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Imaged capillary isoelectric focusing (icIEF) tandem high resolution mass spectrometry for charged heterogeneity of protein drugs in biopharmaceutical discovery

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ABSTRACT

Since the first commercial imaged capillary isoelectric focusing (icIEF) instrument was developed twenty years ago, the technology has become the gold standard of quality and manufacturing process control in the biopharmaceutical industry. This is owing to its high-resolution and high-throughput characterization of protein charge heterogeneity. In addition to a charge variant profiling, mass spectrometry (MS) analyses are also desirable to obtain an in-tact molecular weight (MW) and further identification of these charged species. While offline fractionation technologies including isoelectric focusing (IEF) and free flow electrophoresis (FFE) followed by liquid chromatography (LC)-mass spectrometry (MS) coupling have been employed for this purpose, there have been much fewer reported applications of icIEF-based MS connection and fraction collection. Factors that have impeded the development of these icIEF applications include difficulties with a direct connection to the MS interface as well as high background signal of carrier ampholytes and incompatible coated capillary cartridges. In this work, we developed a robust and flexible icIEF-MS platform which overcomes these challenges to achieve both the rapid icIEF separation and high-resolution MS (HRMS) identification of protein charged variants simultaneously. We demonstrate how this methodology proves highly-sensitive and highly reliable for the characterization of commercial monoclonal antibodies (mAbs) and antibody-drug-conjugates (ADCs). The whole workflow of icIEF-MS for protein heterogeneity is straight forward and accurate and can be performed within 45 min. Furthermore, the developed icIEF-MS configuration can flexibly switch to icIEF-based fraction collection model allowing the user to perform additional in-depth characterization such as peptide mapping by high performance liquid chromatography (HPLC) tandem mass spectrometry (LC-MS/MS).

1. Introduction

Recombinant monoclonal antibodies (mAbs) have been spurring rapid growth in the commercial and clinical products of biotherapeutics across the pharmaceutical industry. 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. The charged heterogeneity of protein drugs requires in-depth structural characterization during critical quality attribute (CQA) assessment to ensure safety, efficacy and potency [1,2]. Furthermore, the monitoring of charge variants is a necessary step for the continuous quality control (QC) of protein drugs. The charged heterogeneity of proteins results from diverse mechanisms including cellular processes, chemical degradation and production conditions during the manufacturing process [3–6]. Currently, there are two main methods for the detection of charge variants of protein drugs: ion exchange (IEX) chromatography and imaged capillary isoelectric focusing (icIEF) or cIEF [7,8], both of which traditionally use UV as the detector.

Abbreviation: icIEF, Imaged capillary isoelectric focusing; MS, Mass spectrometry; , ADC, Antibody-drug-conjugate; , mAbs, Monoclonal antibodies; HPLC, High performance liquid chromatography; , pI, Isoelectric point; , TIC, Total ion chromatography; , AD, Acrylamide derivative.

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Although UV detection has good stability and sensitivity, its selectivity and therefore qualitative capability is limited. In order to analyze the cause of charge isomers, accurate qualitative analysis must be carried out. High resolution mass spectrometry (HRMS) is currently the most powerful means of qualitative protein analysis [9,10]. However, traditionally IEX and icIEF could not be used in conjunction with MS due to the use of less volatile reagents.

Since the first commercial instrument developed, icIEF technology has been becoming the gold standard of the quality and manufactory process control in the biopharmaceutical industry due to its high-resolution characterization of protein drugs with high-throughput. Although a diverse selection of chromatography-mass spectrometry coupling techniques [11–14] have been employed to protein characterization, most of them cannot accurately distinguish the protein variants with tiny isoelectric point (pI) differences. Instead, traditional offline fraction collection technologies such as isoelectric focusing (IEF) and free flow electrophoresis (FFE) [15–18] were performed before LC-MS analysis making charged heterogeneity preparation and analysis a more tedious process. Recently, icIEF or cIEF as a robust separation front-end for MS detection has been demonstrated for the highly efficient characterization of protein charge heterogeneity [19–23]. However, improvements are still required for addressing the challenges in terms of directly connecting MS and fraction collections after the icIEF separation. Otherwise, it will hinder the important applications of protein charge variants driven by icIEF technology in biopharmaceutical industry. Chip-based icIEF-MS reported [19,20] depends on chemical mobilization for MS detection, which tends to result in instability of pH gradient during the chemical mobilization and decreased resolution. Traditional cIEF with single point detection has the disadvantages of low throughput and cumbersome operation due to tedious mobilization process and longtime separation for MS detection after protein focusing [21]. Other critical bottlenecks of icIEF coupled to MS for protein charged variants, include unsatisfactory repeatability, complicated operation frequently involving the trial-and-error optimization and incompatibility with MS resource. Integrating icIEF-MS and protein fraction collection into a unique platform is a valuable alternative to overcome the challenges hindering traditional technologies in characterizing protein heterogeneity.

In this work, a robust iCIEF-MS platform was developed to achieve both of the rapid icIEF separation and reliable high-resolution MS (HRMS) identification of protein charged variants simultaneously. icIEF-MS on the CEInfinite from ASE LTD was constructed with Thermo Fisher Orbitrap Fusion Lumos and Broke QTOF MS was employed for the heterogeneity analysis of therapeutic mAb and ADC [22,23]. The focused peaks from an icIEF system are transferred to a four-port nanoliter valve where peaks of interest are cut and transferred directly (flow injection) or via capillary zone electrophoresis to electrospray mass spectrometry [22]. The direct coupling of an CEInfinite icIEF instrument to MS via the nanoCEasy interface was established for the successful characterization for mAbs, in particular for the narrow charge variant profile of Trastuzumab. In addition, MS-compatible amphoteric electrolytes and both of methylcellulose (MC) and urea-free cartridges in icIEF analysis have been innovatively developed, which can realize zero-volatile reagents in the analysis of protein drug charge variants. In summary, he innovative microliter interface improves the sensitivity of identifying protein drug charge variants. The whole icIEF-HRMS analysis based on seamless MS interface can be solved within 45 min. In addition, just by changing preparative capillary cartridge, the developed icIEF-MS configuration can rapidly and flexibly switch to icIEF-based fraction collection model for protein charged variants following the peptide mapping by high performance liquid chromatography (HPLC) tandem HRMS [24]. The established methodology was high-sensitive and accurate, employing to the heterogeneity characterization of mAb and ADC. The whole workflow of icIEF-MS for protein heterogeneity is straight forward, accurate and high-throughput.

