capillary isoelectric focusing tandem high-resolution mass spectrometry using nano electrospray ionization (ESI) for protein heterogeneity characterization

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A R T I C L E I N F O

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A B S T R A C T

Recombinant monoclonal antibodies (mAbs) have been spurring the rapid growth of commercial biotherapeutics. During production their charge heterogeneity must be assessed as a critical quality attribute to ensure safety, efficacy, and potency. Although imaged capillary isoelectric focusing (icIEF) is a powerful tool for this process, it could be improved further with tandem high-resolution mass spectrometry (HRMS). In this work, a nano-electrospray ionization (nano-ESI) apparatus was constructed to directly couple icIEF to HRMS. The system was evaluated with the standard NISTmAb, as well as more complex mAb, bi-specific antibody, and fusion protein samples. NISTmAb concentrations as low as 0.25 mg/ml demonstrated excellent sensitivity. There were good repeatabilities at 1 mg/ml with 7.58% and 8.01% RSDs for intention time and MS intensity, respectively, and the HRMS signal showed a strong linearity (R = 0.9983) across different concentrations. Meanwhile, the fingerprinting of the complex samples illustrated the versatility and potential of icIEF-HRMS. icIEF-HRMS developed can provide a comprehensive understanding of the underlying structural modifications that impact protein charge heterogeneity. Compared to the traditional ESI, nano-ESI can significantly improve sensitivity while maintaining a reasonable repeatability and throughput. Furthermore, the interface is much easier to connect, and is compatible with many commercial HRMS instruments.

1. Introduction

Recombinant monoclonal antibodies (mAbs) have been driving the rapid growth of commercial biotherapeutics, and have led to the development of more complex antibody-drug conjugates (ADCs), bi-specific antibodies (BsAb), and fusion proteins. During production these molecules are susceptible to chemical modifications due to a variety of cellular processes, manufacturing conditions, and chemical degradation [1–4]. The result is a protein charge heterogeneity resulting from covalent modifications, which requires in-depth characterization during critical quality attribute assessment to ensure safety, efficacy and potency [1,3,5,6]. Furthermore, it is essential to continuously monitor these charge variants throughout manufacturing for quality control purposes.

Besides traditional high performance liquid chromatography (HPLC) and ion exchange chromatography (IEX), capillary electrophoresis (CE) has become an essential technology for the charge variant characterization of mAbs and other complex proteins [7]. Especially, mass spectrometry (MS) is robust for protein identification and CE as separation frontend coupled to MS has attracted many scientists in protein chemistry. Recent reviews comprehensively updated the progress in CE-MS technology [8,9]. Imaged capillary isoelectric focusing (icIEF) is a technology platform developed based on Whole Column Imaging Detection (WCID). The ampholyte in the capillary forms a pH gradient by applying an electric field, and the proteins are distributed along the pH gradient in the capillary according to their respective isoelectric points (pI), thereby realizing the separation of proteins. This method of capillary isoelectric focusing enables extremely efficient separation of

Abbreviations: icIEF, Imaged capillary isoelectric focusing; HPLC, High performance liquid chromatography; IEX, Ion exchange chromatography; MS, Mass spectrometry; ESI, Electrospray Ionization; mAbs, Monoclonal antibodies; BsAb, Bi-specific antibody; pI, Isoelectric point; TIC, Total ion chromatogram; AD, Acrylamide.

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proteins. A detection system consisting of deep UV LEDs and imaging sensor is used for monitoring the separation on the capillary column in real time. Currently, imaged capillary isoelectric focusing (icIEF) has become indispensable to the biopharma industry due to its high analytical throughput, ease of use, fast method development, and excellent reproducibility [10–12]. However, icIEF could become even more powerful if it could reliably couple directly to mass spectrometry (MS) [13–21]. Such a system would enable the intact identification of protein charge variants while providing critical information related to their underlying structural modifications.

The are many practical challenges associated with icIEF-MS that have frustrated analytical chemists for decades. For one, the chemical mobilization of icIEF peaks towards MS is inconsistent and can deteriorate the high separation resolution. Second, many critical icIEF reagents, including carrier ampholytes and capillary coatings, have poor volatility and interfere with the MS signal. Finally, connecting the icIEF-MS interface has a number of compatibility issues due to backpressure, electrical grounding, and capillary blockage. Currently, these challenges are being addressed with the development of new commercial icIEF-MS instruments from Advanced Electrophoresis Solutions Ltd. (AES) and Intabio (now part of Sciex) [13,14].

Recently, in our work, a pressure-driven mobilization technique along with a patented transfer capillary has been developed to maintain the integrity of the icIEF profile for improved resolution and sensitivity [22,23]. Proprietary carrier ampholytes and capillary coatings, along with a makeup solution prior to the MS interface, have effectively mitigated concerns related to background interference and volatility. Overall, it has compatibility with the traditional electrospray ionization (ESI) interface for several commercial MS instruments. Additionally, the robust charge heterogeneity characterization of nine therapeutic mAbs demonstrated improved sensitivity, lower carryover effect, and higher resolution when compared to strong cation exchange (SCX)-MS [24].

In this study, an icIEF-MS system with a nano-ESI interface is presented to enhance the performance further. In general, the reduced flow rates enabled by the nano-ESI can improve the ionization efficiency, lower analyte dispersion in the transfer capillary, and reduce the sample dilution caused by the makeup solution. Compared to the traditional low-flow ESI, the nano-ESI exhibited higher sensitivity and resolution while maintaining outstanding throughput and repeatability. The successful characterization other mAb, BsAb, and fusion protein samples also illustrated the versatility and potential of this system in the increasingly complex landscape of biotherapeutics. Finally, the nano-ESI is much easier to connect and eliminates the need for an auxiliary gas, and combined this makes icIEF-MS more user-friendly for a QC environment.

