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Mass Spectrometry-Based Charge Heterogeneity Characterization of Therapeutic mAbs with Imaged Capillary Isoelectric Focusing and Ion-Exchange Chromatography as Separation Techniques

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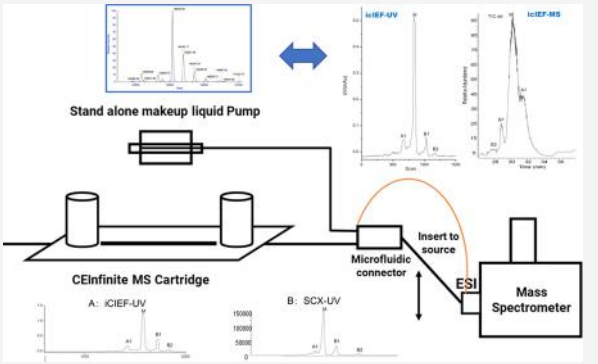
ABSTRACT: Imaged capillary isoelectric focusing (icIEF) and ion-exchange chromatography (IEX) are two essential techniques that are routinely used for charge variant analysis of therapeutic monoclonal antibodies (mAbs) during their development and in quality control. These two techniques that separate mAb charge variants based on different mechanisms and IEX have been developed as front-end separation techniques for online mass spectrometry (MS) detection, which is robust for intact protein identification. Recently, an innovative, coupled icIEF-MS technology has been constructed for protein charge variant analysis in our laboratory. In this study, icIEF-MS developed and strong cation exchange (SCX)-MS were optimized for charge heterogeneity characterization of a diverse of mAbs and their results were compared based on methodological validation. It was found that icIEF-MS outperformed SCX-MS in this study by demonstrating outstanding sensitivity, low carryover effect, accurate protein identification, and higher separation resolution although SCX-MS contributed to higher analysis throughput. Ultimately, integrating our novel icIEF-HRMS analysis with the more common SCX-MS can provide a promising and comprehensive strategy for accelerating the development of complex protein therapeutics.



1. INTRODUCTION

Recombinant monoclonal antibodies (mAbs) have been spurring rapid growth in the commercial and clinical production of biotherapeutics across the pharmaceutical industry. The charged heterogeneity of protein drugs requires in-depth structural characterization for critical quality attribute (CQA) assessment to ensure quality, safety, and efficacy.[1,2] Charge heterogeneity of proteins results from a combination of diverse mechanisms, including cellular processes, chemical degradation, and production conditions during the manufacturing process.[3,4] For example, the occurrence of many post-translational modifications (PTMs) including C-terminal lysine truncation, pyroglutamate formation, deamidation, sialylation, and glycation can result in the formation of charge variants.[5,6] Among the aforementioned modifications, many can cause changes in the isoelectric point (pI) value of the protein, which can have negative impacts on drug stability and solubility. Therefore, it is essential to highlight the need for reliable characterization of charge variants to assess the CQAs and ensure consistent quality during the manufacturing of therapeutic mAbs throughout clinical and commercial development.

Imaged capillary isoelectric focusing (icIEF) and ion-exchange chromatography (IEX) are two essential techniques routinely used for charge variant analysis of therapeutic mAbs during their development and in quality control.[7−15] These two techniques separate mAb charge variants based on different mechanisms and therefore have varying separation selectivities. However, the conditions commonly used in these two methods contain nonvolatile substances and usually cannot be used in conjunction with the most powerful means of mass spectrometry (MS) for protein identification. However, IEX has been developed as a front-end separation technique for MS detection, with much popularity for protein characterization.[16,17] Recently, icIEF-MS has attracted much attention to be utilized for protein charge variant analysis.[18−27] However, the icIEF-MS strategies developed still face huge challenges that need continuous optimizations, limiting the discovery of biopharmaceuticals. Critical bottlenecks of icIEF coupled to MS in the discovery of protein charged variants, including unsatisfactory repeatability, complicated operation frequently involving trial-and-error optimization, and incompatibility of the MS ion source, have been frustrating scientists

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Table 1. Isoelectric Point of Studied mAbs and Related icIEF Solution Conditions

mAb	concentration (mg/mL)	sample (μL)	ampholytes (μL)	H_2O (μL)	pI
Daratumumab	2	100	HR8.5−9.5 (20 μL)	430	8.25−8.70
Bevacizumab	2	100	HR8.5−9.5 (10 μL) HR7−8 (10 μL)	430	7.82−8.70
Pembrolizumab	2	100	HR6−8 (20 μL)	430	7.12−7.58
Infliximab	2	100	HR3−10 (5μL) HR7−8 (15 μL)	430	7.20−7.75
Adalimumab	2	100	HR8.5−9.5 (20 μL)	430	8.30−8.90
Rituximab	2	100	HR9−12 (20 μL)	430	8.70−9.30
Guselkumab	2	100	HR8.5−9.5 (20 μL)	430	8.30−9.10
Denosumab	2	100	HR8.5−9.5 (10 μL) HR7−8 (10 μL)	430	8.35−8.80
Atezolizumab	2	130	HR8.5−9.5(20 μL)	400	8.23−8.76

Table 2. Gradient Programs for SCX-MS Analysis of Studied mAb

mAb	SCX gradient
Infliximab (pI 7.2−7.75)	30−55%B, 0−10 min; 10−10.1, 55−100%; 10.1−12 min, 100%B; 12−12.1 min, 100−0%B; 12.1−14 min, 0%B; 14−14.1 min, 30% B; 14.1−22 min, 30%B
Pembrolizumab (pI 7.12−7.58)	
Adalimumab (pI 8.30−8.90)	40−100%B, 0−10 min; 10−12 min, 100%B; 12−12.1 min, 100−0%B; 12.1−14 min 0%B; 14−14.1 min 40% B; 14.1−22 min, 40%B
Bevacizumab (pI 7.82−8.70)	35−60%B, 0−10 min; 10−10.1, 60−100%; 10.1−12 min, 100%B; 12−12.1 min, 100−0%B; 12.1−14 min, 0%B; 14−14.1 min 35% B; 14.1−22 min, 35%B
Daratumumab (pI 8.25−8.70)	
Atezolizumab (pI 8.23−8.76)	
Denosumab (pI 8.35−8.80)	60−100%B, 0−10 min; 10−12 min, 100%B; 12−12.1 min, 100−0%B1; 2.1−14 min, 0%B; 14−14.1 min, 60% B; 14.1−22 min, 60%B
Guselkumab (pI 8.30−9.10)	
Rituximab (pI 8.70−9.30)	85−100%B, 0−10 min; 10−12 min, 100%B; 12−12.1 min, 100−0%B; 12.1−14 min, 0%B; 14−14.1 min, 85% B; 14.1−22 min, 85%B

in the biopharmaceutical industry. Chip-based icIEF-MS depends on chemical mobilization for MS detection, which tends to result in instability of the pH gradient during chemical mobilization and lead to the risk of decreased repeatability. Traditional cIEF with single-point detection has the disadvantages of low throughput and cumbersome operation due to longtime separation and tedious mobilization process after protein focusing for MS detection. The performance of icIEF-MS highly depends on the comprehensive developments, including the innovations of capillary coatings, MS interface, and carrier ampholytes, which need a high-criterial for being well compatible with an MS instrument.

