Charge variant microheterogeneity explored via icIEF and offline MS analysis of infliximab biosimilars

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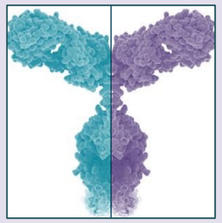
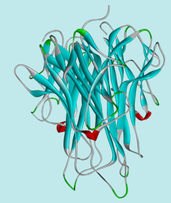


Introduction

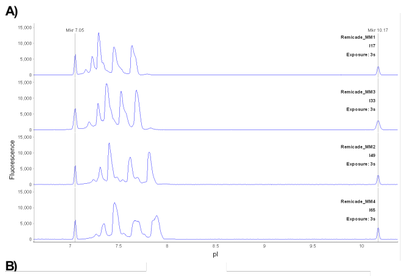
Biosimilarity assessment requires a plethora of analytical assay to decipher the structural microheterogeneity of both target originator and candidate biosimilar. A compulsory assay performed in quality control laboratories is the charge variant analysis, based on the separation of proteoforms with different pI values. The gold standard for charge variant profiling is image-capillary isoelectric focusing (icIEF) although detection methods paired with this technology normally prevent a direct identification by mass spectrometry, due to matrix incompatibility or low recovery of each species. In this study we will develop a complete workflow using icIEF fractionation and analysis of each fraction by intact analysis using microchip CE-MS technology or bottom-up analysis by nano LC-MS/MS workflow using innovator (Remicade®) and biosimilar (Flixabi®) of infliximab as a model analytes[1].

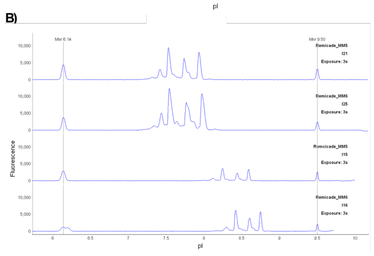
About infliximab

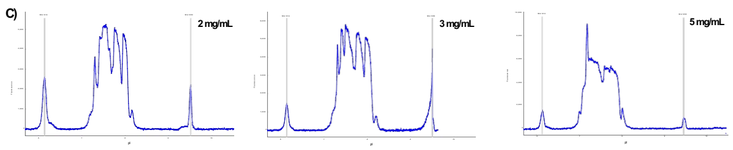


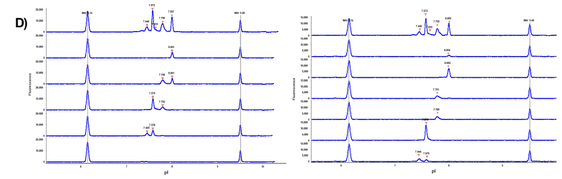
- Infliximab is an anti-TNFα chimeric monoclonal antibody used against inflammatory diseases (Crohn's disease)
- MoA: it binds to TNFα inhibiting its binding to the receptor of immune cells
- Remicade (Janssen) was first approved by FDA in 1998 and by EMA in 1999
- Biosimilars started to appear on the market in 2013.
- 8 biosimilars now present on the market.

icIEF Method Optimization









icIEF Method

MasterMix composition was optimized adjust A) the type and quantity of protein solubilizer; B) the carrier ampholytes and their concentrations. Best condition for separation of infliximab charge variants were obtained using 20% SimpleSol and ampholytes between pH 3 and 10 at 4% concentration. Fractionation method needed further optimization of C) the protein loading concentration, where 2,3 and 5 mg/mL starting concentration were tested. 2 mg/mL was selected to maximize the resolution and purity of the peaks. D) A refocusing step also proved to improve the separation of charge variants providing fractions with higher purity (right) compared to the fractionation performed in without refocusing (left).

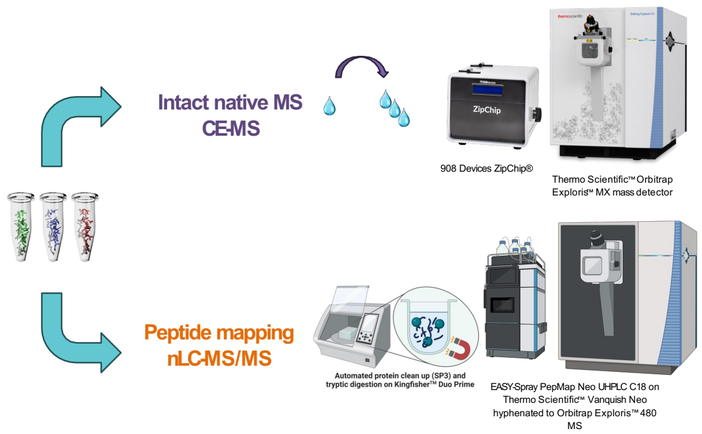
Master Mix

0.35% MC, 4% pH 3-10 ampholytes, 50 mM Arg
20% SimpleSol
1.5% pI markers 6.14, 9.50

