MauriceFlex Fractionation of charge variants accompanied by LC-MS and Digital SPR Analysis correlates Structure to the Function of a mAb

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Abstract

Monoclonal antibodies (mAbs) are important biotherapeutics and are quite expensive to develop. Biosimilars are lower-cost alternatives to the original drug and share the same protein sequence as the innovator but may have differences in post-translational modifications (PTMs) that can affect safety and efficacy. Regulatory agencies require demonstration that biosimilars are clinically similar to innovators and are safe.

This study used Benlysta™ (belimumab Innovator, approved for lupus and lupus nephritis) and a research grade Biosimilar to characterize charge variants by leveraging the MauriceFlex™ instrument which can separate charge variants, mobilize them, and provide fractions for subsequent analyses. A single Flex fractionation run was sufficient to collect fractions for subsequent LC-MS analysis with the BioAccord™ System. This was achieved without additional sample preparation, revealing critical differences between the innovator and biosimilar, such as light chain fragmentation at specific sites and the presence of C-terminal lysine residues. Further, Flex fractions from a single run were extended to digital SPR with the Alto™ system for BLyS and Fc gamma receptor binding studies.

Materials and Methods

All reagents used in the study were of analytical grade unless otherwise specified. All Maurice cartridges, reagents and other consumables were from Bio-techne. The Waters BioAccord LC/MS was used as Mass Spectrometry system. Nicoya Alto system was used for label-free binding analysis.

Figure 1. MauriceFlex System and Flex fractionation Work-Flow: MauriceFlex is used to fractionate Belimumab Innovator and Biosimilar fractions; They are subsequently analyzed by BioAccord system was used for liquid chromatography-mass spectrometry (LC/MS) as well as Nicoya's Alto using Digital Microfluidics.

Samples

Benlysta™ (Belimumab) Innovator and a research-grade biosimilar were obtained from commercial sources. Both mAbs were stressed at 45°C for 5 days and compared to control in this study.

icIEF Analysis Method

The innovator and biosimilar samples were prepared at a final concentration of 0.1 mg/mL in an ampholyte solution containing Pharmalytes (4%) 8-10.5 and 3-10 (4:1), 20% SimpleSol, 5 mM arginine, and Maurice pI markers 7.05 and 9.50. The samples were loaded onto the MauriceFlex instrument along with the Maurice cIEF cartridge and focused for 1 min at 1500 V, then 12 min at 3000 V.

icIEF Fractionation Method

Samples were prepared at a final concentration of 2 mg/mL in an ampholyte solution containing Pharmalytes (4%) 8-10.5 and 3-10 (4:1), 30 mM arginine, 30% SimpleSol, Maurice pI markers 7.05 and 9.50, and Simple Western™ pI markers 7.00 and 9.70. The samples were loaded onto the MauriceFlex instrument along with the MauriceFlex cIEF Fractionation Cartridge and focused for 10 min at 250 V, 10 min at 500 V, 10 min at 1000 V, and 25 min at 1500 V. The detected peaks were mobilized for 25 min at 1000 V, followed by fraction collection for 45 sec at 1000 V. Fractions were collected into plate wells containing 40 µL 5 mM Ammonium Acetate. Arginine was added (Final Conc: 100 mM) to each well after fractionation for Mass Spectrometry analysis only. All data were analyzed using Compass for iCE software.

LC-MS Analysis

A 96-well plate collected from the MauriceFlex was transferred to the BioAccord System for LC-MS analysis without any buffer exchange or additional sample preparation. The BioAccord System was equipped with the Waters MassPREP™ Micro Desalting Column (2.1 x 5 mm, P/N 186004032). Mobile Phase A was water with 0.1% formic acid and Mobile Phase B was acetonitrile with 0.1% formic acid. Separation was achieved using a gradient of 5-90% Mobile Phase B over 1.5 minutes (total method time of 4 minutes). Column temperature was set to 80°C. The RDa™ Detector settings were as follows: default capillary voltage of 1.5 kV, default desolvation temperature of 550°C, and a cone voltage of 70 V. Mass spectra of each fraction were acquired, automatically deconvoluted, and masses matched within 40 ppm via INTACT Mass App within the waters_connect™ Informatics Platform.

SPR Assays

Binding kinetics for the BLyS antigen and Fc gamma receptor IIIa against the biosimilar and innovator antibody fractions in both normal and stressed conditions was performed via capture of the antibody fractions and analyzed using Nicoya's Alto digital SPR. Binding kinetics for the analyte recombinant Human BLyS His-tag Protein, CF (R&D Systems) against biosimilar and innovator antibody fractions was performed via capture by Nicoya's Human IgG/rabbit IgG VHH kit. Binding kinetics for recombinant FcRIIIA/CD16a (R&D Systems) against biosimilar and innovator antibody fractions was performed via capture by Nicoya's Protein A Capture Kit. The sensors of a CBX cartridge were cleaned with 10 mM HCl for 60 sec, followed by 5 min activation with 200 mM EDC and 200 mM NHS. 3 µL of either 2.5 µg/mL human IgG VHH in MES pH 6 or 5 µg/mL Protein A in sodium acetate pH 5.5 was passed over each even channel sensor for 5 min. All sensors were then blocked with 1 M Ethanolamine for 5 min. Next, 2 µL of the biosimilar and innovator fractions were captured on each even channel sensor. Five 3-fold serial dilutions were automatically prepared by Alto™ in 0.01 M Phosphate buffer for both antigens. For kinetic experiments, a single-cycle kinetics (SCK) format was used. The analyte was introduced from lowest to highest concentration with five subsequent association times of 180 sec, followed by a single dissociation time of 600 sec after the analyte titration series. Upon completion of each test, the reference subtracted binding curves were automatically fitted to a 1:1 Langmuir binding model in Nicoya Lifesciences' Nicosystem™ analysis software to determine kinetic and affinity constants.