This study outlines a robust icIEF-MS platform that was developed to achieve both fast icIEF separation and reliable high-resolution MS identification of protein charge variants simultaneously. MS-compatible amphoteric electrolytes and both of methylcellulose (MC) and urea-free cartridges were used in this icIEF analysis to realize zero-volatile reagents in the analysis of protein drug charge variants. The innovative microliter interface improves the sensitivity of identifying protein drug charge variants, and the seamless MS interface ensures that the entire icIEF-HRMS analysis can be solved within 35 min, much faster than traditional cIEF-MS, whose throughput is usually above 60 min per run. Instead of complicated chemical mobilization, the established icIEF-MS platform in our study employed the mobilization solution at a nano flowing rate (30−100 nL/min) using a syringe pump to push the protein peaks focused out of the separation capillary toward the MS ion source or fraction collectors. This guarantees outstanding repeatability in the process of online icIEF-MS including protein focusing with following mobilization, and the entire process can be completed in 35 min, thus enabling high throughput analysis capabilities. In addition, the method development of icIEF is rather fast and straightforward compared to cIEF, and no MC or glycerol is needed as an additive or precondition that is commonly used in cIEF-MS analysis to decrease the protein aggregation. In addition, the established icIEF-MS platform can flexibly switch into the icIEF fractionation mode by just changing the capillary cartridge for μg-level preparative icIEF.[18] Using this novel system, the charge variants of diverse therapeutic mAbs were characterized, and the methodology of icIEF-MS developed was systematically validated including sensitivity, repeatability, carryover effect, and coated capillary lifetime. Meanwhile, the charged heterogeneities of the studied mAbs were also analyzed by strong cation-exchange (SCX)-MS. The results of the two unique front-end separation tools coupled to MS were compared, revealing that icIEF has obvious advantages over SCX-MS for protein charge variants in terms of separation resolution, sensitivity, low carryover effects, and MW measurement accuracy, despite the higher analysis throughput of SCX-MS.

2. EXPERIMENTAL SECTION

2.1. Chemicals. All ampholytes (AESlytes) were obtained from Advanced Electrophoresis Solutions Ltd. (AES, Cambridge, Ontario, Canada). The nine therapeutic mAbs studied in this work (listed in Table 1) were retained samples from the authors' organization (National Institutes for Food and Drug Control). Mass spectrometry-grade acetonitrile (ACN), formic acid, acetic acid, ammonium bicarbonate, and ammonia were purchased from Fisher Scientific (Hampton, NH).

2.2. Conditions for icIEF and SCX Coupled to MS. For icIEF separation, the CEInfinite icIEF (Advanced Electrophoresis Solutions Ltd, Cambridge, Canada) was utilized with on-column UV detection at 280 nm. The sample information

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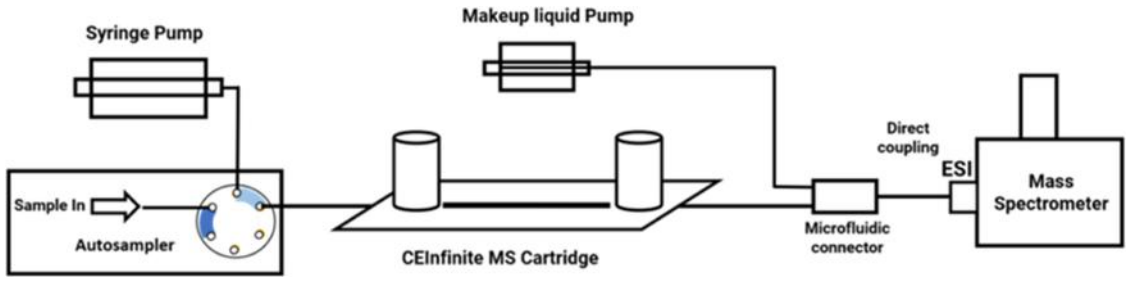

Figure 1. Schematics of the icIEF-MS strategy.

of all nine employed mAbs is indicated in Table 1, and the icIEF analysis for their pIs measurement by icIEF is shown in Supporting information 1. The related information of light and heavy amino acid sequences for nine studied mAbs is indicated in Supporting information 2. The 200 μm ID acrylamide derivative-coated (AD) capillary cartridges (AES, cat. no. CP00303) with 5 cm separation length and micro-tee integrated (AES, cat. no. CP00303M) were used for icIEF-MS. For icIEF separation, the focusing was performed using 1 min at 1000 V, 1 min at 2000 V, and 10 min at 3000 V. For icIEF-MS coupling, 3000 V was applied during mobilization of focused protein bands; the mobilization speed was 80 nL/min with water containing 0.1% (v/v) formic acid, across the separation capillary, and 5 μL/min makeup solution (water/ACN = 1:1, v/v, containing 1% formic acid, v/v) was added through a micro-tee. The mobilization time was 15 min.

For SCX separation, the Thermo Scientific Vanquish UHPLC system was employed with mobile phases composed of relative combinations of A (25 mM ammonium bicarbonate, pH 5.3 adjusted by acetic acid) and B (10 mM ammonia, pH 10.9). The gradient elution programs for studied mAbs are listed in Table 2 with the pIs of studied mAbs. The flowing rate was set at 0.3 mL/min, the column temperature was 30 °C, the UV wavelength was 280 nm, and the sample injection was 20 μg.

2.3. High-Resolution Mass Spectrometry. As for icIEF-MS, a Thermo Q Exactive Plus mass spectrometer equipped with a Biopharma platform, an Ion Max ESI ion source, and a 34-gauge needle (Thermo Fisher Scientific, Bremen, Germany) was used for mass measurement. The spray voltage was 3.8 kV, sheath gas was 5 L/min, auxiliary gas was 15 L/min, S-lens RF was 70 eV, capillary temperature was 300 °C, resolution was 15,000@m/z 200, scan range of the precursor ion was 1500−5000 m/z, in-source CID value was 75 V, and maximum injection time was 200 ms.

As for SCX-MS, a Thermo Q Exactive OE 240 mass spectrometry equipped with a Biopharma platform, an Ion Max ESI ion source, and a 34-gauge needle (Thermo Fisher Scientific, Bremen, Germany) was used for mass measurement. The spray voltage was 3.6 kV, sheath gas was 20 L/min, auxiliary gas was 5 L/min, S-lens RF was 70 eV, temperature was 270 °C, resolution was 15,000@m/z 200, scan range of the precursor ion was 2000−8000 m/z, in-source CID value was 110 V, and maximum injection time was 200 ms.

Biopharma Finder software (version 4.1) from Thermo Fisher was used for the data analysis, including intact mass analysis.

3. RESULTS AND DISCUSSION

The developed icIEF-MS system and patented capillary cartridges employed here eliminate the need for chemical migration when coupled to online mass spectrometry, as shown in Figure 1. Furthermore, the use of only proprietary capillary-coated cartridges and separation solvents during icIEF separations greatly reduce the need for polymers and urea. These features combined enable the isolated protein charge variants to be directly used for high-sensitivity MS characterization, thus retaining the excellent separation resolution of icIEF for mass spectrometry analysis.

Additionally, the constructed system requires no special modifications to the ionization source and can be directly connected to the mass spectrometer from the different leading mass spectrometer brands. After protein focusing is completed along the separation capillary, water containing 0.1% (v/v) formic acid as the mobilization solvent from a syringe pump drives the focused protein bands out of the separation capillary toward the MS ion source (ESI) at an 80 nL/min flowing rate using a 200 μm I.D. AD-coated capillary cartridge with a 5 cm separation length. Sheath liquid or makeup solution (water/acetonitrile = 1:1 v/v, containing 0.5% v/v formic acid) helps the effluents direct into ESI. The seamless interface to MS based on microfluidity prevents sample loss, enhancing the sensitivity of MS detection of proteins. The whole process is automatic and highly user-friendly.

