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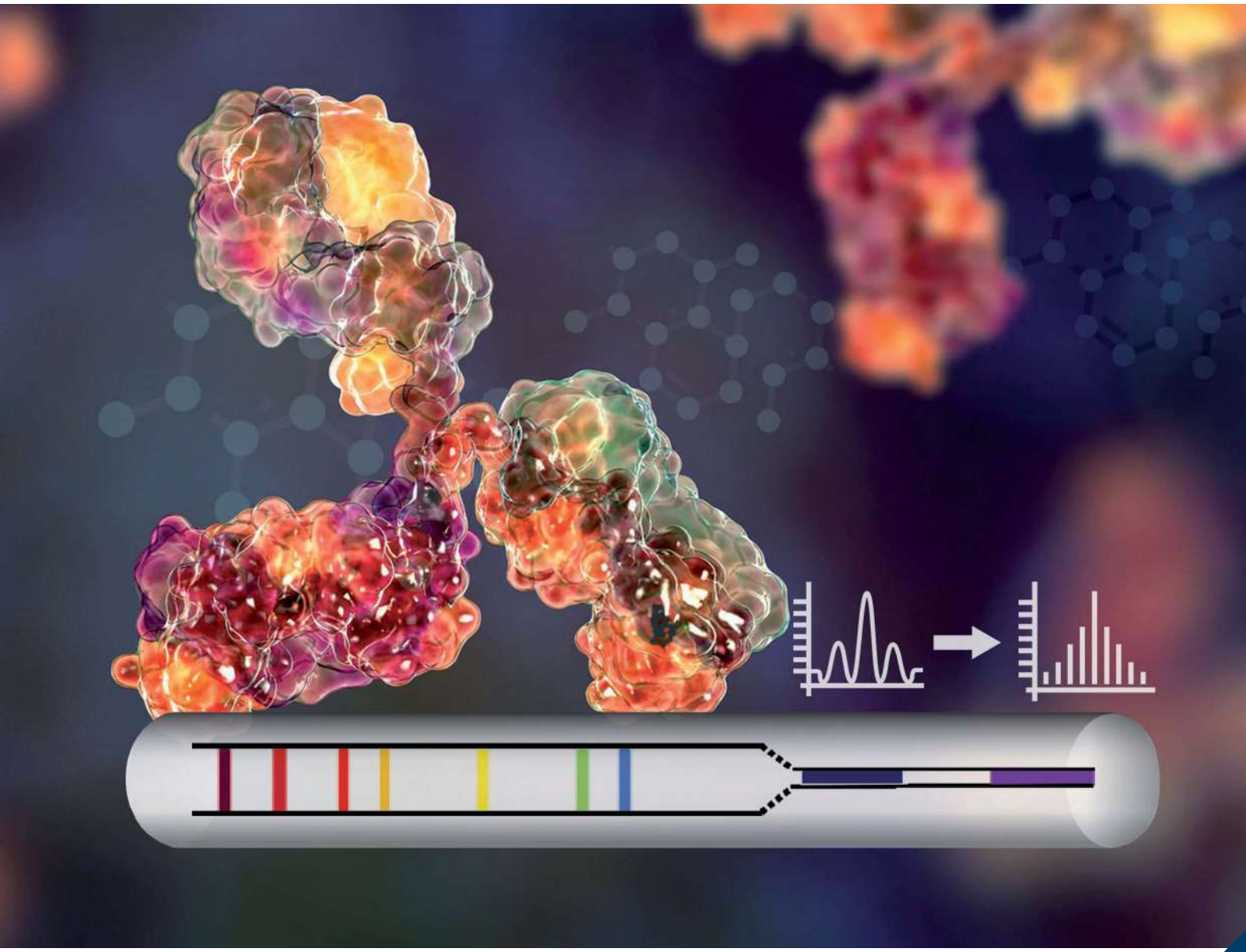

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Fractionation and online mass spectrometry based on imaged capillary isoelectric focusing (icIEF) for characterizing charge heterogeneity of therapeutic antibody

Teresa Kwok, Mike Zhou, Anna Schaefer, Tao Bo, Victor Li, Tiemin Huang and Tong Chen *

