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Cutting-edge mass spectrometry strategy based on imaged capillary isoelectric focusing (icIEF) technology for characterizing charge heterogeneity of monoclonal antibody

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ABSTRACT

Imaging capillary isoelectric focusing (icIEF) technology has been becoming the gold criteria of monitoring monoclonal antibody (mAb) charge heterogeneity that is one of the major product-related variants in recombinant biopharmaceuticals, since the first commercial instrument developed twenty years ago. However, the protein identification in icIEF separation is just based on isoelectric point (pI) measurement of protein. Although high resolution mass spectrometry (HRMS) is currently the most powerful means of qualitative protein analysis, traditional icIEF cannot compatibly be used in conjunction with MS due to the use of less volatile reagents. In addition, protein heterogeneity characterization in depth such as peptide mapping by high performance liquid chromatography (HPLC) requires the focused protein bands to be collected as fractions after the icIEF separation, which is a great challenge in biopharmaceutical discovery. In this work, pembrolizumab was employed as targeting mAb (a highly selective anti-PD-1 humanized mAb), an integrated icIEF platform was developed including analytical profiling, MS coupling and fraction collections for charged variant preparation. Multiple operation modes can be rapidly and flexibly switched just by changing customized capillary separation cartridges without more configurations. Main component, four acidic variants (A1-A4) and three basic variants (B1–B3) were baseline separated then directly detected by icIEF-HRMS online coupling for rapid screening of intact protein heterogeneity where reliable and accurate molecular weight of protein charged variants were obtained. Next, by installing preparative capillary separation cartridge, fractions of major charge variants (A2-3 and B1-2) and main component were collected for following LC-MS peptide mapping characterization. The whole workflow of icIEF-based MS strategy for protein heterogeneity is straight forward, reliable and accurate, which provides a comprehensive and revolutionary technology for protein drug quality control (QC) monitoring, MS coupling for fingerprinting intact protein and HPLC-MS peptide mapping in depth.

1. Introduction

Recombinant monoclonal antibodies (mAbs) across the pharmaceutical industry have been spurring rapid growth in the commercial and clinical products of biotherapeutics. Analyzing charge variants of therapeutic proteins is essential for the characterizing and monitoring of critical quality attributes (CQAs) such as physicochemical and immunochemical properties, biological activity, and quantity during development and manufacturing. The charge heterogeneity of protein drugs (resulting from several mechanisms including chemical degradation, cellular processes, and production conditions during the manufacturing process) requires in-depth structural characterization for CQA assessment to ensure safety, efficacy and potency [1–4]. Post translational modifications (PTMs) including C-terminal lysine truncation, pyroglutamate formation, deamidation, sialylation and glycation occurred could result in the formation of charge variants [5,6]. Among

Abbreviations: icIEF, Imaged capillary isoelectric focusing; MS, Mass spectrometry; mAbs, Monoclonal antibodies; HPLC, High performance liquid chromatography; pI, Isoelectric point.

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the modifications of these proteins, many modifications cause changes in the pI value of the protein, resulting in charge variants. Therefore, the monitoring of charge variants becomes necessary step for the quality assessment of protein drugs. Imaged capillary isoelectric focusing (icIEF) has become an indispensable tool in therapeutic protein development and manufacturing because of its high analytical throughput, ease of use, fast method development, and excellent reproducibility [7,8], however, traditional icIEF use UV as the detector. Although UV detector has good stability and sensitivity, its qualitative capability is rather limited. Hence, high resolution mass spectrometry (HRMS) is the desirable detector for icIEF to provide the most powerful means of qualitative protein characterization including the cause of charge isomers and accurate qualitative analysis of charge isomers. However, traditional icIEF cannot be directly connect to MS due to the use of non-MS friendly reagents. In addition, in-depth peptide mapping analysis by HPLC-MS needs the fraction collections of focused protein bands in icIEF separation, which is extremely challenging. Although a diverse of chromatography-mass spectrometry coupling [11–14] and separation-based fraction collection [15–18] technologies including Ion exchange (IEX) chromatography, isoelectric focusing (IEF) and free flow electrophoresis (FFE) have been involved in protein charged variant characterization, most of them are low-throughput, poor repeatability, tediousness and incompatible reagents used for MS coupling. Also, the protein variants tend to be denatured during the traditional chromatography separation, which is incomparable with those in icIEF analysis. So, icIEF routine analysis, icIEF-MS tandem technology and the fraction collections for preparative icIEF in an integrated platform is the highly desirable method, which can sharpen biopharmaceutical discovery in width and depth.

In this work, a cutting-edge icIEF-MS strategy was developed for achieving both of the rapid icIEF separation and reliable HRMS identification of protein charge variants simultaneously. MS-compatible amphoteric electrolytes and both of polymer (such as methylcellulose, MC) -free and urea-free cartridges in icIEF analysis have been innovatively developed, which can realize zero-volatile reagents in the analysis of protein charge variants. The innovative microliter interface improves the sensitivity of identifying protein charge variants. The whole icIEF-HRMS analysis based on seamless MS interface can be solved within 60min. The established methodology has high sensitivity, high throughput and good repeatability, which was employed to the charge heterogeneity characterization of pembrolizumab (a highly selective anti-PD-1 humanized mAb). In addition, the developed icIEF-MS configuration by changing preparative capillary separation cartridge in our laboratory [19] can rapidly and flexibly switch to icIEF-based fraction collection mode for protein charge variants following the peptide mapping by high performance liquid chromatography (HPLC) tandem HRMS. The whole workflow of icIEF-MS for protein charge heterogeneity analysis is straight forward, accurate and reliable.

2. Materials and methods

2.1. Materials

All ampholytes (AESlytes) were obtained from Advanced Electrophoresis Solutions Ltd (AES, Cambridge, Ontario, Canada). Pembrolizumab employed in this study was kindly donated from Thermo Fisher Scientific (China). Mass spectrometry grade acetonitrile (ACN) and formic acid were purchased from Fisher Scientific (Hampton, NH).

2.2. Solutions for icIEF

For icIEF-MS of pembrolizumab: HR AESlyte 6–8 (carrier ampholytes) was added into commercially available pembrolizumab (25 mg/mL) and diluted using deionised water. Final protein concentration is 1 mg/mL with 2% HR AESlyte 6–8 *(v/v)* mixed in deionised water.

For preparative icIEF of pembrolizumab: HR AESlyte 7–8 (carrier ampholytes) was added into commercially available pembrolizumab (25 mg/mL) and diluted using deionised water. Final protein concentration is 2 mg/mL and 4%HR AESlyte 7–8 (*v/v*). 5 peaks were picked up and collected. Each vial was a combination of 15–20 runs (8μg/run). The fractions were reduced, alkylated then digested with trypsin for following LC-HRMS peptide mapping analysis.

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 coated (AD) capillary cartridges (AES, cat. no. CP00303) and micro-tee integrated (AES, cat. no. CP00303 M) 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 transfer capillary to MS ion source. Both the make-up solution capillary and inlet capillary have a 100 μm ID.