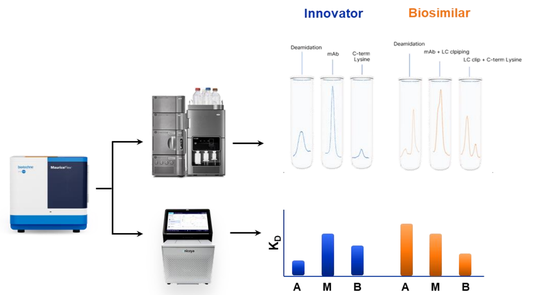
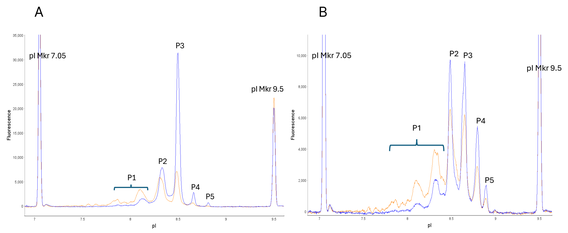


Figure 2. Work-Flows showing MauriceFlex System, Waters BioAccord System and Nicoya's Alto. MauriceFlex was used to fractionate the Belimumab Innovator and Biosimilar. Fractions are subsequently analyzed by BioAccord LC/MS System and Nicoya's Alto using Digital Microfluidics.

Results

Imaged cIEF Reveals Different Charge Variants



Belimumab Innovator: Control % PA					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Average	7.18	27.38	60.55	4.23	0.70
SD	0.21	0.26	0.26	0.05	0.00
%RSD	2.87	0.96	0.44	1.18	0.00

Belimumab Biosimilar: Control % PA					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Average	15.35	34.45	30.98	15.55	3.75
SD	0.52	0.31	0.35	0.13	0.17
%RSD	3.39	0.90	1.13	0.83	4.62

Belimumab Innovator: Stressed % PA					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Average	38.70	34.58	23.55	2.93	0.25
SD	0.41	0.49	0.26	0.15	0.06
%RSD	1.05	1.42	1.12	5.13	23.09

Belimumab Biosimilar: Stressed % PA					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Average	43.25	27.50	19.60	8.00	1.73
SD	0.26	0.23	0.14	0.14	0.05
%RSD	0.61	0.84	0.72	1.77	2.90

Figure 3. Imaged cIEF analysis of Belimumab Innovator and Biosimilar under control and stressed conditions. Using the method described above, we used both control and stressed conditions for Innovator (A) and Biosimilar (B) samples; the Control (blue) and Stressed (orange) samples were analyzed and overlayed. Five unique peaks were identified, P1 (Acidic 2), P2 (Acidic 1), P3 (Main), P4 (Basic 1), and P5 (Basic 2). One additional acidic peak is labeled as P1*; N=4.

cIEF Fractionation with MauriceFlex

The Benlysta (Belimumab) innovator and biosimilar samples were analyzed for charge heterogeneity using MauriceFlex icIEF Fractionation Method, where five major peaks were detected for each (Figure 4). It is interesting to note that there is both an apparent pI value shift, as well as an overall different ratio, of the mAb species. These observations underscore the need for further investigation. The ability to collect these fractions into MS-compatible ammonium acetate buffer enables the user to analyze them with LC-MS without any additional sample preparation, which decreases the overall time from question to results. Each collected fraction was reinjected to MauriceFlex using the icIEF Analysis Method to compare to the unfractionated Innovator or Biosimilar sample, to confirm charge variant species (estimated pI) and to estimate a concentration for each fraction (Figure 5).

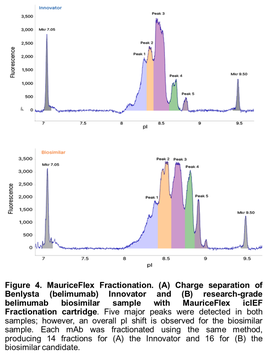


Figure 4. MauriceFlex Fractionation. (A) Charge separation of Benlysta (belimumab) Innovator and (B) research-grade belimumab biosimilar sample with MauriceFlex icIEF Fractionation cartridge. Five major peaks were detected in both samples; however, an overall pI shift is observed for the biosimilar sample. Each mAb was fractionated using the same method, producing 14 fractions for (A) the Innovator and 16 for (B) the biosimilar candidate.