Imaged capillary isoelectric focusing (icIEF) technology has been proved to be robust for the characterization of protein charge heterogeneity due to its high-resolution pI discrimination and high-throughput. Although high performance liquid chromatography (HPLC) tandem mass spectrometry (MS) and offline fraction collection technologies including isoelectric focusing (IEF), ion exchange chromatography (IEX) and free flow electrophoresis (FFE) have been widely utilized for protein charge variant characterization, there are a few applications of MS coupling with icIEF as a front-separation technique and related fractionation technologies for protein charge heterogeneity. However, the application of icIEF-MS has been much less frequent due to difficulties in MS interface, compatible ampholyte and coated capillary cartridge designation, ultimately impeding the breadth of icIEF applications in protein charge heterogeneity. In this study, a therapeutic monoclonal antibody (mAb-M-AT) was used for its charge variant characterization on an integrated icIEF platform with functions including analytical profiling, MS online coupling and fraction collection for charge heterogeneities. The main protein component and its four charge variants were identified using direct icIEF-MS coupling. Additionally, the two major acidic and basic charge variants were collected using preparative fractionation after the protein focused in the separation capillary. The identity of the fractions was confirmed by LC-MS at intact protein level and the results were consistent with those using icIEF-MS online coupling. The multiple operation modes of the icIEF platform described above can be rapidly and flexibly switched just by changing customized capillary separation cartridges without drastically altering instrument configuration. The whole workflow of icIEF-based profiling, fractionation and MS online coupling for protein heterogeneity is straightforward, reliable, and accurate, thus providing comprehensive solutions for in-depth protein heterogeneity characterization.

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1. Introduction

Charge heterogeneity refers to the presence of related species in biopharmaceutical production, which differ from the main product in terms of charge.[1–4] These species can arise due to post translational modifications (PTMs), degradation reactions such as deamidation, C-terminal lysine processing, and glycation.[5,6] Their presence may result in altered product efficacy and pharmacokinetics, complete product inactivation, or in the worst-case enhanced immunogenicity.[7] Additionally, as the patents of many biologicals have expired or are on the verge of expiration, there has been a recent resurgence of the biosimilar industry.[8] Biosimilars are biopharmaceuticals that have demonstrated similarity in their structure, function, quality, safety, and efficacy to the initial product.[9] Since biosimilars are also produced by living organisms, extensive analytical characterization is necessary to prove their similarity to the reference product as minor alterations during manufacturing may lead to significant implications on the quality of the product.

The most common methods for charge variant monitoring in the biotechnology industry include ion exchange chromatography (IEX), traditional isoelectric focusing (IEF) and imaged capillary isoelectric focusing (icIEF).[10–21] Specifically, icIEF technology based on isoelectric point (pI) differentiation is becoming the gold standard across the pharmaceutical industry for protein charge variant characterization. Furthermore, subsequent MS analysis would provide a powerful and comprehensive analysis of the charge variants and their structure.[22,23] Although diverse chromatography-mass spectrometry couplings have been employed for this purpose,[24–27] most cannot accurately distinguish the protein variants with tiny

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isoelectric point (pI) differences. However, the value of online coupling capillary electrophoresis (CE) with MS has long been recognized as a valuable analytical strategy for biopharmaceuticals,[28–30] including capillary zone electrophoresis, cIEF and microfluidic native capillary electrophoresis as separation-fronts. Specifically, the combination of rapid, high-resolution separations by icIEF and cIEF with molecular mass identification by MS would provide a far-reaching analysis of the charge variants and their structure. Recently, icIEF or cIEF as a robust front-end separation for MS detection have been demonstrated,[31–35] but improvements are still required for addressing the challenges in terms of higher-sensitivity, higher-throughput and more user-friendly operation.

As for protein charge variant preparation, traditional offline fraction collection technologies such as isoelectric focusing (IEF) and free flow electrophoresis (FFE) are a more tedious process for charged heterogeneity preparation.[36–38] In addition, the amount and purity of charge variant isomers collected by the traditional slab gel IEF method is usually insufficient for further characterization. Nowadays, IEX is exploited for protein charge variant fractionation. Although both IEX and IEF are charge-based protein separation methods, they rely on different separation principles and as a result, protein charge variants may not correlate well between them. Since IEX provides lower resolution separation, unsatisfactory purity and high salt content are usually expected of the IEX fractions.[39] Fractionation and collection of sample zones in traditional cIEF is unpractical on conventional capillary electrophoresis (CE) instruments and commercially not available. An integrated platform for icIEF analytical profiling, icIEF-MS tandem technology and the fraction collections for preparative icIEF is therefore a highly desirable method, which can sharpen charge variant characterization of protein drugs.

In this study, a therapeutic monoclonal antibody (mAb-M-AT) was used for its charge variant characterization on an integrated icIEF platform. The main protein component and its four charge variants were profiled using analytical icIEF and two major acidic and basic charge variants were fractionated in preparative mode after the protein focused along the separation capillary. The collected charge variant fractions were characterized by LC-MS at intact protein level and the results were consistent with those using icIEF-MS online coupling. This demonstrates that three icIEF operation modes developed in our laboratory can be rapidly and flexibly switched just by changing customized capillary separation cartridges.[40,41] The whole strategy for protein heterogeneity is straightforward, reliable and accurate, which provides comprehensive solutions for protein heterogeneity characterization in the biopharmaceutical industry.

2. Materials and methods

2.1. Materials

Ampholytes (AESlyte HR 7–8) and pI markers (5.12 and 9.46) were obtained from Advanced Electrophoresis Solutions Ltd. (AES, Cambridge, Ontario, Canada). Pharmalyte 3–10 and Pharmalyte 8–10.5 were purchased from GE Healthcare. Therapeutic mAb (mAb-M-AT) employed in this study was kindly donated from Thermo Fisher Scientific (China). Mass spectrometry grade water, acetonitrile (ACN) and formic acid were purchased from Fisher Scientific (Hampton, NH).

