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Spotlight

A Novel Platform for icIEF Fractionation of Antibody Charge Variants

While imaged capillary isoelectric focusing electrophoresis (imaged cIEF or icIEF) has become the method of choice for monitoring charge variant levels of biotherapeutics, the in-depth characterization of charge variants has been carried out mostly by mass spectrometry (MS).

The MauriceFlex™ system is a new and innovative Maurice platform featuring icIEF-based fractionation for collecting protein charge variants, enabling downstream analysis of these variants with techniques such as MS. The fractionation utilizes a special cartridge to perform isoelectric focusing to separate protein charge variants, and once focused and separated, the charge variants are eluted via chemical mobilization for fraction collection. Compared to ion-exchange chromatography (IEX)-based fractionation, MauriceFlex™ fractionation is fast, with pI-based resolution, and overcomes some of the limitations of direct coupled cIEF-MS, such as the need for a dedicated interface and incompatibility of the background electrolytes with the MS. Moreover, offline fractionation and potential enrichment of charge variants pooled from multiple fractionations offer more flexibility for mass spectrometry characterization, including intact mass, reduced mass and peptide mapping.

In addition to fraction collection, MauriceFlex™ can perform routine CE-SDS and cIEF analysis using the relevant Maurice cartridges, and all data is analyzed using the 21CFR Part 11 compliant Compass for iCE software.

In this spotlight, we demonstrate the fractionation workflow and intact and peptide mapping analysis by liquid chromatography and mass spectrometry (LC-MS) on the NIST mAb (RM 8761 from NIST), which is a common reference standard for assessing new analytical technologies for characterizing monoclonal antibodies (mAbs).

Experimental Methods

The stepwise fractionation workflow on MauriceFlex™ is illustrated below:

1 Setup (~30min)

- Load reagents (kit provided) and NIST mAb (1 mg/mL)
- Insert MauriceFlex cartridge

2 Focusing (45min)

Voltage

- 10 min @500 V
- 10 min @1000 V
- 25 min @1500 V

3 Mobilization (25min)

- Mobilizer: 5 mM NH_4Ac
- Voltage: 1000V

4 Elution (20min)

Collect fraction

- 36 fractions at 25 second/fraction on a 96-well plate
- Each well contains 30 uL 5 mM NH_4Ac

Voltage

- 1000 V (off during transition from well to well)

5 Verification (4-5 hours)

- Check identify and purity of fractions with analytical Maurice icIEF
- 16 fractions checked

For intact mass, the fractions were analyzed directly. For peptide mapping, the charge variant fractions from 10 fractionation runs were pooled, lyophilized on a SpeedVac, and reconstituted prior to tryptic digestion. The digested samples were lyophilized and reconstituted in 40 µL 5 mM ammonium acetate solution.

The LC-MS characterization was performed with a Thermo Scientific™ Vanquish UHPLC coupled to a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer. Reverse phase LC separation was used for intact mass analysis and peptide mapping with appropriate gradients of 0.1% formic acid in water and acetonitrile at 0.3 mL/min. The injection volume was 10 µL for both intact and peptide mapping analysis. The data were analyzed using BioPharma Finder 4.1 software.

Results

The method for fractionation of the NIST mAb sample using the MauriceFlex cIEF fractionation cartridge can be developed quickly based on the analytical method on a Maurice cIEF cartridge. **FIGURE 1** shows profiles of analytical icIEF and fractionation focusing runs of NIST mAb. Five charge variants (B2, B1, M, A1, A2) were identified for NIST mAb and their relative abundances were obtained (**FIGURE 1A**). Note that fractionation separation with cIEF fractionation cartridge is designed for maximizing the yield of the fraction collection, and for this purpose, a higher concentration (1 mg/mL) of NIST mAb was loaded. While this resulted in apparently lower resolution with overlapped peaks (**FIGURE 1B**) when compared to charge separation with the regular Maurice cIEF cartridge (**FIGURE 1A**), the same number of charge variants were detected, as seen in **FIGURE 1B**, and were well separated.

As shown in the workflow, the mobilization and elution steps take 45 minutes in total when collecting 36 fractions on a 96-well plate. The Compass for iCE software has a peak prediction feature that provides an estimated range of wells that contain the eluted charged variants. Alternatively, a fluorescence plate reader can be used to select the wells that contain the most abundant charge variant. For NIST mAb, a total of 16 wells of fractions were selected, and their identity and purity were verified with analytical Maurice icIEF. Among the 16 fractions analyzed, 12 were found to contain the charge variants.

FIGURE 2 shows icIEF electropherograms of fraction wells containing individual charge variants with the highest purity. As shown, except for low abundant B2 (0.9%) at 65% purity in fraction #10, fractions containing 100% purity for the four other charge variants were obtained.