icIEF – Optimized Conditions

Separation: 10 min 500 V, 10 min 1,000 V, 25 min 1,500 V
Fluorescence detection: 0.2 sec
Sample Load: 20 sec
Mobilisation: 30 min 1,500 V
Refocus: 5 min 1,500 V
Fractionation 25 s, 1,500 V
pI Markers 6.14, 9.50

Offline fractions analysis



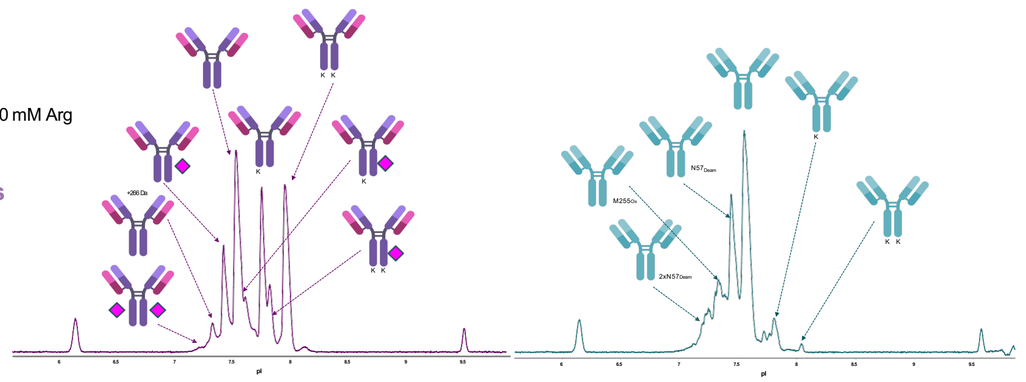
CE-MS Method[2]

5 µL from each fraction were directly diluted three times with CVA diluent provided in the CVA kit (908 Devices), 10 µL are transferred on chip from the autosampler and 2 nL injected on chip.

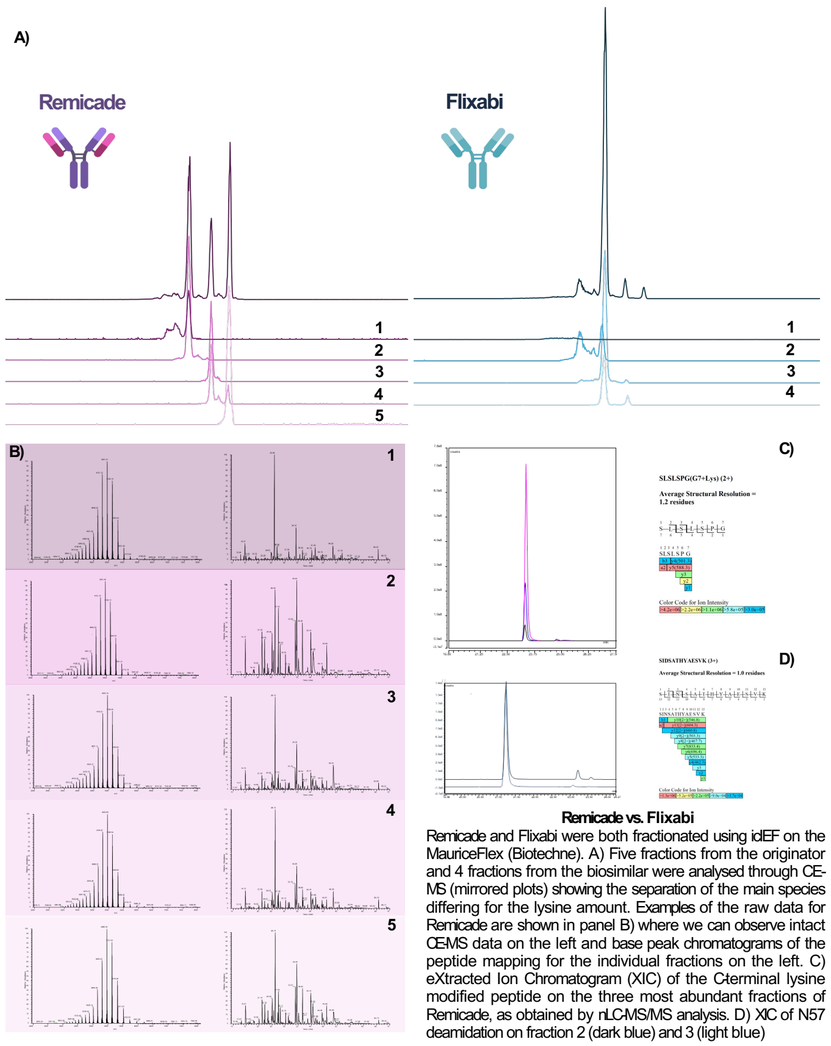
nLC-MS/MS Method[3]