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 15 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: 3.9 kV, sheath gas: 10 L/min, auxiliary gas: 0L/min, S-lens RF 60 eV, microscan: 10, capillary temp: 275 °C, Aux gas temp.: 100 °C, resolution 35,000@*m/z* 200, scan range of precursor ion 1500–5000 m/z and maximum injection time 200 ms.

2.5. UHPLC-MS for peptide mapping of collected protein variant fractions

The collected protein variant fractions were digested as follow: The collected fractions were transferred to centrifugal filter insert tube (Millipore, 3 KDa cutoff) respectively, then 200 μL denaturing buffer (7.0 M Guanidine HCl, 100 mM Tris, pH 8.3) was added into each tube. The centrifugal filter unit was centrifuged at 21,000 g *10min, then the liquid in outer tube was discarded and 200 μL denaturing buffer was added into insert tube. The exchange step was repeated 2 times (3 times in total) to change to denaturing buffer system. The sample volume is around 70 μL after buffer exchange.

The denatured samples were then reduced using 10 mM DTT at 37 °C for 30min, followed by 20 mM IAC incubation at room temperature for 20min in the dark. After that, 200 μL digestion buffer (50 mM Tris-HCl, pH = 7.9) was added into each tube. The centrifugal filter unit was centrifuged at 21,000 g *10min, then the liquid in outer tube was discarded and 200 μL digestion buffer was added into insert tube. The exchange step was repeated 2 times (3 times in total) to change to digestion buffer system.

Trypsin (Fisher, China, MS grade) was used for protein digestion (Trypsin:sample = 1:10, w/w, 37 °C, 30min). Formic acid was used to quench digestion (final concentration 1%). Final sample volume is about 50–60 μL, can be transferred to sample vials for peptide mapping.

The digested peptide samples were separated on a Thermo Scientific™ Vanquish™ UHPLC system (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile using an Acclaim Vanquish C18 column (120 Å; 2.2 μm, 2.1 × 150 mm, P/N 071399-V, 40 °C, 300 μL/min) with a linear gradient (10%-35%B, 6–70min) and detected by a Q Exactive Plus

Biopharma mass spectrometer for peptide mapping. For each sample, four injections were carried out using same LC gradient, one ddMS2 top5 run for identification and three MS1 only runs for quantification. Data acquisition was performed by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.10.

2.6. Data analysis

Data analysis of intact protein deconvolution and peptide mapping was performed using Thermo Scientific™ Biopharma Finder™ software (BPF 4.1) and Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.10.

3. Results and discussions

3.1. MS strategy based on icIEF technology for characterizing charged heterogeneity of protein drugs

As seen in Fig. 1, an innovative MS strategy based on icIEF technology was demonstrated in an integrated platform for comprehensive characterization of mAb charge variants including rapid intact protein identification by icIEF-MS coupling and following HPLC-MS peptide mapping of collected fractions from preparative icIEF. The whole workflow can unity two essential solutions above on a unique icIEF system to carry out in-depth characterization of observed protein peaks in the icIEF-UV analysis.

3.2. icIEF-UV for pembrolizumab

Fig. 2 (A) demonstrated that main component, acidic variants (A1-A4) and basic variants (B1–B3) of pembrolizumab were baseline separated within 10min and the percentages based on peak area of each peak was illustrated in Fig. 2 (B). It was observed that acidic peak A1 and basic peak B3 were rather trace with very low UV abundance, which percentages were 0.76 and 0.30, respectively. How to identify protein charge heterogeneities with extremely low concentrations is critical so an icIEF-HRMS method was developed for addressing such a challenge as follows.

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

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. 3 (A), while only using proprietary coated capillary and separation solvents during icIEF separations greatly reduce the need for polymers and urea. It enables the isolated protein charge variants to be directly used for high-sensitive MS intact analysis, thus retaining the excellent separation resolution of icIEF for mass spectrometry analysis. Seamless interface to MS based on micro-fluidity, to enhance the sensitivity of MS detection of proteins. The constructed system requires no special modifications to the ion source and can be directly connected to the mass spectrometer ion source from the