2.2. Solutions for icIEF

For icIEF-UV profiling: the sample solution contained 0.35% (v/v) methylcellulose solution (MC), 3.5% (v/v) Pharmalyte 3–10, 0.5% (v/v) Pharmalyte 8–10.5, 0.4 mg mL^{-1} mAb-M-AT, 0.5% (v/v) pI marker 5.12 and 0.5% (v/v) pI marker 9.46. The deionised water was used for dilution.

For icIEF-MS coupling: the sample solution contained 3.5% (v/v) Pharmalyte 3–10, 0.5% (v/v) Pharmalyte 8–10.5 and 1 mg mL^{-1} mAb-M-AT (1 mg mL^{-1}) with deionised water used for dilution.

For icIEF fractionation: the sample solution contained 4% AESlyte HR 7–8 and 1 mg mL^{-1} mAb-M-AT with deionised water used for dilution.

2.3. Imaged capillary isoelectric focusing (icIEF)

CEInfinite icIEF (Advanced Electrophoresis Solutions Ltd., Cambridge, Canada) with on-column UV detection at 280 nm was employed in this study. 100 μm ID fluorocarbon (FC) coated capillary (AES, cat. no. CP00201) are utilized for icIEF-UV analysis. 200 μm ID MC coated capillary (AES, cat. no. CP00503) and micro-tee integrated AD coated cartridges (AES, cat. no. CP00303M) are used for icIEF-MS; 320 μm ID AD coated cartridges (AES, cat. no. CP00307) are used for preparative icIEF. All these WCID (Whole Column Imaging Detection) cartridges have a 5 cm long separation capillary, and 50 μm ID transfer capillary is assembled for both icIEF-MS and preparative icIEF cartridges. The 200 μm AD coated cartridge used for icIEF-MS includes a quartz union (works as a micro-tee), connecting the make-up solution and FC coated transfer capillary to MS ion source. Both the make-up solution capillary and inlet capillary have a 100 μm ID.

As for analytical icIEF-UV, the focusing was 0–1 min at 1500 V and 1–8 min at 3000 V.

As for icIEF-MS, the focusing was 0–1 min at 1500 V, 1–8 min at 3000 V, and 3000 V during mobilization; the mobilization speed was 50 nL min^{-1} with water containing 0.1% (v/v) formic acid, across the separation capillary, and 5 μL min^{-1} make up solution (water : ACN = 1 : 1, v/v, containing 0.1% formic acid, v/v) added through a micro tee. Mobilization time was 15 min.

As for icIEF fractionation, the focusing was 0–2 min at 1000 V, 2–10 min at 2000 V, and 3000 V during mobilization; the mobilization speed was 120 nL min^{-1} with water containing 10 mM iminodiacetic acid, and 2.4 μL min^{-1} make up solution (water) added through a micro tee. Mobilization time was 25 min.

2.4. High-resolution mass spectrometry for icIEF-MS

A Thermo Q Exactive Plus mass spectrometer with Biopharma option equipped with an Ion Max ESI Ion Source with a 34-gauge needle (Thermo Fisher Scientific, Bremen, Germany) was used for mass measurement. The spray voltage: 3.8 kV, sheath gas: 5 L min^{-1}, auxiliary gas: 5 L min^{-1}, S-lens RF 70 eV,

microscan: 10, capillary temp: 300 °C, aux gas temp.: 100 °C, resolution 17 500 @ m/z 200, scan range of precursor ion 1500–5000 m/z and maximum injection time 200 ms.

2.5. UHPLC-MS for collected protein variant fractions

The collected fractions were analyzed at intact protein level on a Thermo Scientific™ Vanquish™ UHPLC system (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile; $A:B = 1:1$, v/v) using an Acclaim Vanquish C18 column (120 Å; 2.2 μm, 2.1 × 150 mm, P/N 071399 V, 40 °C) at 300 μL min^{-1}.

The mass spectrometry instrument used with parameters in UHPLC-MS was the same as those in icIEF-MS.

2.6. Data analysis

Biopharma Finder (BPF 5.0) from Thermo Fisher was used for the data analysis including intact protein deconvolution.

3. Results and discussions

3.1. Configurations of fraction collections for preparative icIEF and icIEF-MS online coupling

Our icIEF preparative technology can realize the separation, preparation and collection of high-purity, heterogeneous, charged protein products as illustrated in Fig. 1A. Based on a straight forward icIEF method, minimum method optimization is required to test a new biopharmaceutical product.[41] After protein focusing is completed, low concentraion acid serves as a mobilization solvent, flowing from syringe pump at 100–160 nL min^{-1} flowing rate to drive the focused protein bands out of the separation capillary for the fraction collections while maintaining the high voltage. By the continuous migration of hydroxide ions from the cathodic side and protons from the anodic side of the separation capillary, the charge variant peaks in the separation capillary remain focused under the electric field. Patented capillary cartridges ensure the high purity of each charge isomer.

