Therapeutic Protein Charge Variant Characterization with Intact Mass and Peptide Mapping Following Microgram Preparative Capillary Isoelectric Focusing Electrophoresis Fractionation

Thomas Menneteau[1]; Claire Butré[1]; Géry Van Vyncht[1]; Arnaud Delobel[1]; Marion Galaup[2]; Kristell Lebozec[2]; Laurence Talbot[3]; Kefei Wang[4]

[1]Quality Assistance, Donstiennes, Belgium; [2]Acticor Biotech, Paris, France; [3]Bio-Techne, Noyal Châtillon sur Seiche, France; [4]Bio-Techne, San Jose, CA

Introduction

While the imaged isoelectric focusing capillary electrophoresis (icIEF) has become the method of choice for monitoring the levels of charge variants in biotherapeutics, the new MauriceFlex™ is an innovative system that enables high resolution icIEF-based fractionation of protein charge variants for offline characterization by mass spectrometry.[1]

The main advantage of offline fractionation and potential enrichment of charge variants from pooling offer more flexibility for a full range of MS characterization, including intact mass, subunit mass and peptide mapping.

In this study, we present the fractionation of glenzocimab, a Fab fragment (MW 48.2 kDa) of humanized anti-GPVI monoclonal antibody.[2] The charge variants from three pI clusters were fractioned for intact mass and peptide mapping analysis to identify the possible modifications.

Experimental

MauriceFlex fractionation and verification of glenzocimab

The fractionation and verification of the fraction were performed on MauriceFlex™ using the following workflow and conditions:

Glenzocimab was mixed in 4% ampholytes Pharmalyte 3-10 (1.5%), Pharmalyte 8-10.5 (2.5%), 1.5% pI marker 8.40, 1.5% pI marker 9.99, 25mM Arginine, and 0.35% methyl-cellulose. Approximately 6 µg of protein were loaded onto the MauriceFlex fractionation cartridge. Detection mode was native fluorescence (Ex280, Em320-450, 0.2 s exposure time).

For verification of fraction identify and relative impurity by analytical icIEF, 8 µL of each fraction were mixed with 32 µL of master mix containing 4% ampholytes Pharmalyte 3-10 (1.5%), Pharmalyte 8-10.5 (2.5%), 0.5% pI marker 8.40, 0.5% pI marker 9.99, 10 mM Arginine, and 0.35% methyl-cellulose. Separation was performed with an analytical icIEF cartridge. Detection mode was native fluorescence (Ex280, Em320-450, 20, 40 and 80s exposure time).

To collect more samples for peptide mapping, fraction wells containing the protein charge variants from 4 fractionation runs were pooled based on their well positions determined by the above-mentioned verification as well as by comparison of intact mass profiles of fractions

Intact mass spectrometry analysis

Intact mass analysis was performed by UPLC-MS using an Acquity H-Class system coupled to a Xevo G2 XS Q-Tof high resolution mass spectrometer controlled by UNIFI 1.9.4 (Waters Corporation). The column was a Waters Bioresolve mAb polyphenyl (50 x 2.1 mm, 1.7 µm) with a gradient of water/acetonitrile containing 0.1% FA. 16 µL of each fractions were loaded.

Peptide mapping analysis

The pooled fractions were prepared for peptide mapping analysis first with denaturation and reduction using Rapigest and dithiothreitol followed by alkylation using iodoacetamide. Tryptic digestion was performed with RapiZyme at 37°C for 1 hour, and the resulting peptide mixtures were dried using a speed-vacuum and reconstituted in 40 µL of Water/ACN (99/1) with 0.1% FA, from which 38 µL was injected. LC-MS analysis was performed on the same system as Intact mass analysis in MS^E mode. The separation was on a Waters PREMIER BEH C18 (100 x 2.1 mm, 1.7 µm) with a water/acetonitrile gradient with 0.1% FA.

Results & Discussion

MauriceFlex is designed for microgram level fractionation of protein charge variants. The system utilized a special fractionation cartridge based on chemical mobilization which is known to preserve the good icIEF resolution, as shown with well separated three clusters of charge variants of glenzocimab (Figure 1).

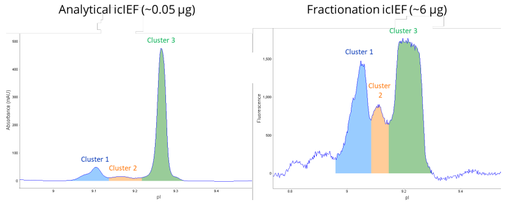

Figure 1. Electropherograms of glenzocimab on analytical cartridge (left) and fractionation cartridge (right). Signal saturation in fractionation is due to the overload of samples to increase the amount of the protein sample collected.