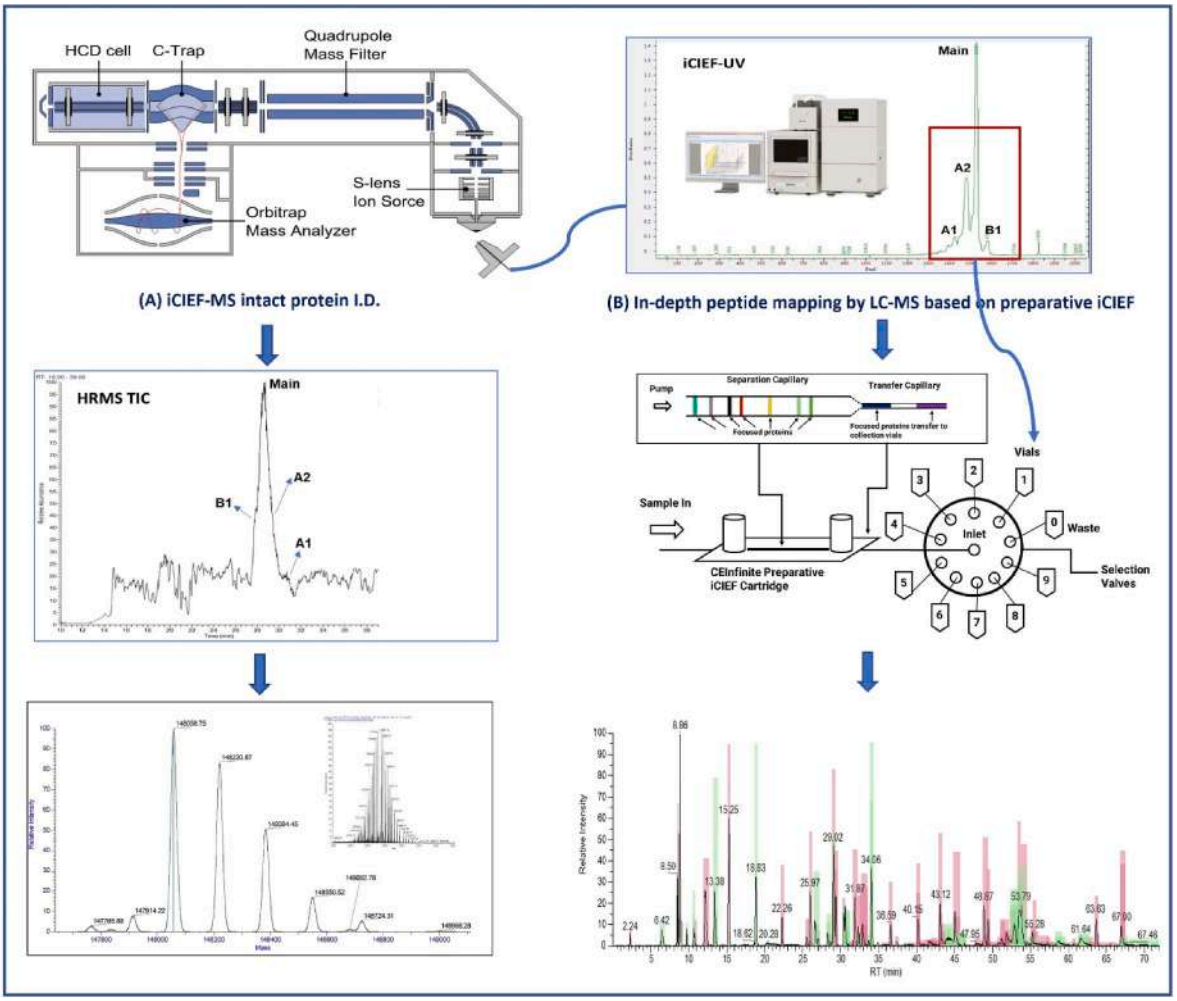


Fig. 1. MS strategy based on icIEF technology for the characterization of protein charged heterogeneity (A) icIEF-MS coupling for intact proteins and (B) HPLC-MS peptide mapping of collected fractions by preparative icIEF based on pI differentiation.

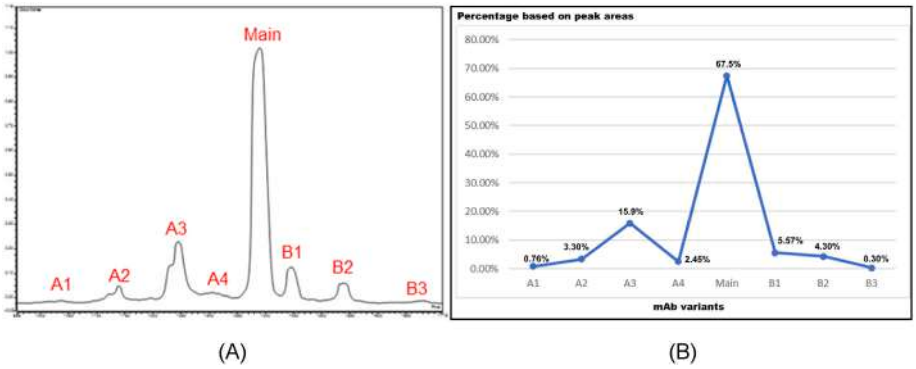
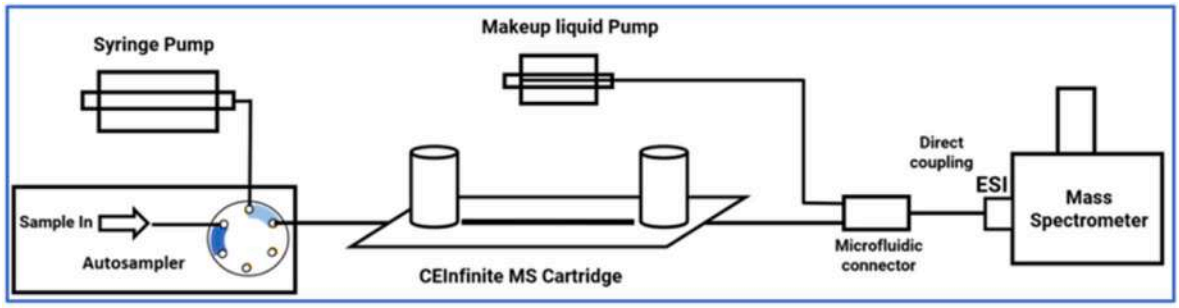


Fig. 2. (A) icIEF- UV profile of pembrolizumab and (B) percentage of mAb variants. 1.6 μg sample was loaded on the column. A1-A4, acidic peaks; Main, main peak; B1–B3, basic peaks.



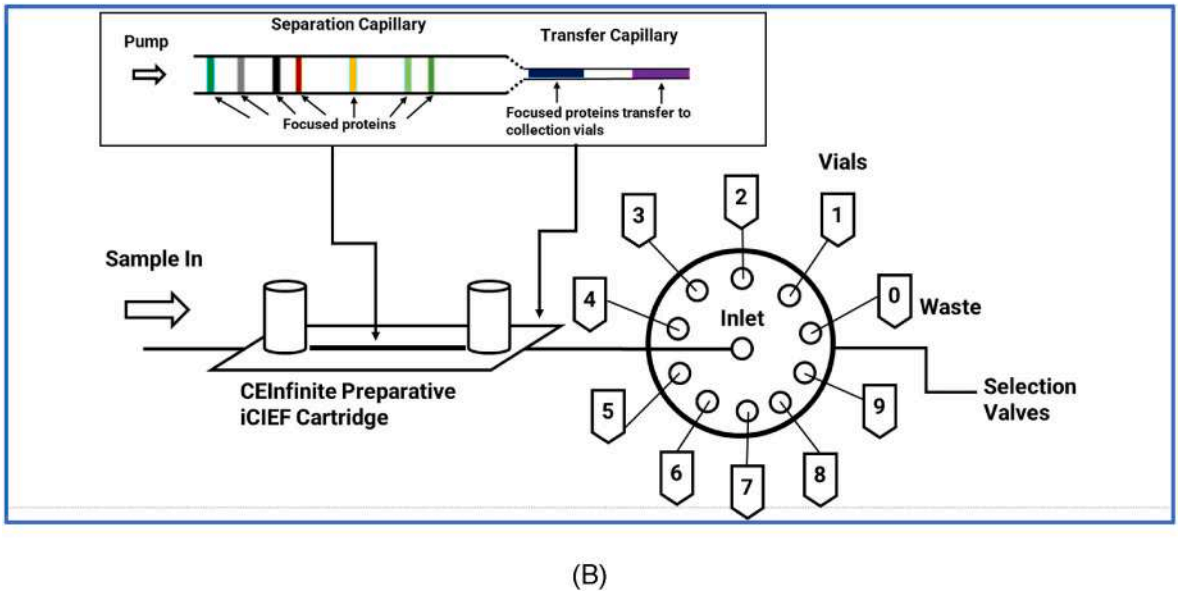


Fig. 3. Schematics of (A) icIEF-MS coupling and (B) fraction collections for preparative icIEF.

different mass spectrometry brands [19].

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 at 50–200 nL/min flowing rate depending on selected separation capillary cartridge I.D. (typically 50 nL/min for 200 μm I.D. cartridge; and 160 nL/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 MS ion source though a seamless interface. The whole process is automatic and with minimum user interference.

As illustrated in Fig. 3B, the developed icIEF preparation technology can realize the separation, collection and preparation of high-purity protein-charged heterogeneous products. Based on the simple and

easy-to-use icIEF method for the isolation of protein-charged heterosomes, minimum method development is required. Fully automated fraction collection, which can separate about 16 μg protein in 45 min for further mass spectrometry characterization or MALDI-MS analysis in just one run, can be repeatedly collected to relatively high amounts of isomers for peptide mapping analysis or activity detection. The use of customized cartridges eliminates the need for polymers and reduces methylcellulose contamination, which is further compatible with the mass spectrometry tandem detection.