15 µL from each fraction were directly mixed with TRIS buffer and underwent standard reduction and alkylation reactions. To improve protein recovery pre-digestion and eliminate excess reagents, automatic clean-up was performed using SP3 magnetic beads. Trypsin digestion was carried at 37 °C for 1h.

Separation and MS Settings	
CE-MS	**CE:** sample transfer 10 µL, injection 2 nL, field strength 500 V/cm, pressure assist start time 0.5 min, replicate delay 20 s. **MS:** Application Mode: Intact Protein, High Pressure Tune Settings - Sheat gas: 2 arbitrary units (au), ion transfer tube: 300 °C Scan Parameters - Resolution: 30K (at m/z 200), Scan range: 4,000-8,000 m/z, RF lens 200%, acquisition gain control (AGC) 100%, Maximum injection time: 20 ms, microscan: 3, in-source CID: 135 eV
nLC-MS/MS	**nLC:** A – 0.1% formic acid in MS grade water, B – 0.1% formic acid in MS grade acetonitrile Flow rate 0.250 µL/min. Gradient 2% - 35% B in 55 minutes, 40%-45% B in 5 minutes. Column wash 80% B for 5 min Column temperature 45 °C **MS:** Application Mode – Peptide Scan Parameters – Resolution 120K (at m/z 200), Scan range: 200-2,000 m/z, RF lens %70, AGC 100%, Maximum injection time 100 ms, microscan 1 **MS/MS:** DDA, Top 5. Isolation window 1,2 m/z, Collision energy (HCD) 28%, Resolution 15K (at 200 m/z), AGC 100%, maximum injection time 250 ms, intensity threshold 5e3, dynamic exclusion 7s, charge states 2-7



Results



Remicade vs. Flixabi

Remicade and Flixabi were both fractionated using icIEF on the MauriceFlex (Biotechne). A) Five fractions from the originator and 4 fractions from the biosimilar were analysed through CE-MS (mirrored plots) showing the separation of the main species differing for the lysine amount. Examples of the raw data for Remicade are shown in panel B) where we can observe intact CE-MS data on the left and base peak chromatograms of the peptide mapping for the individual fractions on the left. C) eXtracted Ion Chromatogram (XIC) of the C-terminal lysine modified peptide on the three most abundant fractions of Remicade, as obtained by nLC-MS/MS analysis. D) XIC of N57 deamidation on fraction 2 (dark blue) and 3 (light blue)

Conclusions

- Charge variant analysis remains a key analytical tool to solve the complex structural heterogeneity of biotherapeutics.
- icIEF is a robust and reliable platform for charge variant analysis and the fractionation feature of the new MauriceFlex ™ allows direct offline MS analysis using both intact and bottom-up approach.
- Although the differences highlighted in infliximab biosimilars are not clinically meaningful, the icIEF fractionation allowed to confidently distinguish acidic variants generated from sialylated N-glycans and acidic variants derived from deamidation events in the CDR region.
- The fractionation of species not allowing easy identification with commonly known modifications allows further studies to be carried for complete analysis.

1) Hong J. MAbs. 2017 Feb/Mar;9(2):364-382. doi: 10.1080/19420862.2016.1264550.
2) Carillo S. J Pharm Biomed Anal. 2020 Jun 5;185:113218. doi: 10.1016/j.jpba.2020.113218.
3) Smith J. Eur J Pharm Biopharm. 2023 Aug;189:276-280. doi: 10.1016/j.ejpb.2023.06.019.