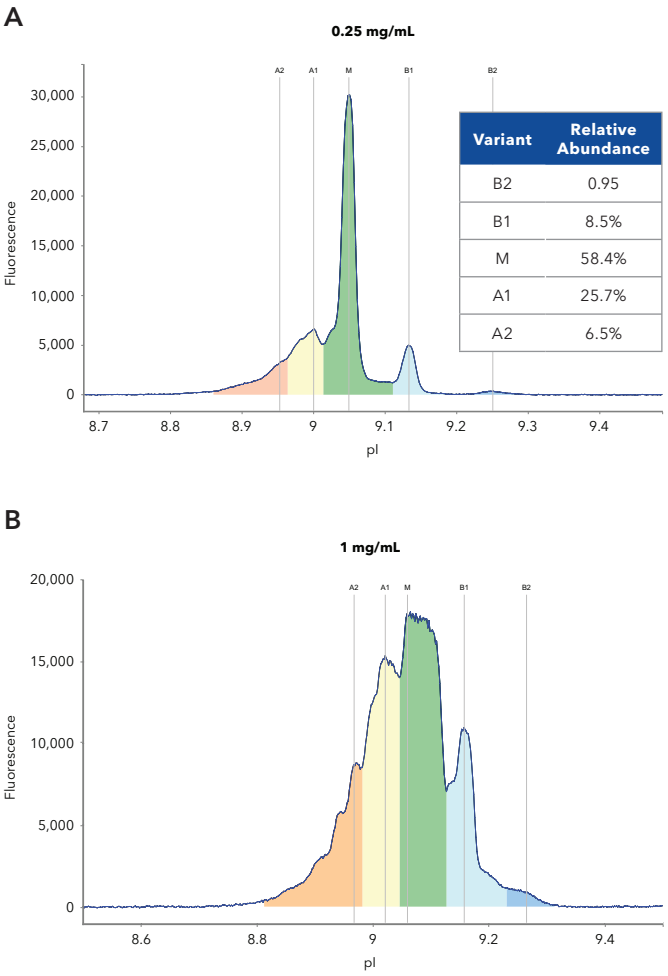

FIGURE 1. icIEF separation of NIST mAB charge variants with the Maurice cIEF cartridge (A) and MauriceFlex™ cIEF Fractionation cartridge (B). Both electropherograms show the same number of charge variants. The relative abundance (%) of each variant was calculated from the peak areas in the analytical run (A) and listed in the table insert.

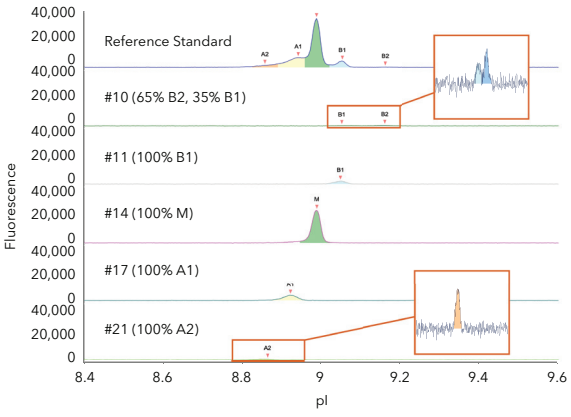

FIGURE 2. Verification of charge variants of representative fractions for identity and purity by Maurice icIEF analytical runs. The numbers assigned represent the fraction numbers.