3.1. icIEF and SCX Separations for Nine Studied mAbs. Under the most optimized conditions, the charge variant separations of nine studied mAbs by icIEF-UV and SCX-UV were obtained and compared as illustrated in Supporting information 3. Both separation tools achieved high-throughput separation within 10 min, and the same peak sequences were acquired for the studied mAbs. The most acidic variants were first eluted, followed by the main peak and the basic variants. For most heterogeneous mAb mixtures, the separation resolutions based on pI differentiation were significantly higher for icIEF than those of SCX. In some cases, such as for infliximab, baseline separation of four charge variants and a main component can be achieved by icIEF, but the separation resolution using SCX was unsatisfactory. Similarly, for pembrolizumab, atezolizumab, and denosumab, all charge variants could be detected with good resolution by employing icIEF; however, some of the charge variants were lost with the use of SCX. In terms of separation selectivity, icIEF demonstrated superperformance due to its subtle pI difference despite icIEF having wider peak widths than SCX due to different separation mechanisms. In this study, as listed in Table 2, the elution gradients were optimized for separation selectivity and analytical time. The SCX was highly dependent on the comprehensive separation mechanisms, including charge interaction and hydrophobic interaction between the studied mAb and the stationary phase, and pI was an essential factor for separation resolution and elution time. Especially for Rituximab with more basic pI (8.70−9.30) among the nine

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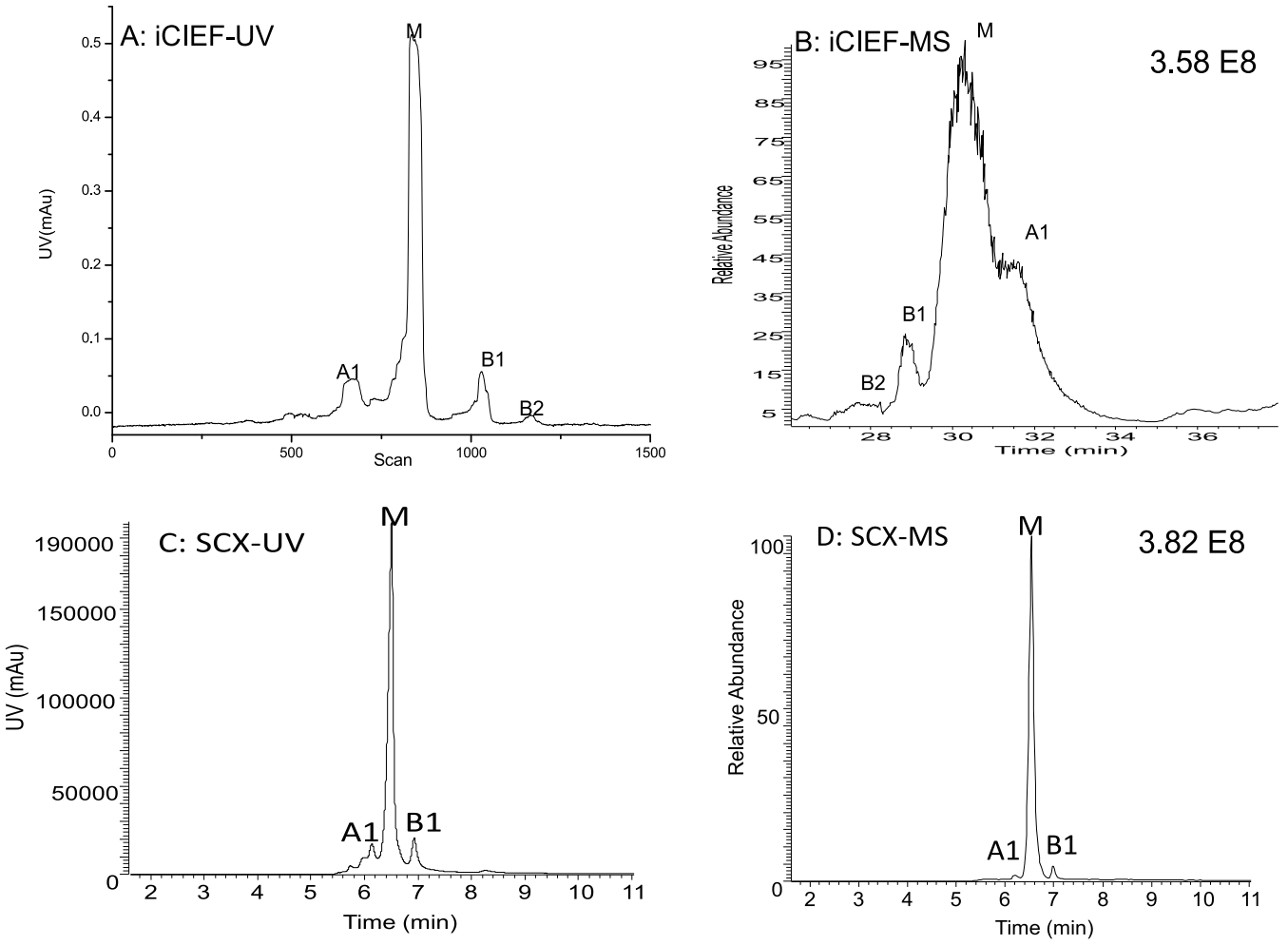

Figure 2. Comparing separation of icIEF-MS and SCX-MS using atezolizumab as the studied mAb. UV profiles (left) and corresponding TIC from tandem MS analysis (right).

studied mAbs, a higher 85% percentage of high pH mobile phase B (10 mM ammonia, pH 10.9) was used as the starting elution point for avoiding too slow elution meanwhile remaining resolution because of reduced charge interaction.

3.2. Comparison of icIEF and SCX Coupled to MS. As shown in Figure 2, charged variants of atezolizumab were identified by icIEF-MS and SCX-MS, and their results were compared. In the SCX separation, the order of acid−base peaks detected by MS is the same as that of UV. However, in icIEF separation, the basic peak is pushed toward the ESI source first and therefore the order of the acid−base peak in MS detection is opposite to that of UV. Another notable difference is that because SCX-MS used a higher flow rate, the extra-column band broadening from UV to MS is less severe, meaning the UV peak shape is more consistent with the MS peak shape. In contrast, the much lower mobilization flowing rate of icIEF-MS means that extra-column effects had a more important influence on icIEF separation; from UV detection to MS detection, the peaks appear more obviously broadened, especially the main peak, which has a higher intensity and partially overlaps with the acidic peaks of slightly lower pIs. Currently, narrow ampholytes and innovative coating of separation capillary in our laboratory are being developed for further improving the separation resolution in icIEF-MS to overcome the lost resolution from peak broadening.

