Novel icIEF fractionation coupled with ZipChip CZE-MS for rapid charge variant characterization

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Poster #P-113

INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) make up a large portion of the rapidly growing drug market. Ensuring safety and efficacy through comprehensive understanding of these products' critical quality attributes (CQAs), including charge heterogeneity, is a regulatory requirement. Various charge isoforms of mAbs can result from cell culture or production processes, potentially affecting the mAb structure and function. While imaged capillary isoelectric focusing (icIEF) is the preferred method for charge profiling, ion-exchange chromatography (IEC) has been the major tool for fractionation combined with characterization. However, IEC is not compatible with certain types of molecules, hydrophobic antibody drug conjugates (ADCs) for example, and icIEF typically provides higher separation resolution. Moreover, an individual charge variant obtained from IEC fractionation may not be comparable to the variant peak in the icIEF profile. Therefore, there is an unmet need for IEF-based fractionation of charge variants for characterization.

We have developed a novel icIEF fractionation solution, which involves icIEF separation and collection of charge variants. This solution enables Maurice icIEF-based peak identification followed by downstream analysis. Here we report icIEF fractionation followed by ZipChip-based mass spectrometry (MS) characterization of the NIST mAb and XMT-1535 mAb. ZipChip (CE-ESI) was utilized for mass spectrometry characterization of the fractions due to its broad sample matrix compatibility, easy sample prep, and fast mass spectrometry analysis time. Individual charge variants of each antibody were successfully collected in less than 2 hours with purity > 80% using icIEF separation conditions with or without urea. Rapid analysis using ZipChip showed the mass spec identification of major and minor isoforms correlated well with reported mass spec data (literature and report). Urea in icIEF separation did not affect the quality of fractionation nor the mass spec result. Multiple fractionation runs of the NIST mAb suggested good reproducibility of the system. We believe this novel icIEF fractionation solution coupled with other analysis methods, such as mass spectrometer, will deliver a powerful charge variant characterization tool for biotherapeutic analytical tool kit.

METHODS

icIEF separation and fraction collection:

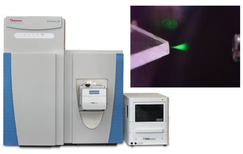
Samples were mixed with ampholytes, pI markers and arginine as described in figures below. Samples were loaded into the Fractionation cartridge and icIEF separation were performed under defined voltage steps. At the end of icIEF separation, mobilization was initiated by introduction of mobilization buffer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

Peak identification:

Peak identification was performed using a single fraction from a single fractionation run. If needed, higher quantity of charge variant can be obtained by combining fractions from multiple fractionation runs (*i.e.*, pooling), however no pooling was needed for this study. A portion of each fraction was checked on Maurice icIEF using conditions described in figures below to confirm the charge variant (pI and purity) present in each fraction. Mass spectrometry analysis of each fraction was performed using a ZipChip system (908 Devices Inc.) coupled to a Thermo Exploris 240 mass spectrometer. An HSN chip (High Speed Native) was used with "Peptides" BGE following the vendor's recommendation. The separation field strength was 1000 V/cm, injection volume was 2 nL, and pressure assist started at 0 min. No buffer exchange was performed prior to analysis.

Schematic of the icIEF fractionation system (left). The fractionation system could be a stand-alone instrument or a Maurice add-on consisting of a Fractionation cartridge and a sample tray that contains (a) Sample, (b) Reagents needed for icIEF separation, (c) Reagents needed for fraction collection, and (d) fraction collection vials or plate

ZipChip® on Thermo Scientific™ Q Exactive™ HF



RESULTS

icIEF fractionation of NIST mAb

1A) Maurice icIEF profile | **1B) Fractionation icIEF profile**

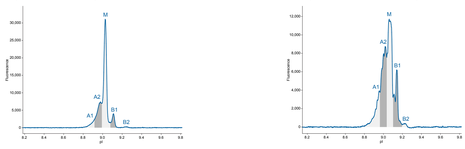


FIGURE 1. (A) icIEF separation of NIST mAb on Maurice icIEF cartridge using native fluorescence detection (B) icIEF separation of NIST mAb on Fractionation cartridge using native fluorescence detection. NIST mAb was run at 0.1 mg/mL on Maurice icIEF and 0.5 mg/mL on Fractionation icIEF. Both samples included 4% Pharmalyte (3-10:8-10.5=1:3) and 0.28% methylcelluose, except the fractionation sample also included 22.5 mM arginine. The separation profile between the two types of cartridges are comparable for the number of peaks, however, different peak ratios were observed on the Fractionation cartridge as high sample concentration caused self-quenching of the fluorescent signal. (B2 = Basic peak 2, B1 = Basic peak 1, M = Main peak, A2 = Acidic peak 2, A1 = Acidic peak 1).

