Highly Sensitive Charge Variant Analysis Of Complex Biopharmaceuticals

Application Note

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Highlights

- Performing highly selective charge variant separation of complex pharmaceuticals using the PATsmart™ ZipChip® System for native intact antibodies.
- Generation of excellent quality MS data which allows for in-depth analysis of also extremely low abundant charge variants.
- Demonstration of outstanding method sensitivity based on the identification of more than 200 Cetuximab isoforms with a sample consumption of only 2 ng of protein.

Introduction

With the rise of precision medicine, the monoclonal antibody (mAb) market has expanded enormously during recent years. Monoclonal antibodies are large biomolecules that are produced by recombinant means and therefore can be highly heterogeneous. For therapeutic use, knowledge of this microheterogeneity is mandated by authorities to guarantee patient safety.

Charge variant analysis on the intact protein level represents an extremely powerful tool to distinguish protein isoforms based on differential surface charge, requiring minimal sample preparation and short analysis times. Recently, efforts were undertaken to combine charge variant separation of proteins with powerful detection modes such as mass spectrometry, creating new and exciting methods for protein characterization.[1,2] Here, we explore the potential of ZipChip System for the in-depth analysis of Cetuximab, a mAb that exceeds conventional mAbs in terms of complexity, as it contains four N-glycans, two in the conserved Fc region and another two in the Fab region (Figure 1) which can be complex and heavily sialylated. These additional Fab glycans are the cause of extremely high glycan heterogeneity and along with other charge altering modifications of the primary sequence are responsible for the complex peak pattern usually observed during

The ZipChip System is particularly **beneficial** in cases where only **very limited quantities of sample are available.**

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charge variant analysis of Cetuximab. This charge variant and glycoform complexity makes Cetuximab a highly challenging molecule for in-depth characterization on the intact protein level.

Materials and Methods

Cetuximab was buffer exchanged to intact antibody sample diluent using Amicon Ultra-0.5 mL spin filters with a 30 kDa size cut-off to yield a 1 mg/mL concentration.

Specification	Value
ZipChip System Protocol	Boosting Sensitivity for Intact Antibody Charge Variant Analysis[3]
Assay Kit	ZipChip System Native Antibody Kit*
Chip Type	ZipChip System HRN
Injection Volume	2 nL
Field Strength	500 V/cm
Run Time	20 minutes
Mass Spec Type	Thermo Scientific QExactive™ Plus Hybrid Quadrupole Orbitrap with BioPharma Option

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

Results

Analysis of Cetuximab was performed using generic instrument parameters without the need for molecule specific optimization. The complex charge variant separation obtained can be seen in Figure 2. In total 8 Cetuximab charge variant peaks were distinguished, varying in abundance. Notably, all peaks are base-line separated which is important for subsequent MS-based isoform annotation and quantification. The averaged mass spectrum of the main charge variant peak

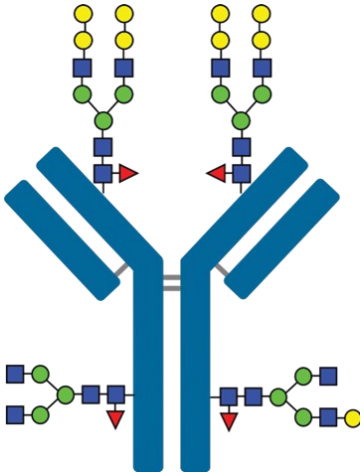

Figure 1. Base General structure of Cetuximab. The Fc region contains simple, bi-antennary glycans also found on other mAbs whereas the Fab glycans can be larger, more complex and sialylated.

is shown in blue in Figure 2. The spectral quality is excellent and allows for deconvolution and the characterization of even low abundant glycoforms. The averaged mass spectrum of the lowest abundant charge variant peak is shown in red in Figure 2. Spectral quality is sufficient for analysis, even for isoforms with relative abundance values of <1%.

Spectral deconvolution and analysis confirmed the expected charge variant migration in order of increasing negative protein net charge. The net charges are shown above each peak in Figure 3. Positive charges are caused by mis-cleaved C-terminal lysine, either on one or both heavy chains whereas negative charges are due to the presence of N-*glycolyl* neuraminic acid (NGNA) residues on the Fab glycans, as indicated in Figure 3. Net charges are ranging from +2, which corresponds to Cetuximab with 2 C-terminal lysine residues and no NGNA, to -5, which represents Cetuximab without

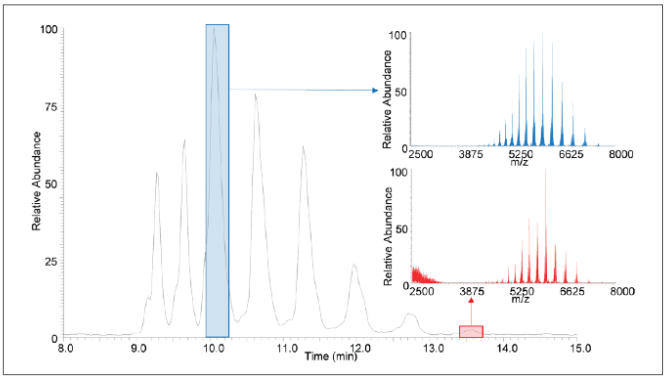

Figure 2. Charge variant separation of Cetuximab. The averaged spectra of the most (blue) and least (red) abundant charge variant peaks is shown in the right panel.

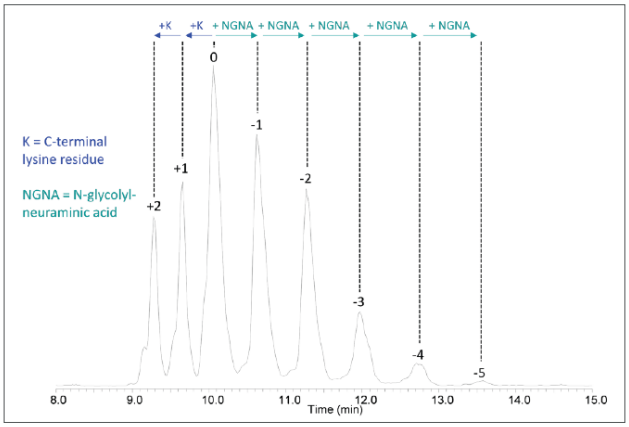

Figure 3. Assignment of protein net charge states to individual charge variant peaks. C-terminal lysine residues are labelled "K" and are shown in blue, N-glycolylneuraminic acids are labelled "NGNA" and are shown in turquois.

C-terminal lysine residues and five NGNA units attached. Importantly, each charge variant peak contains the characteristic glycosylation pattern of Cetuximab, giving rise to a range of isoforms found across the electropherogram.

More than 200 different isoforms were detected and quantified with relative abundance values of down to 0.1%. Considering that the run time required is only 15 minutes and a sample consumption of 2 ng, these results clearly demonstrate the power of the ZipChip System for in-depth analysis of biopharmaceuticals. While the ZipChip System based CE-MS can be effectively employed for mAb characterization, it is in particular beneficial in cases where only very limited quantities of sample are available. This makes microfluidic CE-MS highly attractive for application in early stages of the biopharmaceutical production pipeline.

References

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