3.4. icIEF-MS for pembrolizumab

High sensitivity of high-resolution mass spectrometry allows for sensitively identifying trace protein variants. Especially, as demonstrated in Fig. 4, basic variant peak B3 with trace concentration (~0.3% of all peaks, ~4.8 ng loading) of pembrolizumab can be well detected with good signal to noise ratio with the use of icIEF-MS. The comparison of raw spectra of basic peaks and main peak were achieved (Fig. 4A) and it is easily to observe mass shift even at raw spectrum level since basic variants are mainly produced by heavy chain C-terminal truncation,

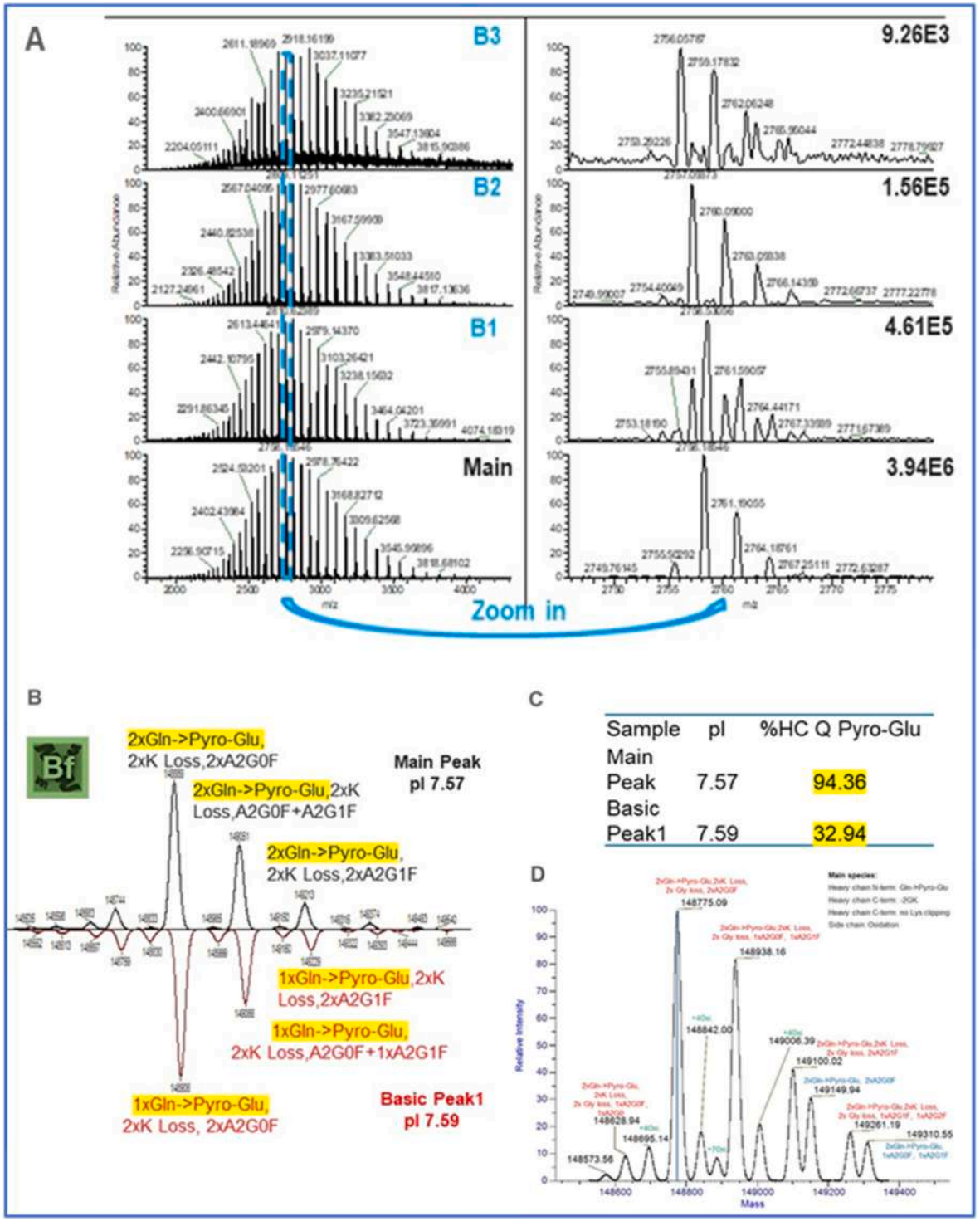


Fig. 4. icIEF-MS online coupling results of pembrolizumab charge variants.

changing in percentage of heavy chain N-terminal pyroglutamate cyclization and oxidation.

The mirror plot of main peak (pI = 7.57) and basic peak1 (pI = 7.59) was shown in Fig. 4B for the deconvolution results. A series of 17Da mass shift were detected for each glycoform. The reason is that comparing to main peak, only one glutamine at N-terminal of heavy chain was cyclized to form pyroglutamate. And this intact protein analysis results correspond with the reported peptide mapping result as follows.

All charge variants identification results were summarized in Table 1. For basic peaks, the main PTMs caused charge heterogeneity are heavy chain C-terminal truncation, changing in percentage of heavy chain N-terminal pyroglutamate cyclization and oxidation. All the modification details could be clearly read out in the MS intact results. For example, -GK truncation (−185Da) was identified on C-terminal of both heavy chains in the lowest basic peak (B3), which is two orders lower than main peak (Fig. 4D). In acidic peaks, series of deamidation and different sialic acid modifications were identified. More details about PTMs will be discussed in the following peptide mapping part.

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

In this work, the preparative icIEF shown in Fig. 3B was used for offline fractionation of charge variants of pembrolizumab, followed by comprehensive peptide mapping analysis using ultra HPLC-HRMS system.

In this study, five major peaks observed in Fig. 1 were successfully collected - two acidic peaks (A2 and A3), two basic peaks (B1 and B2) 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 (Fig. 5 A and 5 B), and the purity of each peak was confirmed on the same instrument using analytical cartridge (Fig. 5C and Table 2).

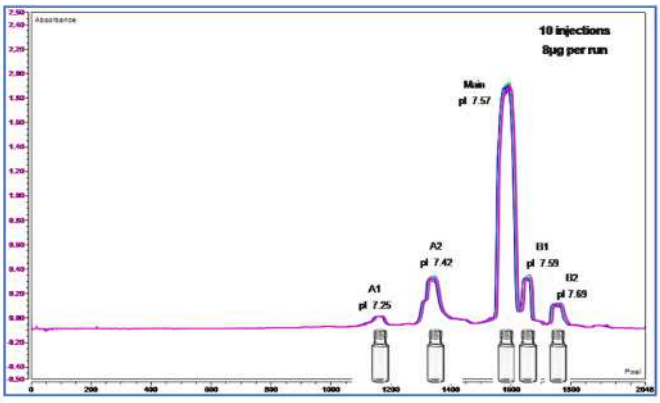
3.6. HPLC-HRMS for peptide mapping analysis of five collected fraction by preparative icIEF

For all five peaks, although the relative abundance of charge variants is quite low listed in Fig. 2(B) (3.30% of acidic peak A2, 5.57% of basic peak B1 and 4.30% of basic peak B2), the sequence coverages are greater than 98% (Fig. 6), which proves the excellent sensitivity of HPLC-MS platform.