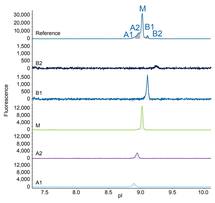


FIGURE 2. Maurice icIEF profile of unfractionated (intact) NIST mAb and fractions collected for NIST mAb charge variants using the same conditions as Maurice icIEF from Figure 1. The five charge variants were collected with > 90% purity based on the IEF peak profiles and shown in Table 1. All charge variant fractions in this image were collected from a single fractionation run. (B2 = Basic peak 2, B1 = Basic peak 1, M = Main peak, A2 = Acidic peak 2, A1 = Acidic peak 1).

ZipChip-based MS characterization of NIST IEF fractions

3A) CZE profile | **3B) Raw mass spectra** | **3C) Deconvoluted mass spectra**

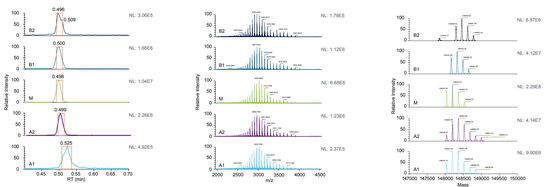


FIGURE 3. (A) Base peak CZE e-gram of NIST mAb fractions from the ZipChip system. Single peaks were detected for all five charge variants. (B) Raw mass spectra of thbg he NIST fractions. (C) Deconvolution mass spectra of NIST mAb fractions. Four major glycoforms were identified for all charge variants. The charge variant identities are shown in Table 1 below.

PEAK	AVERAGE FRACTION PURITY (%)	DECONVOLUTED MASS (DA)	MASS SHIFT (DA)	MODIFICATION
B2	100	148459.06	255.83	C-term K × 2
B1	100	148330.38	127.15	C-term K
M	93	148203.23	0.00	G0F/G1F
A2	100	148364.03	160.80	Glycation
A1	100	148363.08	159.85	Glycation

TABLE 1. Charge variant identifications of NIST mAb fractions. High purity (> 90%) was achieved for each charge variant in the fractions. The deconvoluted masses listed in the table are for the G0F/G1F glycoform of each charge variant. The mass shifts listed for each charge variant are in reference to the G0F/G1F glycoform of the main charge variant.

icIEF fractionation and ZipChip-based MS characterization of XMT-1535 mAb before and after heat stress

Maurice icIEF profile | **Fractionation icIEF profile**

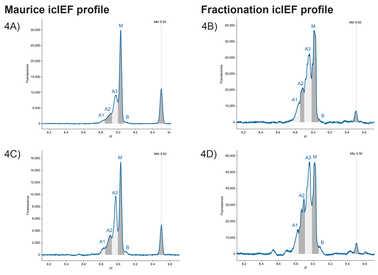


FIGURE 4. icIEF separation of XMT-1535 mAb on Maurice icIEF cartridge (A) and Fractionation cartridge (B). icIEF separation of heat stressed XMT-1535 mAb on Maurice icIEF cartridge (C) and Fractionation cartridge (D). XMT-1535 was run at 0.1 mg/mL on Maurice icIEF and 0.5 mg/mL on Fractionation icIEF. Both samples included 4% Pharmalyte (3-10:8-10.5=1:3) and 0.35% methylcellulose, 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, however, different peak ratios were observed on the Fractionation cartridge as high sample concentration caused self-quenching of the fluorescent signal. As expected, the heat stressed XMT-1535 shows increased peak ratios for A1, A2, A3 and B peaks compared to XMT-1535 on Maurice icIEF and a similar trend is observed for the Fractionation icIEF profile.