Despite the disadvantages of peak broadening and lower analysis throughput as compared to SCX-MS, the sensitivity of icIEF separation is still much higher than that of SCX-MS. On analysis of mAbs using the SCX-MS method on QE Plus, the TIC response was found to be very low. Taking atezolizumab as an example, even when 100 μg of mAb was loaded on the SCX column, the TIC response of the main component was 5 E5, as indicated in Supporting information 4, and the charge variance could not be detected. The response of intact mass for mAbs on Orbitrap Exploris 240 was improved a lot. As shown in Figure 2, loading 20 μg of atezolizumab, the TIC response of the main component was 3.82 E8. Thus, SCX-MS analysis for all mAbs was carried out on Orbitrap Exploris 240 with a higher sensitivity. Compared to SCX-MS, the response of icIEF-MS is much higher. Although the sample load of icIEF-MS is only one-tenth of that of SCX-MS, the TIC intensity on icIEF-QE Plus MS is equal to that on SCX-OES MS, as shown in Figure 2. Two factors contributed to the much higher sensitivity of icIEF-MS than SCX-MS. First, a much lower flow rate was used in icIEF-MS (less than 6 μL/min) than the one used in SCX-MS (300 μL/min). Lower flow rate leads to higher evaporation efficiency and MS efficiency.[28] The low flow operation of the microfluidic systems for icIEF-MS significantly boosts MS sensitivity and increases the dynamic range, even with sample amounts as low as 1 ng. Moreover, the focusing-driven preconcentration in icIEF separation was

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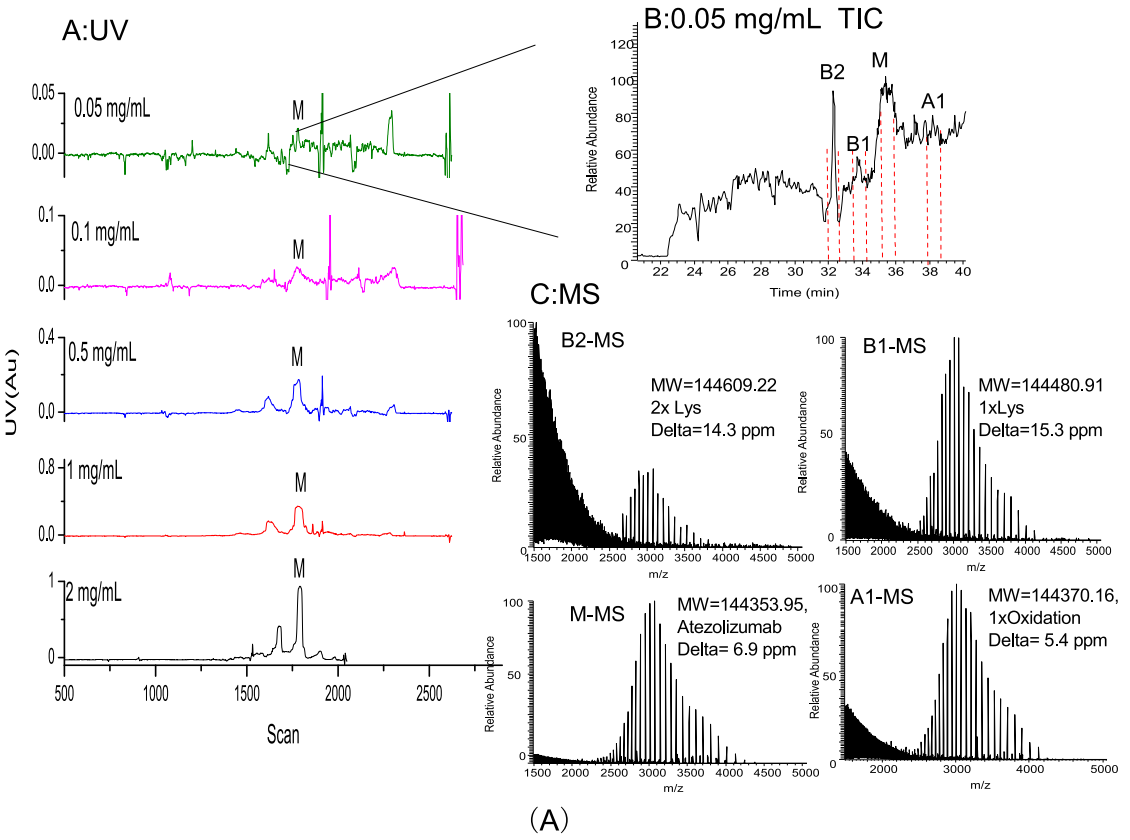

Figure 3. High-sensitivity icIEF-MS characterization of atezolizumab. UV separation profile of varying concentrations (0.05−2 mg/mL) of atezolizumab (A) with TIC (B) and MS (C) profiles of 0.05 mg/mL illustrating the extreme sensitivity of this novel icIEF-MS strategy. The plot of the charge variant concentration vs MS intensity is shown in (D).

helpful for increased sensitivity. Second, the pH of the elution mobile phase used for SCX-MS in this study is near the pI point of the mAb (pH 7−10), so the elution was under neutral-basic conditions. In icIEF-MS, 50% acetonitrile containing 0.5% FA was used for the makeup solution, with the flow rate being 5 μL/min and the flow rate of the preparation pump being 80 nL/min. With such a ratio, the final mobile phase entering the MS was in an acidic condition. Hence, SCX-MS is a neutral/basic mobile phase into the MS, while icIEF-MS is an acidic mobile phase into the MS. Under neutral/alkaline conditions, the ability of proteins to bind protons is much weaker than under acidic conditions, similar to native mass, so the number of charges is less with a lower charge state for SCX-MS as compared to icIEF-MS, as shown in Supporting information 5. Protein at lower charge states becomes more prone to binding adducts. Thus, there are more adducts in proteins eluted from SCX-MS than the one eluted from icIEF-MS. The adducts to proteins suppress ionization and result in a poorer MS efficiency of SCX-MS than the one of icIEF-MS.

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Table 3. Comparing Charge Variants of Atezolizumab by icIEF-MS and SCX-MSC

			icIEF-MS		SCX-MS	
peak name	MW (theoretical)	modification	MW	modification delta (ppm)	MW	modification delta (ppm)
A1	144,370.94	oxidation	144,370.2	5.4		
A1	144,517.08	glycation	144,516.8	1.9	144,518.1	6.5
M	144,354.94	N/A	144,354.5	2.7	144,355.9	5.4
B1	144,483.12	1xLys	144,482.6	3.8	144,484.4	8.6
B2	144,611.29	2xLys	144,610.7	4.4		

Different concentrations of atezolizumab were detected in icIEF-QE plus MS with high sensitivity, as shown in Figure 3. As seen from the TIC and MS spectra of the acidic and basic variants, even at the minimum concentration of 0.05 mg/mL (UV signal of 3 S/N), TIC still detected clear signals for all charge heterogeneities. However, SCX-QE plus MS could not achieve the detection limit at 0.05 mg/ml. In the reported cIEF-MS studies for protein analysis,[21,25−27] sheath liquid flow (flowing rate 0.10−1μL/min) was employed, and the ID of the separation capillary was usually 50 μm with above 40 cm length and typical concentrations of proteins analyzed were 0.1−2 mg/mL. The established icIEF-MS platform in this work used a larger 200 μm ID with just 5 cm length for the separation capillary, which contributed to sensitive MS detection due to a higher sample loading even if a 5 μL/min flow rate of sheath solution with a higher dilution effect was used. In addition, due to the AD-coated capillary, MC and glycerol commonly used in cIEF-MS were free from icIEF-MS developed in our work, which could further reduce the ion compression effect. As mentioned above for the sensitivity test, 0.05 mg/mL mAb concentration achieved the detection limit at the ng-level sample loading.