2. Materials and methods

2.1. Materials

All ampholytes (AESlytes) were obtained from Advanced Electrophoresis Solutions Ltd (AES, Cambridge, Ontario, Canada). Monoclonal antibody NISTmAb was Purchased from Millipore Sigma (cat. no. NIST8671). Other mAbs (bevacizumab and pembrolizumab) and ADC (T-DM1) employed in this study were kindly donated from Thermo Fisher Scientific (China). Mass spectrometry grade acetonitrile (ACN), formic acid and formamide were purchased from Fisher Scientific (Hampton, NH).

2.2. Solutions for iCIEF

For icIEF-MS of bevacizumab: 1 mg/mL of mAbs in 2 % (*v/v*) HR AESlytes 3–10 %, and 10 % formamide are mixed in deionised water.

For icIEF-MS of NISTmAb: 1 mg/mL of mAbs in 0.5 %(*v/v*) HR AESlytes 3–10, 1.5 % (*v/v*) HR AESlytes 8–10.5 %, and 10 % formamide are mixed in deionised water.

For icIEF-MS of pembrolizumab: 1 mg/mL of mAbs in 2 %(*v/v*) HR AESlytes 6–8 are mixed in deionised water.

For icIEF-MS of ADC (T-DM1): commercially available T-DM1 was dissolved in deionised water with HR AESlyte 3–10, HR AESlyte 8.5–9.5 and formamide added. Final protein concentration is 1 mg/mL with 4 % (*v/v*) ampholytes (2 %HR AESlyte 3–10 % and 2 %HR AESlyte 8.5–9.5) and 10 % formamide mixed in deionised water.

For preparative icIEF of pembrolizumab: 2 mg/mL of mAbs in 4 %(*v/v*) HR AESlytes 7–8 are mixed in deionised water.

2.3. Imaged capillary isoelectric focusing (icIEF)

For icIEF separation, the CEInfinite iCIEF (Advanced Electrophoresis Solutions Ltd, Cambridge, Canada) was utilized with on-column UV detection at 280 nm. 200 μm ID acrylamide derivative (AD) coated capillary cartridges (AES, cat. no. CP00303) and micro-tee integrated (AES, cat. no. CP00303M) are used for icIEF-MS; 320 μm ID AD coated cartridges (AES, cat. no. CP00307P) are used for preparative icIEF. All these WCID (Whole column imaging detection) cartridges have a 5 cm length separation capillary, and 50 μm ID transfer capillary is assembled for both icIEF-MS and preparative icIEF cartridges. The icIEF-MS cartridge used for icIEF-MS includes a quartz union (works as a micro-tee), connecting the make-up solution and transfer capillary to ESI of MS. Both the make-up solution capillary and inlet capillary have a 100 μm ID.

For bevacizumab and NISTmAb, the focusing was 1 min at 1000 V, 1 min at 2000 V and 10 min at 3000 V, and 3000 V during mobilization; the mobilization speed was 50 nL/min with water containing 0.1 % (*v/v*) formic acid, across the separation capillary, and 5 μL/min make up solution (water: ACN = 1: 1, *v/v*, containing 0.5 % formic acid, *v/v*) added through a micro tee. Mobilization time was 15 min

For pembrolizumab, the focusing was 1 min at 1500 V, 10 min at 3000 V, and 3000 V during mobilization; the mobilization speed was 40 nL/min with water containing 10 mM acetic acid, across the separation capillary, and 5 μL/min make up solution (water: ACN = 1: 1, *v/v*, containing 0.1 % formic acid, *v/v*) added through a micro tee. Mobilization time was 45 min

For T-DM1, the focusing was 1 min at 1000 V, 9 min at 3000 V, and 3000 V during mobilization; the mobilization speed was 50 nL/min with water containing 0.1 % (*v/v*) formic acid, across the separation capillary, and 5 μL/min make up solution (water: ACN = 1: 1, *v/v*, containing 0.1 % formic acid, *v/v*) added through a micro tee. Mobilization time was 45 min

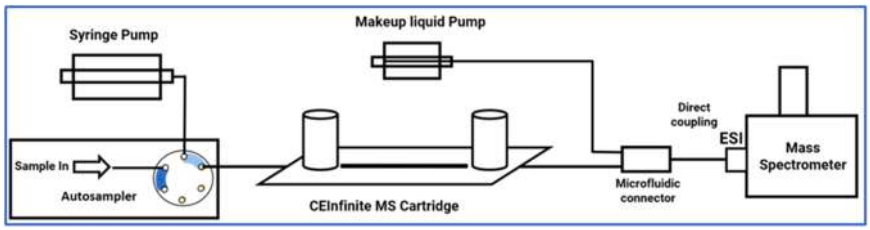


Fig. 1. Schematics of icIEF-HRMS.

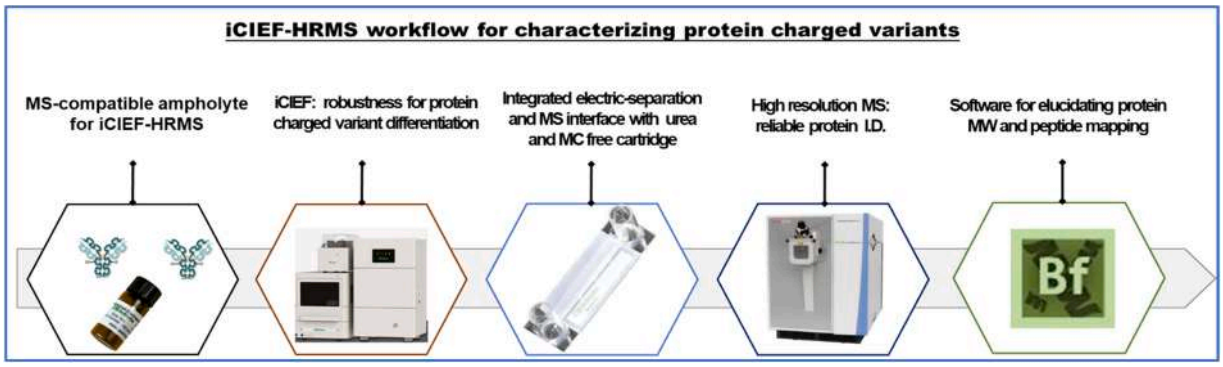


Fig. 2. Workflow of icIEF-HRMS for characterizing protein charged heterogeneity.