The same pressure mobilization technique is employed for the developed icIEF-MS system. Patented capillary cartridges eliminate the need for chemical migration when coupled to online mass spectrometry as shown in Fig. 1B. The use of only proprietary coated capillary and separation solvents during icIEF separations also greatly reduce the need for additional polymers and urea in the sample mix. Mobilization solution containing 0.1% formic acid at 50 nL min^{-1} flowing rate and sheath liquid (water : acetonitrile = 1 : 1 v/v, containing 0.5% v/v formic acid) helps the effluents direct into MS ion source

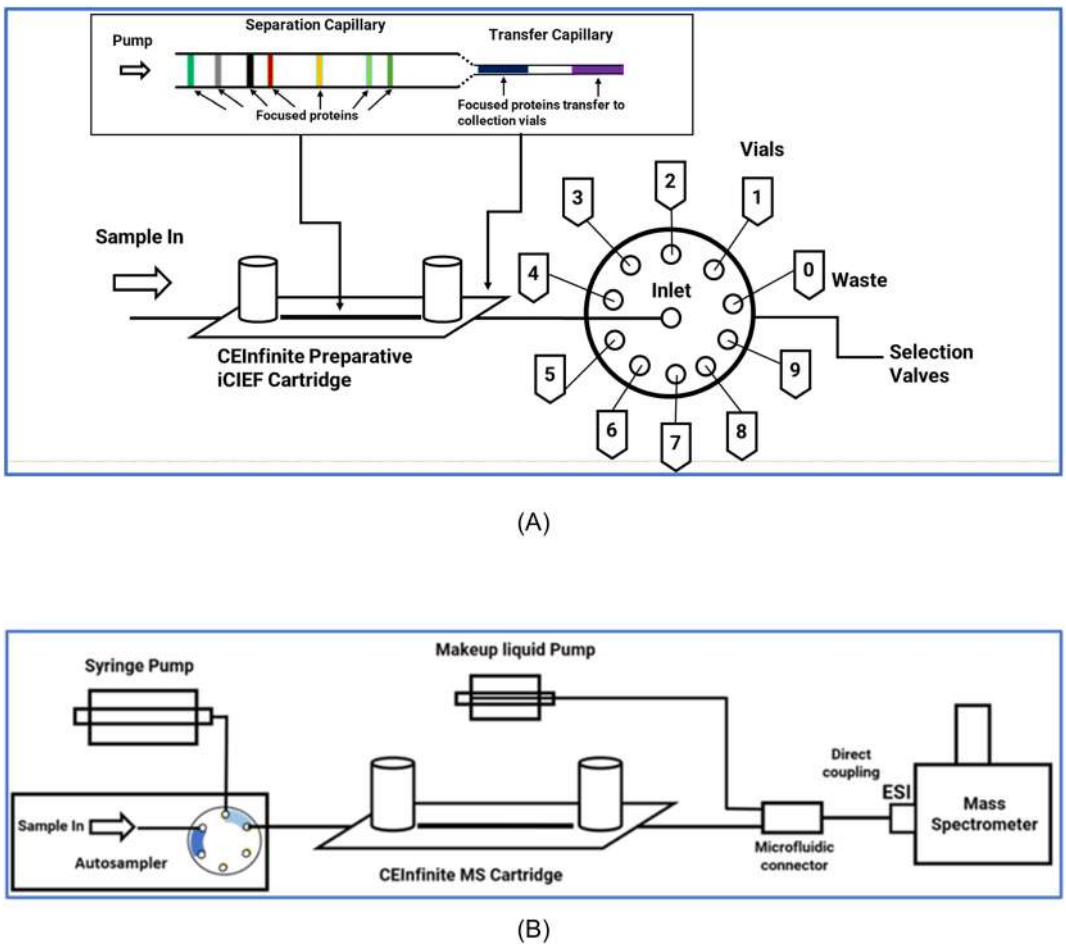


Fig. 1 Configurations of (A) fraction collections by preparative icIEF and (B) online icIEF-MS.

though a seamless interface. The constructed system requires no special modifications to the ion source and can be directly connected to the mass spectrometer ion source from the different mass spectrometry brands.

Fraction collection and online MS modes in icIEF can be rapidly and flexibly switched just by changing customized capillary separation cartridges without altering instrument configuration.

3.2. icIEF-UV for mAb studied

Fig. 2 reveals that the main component, acidic variants (A1–A2) and basic variants (B1–B2) of studied mAb were well separated with the use of 100 μm ID FC coated capillary on an analytical icIEF system in under 10 minutes. The species percentage based on peak area and pIs of protein components are given in Table 1. The pIs of main protein and four charge variants were in the range of 6.5–8.5 pH and the repeatability of peak areas were outstanding below RSD 5.0% ($n = 6$). It was observed that acidic peak A1 and basic peak B2 were trace with the percentages of 5.4% and 2%, respectively. Acidic A2 and basic B1 are the major charge variants with percentages of 16.7% and 21.0%, respectively.