2. Materials and methods

2.1. Chemicals

All ampholytes (AESlyte™) were obtained from Advanced Electrophoresis Solutions Ltd. (AES, Cambridge, Ontario, Canada). Monoclonal antibody NISTmAb was purchased from Millipore Sigma (cat. no. NIST8671), and mAb (USP-MAB-002) was kindly donated by the United States Pharmacopoeia. The BsAb and Fusion Protein-EX employed in this study were retained samples from the authors' organization (AES). Mass spectrometry grade acetonitrile (ACN), formic acid, and formamide were purchased from Fisher Scientific (Hampton, NH).

2.2. Solutions for icIEF

The NISTmAb was mixed with 2% (v/v) UR AESlyte™ 8.5–10 and 10% formamide at concentrations of 0.25, 0.5, or 1 mg/ml. USP-MAB-002 mAb was mixed with 2% (v/v) UH AESlyte™ 7–9. The BsAb was mixed with 2% (v/v) HR AESlyte™ 8–10.5 and 5% formamide. The Fusion Protein-EX was mixed with 2% (v/v) UH AESlyte™ 3–10. The concentration of the aforementioned protein samples was 1 mg/ml, and all icIEF solutions were prepared using deionized water.

2.3. Imaged capillary isoelectric focusing (icIEF)

For icIEF separation, the CEInfinite (Advanced Electrophoresis Solutions Ltd, Cambridge, Canada) was utilized with on-column UV detection at 280 nm. A 200 μm ID acrylamide derivative (AD) coated capillary cartridge (AES, cat. no. CP00303) and micro-tee connector (AES, cat. no. CP00303 M) were used for the icIEF-MS. The whole column imaging detection (WCID) cartridges in icIEF-MS analysis had a 5 cm length separation capillary while a 50 μm ID transfer capillary was used for the MS coupling. The icIEF-MS cartridge included a quartz union (works as a micro-tee) to connect the make-up solution and transfer capillary to ESI. Both the make-up solution capillary and inlet capillary had a 100 μm ID.

For icIEF-MS (Low-flow ESI), the focusing was 1 min at 1000 V, 1 min at 2000 V, and 10 min at 3000 V. Then, the voltage remained at 3000 V during mobilization. The mobilization solution contained 0.1% (v/v) formic acid and its flow rate was 50 nL/min. The makeup solution contained 50% ACN and 0.5% (v/v) formic acid and its flow rate was 5 μL/min. The mobilization time was 20 min. For icIEF-MS (nano-ESI), the same experimental conditions were used, except 0.5% (v/v) formic acid was replaced with 50% ACN and 5% (v/v) acetic acid with 2 μL/min flowing rate in the makeup solution.

2.4. High-resolution mass spectrometry

For icIEF-MS with the low-flow ESI, the Thermo Q Exactive Plus mass spectrometer used an Ion Max ESI Ion Source with a 34-gauge needle (Low-flow ESI, Thermo Fisher Scientific, Bremen, Germany). The MS parameters were: spray voltage: 3.8 kV, sheath gas: 5 L/min, auxiliary gas: 15L/min, S-lens RF: 70 eV, capillary temp: 300 °C, resolution: 17,500@*m/z* 200, scan range of precursor ion: 1500–5000 m/z, and maximum injection time: 200 ms.

For icIEF-MS with the nano-ESI, the Thermo Q Exactive Plus mass spectrometer used an Easy-Spray Ion Source (Nano ESI, Thermo Fisher Scientific, Bremen, Germany). The nano-spray emitter was from Advanced Electrophoresis Solutions Ltd. (AES). The MS parameters were: spray voltage: 2.0 kV, S-lens RF: 50 eV, capillary temp: 250 °C, resolution: 17,500@*m/z* 200, scan range of precursor ion: 2000–5000 m/z, and maximum injection time: 200 ms.

The Biopharma Finder (BPF 5.0) from Thermo Fisher was used for intact protein deconvolution.

3. Results and discussions

3.1. Workflow of icIEF-HRMS for protein charged heterogeneity

Fig. 1A illustrates the configuration and workflow of the icIEF-MS with a nano-ESI including coated capillary separation cartridge that includes a quartz union (works as a micro-tee) to connect the make-up solution and transfer capillary to ESI, nano-flow pump for the mobilization of focused protein bands, and the microliter interface (50 μm ID nano-emitter constructed by fused silica capillary) with built-in platinum electrode designed and a beveled-tip (50° slope angle). The beveled emitter geometry maintains stable electrospray over a wide flow rate range. Note the presence of the nano-spray emitter, which was specifically built to make icIEF compatible with many commercial nano-ESI interfaces, including the Thermo Fisher Easy-Spray shown in Fig. 1B. Altogether, the system can improve the sensitivity MS while retaining to the excellent separation resolution of icIEF.