Besides the difference in sensitivity, the mass accuracy obtained in icIEF-MS is also better than the one obtained in SCX-MS. The accuracy of MS detection depends on the resolution of the MS spectrometer. The attainable mass resolving power is not only dependent on the instrumental mass resolution limits but also heavily affected by the ionization process. The adducts make ion signals become broadened because they originate not only from the multiply protonated analytes but also from analytes carrying adducts.[29] Thus, more adducts lead to a poorer resolution and a larger mass error (poorer accuracy). Although in-source collision-induced dissociation (CID) can reduce the adducts to proteins, not all adducts can be removed. As shown in Table 3, although 110 V of in-source CID value was used, the mass spectrometric peaks obtained in SCX-MS are still wider than those obtained in icIEF-MS, resulting in a larger MW deviation on SCX-MS (5.4 ppm) than that on icIEF-MS (2.7 ppm) for the main component. Another example is bevacizumab (Supporting information 6). Without desalting pretreatment, the MW deviation of bevacizumab by SCX-MS was as large as 33.9 ppm. When analyzed after desalting, the deviation of bevacizumab reduced to 12.8 ppm. It was observed that the deviation of bevacizumab obtained by icIEF-MS was 9.6 ppm even without desalting due to less adduct ion formation. Similar phenomena were also reported in a previous study where the mass accuracy obtained in denature analysis was better than the one obtained in native analysis..[30,31]

3.3. Carryover Effect in icIEF-MS and SCX-MS. Supporting information 7 demonstrated the much smaller carryover effect of icIEF-MS relative to SCX-MS. After the analysis of atezolizumab, water as the sample was used for SCX-MS for analysis and the aqueous solution containing 4% HR8.5−9.5 was used for icIEF-MS. There was no obvious signal on icIEF-UV, but a small signal was detected by SCX-UV at the peak of atezolizumab. Comparing SCX-TIC and icIEF-TIC, it could be observed that both have relatively obvious signal peaks with retention times of 6.82 and 21.5 min, respectively. The MS spectra extracted from these two signal peaks are shown in Supporting information 7 (C and F). Using deconvolution, it was determined that the residual signal detected by SCX-TIC was atezolizumab, while the signal detected by icIEF-TIC was simply the amphoteric electrolyte, meaning no residual analyte signal was detected in icIEF-MS. Low carryover makes the icIEF-MS more accurate and reliable for trace protein charge variants, avoiding false-positive results.

As shown in Figure 3 and Supporting information 7, a background signal between 1500 and 2500 m/z was observed, and the MS information verified that they were not proteins but from carrier ampholytes and the solvent background. The background ions did not interfere with the identification of mAb charge variants.

3.4. Repeatability of Protein Identification by icIEF-MS with the Use of Atezolizumab as the Studied mAb. As demonstrated in Supporting information 8 and 9, the icIEF-MS platform developed here exhibited excellent repeatability of characterizing charge variants of atezolizumab based on five replicates (two analyses on one day and the other three analyses on the following day). Identical charge variants were detected with very low mass deviations (<10 ppm) among all runs, which can guarantee reliability and consistence by employing icIEF-MS for lot-to-lot protein sample characterization.

In this study, using standard NISTmAb, the investigation of repeatability was systematically carried out. As demonstrated in Supporting information 10, the charge variants exhibited outstanding repeatability in terms of the retention time, the accuracy of the measured molecular weight, and the ion intensity of the apex. Supporting information 11 shows good reproducibility of icIEF-MS using two batches of the AD-coated capillary cartridge. The coating of AD for the 200 μm ID capillary was rather stable for icIEF analysis with a satisfactory lifetime. As demonstrated in Supporting information 12, icIEF-UV separation of NISTmAb was identical during 100 consecutive runs and retained the same sensitivity, resolution, and profile.

3.5. icIEF-MS for a Diverse Set of mAbs. Finally, the full set of diverse mAbs listed in Table 1 was characterized for their charge heterogeneities, whose important glycations and modifications were confirmed. Figure 4 shows icIEF-UV profiles and MS TICs, while Table 4 lists the identifications of the native charge variants of all nine studied mAbs. These analyses in our study illustrate the robustness, outstanding repeatability, high accuracy, and sensitive detection of icIEF-MS for a diverse set of protein charge variants. IEX, unlike

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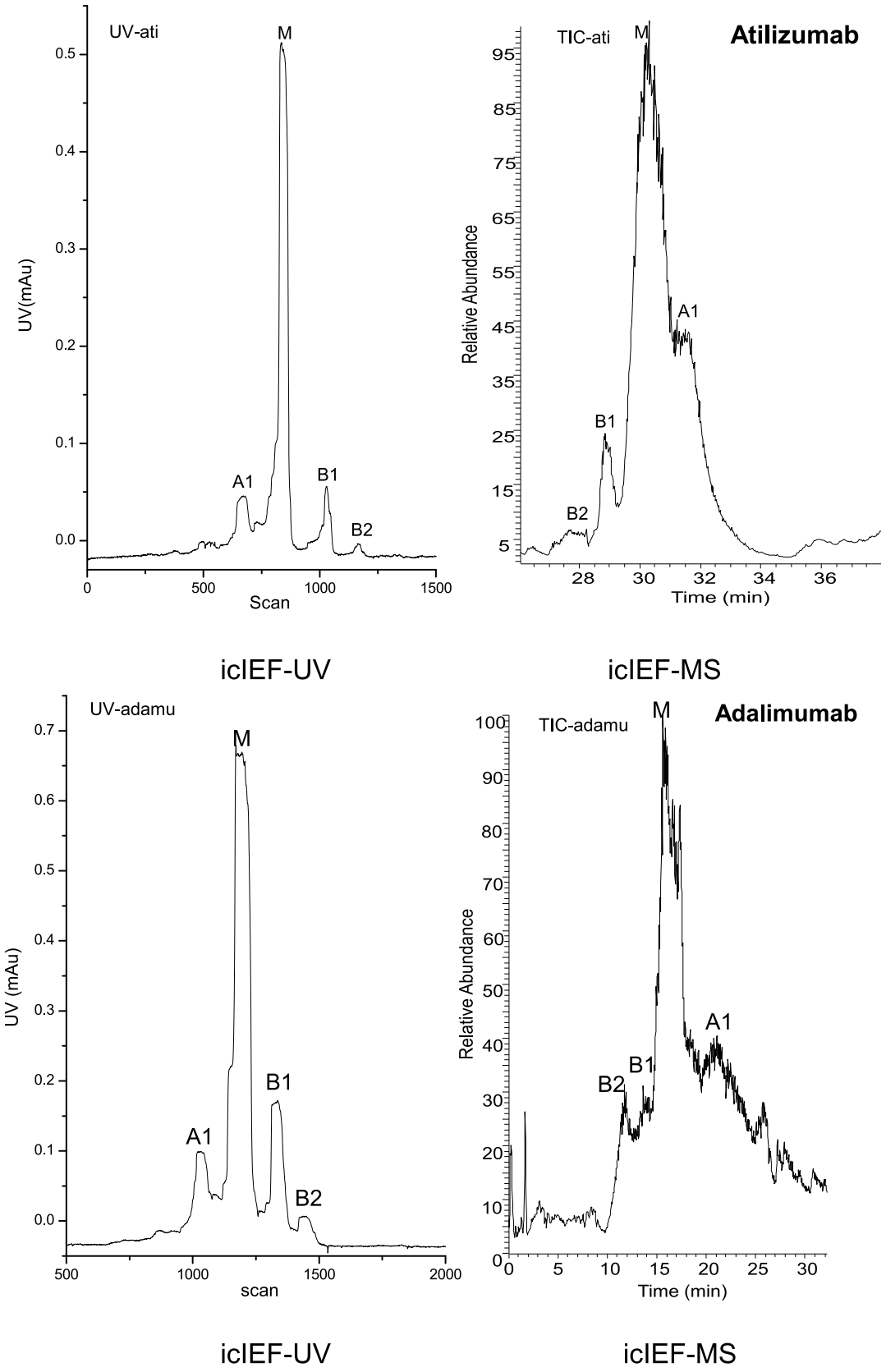