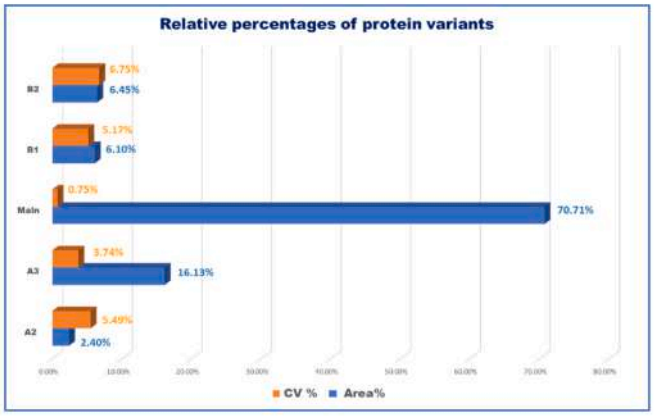
In this study, selected CQAs including terminal modifications, N-glycans and side chain modifications such as deamidation, succinimidation and oxidation were identified and relatively quantified in all acidic, basic and main peak. More meaningfully, %modification varies

Table 1
Identification pembrolizumab' charge variants. HC = heavy chain.

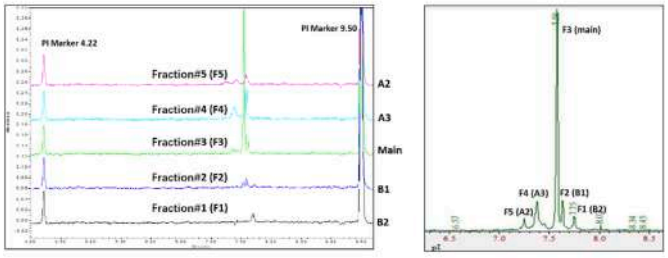
Peak	Main	B1	B2	B3
Modifications	HC N-term: Gln- > Pyro-Glu HC C-term: 2K	HC N-term: Gln- > Pyro-Glu (~30%) HC C-term: 2K	HC N-term: Gln- > Pyro-Glu HC C-term: -1GK&-1K HC C-term: one Lys clipping Side chain: Oxidation	HC N-term: Gln- > Pyro-Glu HC C-term: 2 GK HC C-term: no Lys clipping Side chain: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



(A)



(B)



(C)

Fig. 5. Offline fractionation and confirmation. (A) Overlapped electropherograms of ten consecutive injections were in the offline fractionation; and; (B) Relative percentages of protein variants. (C) Peak purity confirmation with reinjection of the 5 fractionated samples.

Table 2
Confirmation of peak purity with reinjection of the 5 fractionated samples.

pI		7.25	7.42	7.57	7.59	7.69
Fraction/Component		Acidic peak 1	Acidic peak 2	Main peak	Basic peak 1	Basic peak 2
Relative Abundance %	B2	–	–	–	–	100
	B1	–	–	23.51	56.61	19.88
	Main	–	3.35	90.45	6.60	–
	A3	–	42.86	57.14	–	–
	A2	20.06	36.40	43.53	–	–

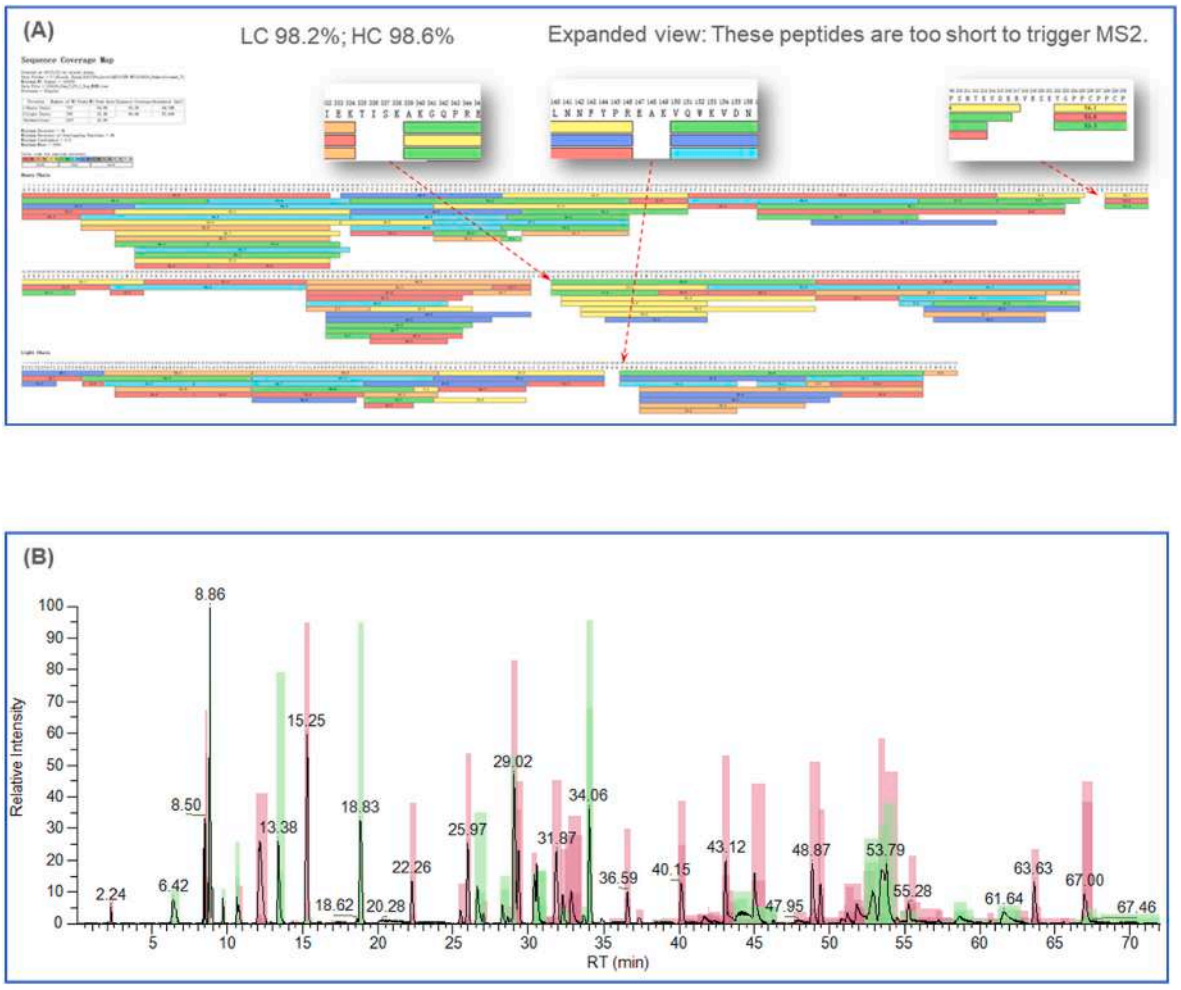


Fig. 6. Sequence coverage map and base peak chromatogram of main peak (pI = 7.57). (A) Sequence coverage map and (B) Base peak chromatogram. Green shade, light chain peptides. Pink shade, heavy chain peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

among different variants were also quantified. Figs. 7–10 show the relative abundance% of each CQA in different peaks.