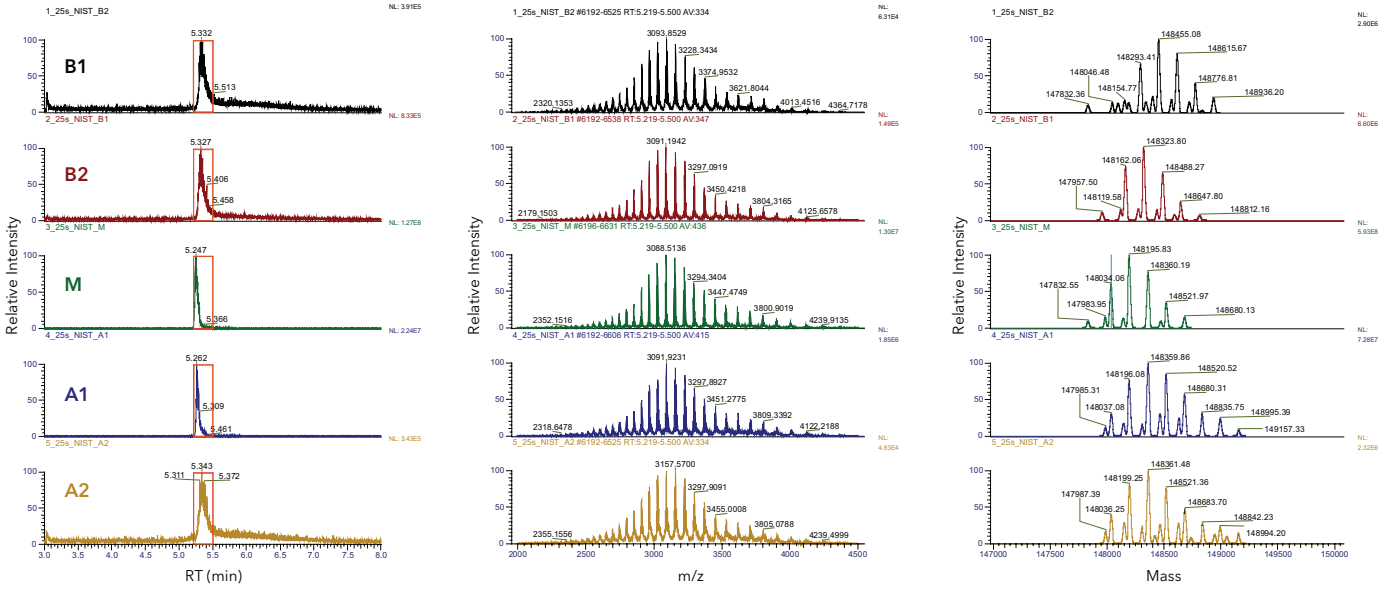

FIGURE 3. LC-MS intact mass analysis of charge variants showing total ion current (TIC) chromatograms (left), mass spectra of the peaks (middle) and the deconvoluted spectra (right).

The LC-MS intact mass analysis on the high purity charge variant fractions are shown in **FIGURE 3** and the modifications identified from the deconvoluted mass spectra for each charge variants are summarized in **TABLE 1**. The results are consistent with the established knowledge of PTMs of the charge variants of NIST mAb.

Peak	Deconvoluted Mass (Da)	Mass Shift (Da)	Modification
B2	148455.08	+259.25	2xC-term K
B1	148323.80	+127.97	C-term K
M	148195.83	0.00	G0F/G1F
A1	148359.86	+164.03	Glycation
A2	148361.48	+165.65	Glycation

TABLE 1. Summary of the identified modifications from intact mass analysis. The mass shifts of each variants are relative to the G0F/G1F glycoform of the M peak.

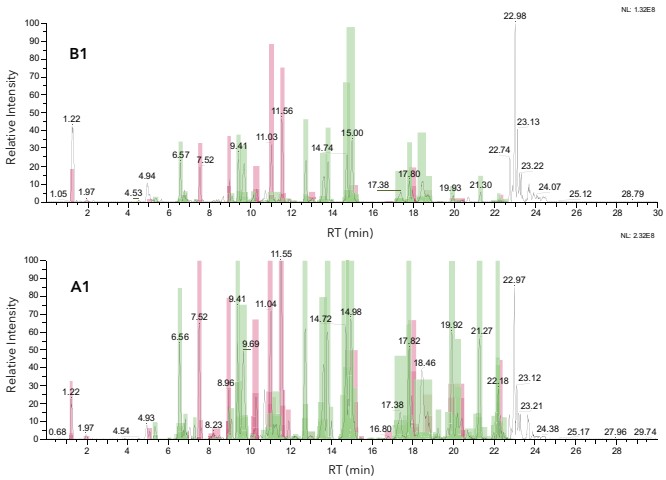

FIGURE 4. Representative chromatograms of the peptide mapping of B1 and A1, and sequence coverage of Light Chain (LC) and Heavy Chain (HC) of all charge variants. The charge variant samples were pooled from 10 fractionation runs.

Peptide mapping is an indispensable tool for characterizing the primary structure of biotherapeutics, and the capability of pooling charge variants from multiple fractionation runs on MauriceFlex™ makes it possible to enrich the charge variants for peptide mapping analysis. **FIGURE 4** demonstrates the peptide mapping results from samples pooled from 10 fractionation runs. As shown, the sequence coverage is above 90% for all variants except for the low abundant B (relative abundance 0.9%) and heavy chain (HC) of the A2 peak.

Sequence Coverage %		
Fraction	LC	HC
B2	84.0	58.9
B1	94.4	92.4
M	95.3	94.0
A1	95.3	92.2
A2	93.9	73.8

Conclusion

By using the NIST mAb sample with the icIEF fractionation feature on the new MauriceFlex™ system, we have demonstrated that:

- High purity fractionation of charge variants can be obtained in a single day
- A single fractionation run provides sufficient charge variant fractions for intact mass analysis
- With enrichment from pooling fractions from multiple runs, charge variants can be analyzed with LC-MS peptide mapping