In the first step, the protein charge variants are separated along the icIEF column according to their isoelectric point. Traditionally, icIEF relies on carrier ampholytes, dynamic polymer coatings, and urea to stabilize the proteins and maintain the quality of the high-resolution

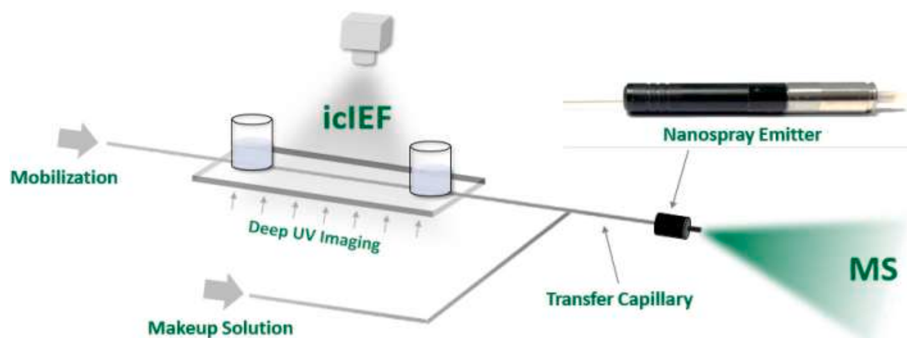


Fig. 1. Schematic of the icIEF-MS system with the Nano-spray Emitter connecting the interface.

separations. However, these reagents can interfere with the electrospray process and impair the MS signal. Therefore, we have developed custom formulations containing proprietary carrier ampholytes and permanent capillary coatings to mitigate these concerns while avoiding the use of urea. AESlyte™ HR (High-Resolution) and UH (Ultra-High Resolution) carrier ampholyte (CA) series developed in our reported study were composed of mixtures of small molecules with specific pKa for forming pH gradient, which demonstrates a reduction in baseline noise and distinguishably increased consistency between lots when compared to other CAs brands of the same pH range [25]. AESlyte™ CAs used in this study are commercial products and can be customized according to application requests by adjusting the compositions and corresponding concentrations with desirable resolutions. In addition, the new methylcellulose coating was developed for icIEF-MS analysis besides the AD coating used in this study [26].

In the second step, the icIEF profile is transferred to the nano-ESI apparatus using pressure-driven mobilization. After proteins' focusing is completed along the separation capillary, water containing 0.1% (v/v) formic acid as mobilization solvent from syringe pump drives the focused protein bands out of the separation capillary towards MS ion source (ESI) at 50 nL/min flowing rate. Make-up solution helps the effluents direct into ESI though a seamless interface. Compared to chemical mobilization, this technique offers better speed and reproducibility while maintaining the focusing mechanism in the icIEF column. When the focused peaks leave this region, they enter a transfer capillary with a reduced diameter to limit their intermixing due to diffusion. A micro-tee positioned along this path adds a makeup solution to improve the volatility and ionization of the sample during the electrospray process. Finally, the icIEF peaks reach the nano-ESI sequentially before being analyzed by HRMS.

The integration of the nano-ESI provides several benefits. First, the innovative microliter interface with built-in platinum electrode design and reduced dead volume helps minimize sample consumption. Second, the reduced flow rates permitted by the nano-ESI can improve the ionization efficiency and eliminate the need for an auxiliary gas. Furthermore, this enables a lower makeup solution flow rate for less sample dilution, and helps reduce the solute dispersion in the transfer capillary. Combined, all of these factors contribute to an improved resolution and sensitivity. Finally, the constructed nano-spray emitter requires no special modifications to the ionization source and can be easily connected to a variety of commercial MS instruments.

3.2. Methodology validation of icIEF-HRMS using nano-ESI and low-flow ESI

The standard NISTmAb was used to evaluate the performance of icIEF-HRMS with the nano-ESI and low-flow ESI interfaces. As shown in Figs. 2 and 1 mg/mL NIST mAb demonstrated excellent sensitivity and repeatability with the nano-ESI, while the five charge variants with subtle pI discrimination could be resolved using the nano-ESI. Fig. 3 illustrated icIEF-MS deconvolution of NISTmAb' charge variants (1 mg/ml, n = 3). Compared to the low-flow ESI results as seen from Fig. 4 that showed the TIC and deconvoluted MS of found proteoforms, the charge variant peaks from nano-ESI appear to be more well-defined thanks to better resolution from lower diffusion. In addition, Table 1 shows that the nano-ESI contributed to higher sensitivity with a 40% increase in MS intensity for the main component due to less dilution effect resulting from lower make-up solution flowing rate than low-flow ESI. The

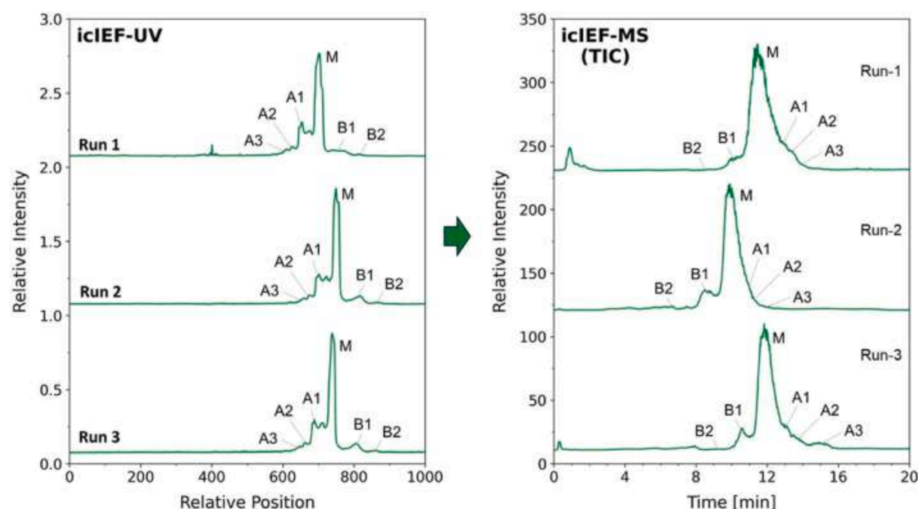


Fig. 2. icIEF-MS characterization of NISTmAb using Nano-ESI.