Figure 4. continued

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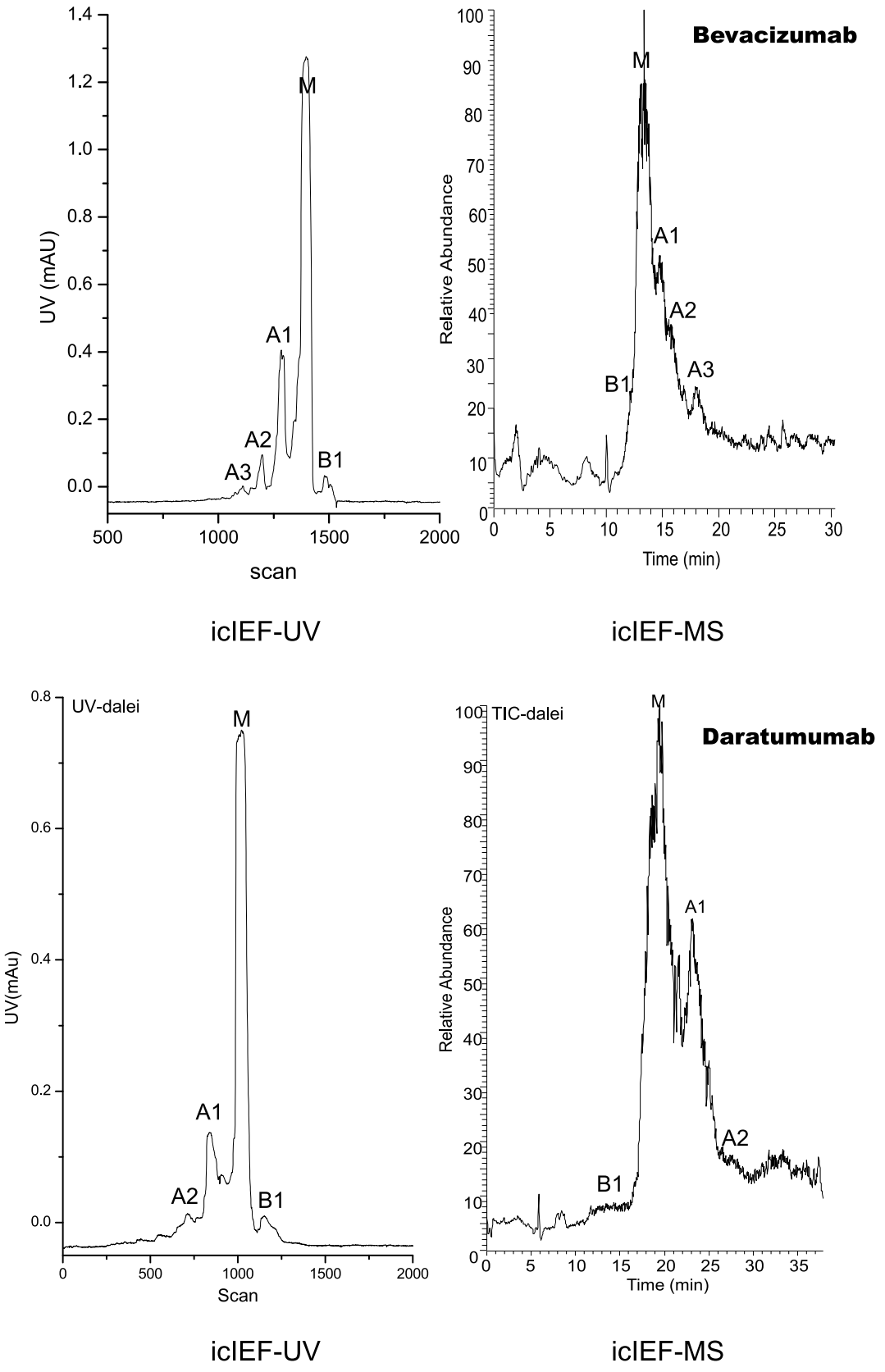

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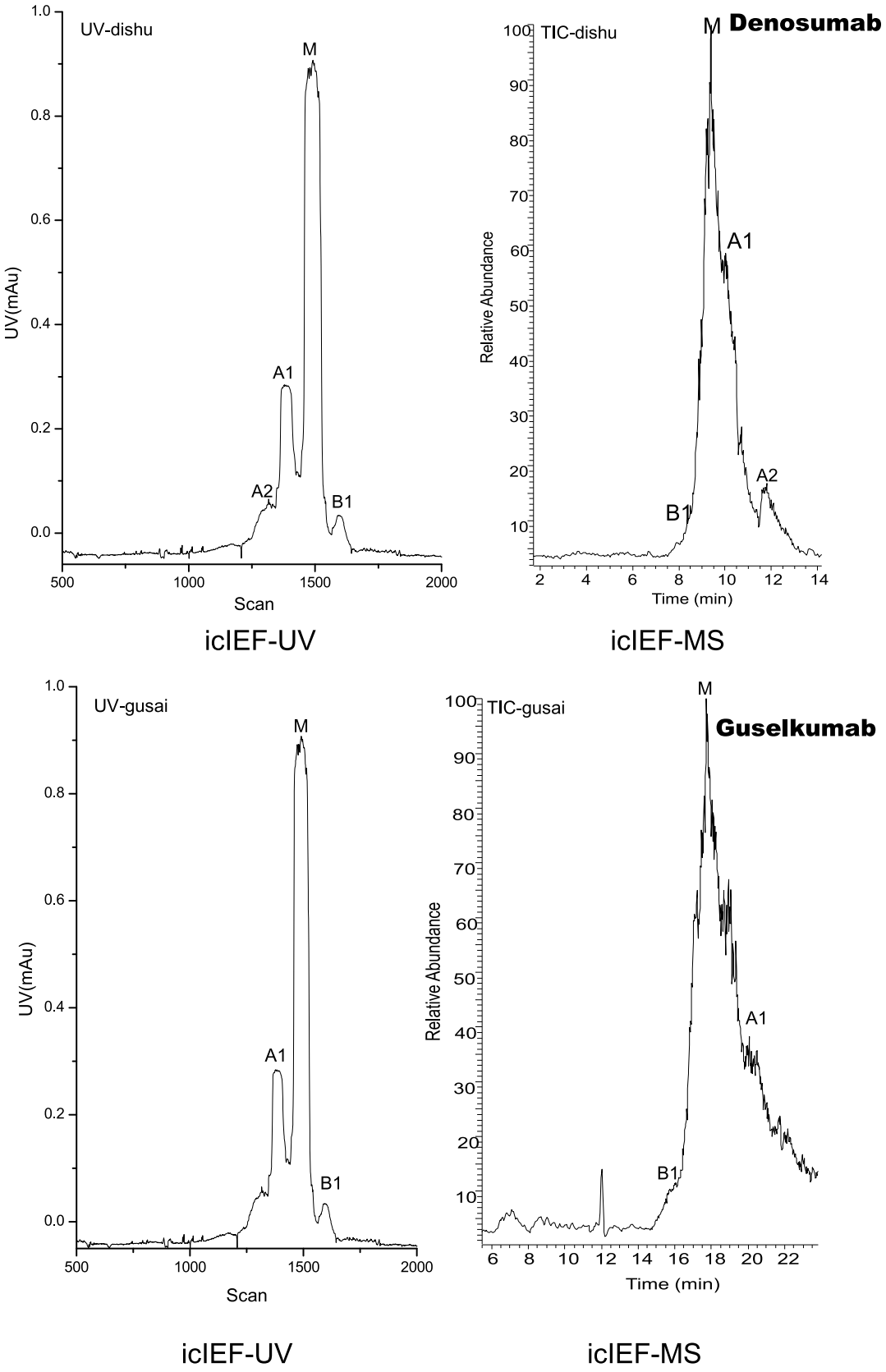

