Charge Variant Analysis of Native Antibodies for In-depth Characterization of mAb-based Biotherapeutics

Application Note

Introduction

The PATsmart™ ZipChip® System is a simple, fast, and powerful technology for performing charge variant separations of biotherapeutic proteins with mass spec characterization. The microfluidic capillary zone electrophoresis (CE) process separates species in an open channel based simply on differences in the charge and size of the molecules. For large proteins like mAbs, common variations to the protein structure have minimal effect on the total size of the molecule, but differences in charge are amplified by performing the separation at a pH close to neutral. These conditions minimize the total charge on the molecule while also keeping the molecule tightly folded in its native structure. The result is a high-resolution charge variant separation combined with sensitive and information- rich, native mass spectrometry. Additionally, because the separation occurs with no stationary phase and no solvent gradients, the ZipChip System is also the simplest method for charge variant-MS analysis. Recent publications by some of our customers provide an excellent demonstration of the capabilities of this technology for the thorough characterization of biotherapeutic mAbs[1,2].

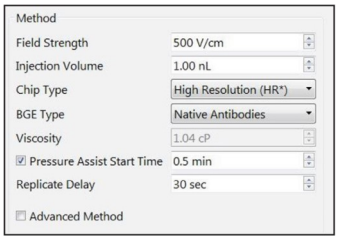
Here we demonstrate the capabilities of this technology with a detailed characterization of the NIST mAb reference material. In addition to the NIST mAb, three other mAbs (Herceptin, USP mAb, and Cetuximab), were analyzed with the same method to demonstrate how high-resolution charge variant separations of a wide variety of mAbs can be achieved with no method development.

Methods

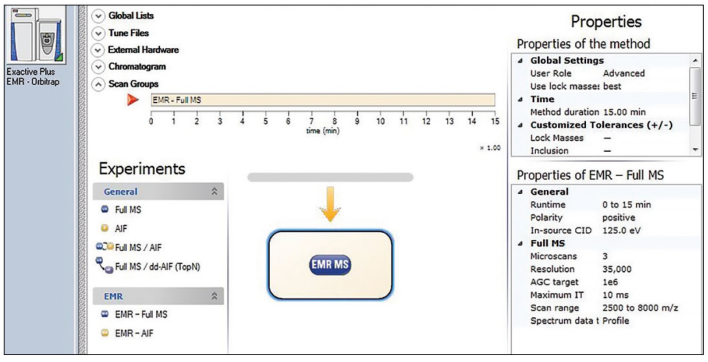
Instrumentation. All work was performed using a commercially available microfluidic CE-MS system (ZipChip System, Repligen) attached to an Exactive Plus EMR mass spec (ThermoFisher Scientific). The microfluidic devices utilize a covalently attached, neutral polymer surface coating to prevent analyte interactions and suppress electroosmotic flow. For this work a “high resolution native” (HRN) chip was used, along with the ZipChip Native Antibodies Kit*.

Samples. NIST Monoclonal Antibody Reference Material 8671, Herceptin (trastuzumab), and USP Monoclonal IgG System Suitability Standard were diluted directly from formulation with the ZipChip Native Antibodies BGE to a concentration of 0.5 mg/mL. Due to its complex glycosylation and relatively low formulation concentration, Erbitux (cetuximab) was buffer exchanged before analysis. Buffer exchange was performed using a Zeba Spin cartridge, exchanging the sample into the ZipChip Native Antibodies BGE to a concentration of 2 mg/mL.

The ZipChip System Method. Samples were loaded via the ZipChip autosampler and analyzed using the method settings shown:



Orbitrap MS Method. Data were acquired in EMR mode with a relative HCD pressure of 1, inlet capillary at 300°C and S-lens RF level of 150. The XCalibur acquisition settings were as shown:



*The Native Antibodies Kit has since been replaced by the Charge Variant Analysis Kit (p/n: 850-00052)

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Analysis. Data were visualized using Thermo Xcalibur QualBrowser. The NIST mAb data was processed with Repligens' Darwin software.

Results and Discussion

Figure 1 shows the ZipChip System native antibody charge variant analysis of the NIST mAb. The electropherogram shows excellent resolution of basic and acidic variants with high signal to noise. The spectra shown below were generated by averaging the MS signal across the full width of the labeled peaks. The full width of each spectrum is shown alongside a zoom in of the most intense (+26) charge state, showing the excellent resolution of glycoform peaks achieved at an orbitrap resolution setting of 35,000.

The glycosylation profile of the molecule is the same for most of the charge variant peaks, as would be expected for a mAb containing neutral glycans. The two basic variant peaks are easily identified as C-terminal lysine variants based on the 128 Da mass shift of each peak and the increase in electrophoretic mobility associated with the addition of a positively charged lysine residue. The most abundant acidic variant peak is a deamidation of the main variant peak. Deamidation shifts the molecular mass by 1 Da, so detecting this modification at the intact level would not be possible without the ZipChip System separation. Each molecule contains an N-glycosylation site on both of its heavy chains, so glycan assignments are made as pairs of glycans which yield the accurate masses observed. Differentiating G1F/G1F from G0F/G2F is not possible at the fully intact level. Table 1 lists the glycan assignments for the 10 most abundant glycoforms observed in the deconvoluted spectrum of the main variant peak. These glycoforms range in fractional abundance from 35% down to below 0.1%. The same glycoforms are observed on the C-terminal lysine variants and the deamidated variants, multiplying the total number of proteoforms identified.