2.4. High-resolution mass spectrometry

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. For bevacizumab and NISTmAb, the MS parameters are as follow: spray voltage: 3.6 kV, sheath gas: 20 L/min, auxiliary gas: 5 L/min, S-lens RF 70 eV, capillary temp: 275 °C, resolution 35,000 @m/z 200, scan range of precursor ion 2000–8000 m/z and maximum injection time 200 ms.

For pembrolizumab and T-DM1, the following parameters were employed: spray voltage: 3.9 kV, sheath gas: 10 L/min, auxiliary gas: 0 L/min, S-lens RF 60 eV, micro scan= 10, capillary temp: 275 °C, Aux gas temp.: 100 ℃, resolution 35,000 @m/z 200, scan range of precursor ion 1500–8000 m/z and maximum injection time 200 ms.

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

3. Results and discussion

The developed icIEF-HRMS system and patented capillary cartridges eliminate the need for chemical migration when coupled to online mass spectrometry as shown in Fig. 1, while only using proprietary capillary-coated cartridges and separation solvents during icIEF separations greatly reduce the need for polymers and urea. It enables the isolated protein charge heterogeneity to be directly used for high-sensitivity MS characterization, thus retaining the excellent separation resolution of icIEF for mass spectrometry analysis. Seamless interface to MS is based on micro-fluidity, to enhance the sensitivity of MS detection of proteins. The constructed system requires no special modifications to the ionization source and can be directly connected to the mass spectrometer ionization source in this study.

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 50–200nL/min flowing rate depending on selected separation capillary cartridge I.D. (typically 50nL/min for 200 μm I.D. cartridge; and 160nL/min for 320 μm I.D. cartridge). Sheath liquid or make-up solution (water: acetonitrile =1:1 *v/v*, containing 0.1–0.5 % *v/v* formic acid) helps the effluents direct into ESI though a seamless interface. The whole process is automatic without user interference.

3.1. Workflow of iCIEF-HRMS for protein charged heterogeneity

With the workflow established here (Fig. 2), data acquisition on the icIEF-HRMS system is straight-forward, concise, and requires little user input. Intact protein characterization and offline iCIEF fractionation followed by peptide mapping analysis can be rapidly and reliably achieved. Customized capillary coatings decrease the need for polymers such as MC, thus simplifying the operation steps and prevent contamination of the ESI ion source. Proprietary SH, HR and UH carrier ampholytes (CAs) (AES Ltd.) allow icIEF-MS to be free from urea, a common denaturant widely used in the icIEF separation, while maintaining the excellent separation resolution of charged variants. Furthermore, these ampholytes can be utilized at much lower concentration when compared to other commercial brands, while maintaining the excellent performance resulting in a lower background signal in MS analysis. Finally, Biopharma Finder (BPF) from Thermo Fisher can offer rapid and accurate processing of protein data including intact protein deconvolution and peptide mapping elucidation.

3.2. Optimization of icIEF for MS detection

Normal pH range 3–10 ampholytes were applied to bevacizumab with satisfactory resolution of charge variants. As for NISTmAb, pembrolizumab and ADC, the use of only pH 3–10 ampholytes could not achieve good separation resolutions, so much narrower pH ranges of ampholytes (pH 8–10.5, pH 6–8 and pH 8.5–9.5) were used or mixed to optimize resolution of more complex protein isomers.

One complication is that proteins tend to precipitate around their pI values during focusing, which directly affects the reproducibility of their charge profiles. Protein concentration, focusing time and various supplementing additives are key parameters to minimize the protein precipitation and aggregation. Urea and sucrose are common additives to

