Glycosylation profiling of Cetuximab subunits with CZE-MS

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Introduction

Monoclonal antibody cetuximab has four N-glycans with two on each heavy chain located in the Fc and CH1 domain. Previous studies have shown that the Fab glycans are complex and multiply sialylated.

The complex glycosylation profile makes the molecule challenging to characterize in detail by traditional reverse phase analysis due to related glycoforms coelution.

ZipChip is a microfluidic device integrating capillary zone electrophoresis (CZE) with electrospray ionization. ZipChip coupled with high resolution mass spectrometry provides charge-based selectivity which can assist with glycoforms separation. This can be used to improve domain specific characterization of proteins with complex glycosylation such as N-glycans.

Methods

Reconstitute the *IdeS* protease in 50 mM ammonium bicarbonate and mix with 2 mg/mL cetuximab for digestion. Incubate the mixture at 37° C for 30 minutes. To the digested sample add DTT to target 10mM addition and incubate at 37° C for another 30 minutes. Cetuximab subunit sample was then buffer exchanged (Micro Bio-Spin™ 6 Columns, Bio-Rad) into premixed native flex BGE for native subunit analysis with high resolution native (HRN) chip and Metabolites BGE for denaturing subunit analysis with high speed (HS) chip. 500 V/cm field strength was applied over the chip and 1nL sample was injected for analysis. Bruker timsTOF Pro 2 mass spectrometer was employed for the MS detection with settings tuned for intact and subunit mAb analysis. Bruker Data Analysis software was used for data processing and mass deconvolution.

Results

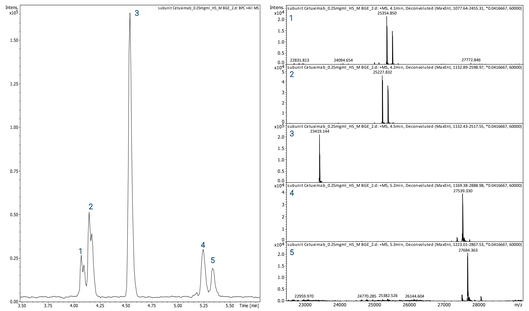

Fig. 1 Charge variant separation of Cetuximab subunits (treated with *IdeS* and DTT) in metabolite background electrolyte with HS chip (left) and deconvoluted mass spectra of corresponding electropherogram peaks (right). Peak 1 and peak 2 were determined to be Fc/2 charge variants. A 128 Da mass shift was observed from peak 1 to peak 2 with highly conserved glycosylation profile. Peak splits within peak 1 and 2 are due to Fc/2 glycosylation (data not shown). Peak 3 is from light chain. Peak 4 and 5 are coming from the Fd with a 145 Da mass delta, which can be attributed to sialylation and corresponds to the mass difference between a galactose and a N-glycolyl neuraminic acid.

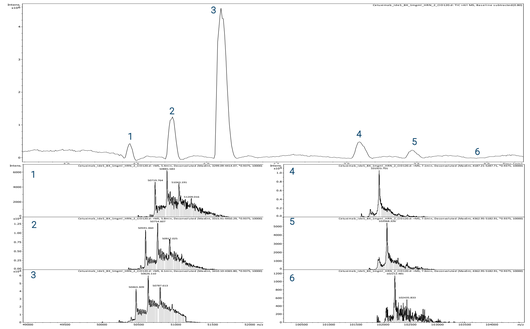

Fig. 2 Charge variant separation of *IdeS* treated Cetuximab in native flex BGE with HRN chip (top). Deconvoluted mass spectra (bottom) are shown for the six electropherogram peaks. Fc charge variants were assigned to the first three eluted peaks . Positive mass shifts of 128 Da was observed from peak 1 to peak 2 and peak 2 to peak 3 with highly conserved MS pattern. Incomplete C-terminal lysine processing was suggested to be the cause for these basic variants. Peak 4, 5 and 6 were determined to be F(ab')2 charge variants. Peak 5 and 6 showed positive mass offsets of up to two times 145 Da when compared to peak 4. Sialylation on the Fab glycans explains this shift of 145 Da.

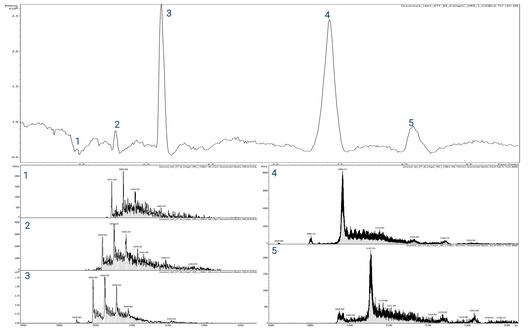

Fig. 3 Top: charge variant separation of Cetuximab subunits (treated with *IdeS* and DTT) in native flex BGE with HRN chip
Bottom: deconvoluted mass spectra for Fc charge variants (left) and Fd/LC complex charge variants (right): two times 128 Da mass shifts were observed from peak 1 to peak 3. One time 145 Da mass offset was found from peak 5 to peak 4.

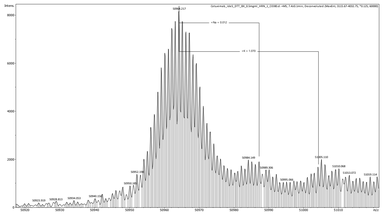

Fig. 4 Isotopically resolved mass spectrum of electropherogram peak 4 from *Fig. 3*. MaxEnt deconvolution shows excellent isotopic resolution for ~50 kDa species observed in Cetuximab subunits.

- Subunit charge variants were well separated in the electropherograms, which was essential for in-depth characterization of the individual domain. Excellent mass accuracy for the resulting data allowed the unambiguous confirmation of the subunits. Under denaturing conditions Cetuximab subunit light chain, Fc/2 and Fd species were well separated within a ~2 min separation window (*Fig. 1*). This allows for quick subunit analysis with confident species assignment.
- A well resolved separation of six peaks under native flex BGE was achieved which could be linked to the F(ab')2 and Fc region of *IdeS* treated Cetuximab (*Fig 2*). Formation of Fc/2 aggregates was found and can be explained by the non-covalent interactions between the two Fc/2 regions. Peak 2 indicates one C-terminal lysine truncation compared to peak 1 and the same applies to peak 3 and peak 2. Peak 5 corresponds to a singly sialylated F(ab')2 and peak 6 corresponds to a doubly sialylated F(ab')2.
- The CZE separation of IdeS treated and reduced Cetuximab under native flex BGE (*Fig. 3*) yields similar separation of Fc charge variants. Charge variants of Fd/LC species reveal one sialylation. Interactions between Fd and LC was preserved under the CZE separation and MS instrumentation conditions.

Summary and Conclusion

- ZipChip combined with high resolution MS offers a highly selective platform for charge variant analysis of mAbs and glycoproteins.with high isotopic fidelity.
- Three CZE-MS runs of Cetuximab subunits under different BGE and chip conditions reveals heterogeneities resulted from incomplete lysine truncation on the Fc/2 region and various levels of sialylation on the Fab glycans. Up to two NeuGc were observed on the Fab glycan, which agrees with the previously published intact analysis.
- Non-covalent interactions between Cetuximab subunits were able to be preserved under current experimental setup. Further activation can be utilized for more in-depth glycosylation profiling of Cetuximab.

Technology