3.3. Fraction collections of main components and two major charge variants for studied mAb

In this study, the preparative icIEF employing 320 μm ID AD coated capillary shown in Fig. 1A was used for fractionation of the main component and two charge variants (acidic A2 and basic B1) of studied mAb, followed by intact protein analysis by HPLC-MS. As illustrated in Fig. 3, peaks for all of the expected protein charge variants were firstly focused along the separation capillary by applying high voltage and then the focused bands were migrated into a transfer capillary driven by nano flowing pump for the fraction collections.[41] The collection procedure is automated and 50–100 μg proteins were fractionated within three days based on the accumulation from consecutive runs. As seen in Fig. 3 (left), the mobility process driven by nano-syringe pump allows the focused bands of proteins to move out of separation capillary for the fractions/collections. During the mobilization process, the high voltage was applied in order to remain the separation resolutions. Fig. 4 demonstrated the excellent repeatability of preparative icIEF for three fractions to

Table 1 pIs and relative peak areas of charge variants by analytical icIEF

Peak	pI Value	Relative peak area	RSD ($n = 6$)
A1	6.97	5.3%	4.65%
A2	7.08	16.6%	0.88%
Main	7.25	54.7%	2.33%
B1	7.59	21.0%	1.93%
B2	8.07	2.4%	3.46%

support the high purity of fractions accumulated. The purity of each peak was confirmed on the same instrument using the analytical cartridge in Fig. 5, illustrating the UV profile of reinjected fractions. The purity was above 70.0% for each of three peaks, with a collected amount above 50 μg and corresponding recovery rate from 20–40% (Table 2). Following fraction collection HPLC-MS analysis for three collected fractions was carried out for molecular mass identification (Fig. 6A). This can be followed by charge modification identification using in-depth peptide mapping by LC-MS/MS which is in progress for this mAb.

3.4. icIEF-MS for studied mAb

IcIEF-MS coupling is a different strategy than icIEF-based fractionation with following LC-MS analysis, allowing rapid screening for the identifications of charge variants at the intact-level of a mAb sample without fractionation required. As illustrated in Fig. 1B, the developed icIEF-MS system enables the isolated protein charge variants to be directly injected into the high-sensitive MS, thus retaining the excellent separation resolution of icIEF for analysis. The seamless interface to MS is based on micro-fluidity, to enhance the sensitivity of MS detection of proteins.

As illustrated in Fig. 6B, using narrow pH range ampholyte (pH 7–8) and 200 μm ID MC coated capillary, the main species and four charge variants (two acidic and two basic variants) of the studied mAb were successfully characterized by icIEF-MS within 45 min meanwhile its icIEF-UV profile before MS detection was simultaneously recorded by whole column imaging detection. Due to novel MC coated separation capillary employed, this icIEF-MS online coupling strategy avoided the

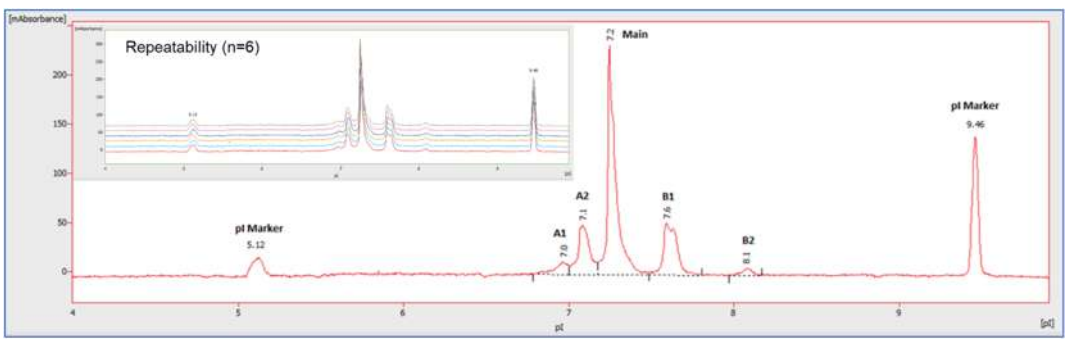


Fig. 2 Analytical icIEF-UV separation for mAb.