5A) Reference XMT-1535 Fractions | **5B) Stressed XMT-1535 Fractions**

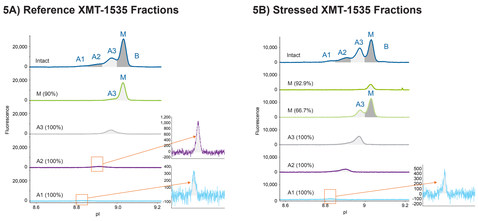


FIGURE 5. Maurice icIEF profile of unfractionated (intact) and fractions collected for charge variants of XMT-1535 mAb (A) and heat stressed XMT-1535 mAb (B) using the same conditions as Maurice icIEF from Figure 4. All charge variants with >5% peak area were successfully collected, and most charge variants were collected in a single fraction with 100% purity. Two fractions were collected with the Main charge variant for the heat stressed XMT-1535 mAb. One fraction contained high purity of Main (> 90%) and a second fraction showed Main co-eluted with the A3 charge variant. All charge variants in a single panel were collected from a single fractionation run. (B = Basic peak, M = Main, A3 = Acidic peak 3, A2 = Acidic peak 2, A1 = Acidic peak 1).

6A) Raw mass spectra | **6B) Deconvoluted mass spectra**

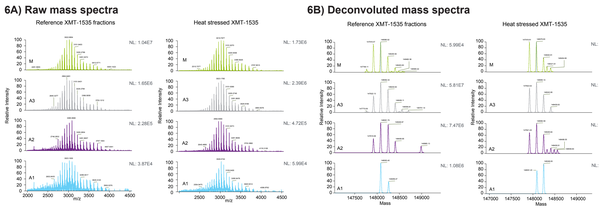


FIGURE 6. (A) The raw mass spectra of XMT-1535 mAb fractions (left) and heat stressed XMT-1535 mAb fractions (right). (B) Deconvoluted mass spectra of XMT-1535 mAb fractions (left) and heat stressed XMT-1535 mAb fractions (right). Four charge variants were detected for both mAb samples. The deconvoluted mass of acidic variants for both mAb samples are shifted by by a few Da compared to the main peaks, suggesting potential deamidation on acidic variants.

XMT-1535 LOT	REFERENCE MAB		STRESSED MAB	
GLYCOSYLATED SPECIES	THEORETICAL MASS (DA)	OBSERVED MASS (DA)	ERROR PPM	OBSERVED MASS (DA)	ERROR PPM
G0F/G0F	147917	147919	13.5	147920	20.3
G0F+G1F	148080	148080	0.0	148080	0.0
G1F/G1F or G0F/G2F	148242	148241	6.7	148243	6.7
G1F/G2F	148404	148403	6.7	148404	0.0

TABLE 2. Glycoform identification in the fractions of the main peak from reference XMT-1535 and heat stressed XMT-1535 mAb. Reference mAb and stressed mAb have the same deconvolution masses, indicating the heat stressing only changed the relative abundance of the charge variants but there were no significant or detectable changes in the modifications on each charge variant. The small mass error (≤20 ppm) of the observed deconvoluted mass for reference mAb and stressed mAb suggests high mass accuracy of the system.

icIEF fractionation of heat stressed XMT-1535 mAb with urea and ZipChip-based MS characterization

7A) | **7B)**

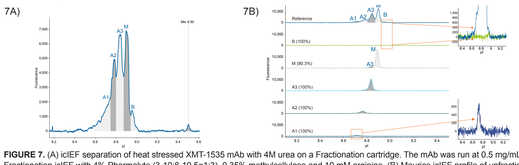


FIGURE 7. (A) icIEF separation of heat stressed XMT-1535 mAb with 4M urea on a Fractionation cartridge. The mAb was run at 0.5 mg/mL on Fractionation icIEF with 4% Pharmalyte (3-10:8-10.5=1:3), 0.35% methylcellulose and 10 mM arginine. (B) Maurice icIEF profile of unfractionated heat stressed XMT-1535 mAb (top trace) and fractions collected for charge variants of the mAb focused and fractionated in the presence of urea. Unfractionated and fraction samples were run using the same Maurice icIEF conditions as Figure 4. All charge variant peaks, including < 2% peak area, were successfully collected into individual fractions with > 90% purity. All charge variant fractions in this image were collected from a single fractionation run. (B = Basic peak, M = Main, A3 = Acidic peak 3, A2 = Acidic peak 2, A1 = Acidic peak 1).