The terminal modifications in all peaks were shown in Fig. 7. Fig. 7A indicated that the % Lysine clipping in basic peak B2 (pI 7.69) is 69.48% and 91.00% in basic peak B1 (pI 7.59) compared to the results that the Lysine clipping in main and acidic peaks are nearly complete. In basic peak B1 (pI 7.59), only ~30% pyroglutamate cyclization was detected while 94.36% in main peak, 92–94% in both acidic peaks and 93% in basic peak B2 (pI 7.69) were detected. Usually pyroglutamate cyclization produces acidic variants [20] so it makes sense that in basic peak B1, the percentage of pyroglutamate cyclization decreased. All relative abundance were averaged across three technical replicates and CV<6%.

As demonstrated in Fig. 8, it is noteworthy that the percentage of sialic acid contained glycoforms increased 5–10 times in acidic peaks compared to main peak. The changing of glycans were observed at both intact and peptide mapping levels. In our previous work [19], the icIEF-MS intact data of acidic peaks included some low abundance components, which made characteristic mass shift of sialic acid. Then the glycan modification searching parameters were set up when processing peptide mapping data. Form peptide mapping result, it was found that on conserved N-glycosylation site (heavy chain N297), besides popular glygoforms such as A2G0F/A2G1F/A2G2F, sialic acid contained species, like A2S1G0F/A2S1G1F/A2S2F were identified and the percentage of these glycoforms increased in acidic peaks, which was corresponding to intact MS result. Also, the relative abundance of A1G0F increased significantly in basic peaks.

Deamidation and oxidation are also critical PTMs effect charge heterogeneity of mAb. In this study, N55 was taken as an example to show the deamidation% and succimidation% variations among acidic peaks and main peak. This site was selected because it is within the Complementarity Determining Region (CDR) domain and it is involved in van der Waals contact with PD-1 [21]. As mentioned in Fig. 9, the level of % HC N55 deamidation is 0.18% in main peak (pI 7.57) compared to 0.93% in acidic peak A2 (pI 7.25) and 0.83% in acidic peak A3 (pI 7.42). It is easily to observe that the area of deamidated peaks increased. HC N55 succinimidation level decreased in acidic peaks compared to main peak. It is reasonable because succinimidation is an intermediate reaction during asparagine deamidation. Oxidation is a common modification at methionine or tryptophan, but not every oxidation variant will involve a change in pI. Thus, we monitored the oxidation% of all methionine and tryptophan in the sequence and focused on those increased significantly in basic peaks across multiple sites compared to their levels in the main peak, as illustrated in Fig. 10. Among these sites, M34, M48 and M105 at heavy chain are within CDR domain and M105 is involved in van der Waals contact with PD-1 [21]. M252, M328 and M428 are located in Fc region. Modifications in the Fc region of IgG-type antibodies can shorten serum half-life or affect effector functions such as antibody dependent phagocytosis (ADCP) [22], which have been employed by antibody-based therapies to target the immune system to pathogens or cancer cells [23]. Cymer et al. discovered that M252 oxidation affects binding to Fc-gamma receptor IIa (FcγRIIa), involved in ADCP [24].

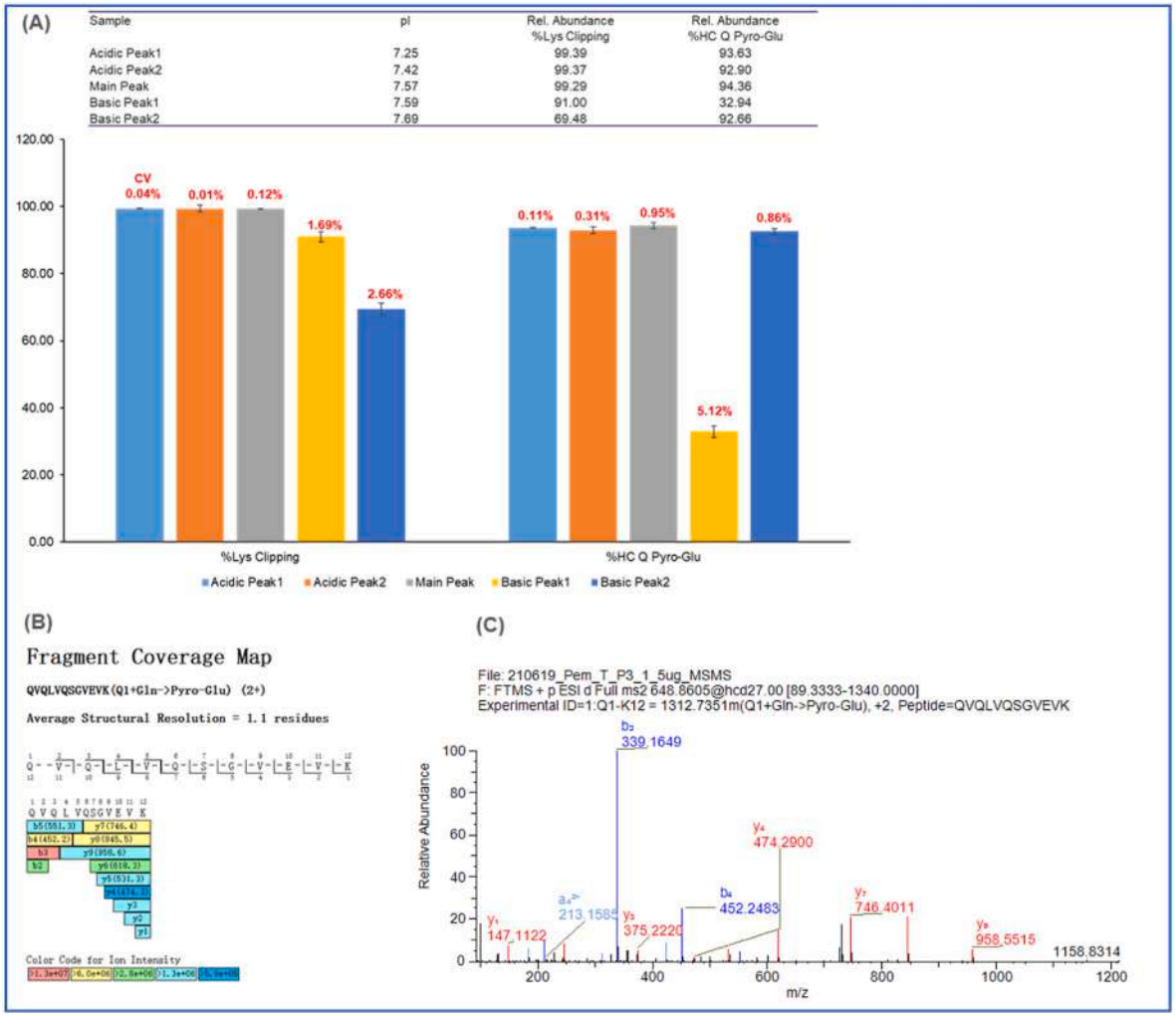


Fig. 7. The terminal modifications in all peaks. (A), the percentage in all peaks. (B), fragment coverage map of pyroglutamate cyclization peptide from main peak. (C), MS2 spectrum of this peptide.