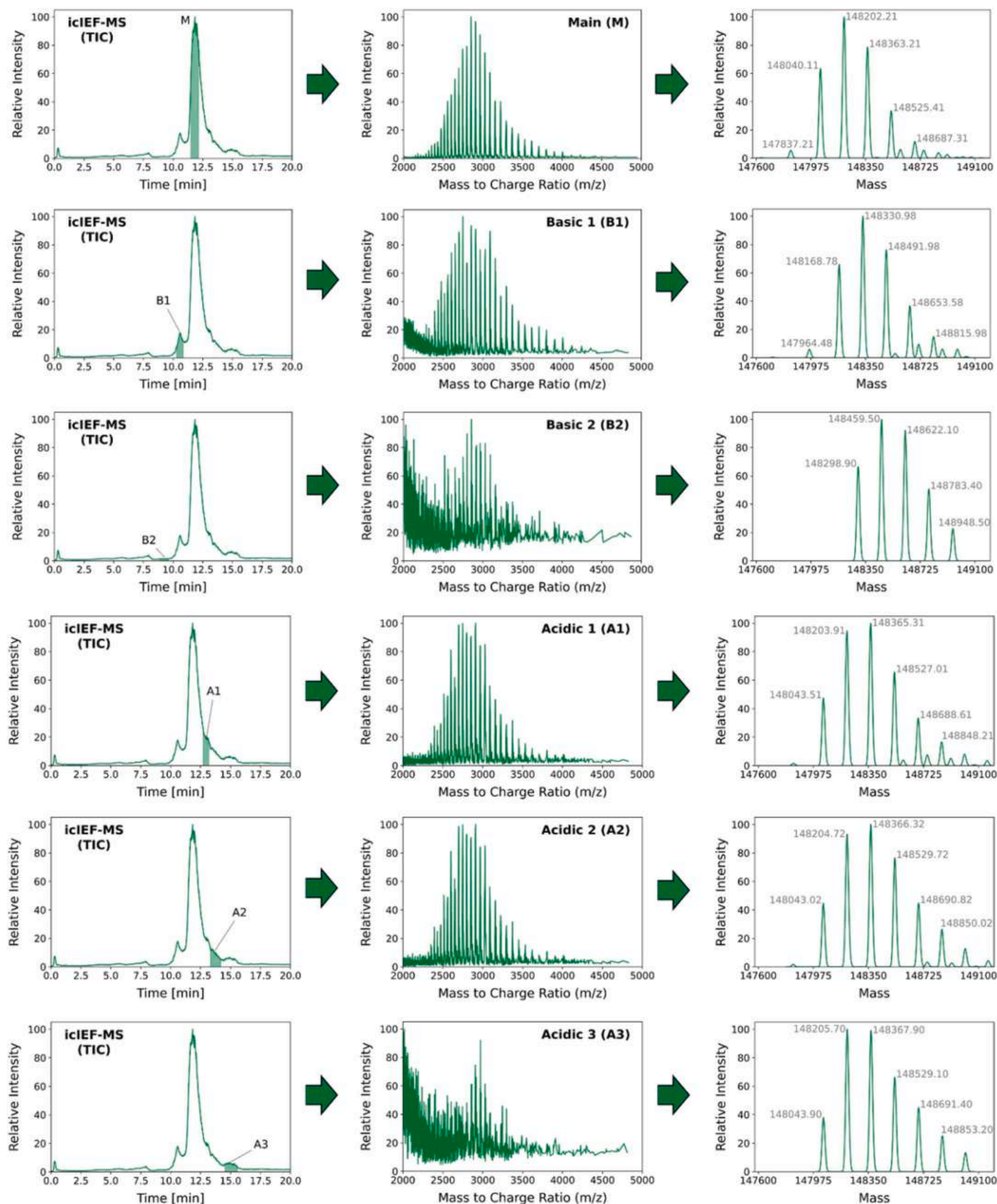
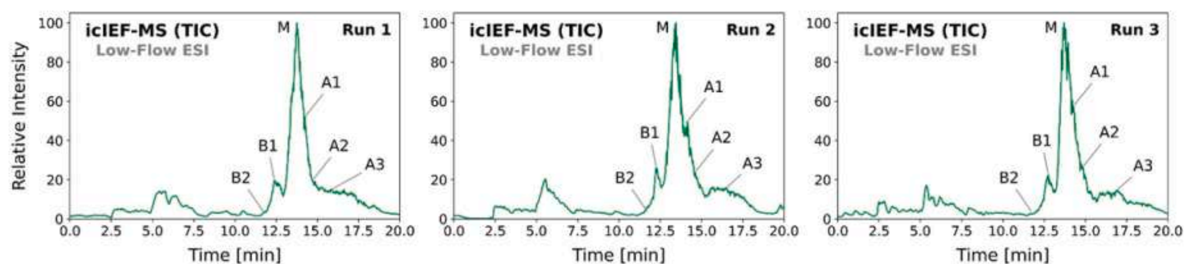
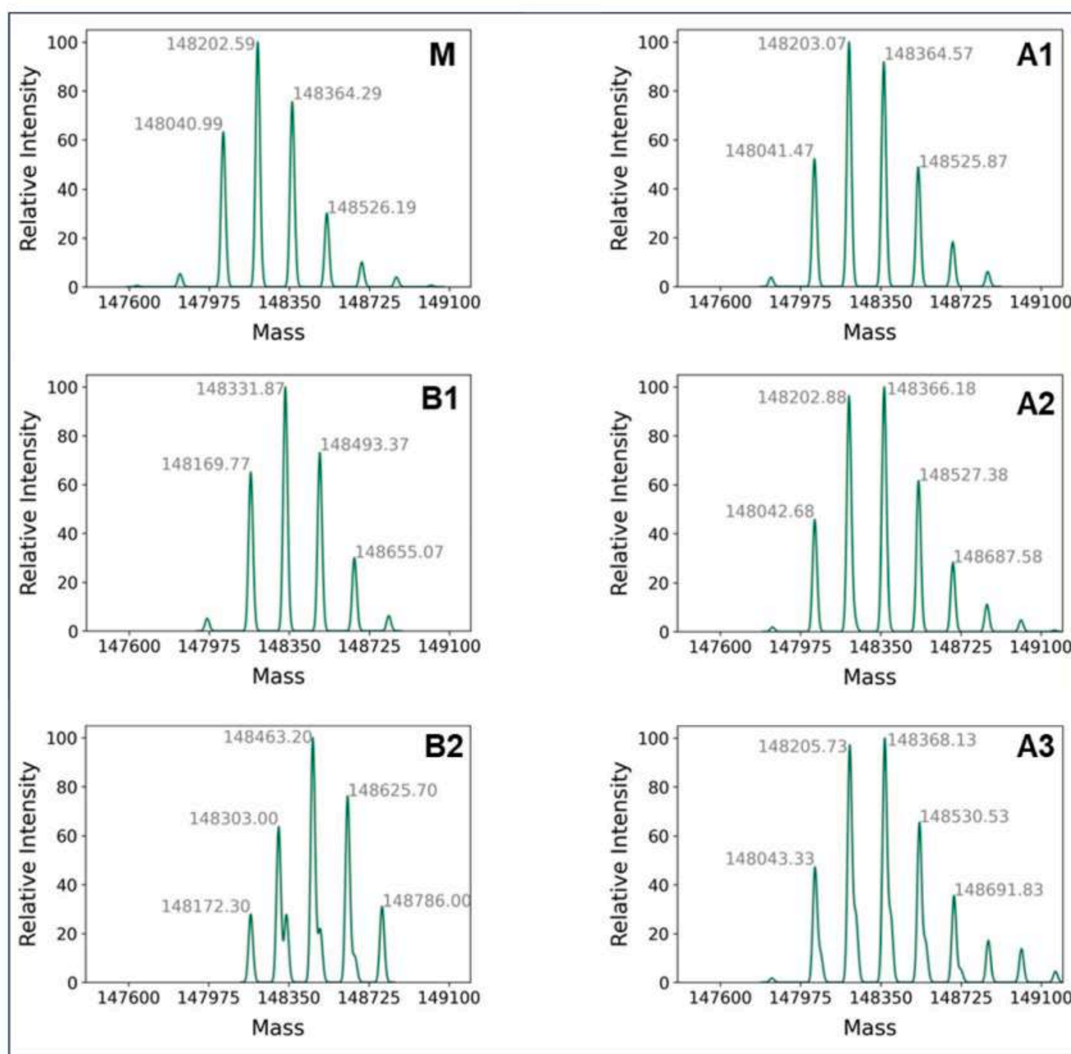