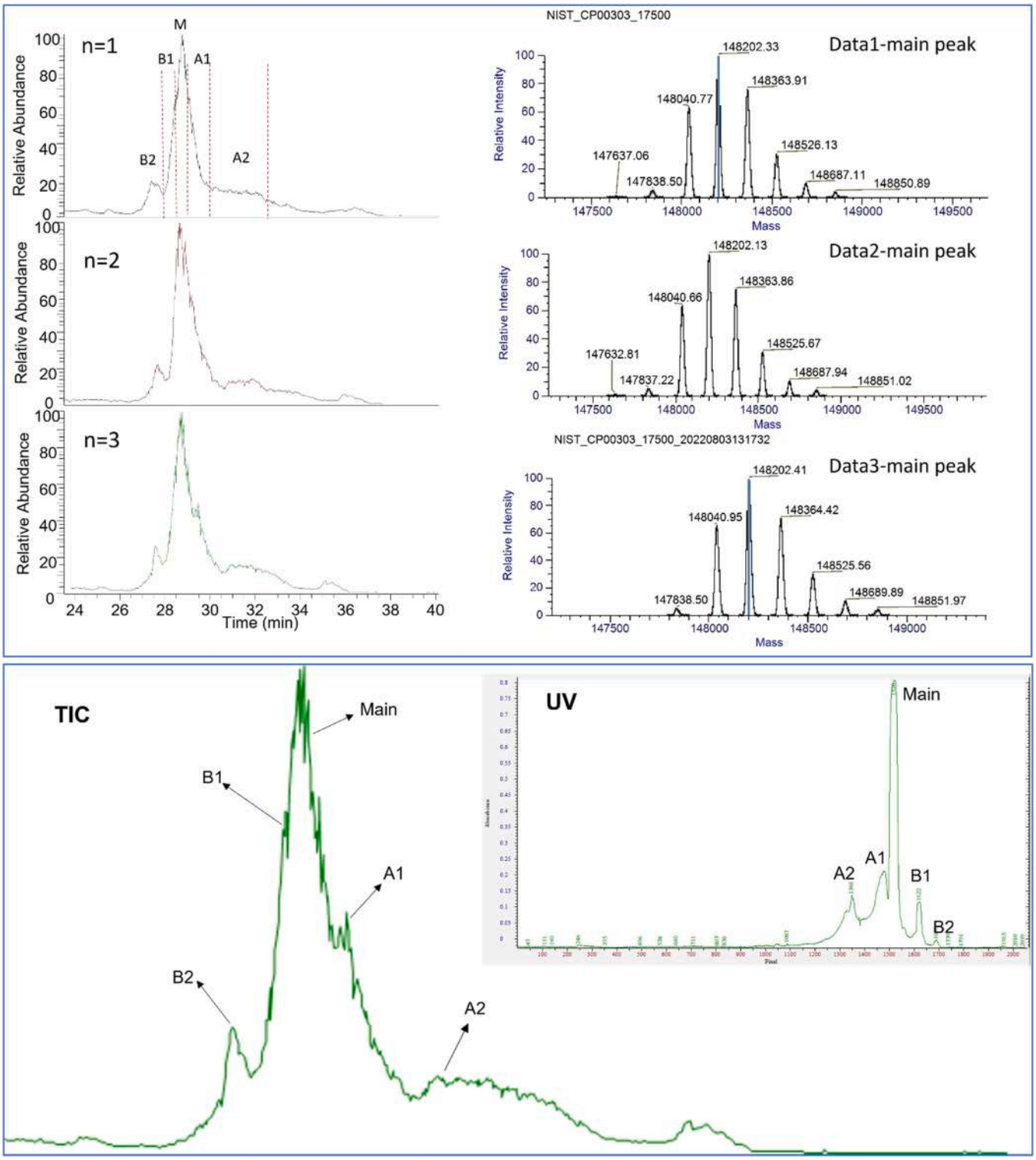


Fig. 3. Methodology repeatability of icIEF-HRMS for Intact NISTmAb.

reduce protein aggregation, solubilize proteins in sample matrix and therefore improve assay repeatability. However, urea is not compatible with MS due to the contamination of ion source. A novel reagent, formamide instead of urea, was reported to significantly improve reproducibility of protein charge profiles [25] and is more compatible with direct MS coupling. In our study, 5 %, 10 % and 20 % formamide was added into the sample solution and both of 10 % and 20 % showed good resolution of charge variants. For further experiments, 10 % formamide was chosen the optimized concentration.

The flow rates of mobilization solution from 30 to 100 nL/min and make-up solution from 1 to 10 μL/min were also tested. It was found that 40–50 nL/min mobilization rate achieved the optimal separation resolution and that the resolution decreases with increasing rate from 50 to 100nL/min. Higher flow rates above 100 nL/min of mobilization tended to result in high system back pressure that interfered with icIEF separation. It was found that 5 μL/min make-up solution was the best flow rate as it resulted in the best sensitivity.

Moreover, a 100 μm ID capillary usually used for routine icIEF-UV

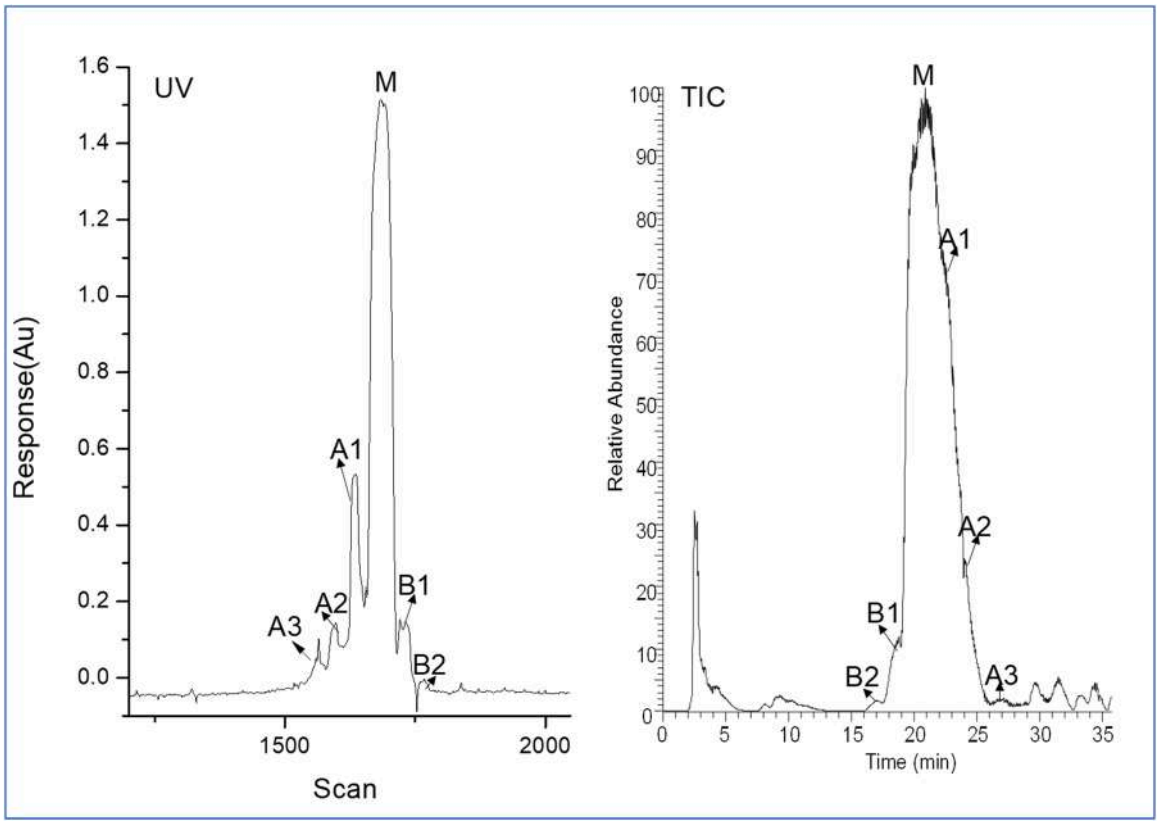


Fig. 4. Electropherogram and total ion chromatogram (TIC) in icIEF-HRMS analysis for bevacizumab. M：main peak； A1–3：acid peak 1, 2 and 3; B1–2: basic peak 1 and 2.

