In Depth Characterization of mAb Charge Variants with iCIEF Fractionation Followed by Peptide Mapping Analysis

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ABSTRACT

Purpose: Applying the world's first Preparative iCIEF solution for charge variants separation and fractionation followed by LC-MS based peptide mapping.

Methods: iCIEF separation for mAb charge variants and peptide mapping.

Results: In this study, we successfully collected five iCIEF peak fractions and analyzed them by peptide mapping to assess and compare the product quality attributes (PQAs) in each charge variant species.

INTRODUCTION

Protein separation, purification and analysis are crucial components in biotherapeutic development, but the process can be complex and challenging. Isolating charge isomer peaks for identification would provide invaluable information for stability studies, as well as formulation and process control. Imaging Capillary isoelectric focusing (iCIEF) is an essential technique widely adopted in QC environment to both visualize and quantify the presence of charge variants in protein samples. Whole column imaging detection (WCID) capillary electrophoresis (CE) systems have revolutionized protein charge related heterogeneity analysis with iCIEF. In this study, a CEInfinite Preparative iCIEF system was used for offline fractionation of charge variants of pembrolizumab, a highly selective anti-PD-1 humanized mAb, followed by comprehensive peptide mapping analysis using UHPLC-HRAM MS system.

MATERIALS AND METHODS

Sample Preparation

HR AESlyte 7-8(carrier ampholytes) was added into commercially available pembrolizumab (25mg/mL) and diluted using ddH_2O. Final protein concentration is 2mg/mL and 4%HR AESlyte 7-8 (v/v) .

Patented cartridge (ID320μm) was used for separation. 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.

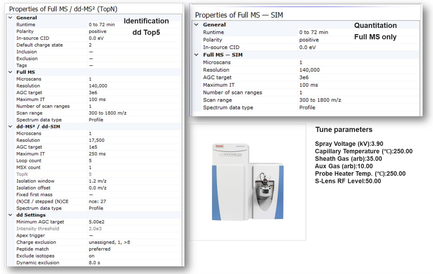
Test Methods

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 Thermo Scientific™ Acclaim™ Vanquish C18 column (120Å, 2.2 μm, 2.1 x 150 mm, P/N 071399-V, 40°C, 300 μL/min) with a linear gradient (10%-35%B, 6-70min) and detected by a Thermo Scientific™ Q Exactive™ Plus Biopharma mass spectrometer for peptide mapping. For each sample, four injections were carried out using same LC gradient, one ddMS^2 top5 run for identification and three MS1 only runs for quantification (Figure1).

Data Analysis

Data analysis was performed using Thermo Scientific™ Biopharma Finder™ software and Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.10.

Figure 1. MS settings.



RESULTS

Automated charge variants separation and collection using CEInfinite Preparative iCIEF System with high reproducibility and efficiency

In this study we successfully collected five peaks - two acidic peaks, two basic peaks and a main peak for subsequent peptide mapping analysis. The collection procedure is automated and ~100μg protein was separated and fractionated in two days (Figure 2). 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 (Figure 3).

Figure 2. CEInfinite Preparative iCIEF System.
(A), The instrument. (B), Capillary diameter transfer technology.

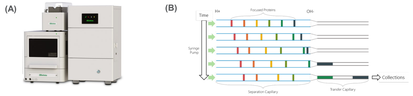
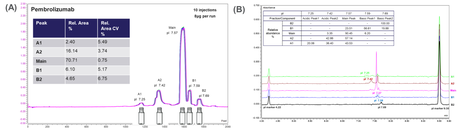


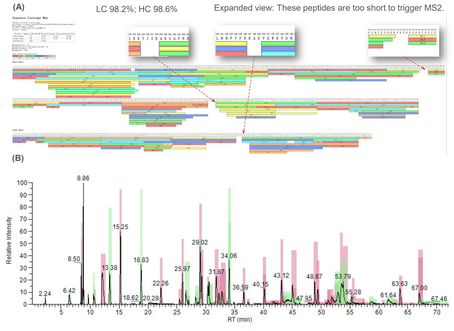
Figure 3. Offline fractionation and confirmation.
(A), Offline fractionation. Ten injections were overlaid and insert table shows relative peak area and CV of each peak. (B), Peak purity confirmation with reinjection of the 5 fractionated samples.



Peptide mapping – sequence coverage

For all five peaks, although the relative abundance of charge variants are quite low (2.40% of acidic peak1, 6.10% of basic peak1 and 4.65% of basic peak2), the sequence coverages are greater than 98% (Figure 4), which proves the excellent sensitivity of UHPLC-MS platform.