Fig. 3. icIEF-MS deconvolution of NISTmAb (1 mg/ml, n = 3) using the nano-ESI.



(A) TIC



(B) Deconvoluted MS

Fig. 4. icIEF-MS characterization of NISTmAb (1 mg/ml, n = 3) using Low-flow ESI.

Table 1
NISTmAb (1 mg/ml) icIEF-MS repeatability with the use of Nano-ESI and Low-flow ESI.

MS ion source	Nano-ESI			Low-flow ESI		
Injections (n = 3)	Run-1	Run-2	Run-3	Run-1	Run-2	Run-3
Retention time (Main peak)	11.5min RSD = 7.58% (n = 3)	10.1min	11.6min	13.6min RSD = 1.10% (n = 3)	13.8min	13.9min
MS intensity (Main peak)	1.94 E8 RSD = 8.01% (n = 3)	1.74 E8	2.04 E8	1.29 E8 RSD = 6.69% (n = 3)	1.46 E8	1.32 E8

consecutive three runs were carried out for the repeatability test. Low-flow ESI exhibited better repeatability of retention time in Total Ion Chromatogram (TIC, RSD<2.0%, n = 3) than the nano-ESI (RSD = 7.58%, n = 3) because a higher flow rate enables a more stable applied pressure from the pump. As for nano-ESI, it was observed that the retention time of Run-2 was a little away as comparing to Run-1 and Run-3. However, as shown in Table 1, its RSD of retention time was 7.58% based on the three runs, which is tolerable for electric-migration CE technology that has more complex factors to influence the chromatographic behavior. In addition, nano ESI showed much more stable baseline than low-flow ESI in icIEF-MS analysis, which contributes to more accurate protein identification with less background interference.