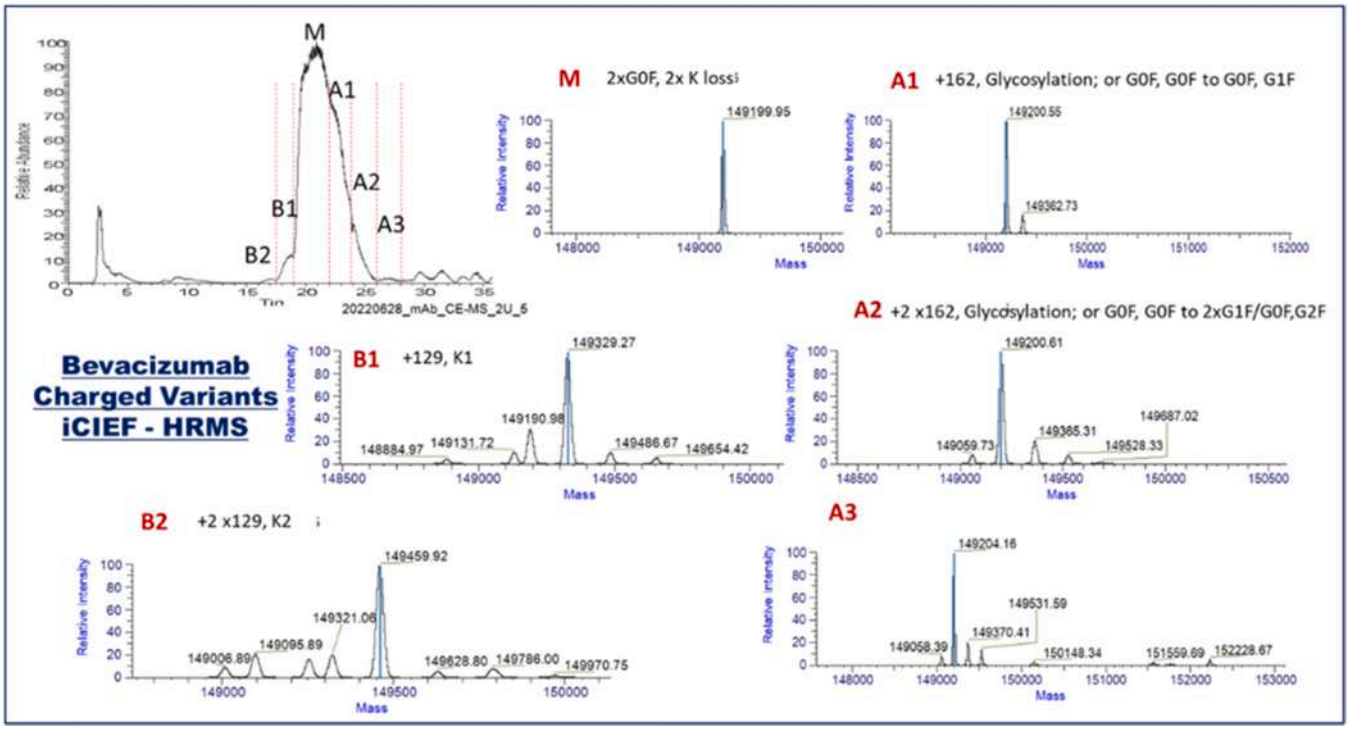


Fig. 5. Structural elucidation of bevacizumab' charged variants by Biopharma Finder 5.0.

cannot achieve the required sensitivity for MS coupling, so 200 μm ID capillary was utilized for icIEF-MS connection meanwhile retaining the separation resolutions.

MS conditions were optimized for the studied protein drugs following the FIA (Flowing Injection Analysis) and are indicated in the Materials and methods section above.

Table 1
Identification of bevacizumab' charged variants by icIEF-MS.

Fraction No.	MW (Da)	Identification
M	149,200	2x G0F,2x K loss
B1	149,329	2x G0F, 1x K loss
B2	149,459	2x G0F
A1	149,362	1x G0F, 1x G1F, 2x K loss/2x G0F, 1x glycation, 2x K loss/
A2	149,528	2xG1F, 2x K loss / 1xG0F, 1xG2F, 2x K loss / 2xG0F, 2x glycation, 2x K loss/
A3	149,204	Probably deamidation, while it should be confirmed by peptide mapping

3.3. Repeatability of icIEF-MS

Intact NISTmAb was utilized for the stability evaluation of methodology with good repeatability as shown in Fig. 3, which overcomes the unsatisfactory repeatability from traditional capillary electrophoresis technologies. Due to nano-flowing mode at 50 nL/min level for mobilization instead of chemical mobilization, the retention analysis time observed from total ion chromatography (TIC), peak shape and MS information is highly repeatable (n = 3) due to coated capillary stability and compatibility of CAs with MS, to guarantee high sensitivity and selectivity in MS detection. This warranties the applicability of icIEF-MS established when applied to protein charge heterogeneity.

3.4. iCIEF-MS for bevacizumab

Fig. 4 demonstrates the icIEF electropherogram and MS TIC for the identification of bevacizumab' acidic (A1 and A2) and basic (B1 and B2) charged variants. Thermo Fisher Biopharma Finder 5.0 (BPF 5.0) was utilized for the accuracy of high-resolution data for confident intact mass analyses as illustrated in Fig. 5 and Table 1. For absolute confidence in deconvoluted molecular weights in basic, acidic and intact conditions, advanced algorithms make the most of high-quality high resolution mass analyzer data. Deconvolution using sequence-specific isotope tables provides accurate results for identification of complex biotherapeutics. Confidently discovery, identification, quantification and product quality monitoring can be achieved with easy-to-understand data visualization within a single software platform.

3.5. icIEF-MS for pembrolizumab

The icIEF profile of pembrolizumab displays a main species as well as three basic peaks and 2 acid peaks (Fig. 6). These charge variants were also characterized using icIEF-MS and BPF 5.0. All charge variants identification results were summarized in Table 2.

Therapeutics with trace variants observed in icIEF-UV benefit from the high sensitivity of high-resolution mass spectrometry. For example, basic peak B3 of pembrolizumab (~0.3 % of all peaks, ~4.8 ng) (Fig. 6) can still be detected with good signal to noise ratio. The raw mass spectra of basic peaks and main peak were also compared (Fig. 7 A). It is easily to observe mass shift even at raw spectrum level because basic variants are mainly caused by heavy chain C-terminal truncation, heavy chain N-terminal pyroglutamate cyclization and oxidation.

For basic peaks, the main PTMs causing charge heterogeneity are heavy chain C-terminal truncation, fewer heavy chain N-terminal pyroglutamate cyclization events and increasing oxidation. All the modification details could be clearly read out in the MS intact results. Fig. 7B shows the deconvoluted results for the main peak (pI=7.57) and basic peak1 (pI=7.59) with similar pI. A series of 17 Da mass shifts can be detected between spectra corresponding to an additional glutamine at the N-terminal of the heavy chain being cyclized to form pyroglutamate in the main species. These intact protein analysis results align with the previously reported peptide mapping result (Fig. 7 C) [19]. Additionally, -GK truncation (−185 Da) was identified on C-terminal of both heavy chains in the lowest basic peak (B3), which is two orders lower than main peak (Fig. 7D).

