Novel icIEF fractionation coupled with different MS systems for rapid charge variant characterization of therapeutic antibodies

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INTRODUCTION

Antibody-drug conjugates (ADCs) are a growing class of biotherapeutics in which a potent small molecule (drug) is chemically linked to an antibody. Ensuring safety and efficacy through comprehensive understanding of these products' critical quality attributes (CQAs), including charge heterogeneity, is a regulatory requirement. While imaged capillary isoelectric focusing (icIEF) is the preferred method for charge profiling, ion-exchange chromatography (IEX) has been the primary tool for charged-based fractionation combined with mass spectrometry (MS) characterization. However, the conjugated linker-drug on ADCs could potentially affect the separation performance leading to difficulties with IEX analysis and fractionation. Moreover, separation resolution is often better using icIEF than IEX due to the inherently different separation modes. Recent discontinuation of other charge-based fractionation technologies has resulted in an unmet need for IEF-based fractionation of charge variants for further characterization.

We have developed a novel icIEF fractionation solution, which involves icIEF separation and the collection of IEF-resolved charge variants. Here we report Maurice icIEF-based peak identification and mass spectrometry characterization of fractions from an ADC parent mAb. Individual charge variants of the mAb were successfully collected in less than 2 hours with purity of 80%–100% using icIEF separation conditions with urea. Without any post-run sample prep, fractions from a single fractionation run were then used for MS characterization. LC-MS and ZipChip-MS setups were utilized, and both analyses identified major and minor isoforms that correlated well with reported mass spec data. Urea and methylcellulose present during the icIEF separation did not affect the quality of fractionation nor the mass spec results. Furthermore, charge variants collected during fractionation were characterized by peptide mapping, allowing unambiguous correlation between post translational modifications (PTM) and the individual charge variant. While peptide mapping data were collected for fractions from a single run, pooling of fractions from multiple runs provides increased protein quantities if needed. IEF-based fractionation provides a significant advantage over IEF-MS direct coupling methods as fractions can be utilized for multiple analyses, including intact mass and peptide mapping. This novel icIEF fractionation solution coupled with other analysis methods, such as mass spectrometry, delivers a powerful charge variant characterization tool for therapeutic antibodies.

WORKFLOW AND METHODS

icIEF separation and fractionation:

Samples were mixed with ampholytes, pI markers, and arginine as described in the figures below. Samples were loaded into the Fractionation Cartridge and icIEF separation was performed under defined voltage steps. At the end of icIEF separation, mobilization was initiated using a mobilization buffer and fractions were collected at defined intervals into a 96-well plate containing buffer. Focusing and mobilization were monitored using real-time fluorescent imaging.

icIEF fraction check:

A portion of the fraction was checked on Maurice using a standard Maurice icIEF cartridge to confirm the charge variant (pI and purity) present in each fraction.

LC-MS and ZipChip-MS fraction characterization:

A portion of the fraction was either analyzed by intact mass spectrometry or digested for peptide mapping as needed. MS characterization was performed using a single fraction from a single fractionation run. If needed, higher quantities of a charge variant can be obtained by combining fractions from multiple fractionation runs (i.e., pooling), however no pooling was needed for this study.

LC-MS analysis: A MAbPac™ Reversed Phase HPLC column (Thermo, PN 303182) was used for separation and MS analysis was performed using Thermo Scientific UltiMate™ 3000 RSLCnano System coupled with Thermo Fisher Q Exactive HF mass spectrometer. Injection volume was 5 µL. Flow rate was 15 µL/min. No buffer exchange was performed prior to analysis.

ZipChip analysis: A ZipChip system (908 Devices Inc.) was coupled to a Thermo Exploris 240 mass spectrometer or a Thermo Fisher Exactive EMR mass spectrometer. An HSN chip type (High Speed Native) was used. The BGE is the commercially available "Peptides" BGE for the ZipChip system. The separation field strength is 1000 V/cm; injection volume is 2 nL; pressure assist starts at 0 minutes.

Peptide mapping: Individual fractions were denatured by 8 M urea, followed by reduction and alkylation with iodoacetamide. After the samples were diluted by 4 times, tryptic digestion were performed at 37 °C overnight with a trypsin-to-protein ratio of 1 to 30. The digestion reaction was quenched by adding formic acid to lower the pH.