Note that with chemical mobilization, neutral molecules like methyl cellulose, which is ESI incompatible, will not be eluted. Thus, fraction sample in ammonium acetate can be injected to LC-MS directly.

The icIEF verification (Figure 2) found a total of 10 fractions containing the charge variants from 3 clusters. The fractions were named after their well positions on the 96-well plate following their eluting order: B8➔ B12➔ C12➔C8. The icIEF verification was also used to align the fraction prior to their pooling. High purity fractions were obtained for each of the three clusters.

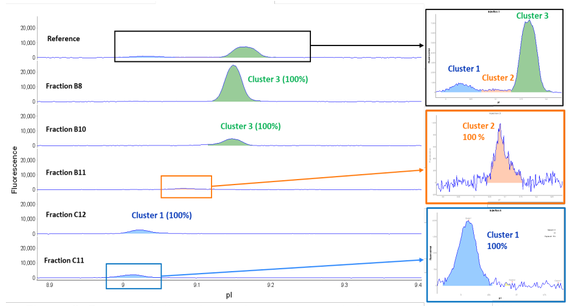

Figure 2. Representative electropherograms and charge variant purity (% in relative abundance) of collected glenzocimab charge variant fractions verified by analytical icIEF on MauriceFlex.

Analysis by intact LC-MS led to the identification of a pyroglutamic acid modification of the mAb fragment (-17 Da), representing the most abundant population of glenzocimab, corresponding to Cluster 3 (Figure 3). Analysis of fractions corresponding to Cluster 2 allowed the identification of a glycation on the mAb fragment (+162 Da).

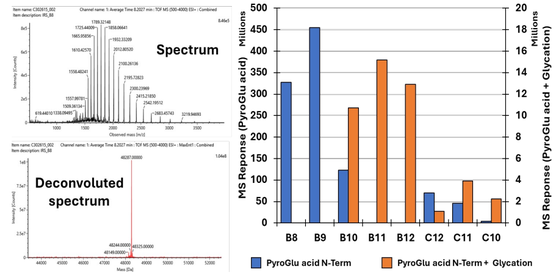

Figure 3. Intact mass analysis of fractionated glenzocimab. MS spectrum and deconvoluted spectrum of a fraction (left). MS responses of pyroglutamic acid mAb fragment and glycated mAb fragment across the fractions (Right)

However, the observed mass in the fractions of Cluster 1 could not lead to the identification of any modification.

In order to identify the modification corresponding to Cluster 1, peptide mapping was used, which allows for identifying and locating modifications of much smaller mass shifts. However, due to the low amount of protein from single fractionation run, pooling (4x) and adjustment of the digestion procedure and LC-MS method were needed for low microgram protein peptide mapping.

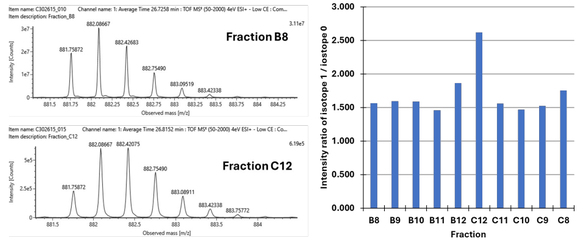

Figure 4. MS spectra of peptide LC 25-47 in fractions B8 and C12 and plot of the MS intensity ratio isotope 1/isotope 0 of peptide LC 25-47 in all the analyzed fractions.

From peptide mapping, the evolution of the ratio between isotope 0 and isotope 1 peak intensities of LC peptide 25-47 strongly suggests the presence of a deamidation on one of the many asparagines on the peptide (Figure 4).

Both N-terminal pyroglutamic acid and glycation of the glenzocimab could also be confirmed from the peptide mapping.

Conclusion

1) Combination of offline MauriceFlex icIEF fractionation with intact mass and peptide mapping led to the identification of three modifications of the glenzocimab from 3 charge variant clusters.
2) icIEF fractionation can be applied to any purified protein to identify charge variants when these variants can be separated on analytical icIEF. However, fractionation requires optimization and fractions need to be verified for identity and purity.
3) A single fractionation run yields enough charge variant fraction samples that can be directly analyzed for intact mass.
4) Pooling from more fractionation runs is needed for peptide mapping, along with adjustment of digestion procedures and LC-MS method according to the sample quantity and modification of interest.
5) A more comprehensive peptide mapping to confirm specific PTM sites would need to optimize the fractionation throughputs to collect more high purity charge variants.

References

1) Applications of capillary electrophoresis for biopharmaceutical product characterization. Kumar *et al.*, Electrophoresis, 2022 Jan;43(1-2):143-166.
2) Targeting platelet GPVI with glenzocimab: a novel mechanism for inhibition. Billiald *et al.*, Blood Adv., 2023 Apr 11;7(7):1258-1268.

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