For acidic peaks, at intact level we could observe + 1/+ 2/+ 3 Da mass shift indicating single or multiple deamidation. A series of deamidation and different sialic acid modifications were deemed possible, which are to be confirmed by subsequent LC-MS peptide mapping analysis.

Table 2
Results of all charge variants identification of pembrolizumab. HC=heavy chain.

Peak	**Main**	**B1**	**B2**	**B3**
Modifications	HC N-term: Gln->Pyro-Glu HC C-term: − 2 K	HC N-term: Gln->Pyro-Glu(~30 %) HC C-term: − 2 K	HC N-term: Gln->Pyro-Glu HC C-term: -1GK&− 1 K HC C-term: one Lys clipping Side chain: Oxidation	HC N-term: Gln->Pyro-Glu HC C-term: − 2GK HC C-term: no Lys clipping Side chain: Oxidation
Peak	**A1**	**A2**	**A3**	**A4**
Modifications	Heavy chain N-term: Gln->Pyro-Glu Heavy chain C-term: − 2 K Side chain: deamidation, sialic acid			

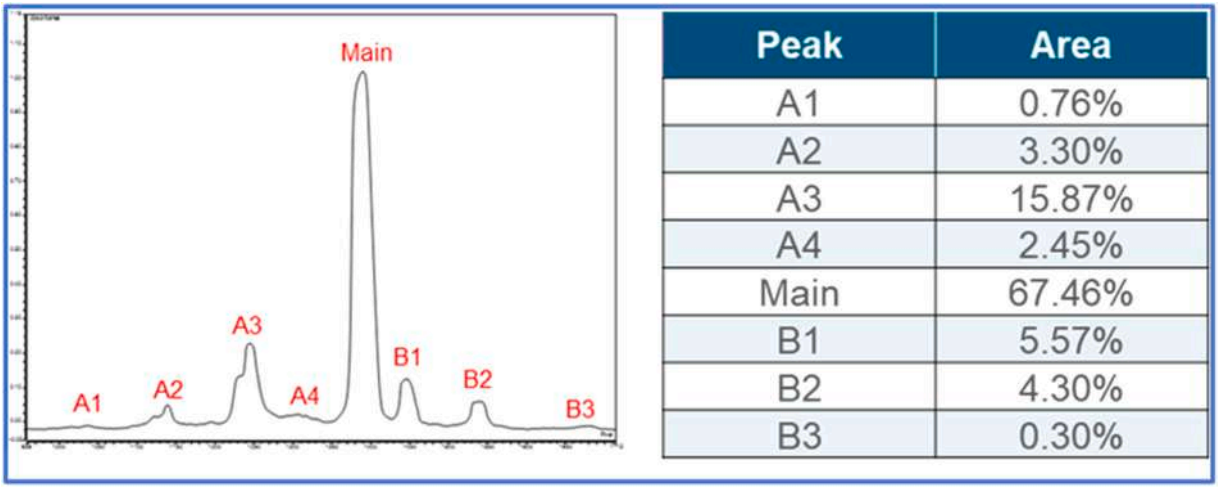


Fig. 6. icIEF- UV profile of pembrolizumab. About 1.6 μg sample was loaded on the column. A1-A4, acidic peaks; Main, main peak; B1-B3, basic peaks.