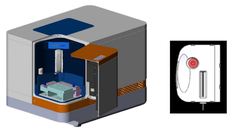
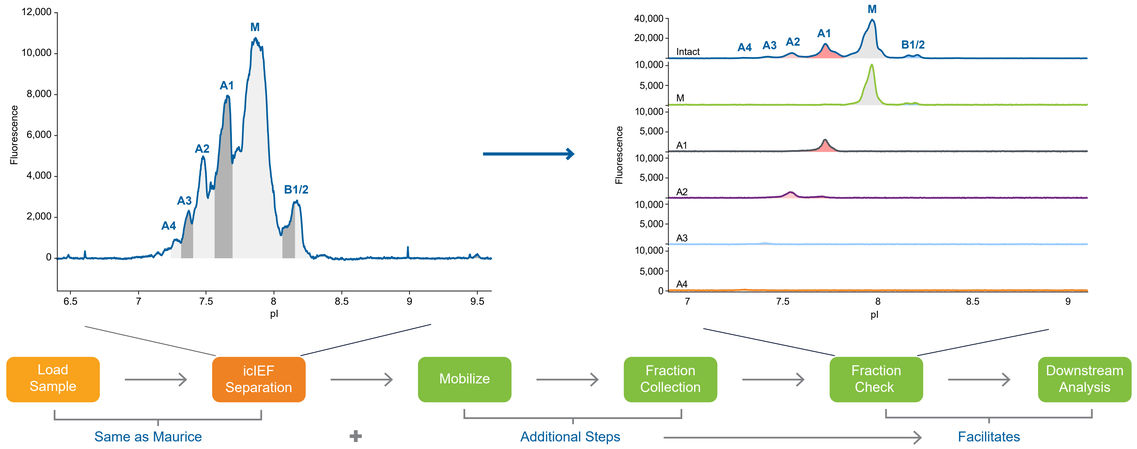


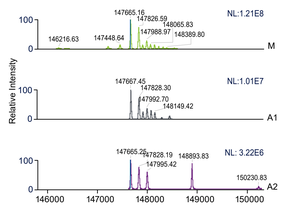
FIGURE 1. icIEF fractionation instrument and cartridge

FIGURE 2. ZipChip-MS *(Note: picture is for illustrative purposes only.)*

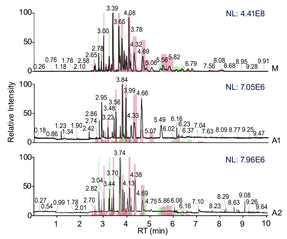
3A) Fractionation workflow with mAb



3B) Intact mass fraction analysis



3C) Peptide mapping fractions



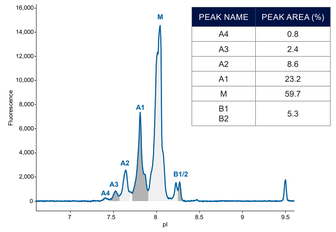
CHARGE VARIANT	HC SEQUENCE COVERAGE (%)	LC SEQUENCE COVERAGE (%)
M	89.0	83.2
A1	70.7	68.7
A2	82.8	83.6

FIGURE 3. (A) icIEF separation of mAb on a Fractionation cartridge using native fluorescence detection. ADC parent mAb was run at 1 mg/mL on Fractionation icIEF using 2.4% Pharmalyte (15%:42.5%:42.5%=3–10:5–8:8–10.5), 0.3% methylcellulose, 3.5 M urea and 10 mM arginine. (B) Intact mass analysis of fractions containing M, A1, and A2 peaks. (C) Peptide mapping analysis of fractions containing M, A1, and A2 peaks. As shown in the table above, sequence coverage for analyzed fractions achieved greater than 70% and 68% for heavy chain (HC) and light chain (LC), respectively.(B1/2=Base peak 1 and Base peak 2, M=Main peak, A1 = Acidic peak 1, A2=Acidic peak 2, A3=Acidic peak 3, A4=Acidic peak 4).

RESULTS

icIEF fractionation of ADC parent mAb

4A) Maurice icIEF profile



PEAK NAME	PEAK AREA (%)
A4	0.8
A3	2.4
A2	8.6
A1	23.2
M	59.7
B1 B2	5.3

4B) Fractionation icIEF profile

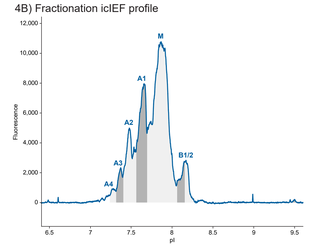


FIGURE 4. (A) icIEF separation of ADC parent mAb on Maurice icIEF cartridge using native fluorescence detection (B) icIEF separation of ADC parent mAb on Fractionation cartridge using native fluorescence detection. ADC mAb was run at 0.4 mg/mL on Maurice icIEF and 1 mg/mL on Fractionation icIEF. Both samples included the same IEF master mix, except the fractionation sample also included 10 mM Arginine. The separation profile between the two types of cartridges are comparable for the number of peaks and corresponding pI of each peak, however, different peak ratios were observed on the Fractionation cartridge as high concentration caused self-quenching of the fluorescent signal.