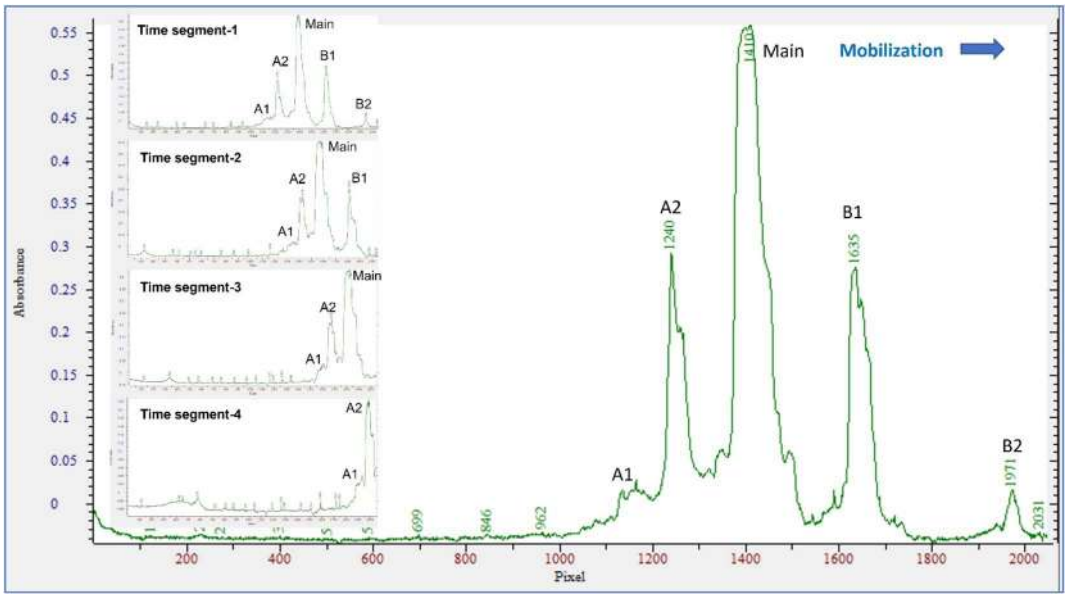


Fig. 3 Fraction collection process with icIEF separation and the mobilization of focused protein bands for charge variant preparation.

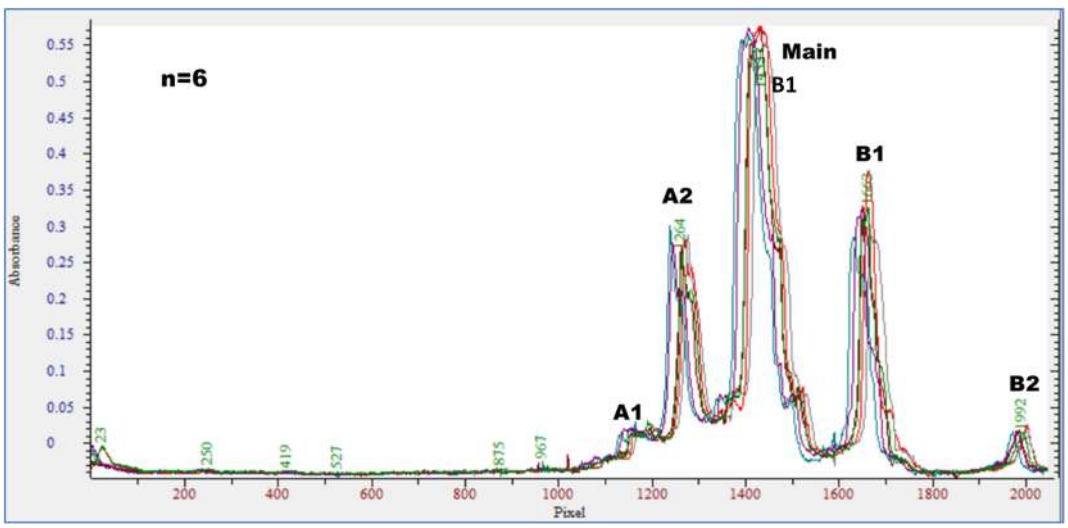


Fig. 4 Repeatability of fraction collections of main component and its two charge variants ($n = 6$).

use of MC solution as additive in the running buffer and preconditioning prior to icIEF separation. Overall, this contributed to the improved compatibility with MS ion source thanks to low contamination with non-volatile salts. The icIEF-MS established in this study demonstrated much higher throughput than the reported cIEF-MS results with single-point detection which analysis times were above 1 hour.[42,43] Also, the established icIEF-MS bigly improves the repeatability by avoiding tedious chemical mobilization the routine cIEF-MS utilizes. Our study indicated that the icIEF-MS in this study were sensitive, especially for trace variant A1 and B2 as shown in Fig. 6B.