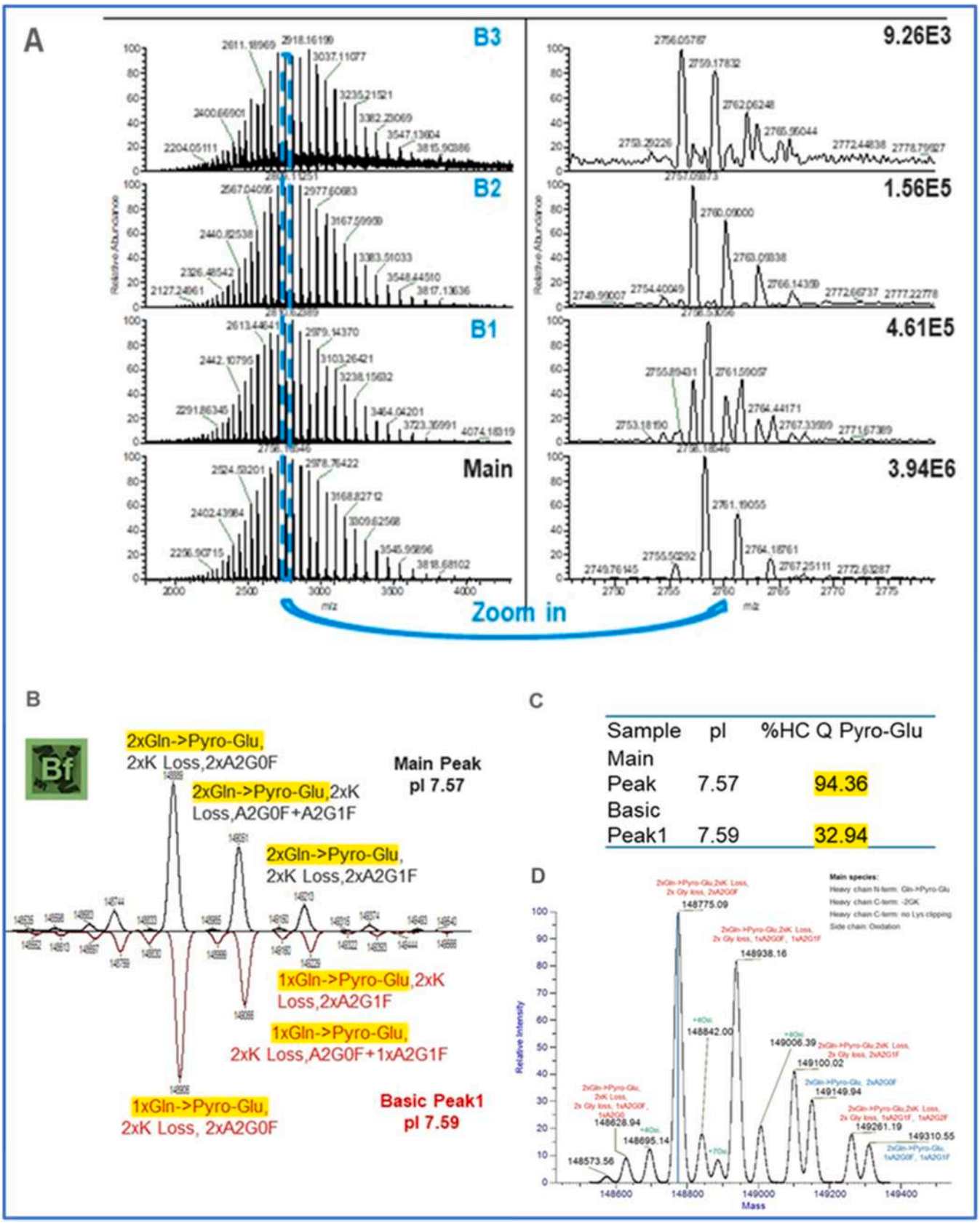
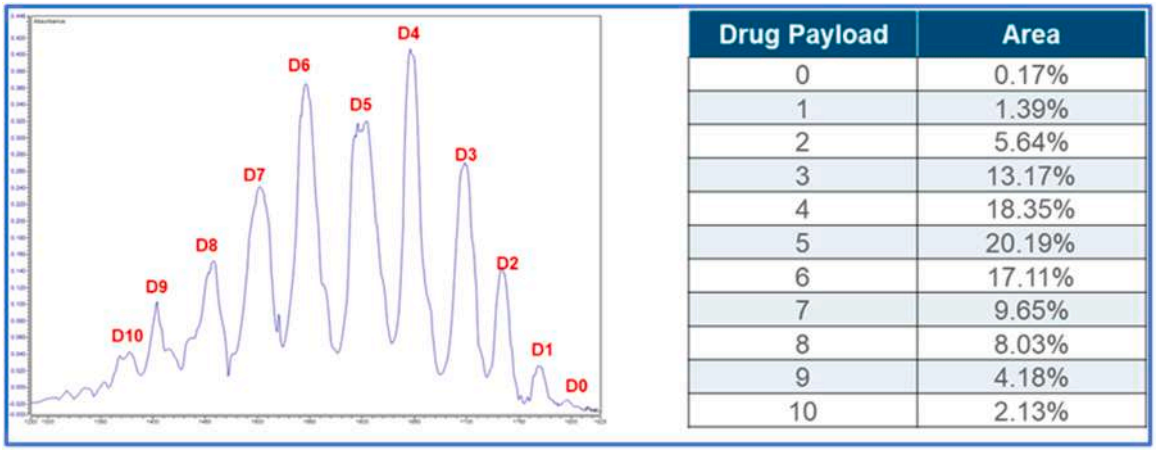


Fig. 7. icIEF-MS online coupling results of pembrolizumab charge variants.
A: raw spectra comparison of basic peaks and main peak. B: the mirror plot of main peak (pI=7.57) and basic peak1 (pI=7.59) deconvolution results. C: The Q pyro-glu % measured by previous offline peptide mapping result. D: deconvolution result of basic peak 3 (B3).



Drug Payload	Area
0	0.17%
1	1.39%
2	5.64%
3	13.17%
4	18.35%
5	20.19%
6	17.11%
7	9.65%
8	8.03%
9	4.18%
10	2.13%

Fig. 8. icIEF-UV profile of T-DM1. About 1.6 μg sample was loaded on the column. D0-D10, different drug payloads.

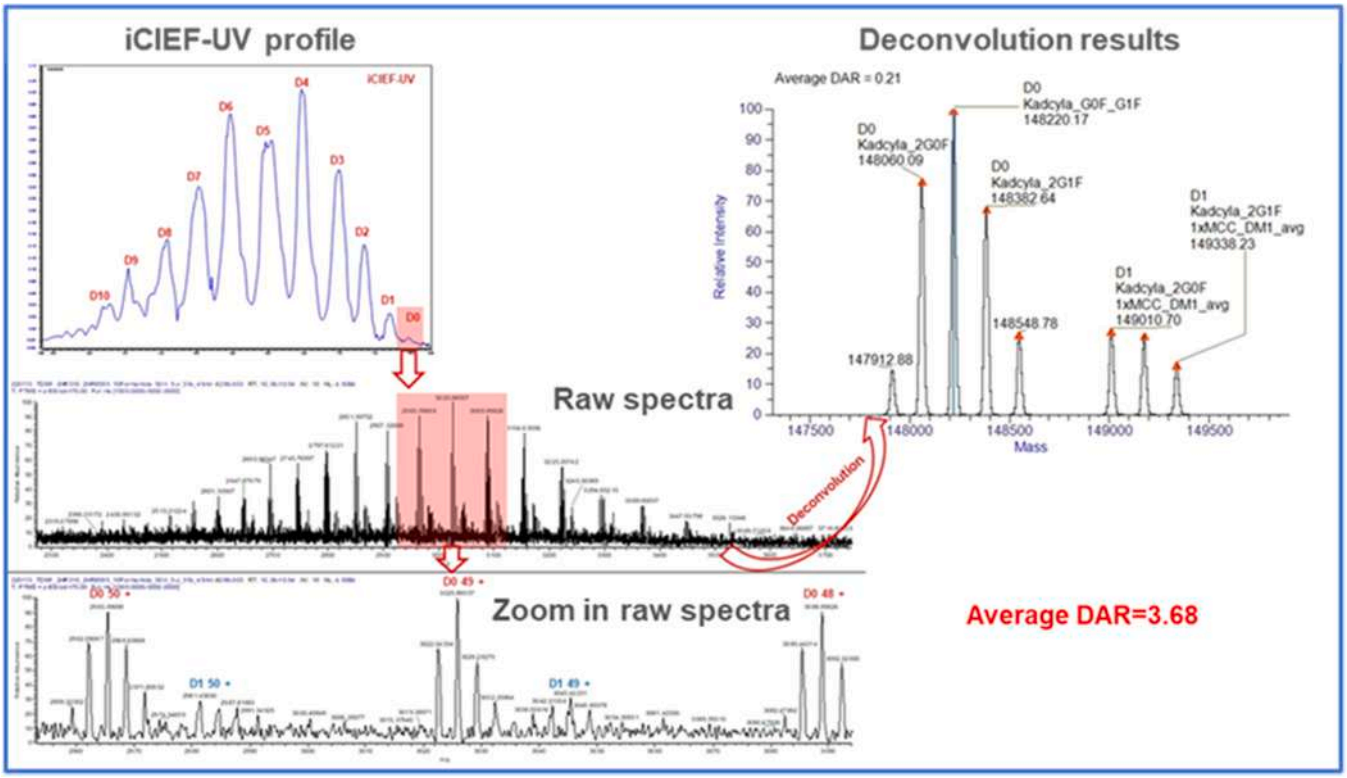


Fig. 9. icIEF-MS spectra and deconvolution results of T-DM1, D0. The complexity of MS spectra was significantly reduced due to iCIEF separation before MS detection.