As shown in Table 1, the repeatability of the MS intensity for the main component (RSD 5.0–10.0%, n = 3) was good for both the nano-ESI and low-flow ESI. Moreover, Fig. 5 demonstrates the excellent sensitivity of the nano-ESI, as minor charge variants B1 and A3 remain visible at NIST mAb concentrations as low as 0.25 mg/ml. Finally, as seen in Table 2, the nano-ESI exhibited outstanding linearity in the range of 0.25–1 mg/ml (r = 0.9983), which is essential to accurate quantitation of protein charge variants.

As demonstrated in Fig. 3, the charge variants of Basic Peak 1 and 2 show +128 and + 256 Da mass increases compared to the main peak, which were respectively identified as C-terminal lysine variants. Additional peaks above G2F/G2F at +162 Da intervals are observed in acidic peaks 1, 2 and 3, identified as putative glycation. A +1 Da mass shift was observed in Acidic Peak 1, A +2 Da mass shift was observed in Acidic Peak 2 and A +3 Da mass shift in Acid Peak 3 for proteoforms with G0F/G0F, indicating a high abundance of proteoforms with 1–3 deamidation events in these three charge variants. As indicated in Fig. 4, low-flow ESI demonstrated the similar deconvoluted MS patterns of found proteoforms to nano-ESI. In addition, more low-abundance proteoforms were detected with the use of nano-ESI due to its higher resolution and sensitivity than low-flow ESI.

3.3. icIEF-HRMS using nano-ESI for the heterogeneity characterization of mAb (USP-MAB-002)

icIEF-HRMS using both nano-ESI and low-flow ESI were used to characterize the heterogeneity of mAb (USP-MAB-002, 1 mg/ml). As seen from Fig. 6A, the nano-ESI revealed the main component and its three charge variants (A1, A2 and B) with satisfactory sensitivity and resolution. The deconvoluted MS information is then shown in Fig. 6B and C, and even basic variant can be detected at low concentrations due to excellent sensitivity of the nano-ESI. Compared to the low-flow ESI shown in Fig. 6A, the nano-ESI demonstrated less TIC baseline fluctuation and less background interference with better peak shape. This enables the identification of the two acidic variants (A1 and A2) with more certainty and contributes to accurate semi-quantitation of charge variants based on peak area percentage. In addition, based on these results, the charge variants can be fractionated with a preparative icIEF instrument developed in our lab for in-depth peptide mapping and intact analyses by HPLC-MS and IEX-MS [27,28]. This work is currently in progress.

Table 2
Linearity of NISTmAb in the range of 0.25–1.00 mg/ml.

Concentration	0.25 mg/ml	0.50 mg/ml	1.00 mg/ml
MS intensity of main peak	0.54 E8	1.08 E8	1.94 E8
Linearity	Y = −0.057X+0.5399, r = 0.9983 (Y- concentration, mg/ml; X- MS intensity)		

3.4. icIEF-HRMS using nano ESI for the heterogeneity characterization of complex proteins

Recently, complex proteins including antibody-drug conjugates (ADCs), bi-specific Abs and fusion proteins have regained the special attention of scientists due to their unique therapeutic effects [29–32]. Few applications of characterizing such complex proteins by CE-MS have been reported. Especially for fusion proteins or recombinant proteins with extremely complex components, both HPLC-MS and CE-MS present significant challenges when dealing with protein samples that exhibit complex and high glycations. In our recent study, we have found that the deglycosylation process of protein samples is absolutely critical for enhancing MS detection, particularly when dealing with very weak signals caused by these complex and extensive glycations [33]. However, the type of fusion protein with low glycations can be directly analyzed by mass spectrometry. In this study, the icIEF-HRMS platform with nano-ESI was applied to complex proteins to demonstrate its wide applicability. As shown in Fig. 7, the Fusion Protein-EX with low glycations demonstrated a fairly complex mapping of protein components with two peak groups and molecular weights ranging from 60 to 100K for all detected proteins. A typical deconvoluted MS from group-2 was shown in Fig. 7. The characterization of a BsAb is also on display, and it contains three major charge variants with rather high pI values greater than 9.5 and the molecular weight around 175K. The sensitivity was

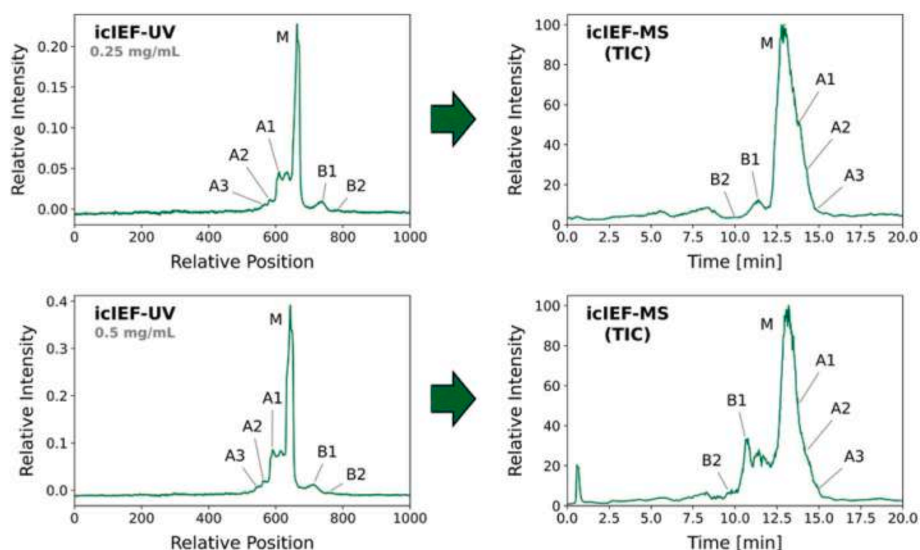


Fig. 5. Sensitivity of icIEF-MS for NISTmAb using Nano-ESI.