8A) Raw mass spectra | **8B) Deconvoluted mass spectra**

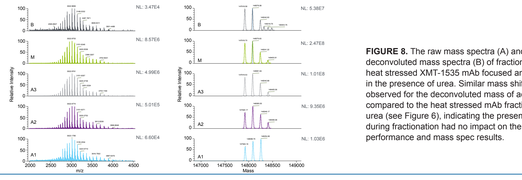


FIGURE 8. The raw mass spectra (A) and deconvoluted mass spectra (B) of fractions collected for heat stressed XMT-1535 mAb focused and fractionated in the presence of urea. Similar mass shifts are observed for the deconvoluted mass of acidic variants compared to the heat stressed mAb fractions without urea (see Figure 6), indicating the presence of urea during fractionation had no impact on the fractionation performance and mass spec results.

Reproducibility of icIEF fractionation system with NIST mAb

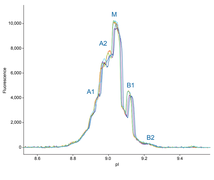


FIGURE 9. Overlay of IEF separation profiles of NIST mAb from five icIEF fractionation runs demonstrate excellent reproducibility. NIST mAb at 0.5 mg/mL was prepared in a mixture of 4% Pharmalyte (3-10:8-10.5=1:3), 0.28% methylcellulose and 22.5 mM arginine. Native fluorescence images were taken at the completion of IEF separation. (B2 = Basic peak 2, B1 = Basic peak 1, M = Main peak, A2 = Acidic peak 2, A1 = Acidic peak 1).

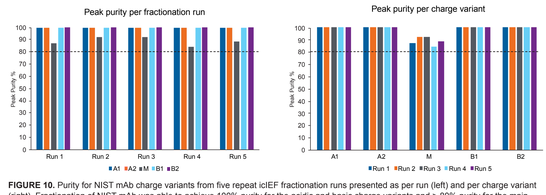


FIGURE 10. Purity for NIST mAb charge variants from five repeat icIEF fractionation runs presented as per run (left) and per charge variant (right). Fractionation of NIST mAb was able to achieve 100% purity for the acidic and basic charge variants and > 80% purity for the main charge variant and can be achieved in a reproducible manner. Purity of a charge variant present in multiple fractions in a single run is the average purity of those fractions.

DISCUSSION

Understanding charge heterogeneity for protein therapeutics is necessary for meeting regulatory requirements and commercialization. The icIEF fractionation system reported here provides a more streamlined workflow for mass spectrometry (MS) characterization of charge variants detected using icIEF. Using NIST mAb as an example, we demonstrated that our fractionation system can collect individual charge variants with ΔpI <0.1 and abundance < 2%. ZipChip-based MS successfully detected each charge variant collected and their mass identification correlated well with reported data. We also used this system coupled with ZipChip to characterize the XMT-1535 mAb before and after heat stress. The results indicated the heat stressing only changed the relative abundance of the charge variants but there were no significant or detectable changes in the modifications on each charge variant. We also performed fractionation of XMT-1535 mAb with and without urea and subjected the fractions for ZipChip-based MS characterization. The charge profile of the individual charge variants and MS identification suggested urea has no impact on the quality of fractionation nor integrity of MS result. In addition, preliminary reproducibility data from multiple fractionation runs of NIST shows good reproducibility of our fractionation system.

CONCLUSION

We present a novel icIEF fractionation solution, in conjunction with down stream analysis methods, such as ZipChip-based MS, making it possible for versatile structural and functional characterization of individual charge variants. Key advantages include, but are not limited to:

- **Easy method transfer:** Use existing Maurice icIEF method on Fractionation cartridge with minimum/no modification
- **Same IEF peaks:** Collected charge variants are identical to those detected in Maurice icIEF profiles
- **Sample flexibility:** Methylcellulose, urea, or different types of additives can be used during fractionation and will either not enter the collected fractions or can be removed before downstream analysis
- **Fast MS analysis:** The speed, sensitivity, and sample matrix compatibility of the ZipChip-MS method make it an excellent platform for mass spec analysis of the icIEF fractions
- **More sample:** Ability to pool charge variant fractions from multiple runs when more sample is required
- **Flexibility of downstream analysis:** Fractions can be characterized using other analysis methods beyond mass spectrometry

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