3.6. icIEF-MS for antibody-drug-complex (ADC)

Trastuzumab emtansine, which is also called T-DM1, is an antibody-drug conjugate consisting of the humanized monoclonal antibody trastuzumab (Herceptin) covalently linked to the cytotoxic agent DM1. For this lysine conjugated ADC, different drug loads from D0 to D10 were clearly separated at icIEF-UV level with relative peak areas (Fig. 8). It is very meaningful because these isoforms cannot be separated on routine Reversed Phase Liquid Chromatography (RPLC)-MS.

ADCs are complex molecules composed of an antibody linked to a biologically active cytotoxic (anticancer) payload or drug, so charge-based separation before intact MS analysis can significantly reduce the complexity of spectrum, making it easier for interpretation. This is critical as different drug loads can affect an ADCs efficacy and safety.

The MS spectra and deconvolution results of RPLC-MS (data not shown) and icIEF-MS were compared and found out that using icIEF-MS strategy, separation of ADC with different drug load reduced the interference of MS signals from adjacent charge states and drug payloads. Fig. 9 shows typical results of T-DM1, D0. Therefore, the deconvolution results were simplified.

3.7. icIEF-based fraction collections for protein charged variants preparation

A preparative icIEF system was rapidly switched from icIEF-MS model just by changing customized capillary cartridge for fraction collections [19]. In this work, the preparative icIEF was used for offline fractionation of charge variants of pembrolizumab, a highly selective

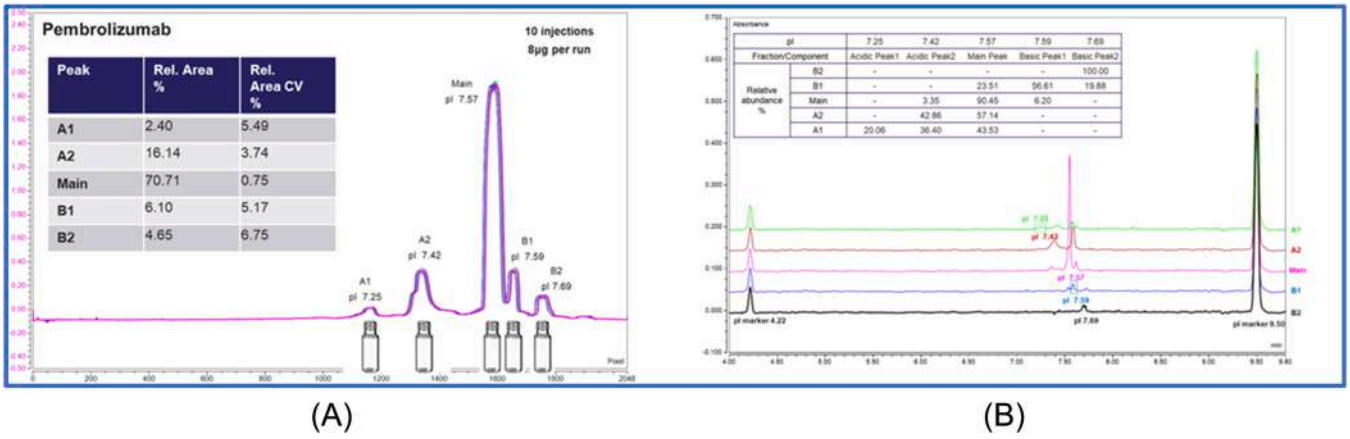


Fig. 10. Offline fractionation and confirmation of pembrolizumab' charged variants.
(A) Offline fractionation. Ten injections were overlaid and insert table shows relative peak area and CV of each peak. (B) Peak purity confirmation with reinjection of the 5 fractionated samples.

anti-PD-1 humanized mAb, followed by comprehensive peptide mapping analysis using ultra HPLC-HRMS system.

Five peaks were successfully collected - two acidic peaks, two basic peaks and main peak for subsequent peptide mapping analysis. The collection procedure is automated and ~100 μg protein was separated and fractionated in two days. The CV of relative area % are lower than 7 % for all five peaks across ten injections, and the purity of each peak was confirmed on the same instrument using analytical cartridge (Fig. 10). The following HPLC-MS peptide mapping analysis for collected fractions is in progress for a comprehensive study, which will be published in future.

4. Conclusion

Rapid and accurate characterization of protein charged heterogeneity is a critical need for therapeutics to support the rapid growth in biopharma industry, but for a long time there has not been a robust analytical platform that could simultaneously provide rapid and high-efficient charge variant separation along with the molecular mass identification of peaks. icIEF-HRMS can provide a promising strategy for differentiation and identification of protein charged variants. The icIEF-HRMS developed in this study has overcome the demerits of reported CE-MS products which are frustrated by poor repeatability and low sensitivity, to realize the highly stable platform and promising workflow based on icIEF-HRMS. In addition, icIEF-MS configuration developed can flexibly and seamlessly switch to icIEF preparative model for the μg level fraction collection of charged variants which can further be LC-MS analyzed at peptide mapping level. The total solutions allow to achieve the QA analysis, MS direct connection and fraction collection of protein charged variants on a "an integrated icIEF".

CRediT authorship contribution statement

Xiaoxi Zhang: Data curation, Formal analysis. **Min Du:** Supervision. **Victor Li:** Investigation. **Tao Bo:** Supervision, Writing - original draft, Writing - review & editing. **Tiemin Huang:** Supervision, Validation. **Tong Chen:** Conceptualization, Supervision, Validation, Writing - review & editing.

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.

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