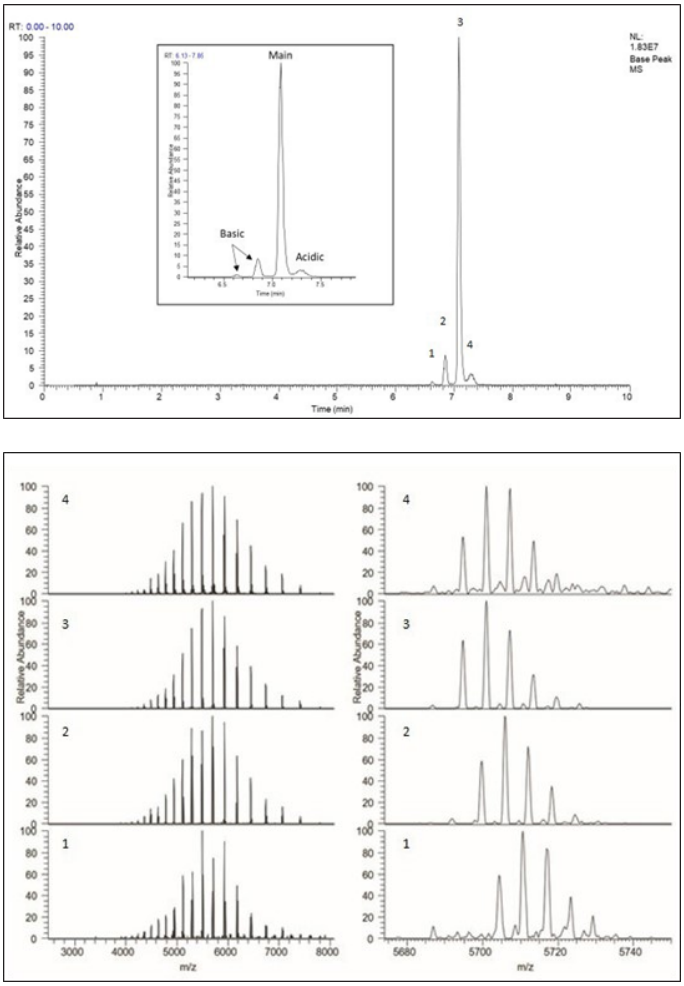

Figure 1. Electropherogram and raw charge variant spectra for the analysis of NIST mAb.

Mass	Glycan ID	Fractional Abundance	Mass Error (ppm)
148197.8	G0F/G1F	35.64%	-2.39
148359.6	G1F/G1F	30.76%	-3.57
148035.4	G0F/G0F	18.81%	-4.42
148522	G1F/G2F	10.24%	-1.76
148684.7	G2F/G2F	2.54%	2.53
147833	Man3GF/G0F	0.62%	0.38
147992.5	Man3GF/G1F	0.50%	-17.23
148846	G2FGal1/G2F	0.48%	-2.11
149010	G2FGal1/G2FGal1	0.24%	10.98
148155.5	Man3GF/G2F	0.09%	-10.8

Table 1. NIST mAb glycoform identifications

To demonstrate the generic utility of this ZipChip System method, three other commonly analyzed mAbs were run via the same exact method as the NIST mAb. The electropherograms for all 4 samples are shown in Figure 2. We observe excellent separation of charge variants in all cases, with charge variant peak profiles closely matching results of other charge variant analysis techniques (such as cation exchange chromatography, capillary isoelectric focusing, and optical CZE).

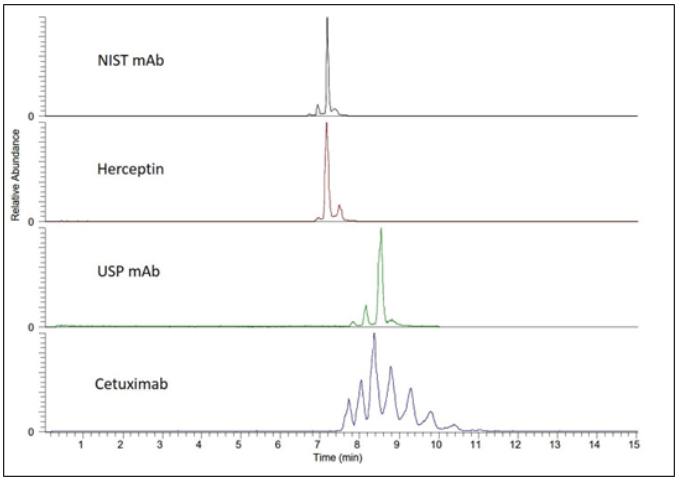

Figure 2. ZipChip Native Antibodies method, electropherograms for 4 different mAbs using the exact sample ZipChip System method and orbitrap MS settings.

Conclusions

The ZipChip Native Antibodies charge variant analysis is a powerful method for characterizing biotherapeutic proteins. The method separates charge variants in a simple background electrolyte that is optimized for mass spec performance. Mass spectra of the fully separated charge variants yield a depth of information from the native structure of the molecules.
Furthermore, the method works for a wide variety of mAbs and related molecules without any need for method optimization, producing charge variant peak profiles that closely correlate with results from other charge variant analysis methods. This indicates that ZipChip System can provide a ready solution to quickly identify unknown peaks from routine charge variant analyses.

References

1. *In-depth analysis of monoclonal antibodies using microfluidic capillary electrophoresis and native mass spectrometry.* Sara Carillo, Craig Jakes & Jonathan Bones, Journal of Pharmaceutical and Biomedical Analysis. 2020, DOI: 10.1016/j.jpba.2020.113218
2. *Comparative Elucidation of Cetuximab Heterogeneity on the Intact Protein Level by Cation Exchange Chromatography and Capillary Electrophoresis Coupled to Mass Spectrometry.* Fussl F, Trappe A, Carillo S, Jakes C, & Bones J., Anal Chem. 2020, DOI: 10.1021/acs.analchem.0c00185

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