The MW identifications of main component and two charge variants (acidic A2 and basic B1) were compared utilizing LC-MS analysis of collected fractions and online icIEF-MS characterization of studied mAb sample (Fig. 7). It was found that the deconvoluted MWs of the highest MS intensity are consistent on both analytical strategies. The MWs for the charge modifications disclosed were also very similar with minor deviations due to their lower concentration. It was observed that there were some glycoforms of the studied mAb with low abundance in the mass spectra of icIEF-MS but these glycosylated species were not detected using the HLPC-MS method. IcIEF-MS is higher sensitivity than HPLC-MS as a much lower flow rate was used in icIEF-MS (less than 5 μL min^{-1}) than the one used in HPLC-MS (300 μL min^{-1}). Lower flow rate leads to higher evaporation efficiency and MS efficiency.[44] Moreover, the focusing-driven preconcentration in icIEF separation was helpful for increased sensitivity. This study demonstrates that

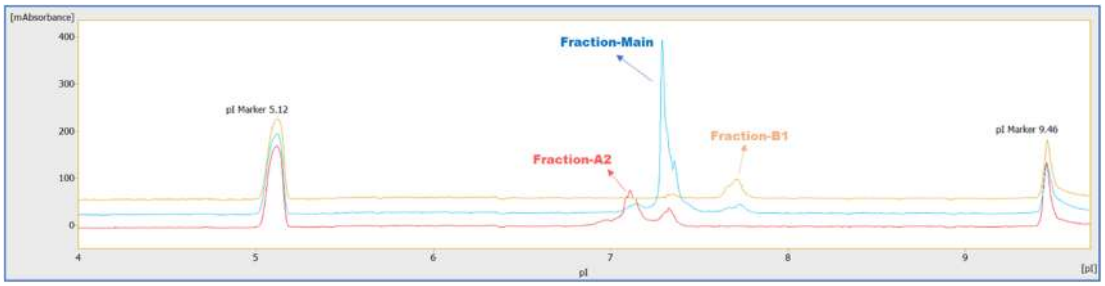


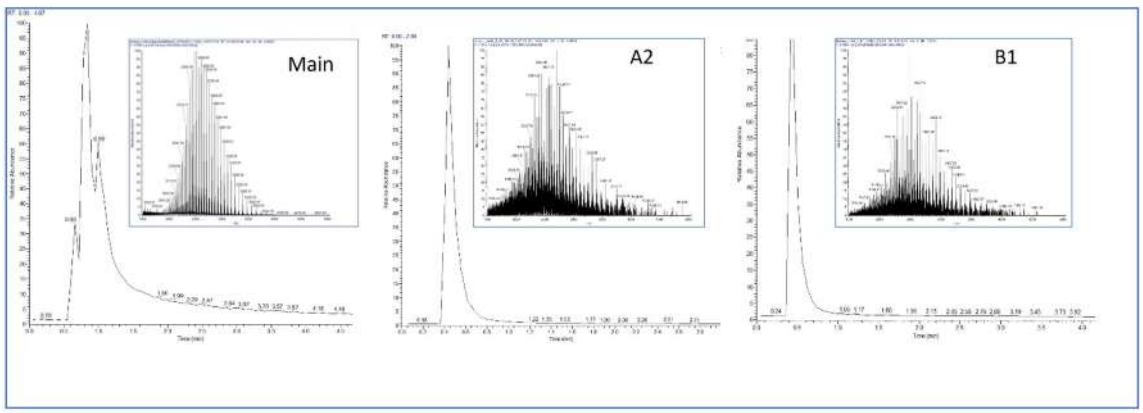
Fig. 5 Impurities of fraction collections of main component, acidic variant A2 and basic variant B1.

Table 2 Fraction purities of main component and two charge variants

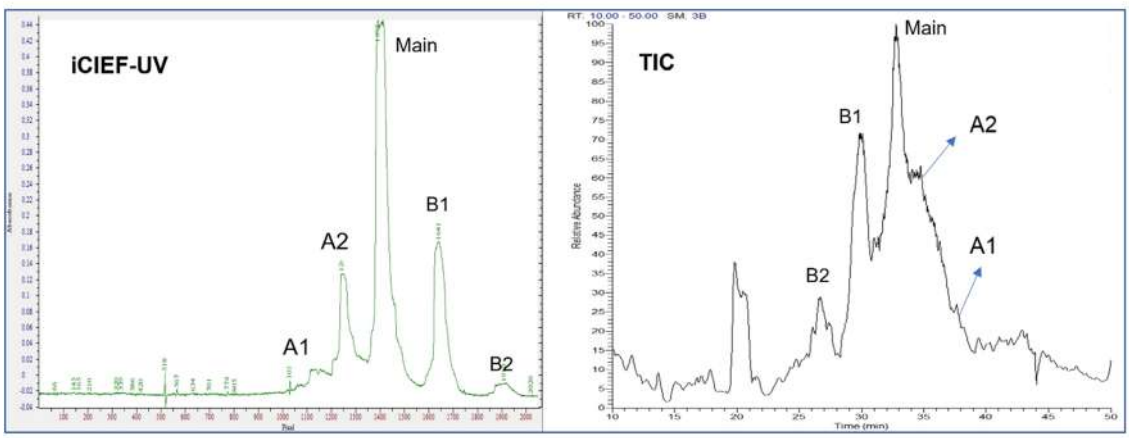
Peak	Purity	Amount	Recovery
Main	91.5%	>50 μg	39.0%
A2	70.8%	~50 μg	40.1%
B1	82.7%	~50 μg	21.4%

this icIEF-MS platform can carry out extremely fast fingerprinting and providing preliminary elucidation of the possible identification of targeted charge variants. LC-MS analysis of collected fractions can contribute to in-depth characterization at intact and peptide mapping levels.