Peak ID for collected charge variants

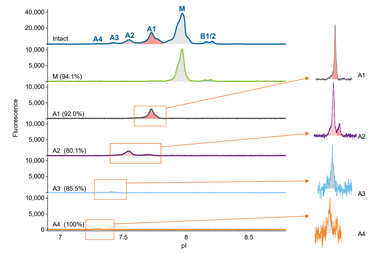
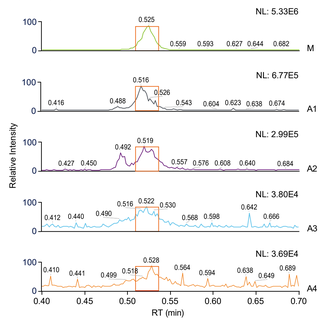


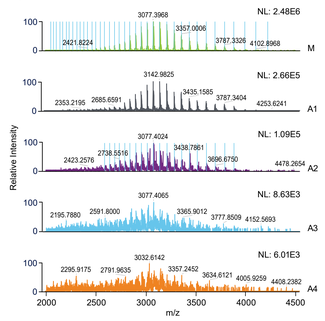
FIGURE 5. Maurice icIEF profile of unfractionated (intact) ADC parent mAb and fractions collected for mAb charge variants using the same conditions as Maurice icIEF from Figure 4. All charge variants except for Basic peaks (B1/2) were successfully collected, and most charge variants were collected with >90% purity.

ZipChip-based MS characterization of fractions of ADC mAb

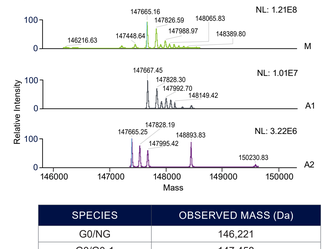
6A) CZE profile



6B) Raw mass spectra



6C) Deconvoluted mass spectra

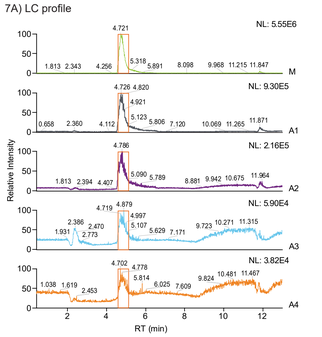


SPECIES	OBSERVED MASS (Da)
G0/NG	146,221
G0/G0-1	147,458
G0/G0	147,665
G0/G1	147,828
G1/G1, G0/G2	147,991
G1/G2	148,152
G2/G2	148,314
G2/G2+2S	148,900
S = SIALIC ACID (NEURAMINIC)	

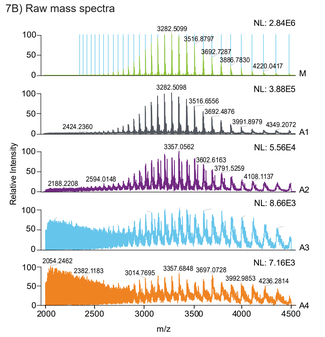
FIGURE 6. Base peak CZE e-gram (A), raw mass spectra of the ADC parent mAb fractions (B), and deconvoluted mass spectra (C) of ADC parent mAb fractions. Deconvolution results of major glycoforms were obtained for M, A1, and A2. Glycoforms identified are shown in the table.

LC-MS characterization of fractions of ADC mAb

7A) LC profile



7B) Raw mass spectra



7C) Deconvoluted mass spectra

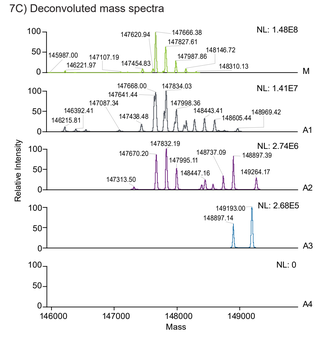


FIGURE 7. Base peak LC e-gram (A), raw mass spectra of the ADC parent mAb fractions (B), and deconvoluted mass spectra (C) of ADC parent mAb fractions. Deconvolution results of major glycoforms were obtained for M, A1, and A2. Some deconvoluted masses were achieved for A3, which roughly match with 2 and 3 sialic acids. Glycoforms identified are shown in the table in Figure 6.

CONCLUSION

The icIEF fractionation system reported here addresses challenges of fractionation of ADCs on IEX and provides a separation mode identical to the analytical icIEF methods traditionally in use today, mitigating the need to conduct bridging studies or compare charge variant data between different separation techniques. Using an ADC parent mAb as a sample model, we collected individual charge variants with ΔpI <0.1 and abundance >1%. ZipChip-based MS analysis detected charge variants with abundance >5% while LC-MS detected charge variants with abundance >2%, and major glycoforms identified correlated well with reported data. The advantages of this icIEF fractionation system include, but are not limited to:

- **Easy method transfer:** Use existing Maurice icIEF methods on the new fractionation system with minimum or no modification
- **Same IEF peaks:** Charge variant peaks detected in Maurice icIEF profile are the same as charge variants collected in fractionation
- **Sample flexibility:** Methylcellulose, urea, or other additives can be used during fractionation and will either not enter the collected fractions or can be removed before downstream analysis
- **More sample:** When needed, fractions from multiple fractionation runs containing the same charge variant can be combined
- **Flexibility of downstream analysis:** Fractions can be characterized using multiple mass spectrometry systems as well as by other analysis methods as needed since charge variant fractions are collected in a non-destructive manner.

CONTACT:
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