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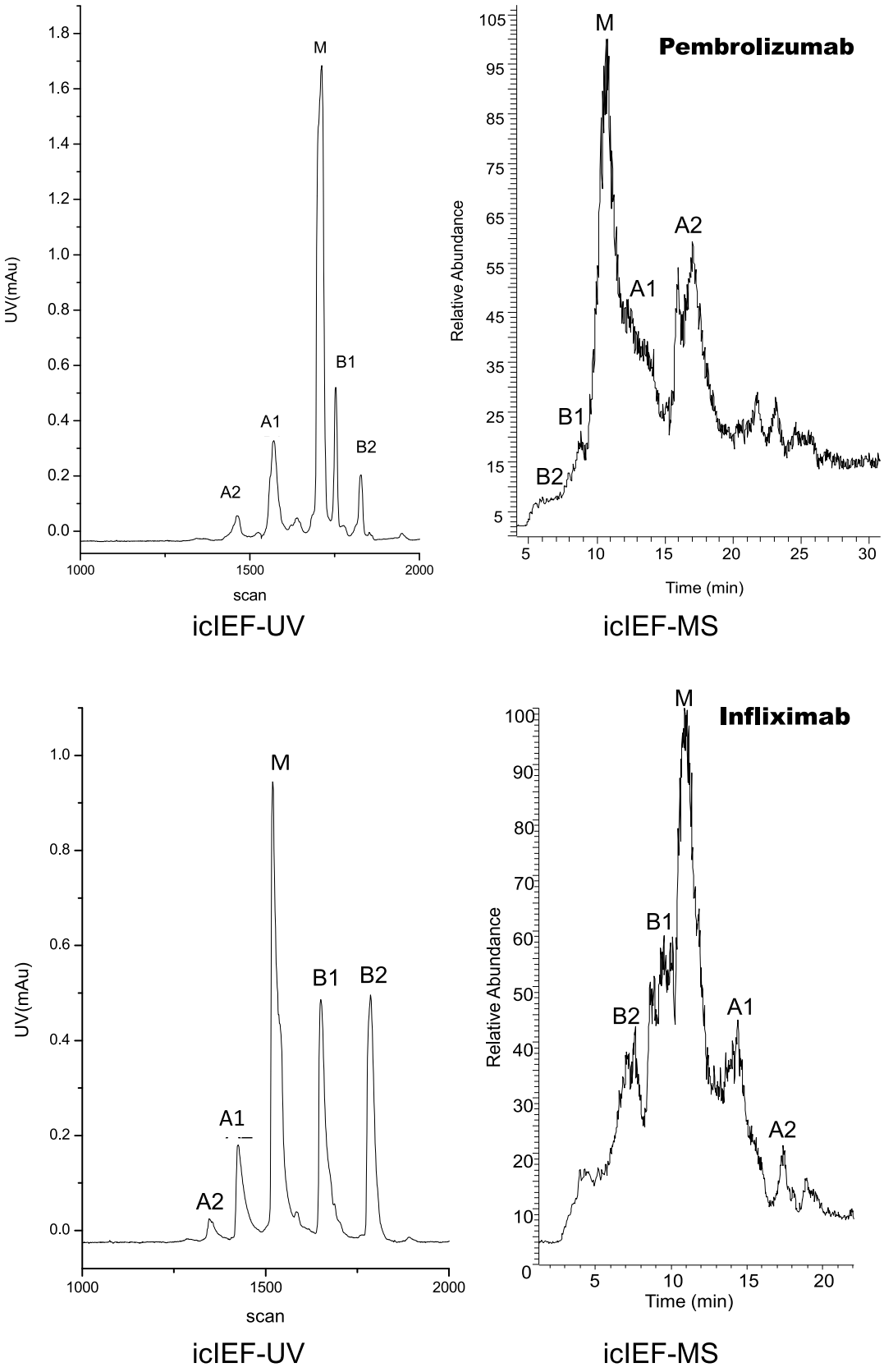

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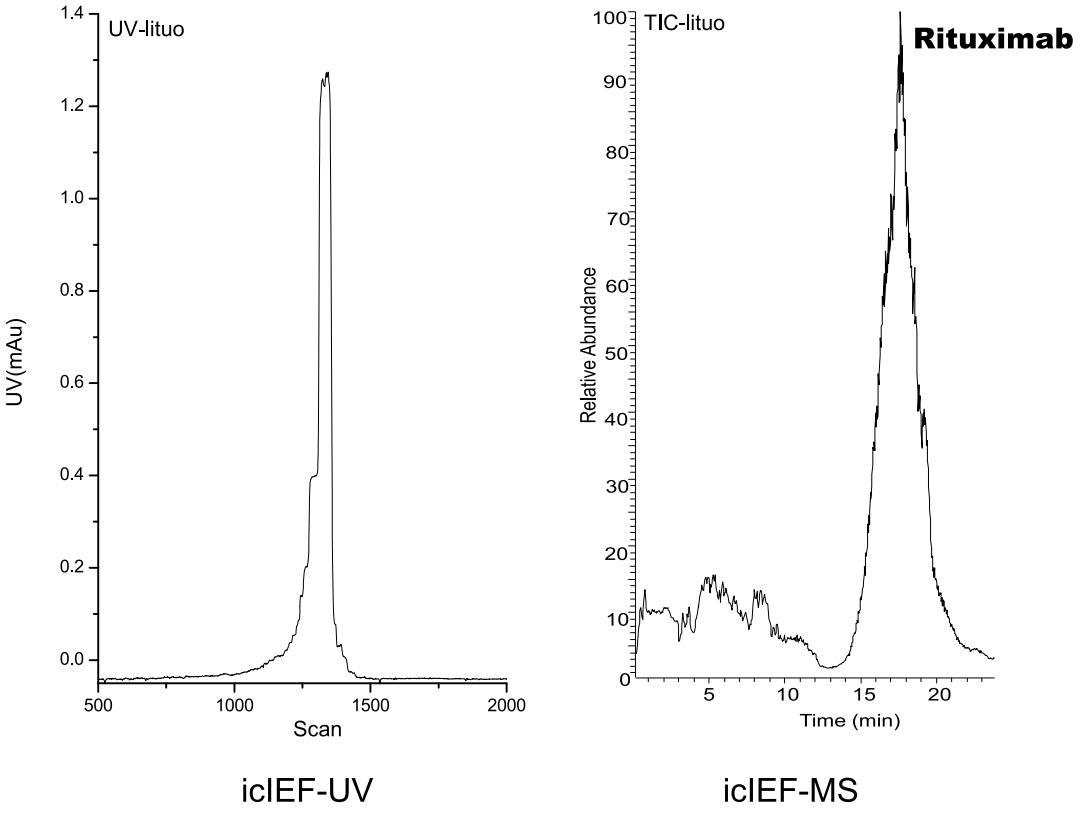

Figure 4. icIEF-MS characterization for a diverse set of therapeutic mAbs (nine studied mAbs).

cIEF/icIEF, does not separate analytes based on overall charge but on the charge available for interaction with the solid phase. Thus, a particular proteoform could be separated by capillary electrophoresis but not by IEX, thereby yielding complementary information as orthogonal techniques.[32]

The characterization of acidic variants is more challenging than that of basic variants since acidic variants have more complicated modifications. Although such acidic modifications usually can be discriminated by pI, their differences in molecular mass are rather minor. icIEF online coupled to high-resolution mass spectrometry can provide a useful tandem platform to address such challenges in acidic charge variants by elucidating protein structural information based on combining intact protein molecular weights and pI values measured.