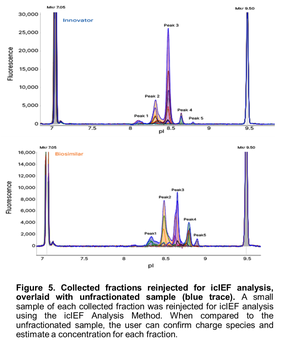


Figure 5. Collected fractions reinjected for icIEF analysis, overlaid with unfractionated sample (blue trace). A small sample of each collected fraction was reinjected for icIEF analysis using the icIEF Analysis Method. When compared to the unfractionated sample, the user can confirm charge species and estimate a concentration for each fraction.

LC-MS Analysis of Belimumab Fractions

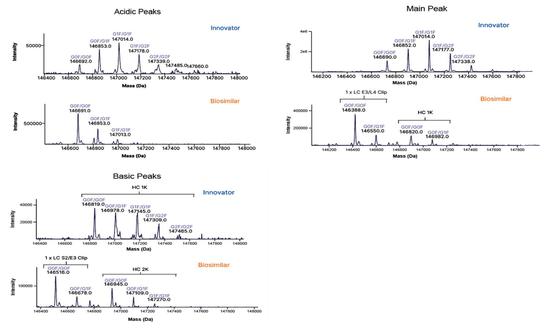


Figure 6. Deconvoluted spectra of charge variant fractions highlight key differences between the innovator and biosimilar. (A) Deconvolution of acidic fractions of the innovator and biosimilar, with revealing potential deamidation. (B) Main peak fractions highlight discrepancies such as unprocessed C-terminal lysine (1K) and a break between glutamic acid and leucine (E3/L4) in the biosimilar. (C) Basic fractions show multiple species with the innovator primarily exhibiting C-terminal lysine and the biosimilar also displaying a break between serine and glutamic acid (S2/E3).

icIEF Peak	Innovator	Biosimilar Candidate
Peak 1	Possible mAb deamidation, free LC, LC dimer*	Possible mAb deamidation, free LC, LC dimer*
Peak 2	mAb, possible mAb deamidation	mAb
Peak 3	mAb, free LC, LC dimer*	mAb + 1 x LC E3/L4 clip, mAb, mAb + 1K species
Peak 4	mAb + 1K species	mAb + 1x LC S2/E3 clip, mAb + 2K species
Peak 5	Insufficient MS signal	mAb + 1 x LC S2/E3 clip + 1K species

Table 1. Summary of LC-MS analysis of select acidic, main, and basic fractions collected after icIEF. Each antibody has unique charge variants.

SPR Analysis of Belimumab Fractions

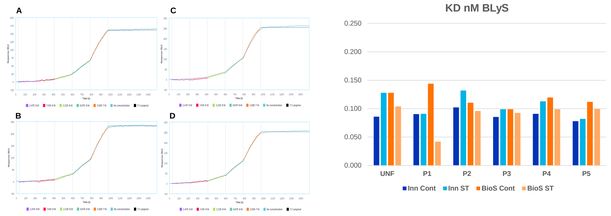


Figure 7. Binding affinity and kinetics measured for different fractions with the BLyS antigen. The figure displays representative SCK binding curves for BlyS binding to a fraction of the (A) Belimumab innovator, (B) Belimumab innovator stressed, (C) Belimumab biosimilar, and (D) Belimumab biosimilar stressed, respectively, captured on an anti-human Fc surface. KD values are in low NM range and were compared for all conditions.

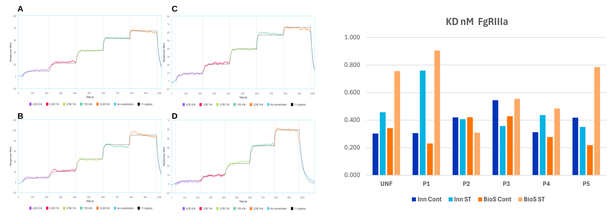


Figure 8. Binding affinity and kinetics measured for different fractions with FcγRIIIA. The figure displays representative SCK binding curves for FcγRIIIA binding to a fraction of the (A) Belimumab innovator, (B) Belimumab innovator stressed, (C) Belimumab biosimilar, and (D) Belimumab biosimilar stressed, respectively, captured on a Protein A surface.

Conclusions

- A novel workflow that leverages the combination of MauriceFlex fractionation, LC-MS analysis, and SPR analysis is described. This workflow offers a robust and efficient approach to evaluating charge variants from a single Flex fractionation run is illustrated.
- This workflow facilitates easy investigation of charge variants between an innovator and a biosimilar candidate of Belimumab. A variety of charge variant species were identified (see Table 1), from unprocessed C-terminal lysine variants to significant mAb clipping species in the biosimilar candidate sample.
- The binding kinetics acidic and basic fractions (P1 and P5) with FcγRIIIA showed some significant differences in the binding kinetics. These differences may be due to possible deamidations in P1 and changes to unprocessed Lysine residues and clips in P5 (See Table 1).

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