3.7. Advantages of developed MS strategy with icIEF as the separation-front

icIEF or cIEF as a robust separation front-end for MS detection have been exhibited high-efficient capacity for the characterization of protein charge heterogeneity [11–14,20], but improvements are still required for addressing the challenges in terms of online MS detection and fraction collections after the icIEF separation in terms of MS compatibility, selectivity and operation flexibility. Chip-based icIEF-MS reported [13,14] depends on chemical mobilization for MS detection, which tends to result in instability of pH gradient during the chemical mobilization. Traditional cIEF with single point detection has the disadvantages of low throughput and cumbersome operation due to tedious mobilization process and long-time separation for MS detection after protein focusing [12]. The established icIEF-MS platform in our study exhibited significant advantages over reported results. Instead of chemical mobilization, the mobilization solution at nano flowing rate (30–100 nL/min) using syringe pump pushed the protein peaks focused out of separation capillary towards MS ion source or fraction collectors. This guaranties better repeatability in the process of online icIEF-MS including protein focusing with following mobilization and adjusting mobilizing rate can more flexibly tune the icIEF-MS selectivity. In the whole process of icIEF-MS, 3 kV voltage applied remained separation resolution to the greatest extent. Moreover, the critical reagents in icIEF play an essential role in high-performance icIEF and icIEF-MS analysis. Developed high-resolution carrier ampholytes with narrow pH range in this study were utilized for effectively improving the separation resolutions. The coatings of capillary are also important for high-efficient online icIEF-MS. Besides routine fluorocarbon (FD) and acrylamide derivative (AD) coated capillary used in this studied, the customized methylcellulose (MC) coated capillary cartridge is developed for polymer-free icIEF and online icIEF-MS to simply the whole workflow. The related studies are in progress and will be published in future.

Mass spectrometers offer class leading accurate mass and resolving power in both intact MS and peptide mapping analysis. Mass analysis of intact proteins is easy, fast and can be high throughput, quickly confirm critical quality attributes assessment and rapid identity confirmation for lead selection studies. For peptide mapping, HRMS provide the most effective way to accurately and confidently identify peptides. The high-resolution Orbitrap mass spectrometer with HPLC give the confidence to perform peptide mapping where full characterization is required and complete sequence information needed.

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, 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. However, due to different separation mechanisms and resolutions of IEX, IEF and FFE from icIEF technology, the fraction collected usually cannot well match with the results from icIEF and the preparative proteins tend

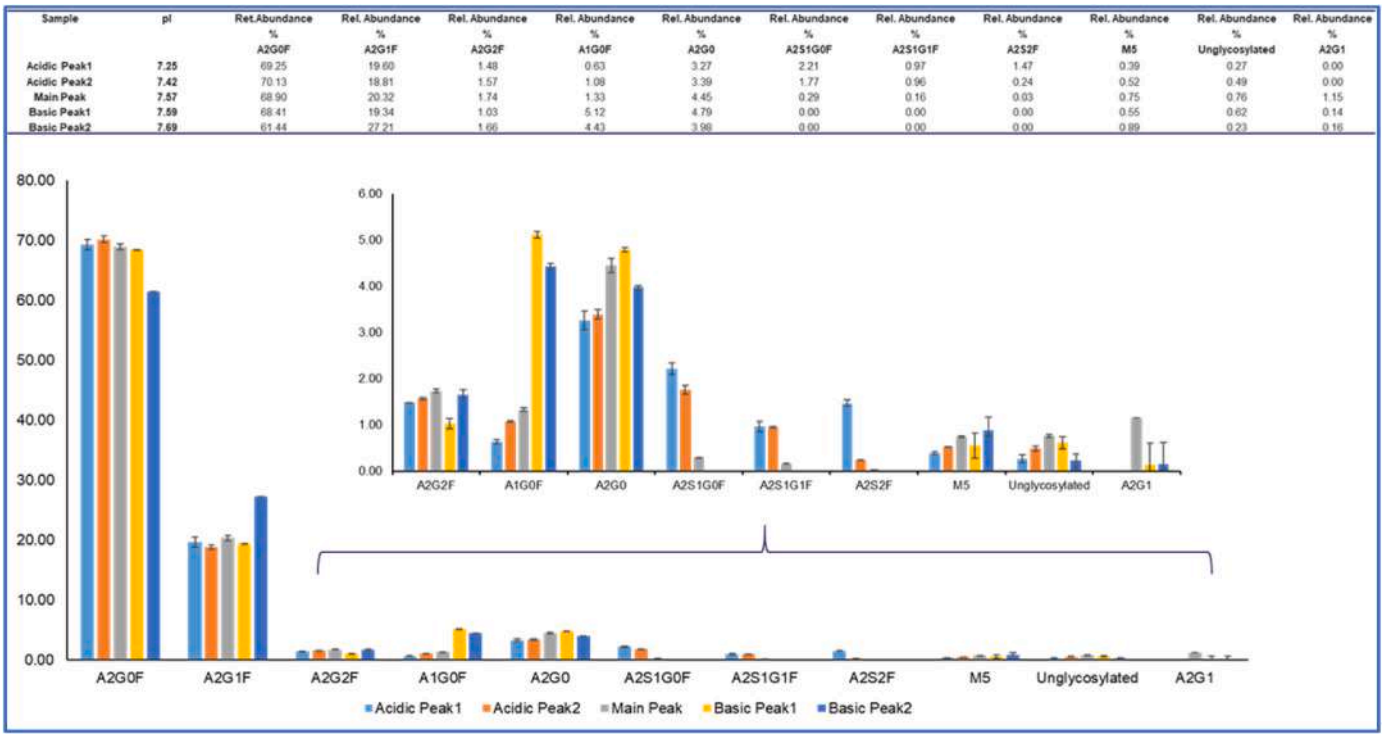


Fig. 8. The percentage of N-glycan in all peaks. Expanded view, N-glycans <6%.