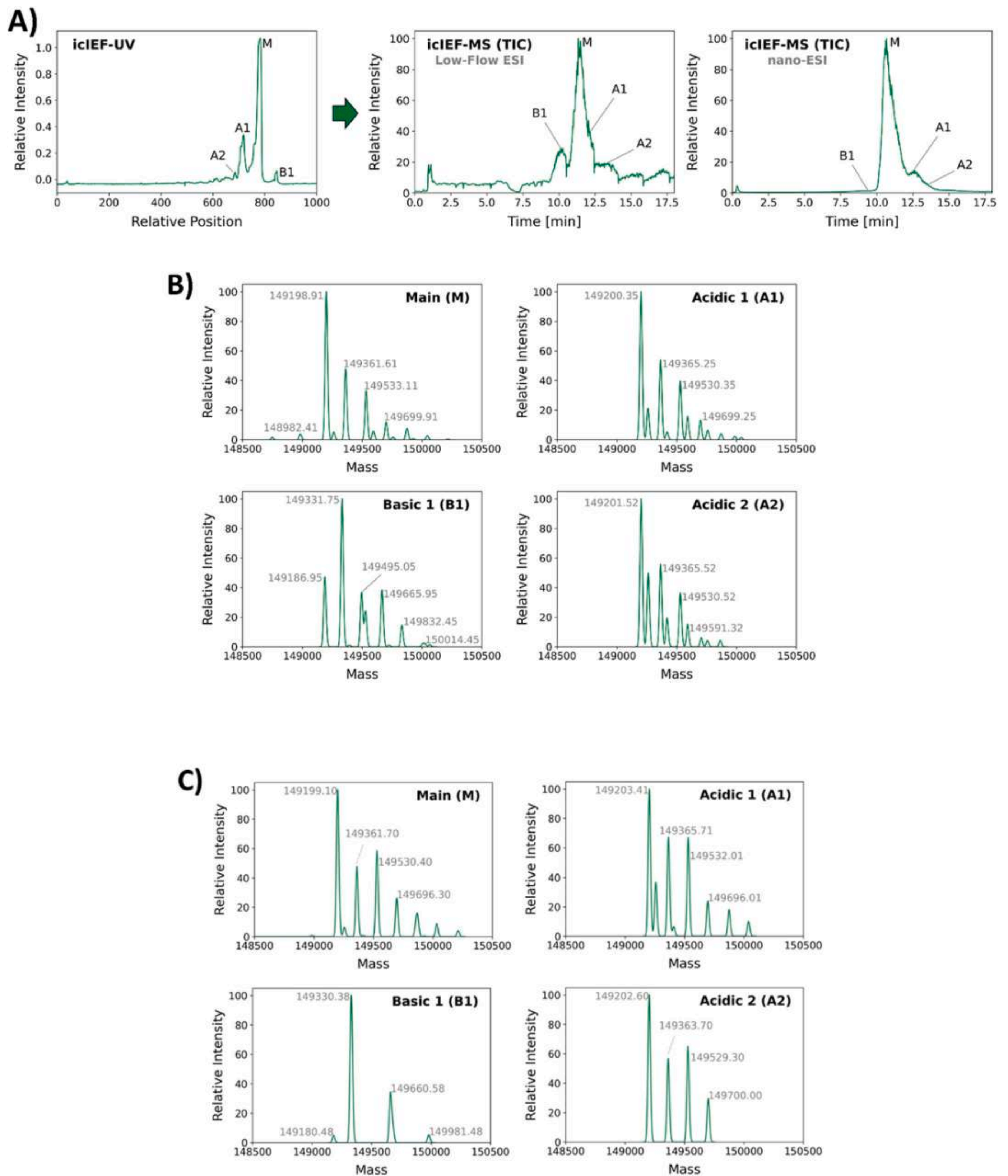
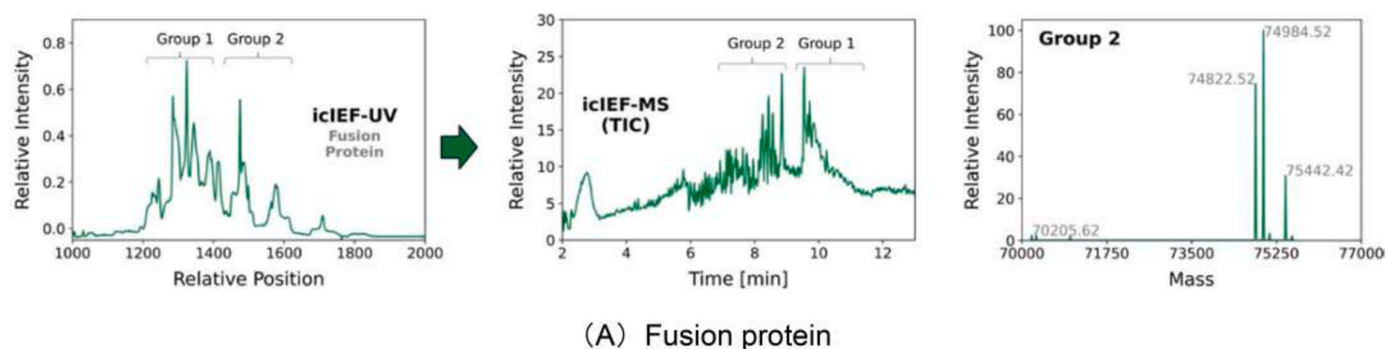
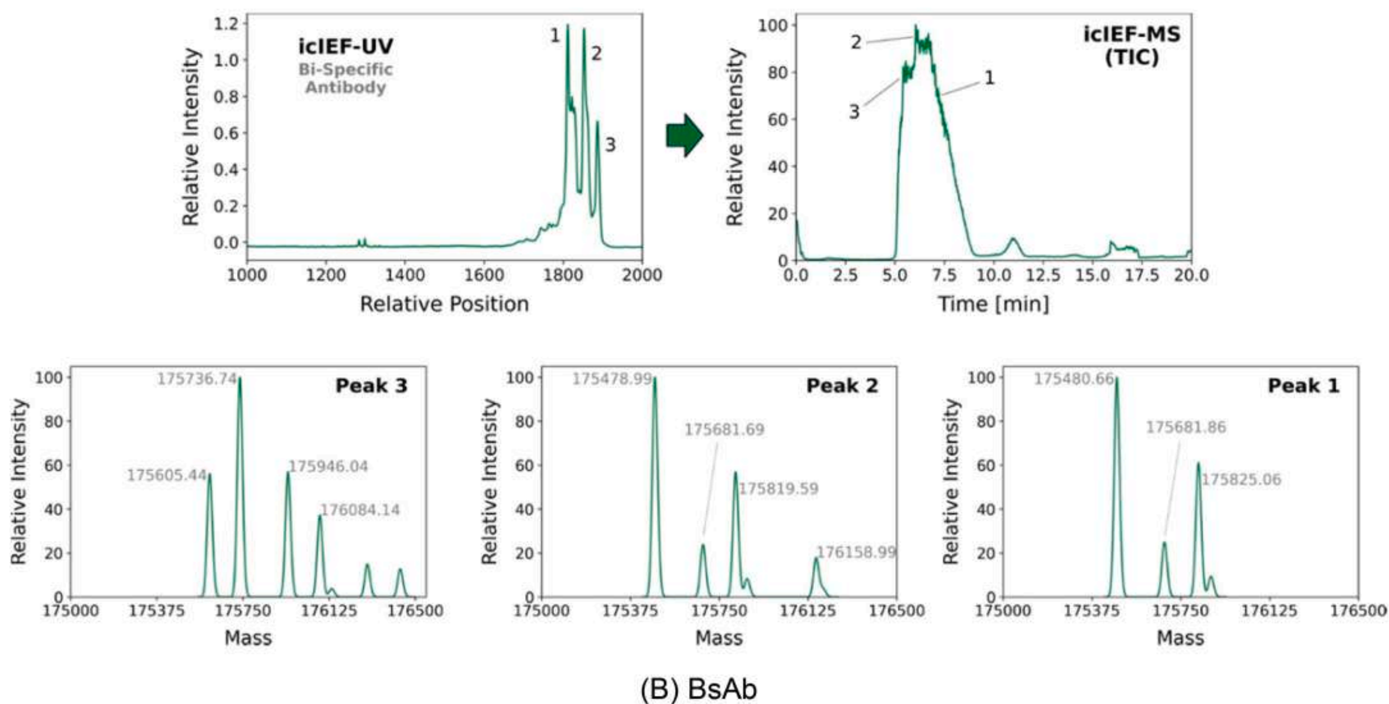