The impact of charge variants on safety/PK/PD is important to rank the critical quality attributes. Semipreparative IEX can be used to separate different charge variants and to assess, for example, the impact on antigen binding by ELISA. However, the traditional icIEF and cIEF cannot achieve the fraction collections of protein charge variants, so the in-depth characterization of protein heterogeneity is rather difficult. In our recent studies,[18,33] an icIEF-based fractionation of proteins was developed and the charge variants could be prepared by autofraction collections at the μg level. The collected fractions were further analyzed by LC-MS peptide mapping, and the developed preparative icIEF technology has a huge potential to be an alternative tool to semipreparative IEX for in-depth characterization of protein charge variants and related study of protein chemistry. In our developed platform, the switch between icIEF-MS coupling and preparative icIEF is flexible and rapid just by changing the different separation capillary cartridges.[18]

4. CONCLUSIONS

In summary, rapid and accurate characterization of protein charged heterogeneity is a critical step in the development of therapeutic mAbs. Advances in icIEF-MS and SCX-MS are essential to support the rapid growth of the biopharmaceutical industry. In this study, icIEF-MS and SCX-MS were employed to characterize the charge variants of a diverse set of therapeutic mAbs, and their results were compared based on methodological validation. This study provides evidence of the comparable results that can be obtained from both separations in tandem with MS. However, icIEF-MS exhibited more advantages than SCX-MS in terms of separation resolution, sensitivity, low carryover effects, and MW measurement accuracy. Therefore, integrating our novel icIEF-HRMS analysis with the more common SCX-MS can provide a promising and comprehensive strategy for the differentiation and identification of charged variants for the purpose of developing protein therapeutics. Also, just by changing the corresponding capillary cartridge, the developed icIEF-MS platform can be flexibly and rapidly switched into the preparative icIEF mode for protein charge variant fractionation that can be followed by in-depth characterization, including LC-MS peptide mapping, biointeractions, and related protein chemistry study. Integrating icIEF-MS and icIEF-based fractionation further contributes to a comprehensive icIEF-based MS strategy in mAb heterogeneity characterization. As reported for capillary electrophoresis-MS technology, the icIEF-MS developed in this study demonstrated a straightforward workflow with a faster method development for the protein heterogeneity study.

The icIEF-MS platform established still needs continuous development in the future. The advantages of the method in

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Table 4. Charge Variant Characterization of the Studied mAbs by icIEF-MS[a]

mAb	A2		A1		M		B1		B2	
	MW	modification	MW	modification	MW	modification	MW	modification	MW	modification
Adalimumab	N/A	N/A	148,053.5	1XA2G0F, 1xA1G0M4, 1XGlycation	148,244.6	1xA2G0F,1xA2G1F	148,209.2	2xA2G0F,1xLys	148,336.7	2xA2G0F, 2xLys
Infliximab	148675.3	2xA2G0F, 1xGlycation	148,516.6	2xA2G0F, deamidation elucidated	148,513.7	2xA2G0F	148,641	2xA2G0F,1xLys	148,769.4	2xA2G0F, 2xLys
Pembrolizumab	149503.9	1xA2G0F, 1XA2S1G0F, 1XGlycation	149,208.9	1xA2G0F, 1xA2G1F, 1xGlycation	148,888.1	2xA2G0F	148,829	2xA2G0F,1xC_amidation	148,891.7	1xM5, 1XA2G1F, 1xLys, 1xC_amidation
*Bevacizumab	149528.3	2xA2G0F,2xGlycation	149,362.7	2xA2G0F, 1xGlycation	149,200	2xA2G0F	149,329.3	2xA2G0F, 1XLys		
Daratumumab	148027.9	2xA2G0F, deamidation elucidated	148,346.4	1xA2G0F, 1xA2G1F, 1xGlycation	148,025.9	2xA2G0F	148,153.9	2xA2G0F, 1xLys		
Atezolizumab	N/A	N/A	144,516.8	1xGlycation	144,354.6		144,482.6	1xLys	144,610.7	2xLys
Denosumab	147675.2	2XA2G0F, 2xGlycation	147,678.3	1xA2G0F, 1xA2G1F, 1xGlycation	147,358.3	1xA2G0F, 1 xA1G1M4F,2xC_amidation	147,477.4	2xA2G0F, 1xLys	N/A	N/A
Guselkumab	N/A	N/A	146,778.1	1xA2G0F, 1xA2G1F,1xGlycation	146,614.9	1xA2G0F, 1xA2G1F	146,580.1	2xA2G0F, 1xLys	N/A	N/A
Rituximab	N/A	N/A	147,562.5	1xA2G0F, 1xA2G1F, 1xGlycation	147,239.8	1xA2G0F, 1xA2G1F	147,204.9	2xA2G0F, 1xLys	N/A	N/A

[a]Asterisk (*) denotes that bevacizumab had an acidic variant (A3; MW: 149204.2; deamidation elucidated) with trace concentration, besides A1, A2, and B1 charge variants listed in table.

this study are high throughput, excellent sensitivity, and satisfactory reproducibility. However, at the same time, the TIC in this study indicated a sacrifice of separation resolution in MS detection due to the analyte diffusion from the outlet of the icIEF separation capillary to the ESI. In our laboratory, more efforts are being carried out for increasing the separation resolution in icIEF-MS analysis through offsetting analyte diffusion, including the use of nano-ESI with a lower flow rate of makeup solution, reduced length of transfer capillary, and new design of grounding electrodes in the separation cartridge in icIEF-MS analysis. In addition, the innovation of the ultrahigh-resolution carrier ampholytes and the new capillary coatings we are developing contributes to optimal separation resolution and selectivity in the icIEF-MS characterization of protein charged heterogeneity. Relevant optimizations are in progress.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c05071.

pIs measurement by icIEF; peptide sequences; icIEF-UV and SCX-UV profiles; SCX-QE Plus for atezolizumab characterization; charge states; effect of desalting on MW measurement; carryover effect; repeatability of icIEF-MS; molecular weight stability by icIEF-MS; repeatability of NISTmAb in icIEF-MS; batch repeatability of the capillary cartridge in icIEF-MS; and long-term repeatability of capillary in icIEF separation (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATION

icIEF	imaged capillary isoelectric focusing
(SCX)	strong cation exchange
(IEX)	ion-exchange chromatography
MS	mass spectrometry
mAbs	monoclonal antibodies
HPLC	high-performance liquid chromatography
pI	isoelectric point
TIC	total ion chromatography
AD	acrylamide
MW	molecular weight

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