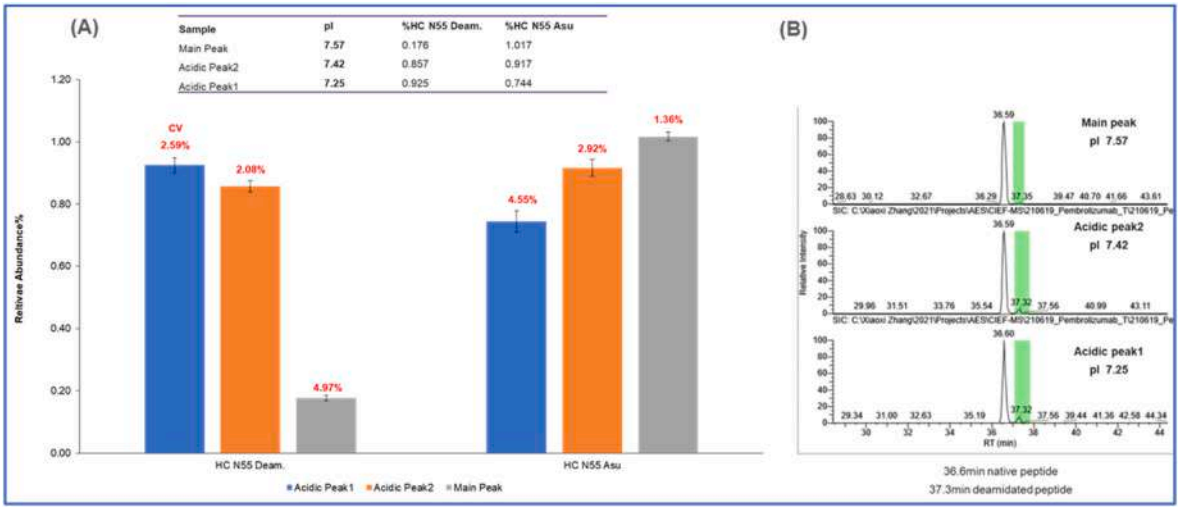


Fig. 9. HC N55 deamidation and succinimidation in main and acidic peaks. (A), the percentage distribution. (B), extracted ion chromatogram of native and deamidated peptides in main and acidic peaks.

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 o protein charge variants.

4. Conclusions

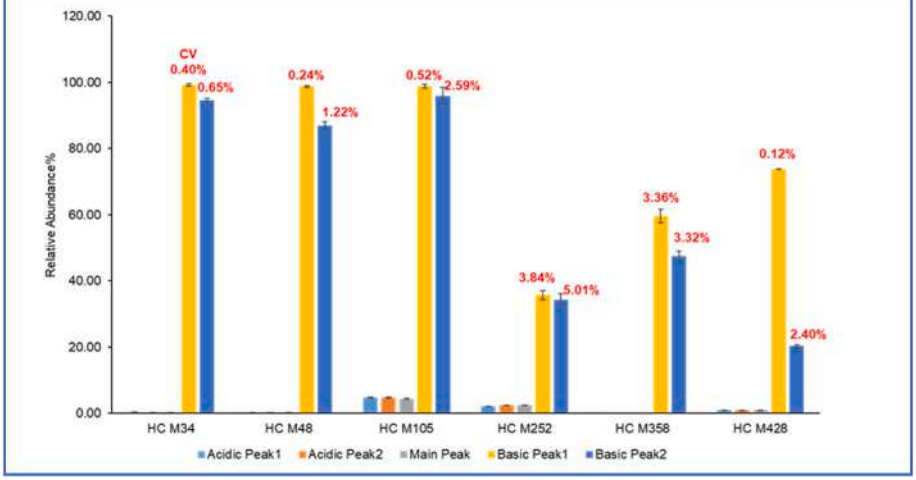
A comprehensive MS strategy based on highly efficient and robust icIEF has been developed for intact protein characterization by icIEF-MS coupling and HPLC-MS peptide mapping analysis for collected fractions of mAb charged variants by preparative icIEF, which can be carried out on the same icIEF platform. The developed icIEF-MS has been introduced and demonstrated that a highly effective icIEF-MS of protein charge variant can be realized within 30min. As for preparative icIEF, the purity of the collected protein variants can be confirmed and used for further in-depth protein characterisation by LC-HRMS peptide mapping. The whole workflow of icIEF-based MS strategy for protein heterogeneity is straight forward, reliable and accurate, which provides a comprehensive and revolutionary technology for protein drug quality control (QC) monitoring, MS coupling for rapid fingerprinting intact protein and HPLC-MS in-depth characterization.

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 peak3 (B3).

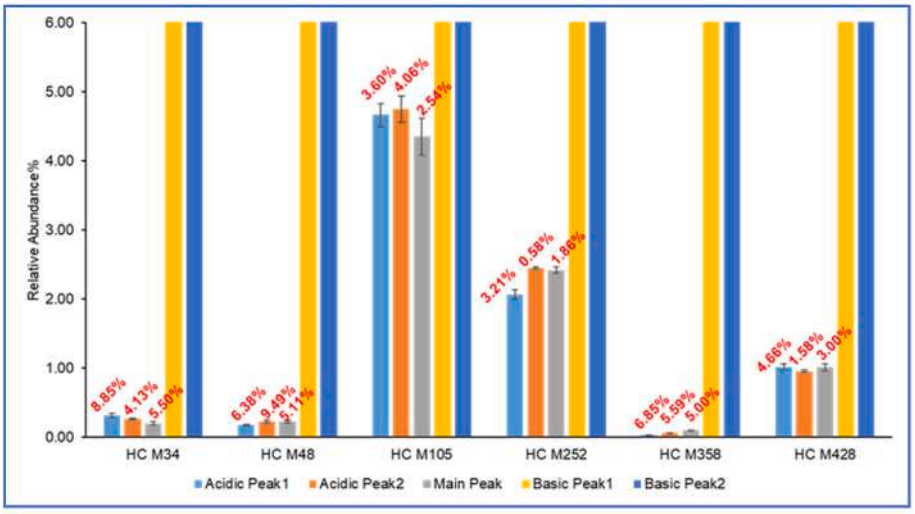
All relative abundance were averaged across three technical replicates.

All relative abundance were averaged across three technical

Sample	pI	Rel. Abundance %HC M34	Rel. Abundance %HC M48	Rel. Abundance %HC M105	Rel. Abundance %HC M252	Rel. Abundance %HC M358	Rel. Abundance %HC M428
Acidic Peak1	7.25	0.32	0.17	4.66	2.06	0.03	1.02
Acidic Peak2	7.42	0.27	0.23	4.74	2.45	0.06	0.95
Main Peak	7.57	0.20	0.22	4.35	2.42	0.10	1.01
Basic Peak1	7.59	99.19	98.80	98.89	35.71	59.63	73.77
Basic Peak2	7.69	94.60	86.99	95.97	34.38	47.45	20.25



(A)



(B)

Fig. 10. The percentage of Oxidation in all peaks. (A) Overview and (B) Expanded view of components <6%.

replicates.

All relative abundance were averaged across three technical replicates.

Data availability

Data will be made available on request.

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