Fig. 6. A) icIEF-MS for mAb (USP-MAB-002) heterogeneity using nano-ESI and low-flow ESI; B) The deconvolution of the icIEF-MS profile for nano-ESI; and C) The deconvolution of the icIEF-MS profile for low-flow ESI.



(A) Fusion protein



(B) BsAb

Fig. 7. icIEF-MS for complex protein heterogeneity using Nano-ESI.

outstanding and the MS TIC demonstrated the discrimination of three variants even though their pI differences are rather subtle. As comparing to the icIEF-MS using low-flow ESI, nano ESI exhibited obvious advantages including higher sensitivity, better resolution and more stable baseline.

4. Conclusions

As an extension of our icIEF-MS platform with low-flow ESI [24–27, 33], a nano-spray emitter to enable nano-ESI was developed as an alternative approach to further improve the icIEF-MS performance in terms of sensitivity and resolution. Compared to routine low-flow ESI, the nano-ESI demonstrated a higher sensitivity and resolution while providing increased compatibility with different MS brands. The workflow provided an innovative strategy for fast characterizing protein heterogeneity, and can be employed throughout all stages of biopharmaceutical development, spanning from drug discovery to manufacturing and quality control. In this study, the developed icIEF-MS using nano-ESI was used for successfully profiling fusion protein and BsAb, underscoring its broad applicability for the analysis of complex proteins.

Notes

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Teresa Kwok: Conceptualization, design, acquisition, Formal analysis, Writing – review & editing. **She Lin Chan:** Investigation. **Matthew Courtney:** Writing – review & editing. **Mike Zhou:** Supervision, Validation. **Tiemin Huang:** Supervision. **Tao Bo:** Writing – original draft, Writing – review & editing. **Victor Li:** Investigation, Supervision. **Tong Chen:** Conceptualization, design, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Data will be made available on request.

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