This is the first analytical platform to integrate icIEF-UV profiling, fractionation and online MS coupling on an instrument. Previously, when an icIEF separation is completed,



(A) HPLC-MS TIC and mass spectrum



(B) icIEF-MS

Fig. 6 (A) HPLC-MS and (B) online icIEF-MS for mAb charge variants.

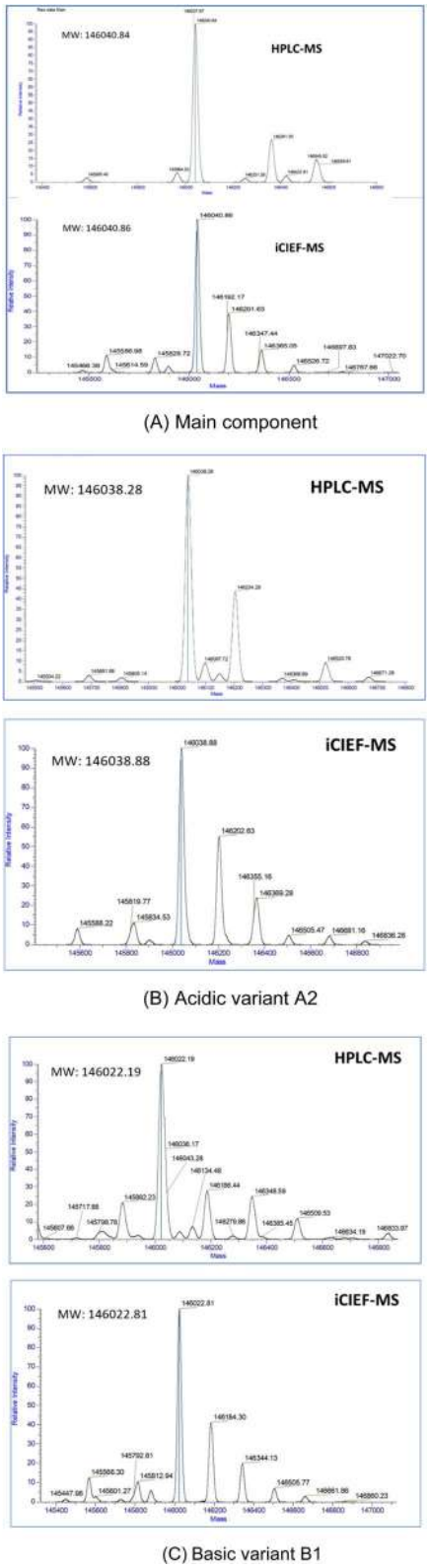


Fig. 7 Deconvoluted molecular weights of charge variants (A) main component (B) A2 (C) B1 by HPLC-MS for the collected fractions and online icIEF-MS.

traditional IEX, IEF and FFE have to be used for the preparation of protein charge variants observed. The proteins peaks in icIEF cannot be directly collected as fractions or online MS detection. However, due to different separation mechanisms and resolutions of IEX, IEF and FFE from icIEF technology, the fraction collected usually cannot well match the results from icIEF and the preparative proteins tend to be denatured during the fraction collection due to long-time process and strongly chemical conditions applied. Established preparative icIEF in our work well addressed such a challenge in the preparation of protein charge variants.

4. Conclusions

In this study, icIEF fractionation and online icIEF-MS were developed for charge variant analysis after analytical icIEF-UV profiling. The established hybrid platform was successfully utilized for in-depth charge variant characterization of a therapeutic antibody. The fractions of a main protein component with its two major charge variants were collected for following LC-MS analysis at intact protein level. Meanwhile, icIEF-MS coupling was performed for rapid identification of protein heterogeneity. The whole workflows of icIEF-based fractionation and MS online detection for protein heterogeneity are straight forward, reliable and accurate, which can provide comprehensive and cutting-edge technologies for protein drug quality control (QC) monitoring, online MS detection for rapid fingerprinting intact proteins and HPLC-MS in-depth characterization of collected fractions.

Abbreviation

icIEF	Imaged capillary isoelectric focusing
MS	Mass spectrometry
mAb	Monoclonal antibody
HPLC	High performance liquid chromatography
pI	Isoelectric point
TIC	Total ion chromatography
FC	Fluorocarbon
AD	Acrylamide
MC	Methylcellulose
CA	Carrier ampholyte

Author contributions

Teresa Kwok: data curation and formal analysis; Mike Zhou: data curation and formal analysis; Anna Schaefer: manuscript review and editing; Tao Bo: supervision and writing–original draft; Victor Li: investigation; Tiemin Huang: supervision and validation; Tong Chen: conceptualization, supervision and validation.

Conflicts of 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.

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