Figure 4. Sequence coverage map and base peak chromatogram of main peak (pI=7.57).
(A), Sequence coverage map. (B), Base peak chromatogram. Green shade, light chain peptides. Pink shade, heavy chain peptides.



The modification% of PQAs in different peaks

In this study, selected PQAs 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 among different variants. Figure 5-8 show the relative abundance% of each PQA in different peaks.

Figure 5. The terminal modifications in all peaks.
(A), the percentage in all peaks. The % Lysine clipping in basic peak2 (pI 7.69) is 69.48% and 91.00% in basic peak1 (pI 7.59) compared to the Lysine clipping in main and acidic peaks are nearly complete. In basic peak1 (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 peak2 (pI 7.69) were detected.
All relative abundance were averaged across three technical replicates and CV<6%.
(B), fragment coverage map of pyroglutamate cyclization peptide from main peak.
(C), MS^2 spectrum of this peptide.

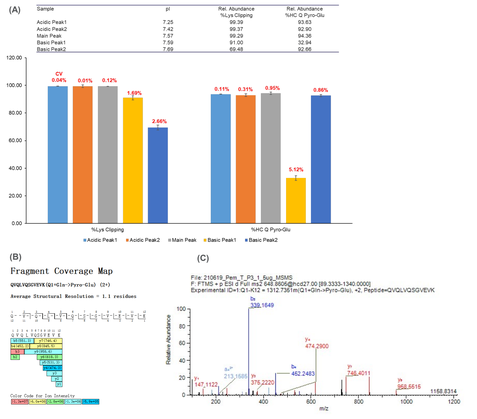


Figure 6. The percentage of N-glycan in all peaks. Expanded view, N-glycans <6%.
It is noteworthy that the percentage of sialic acid contained glycoforms increased 5~10 times in acidic peaks compared to main peak. Also, the relative abundance of A1G0F increased significantly in basic peaks.
All relative abundance were averaged across three technical replicates.

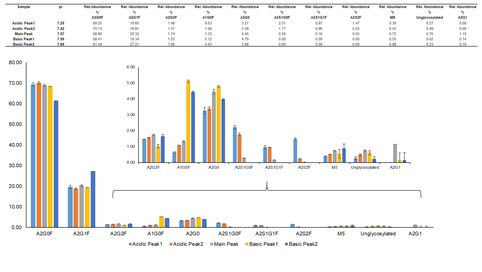


Figure 7. HC N55 deamidation and succinimidation in main and acidic peaks.
(A), the percentage distribution. (B), extracted ion chromatogram of native and deamidated peptides in main and acidic peaks. The level of % HC N55 deamidation within the Complementarity Determining Region (CDR) domain is 0.18% in main peak (pI 7.57) compared to 0.93% in acidic peak1 (pI 7.25) and 0.83% in acidic peak2 (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.
All relative abundances were averaged across three technical replicates.

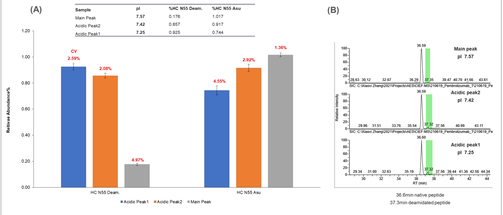
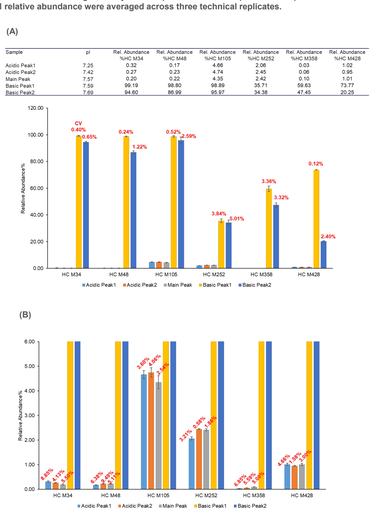


Figure 8. The percentage of Oxidation in all peaks.
(A), Overview. (B), Expanded view of components <6%.
Oxidation increased significantly in basic peaks across multiple sites compared to their levels in the main peak.
All relative abundance were averaged across three technical replicates.



CONCLUSIONS

- In this work we demonstrated outstanding performance of the CEInfinite Preparative iCIEF System on automated charge variants separation and collection.
- LC-MS based peptide mapping analysis provided in-depth characterization of PQAs and comparison of their relative abundance level in acidic/basic peaks.
- The combination of these two technologies enables a comprehensive understanding of charge variants of biotherapeutics.

TRADEMARKS/LICENSING

PO66105 EN0921S