A Novel icIEF and Digital SPR Workflow for Correlating the Charge Structure to the Function of a Bispecific Antibody

Michael Piazza[1], Srinivasa Rao[2], and Chris Heger[2]
[1]Nicoya Lifesciences, Kitchener, ON, Canada; [2]ProteinSimple, a Bio-Techne brand, San Jose, CA USA

Abstract

Biomolecular charge variant characterization has been a challenge that involves a cumbersome and time-consuming process. In this study, we demonstrate the application of the MauriceFlex™ and Alto™ platforms to fractionate and characterize binding kinetics of charge variants from a therapeutic bispecific antibody (BsAb) Mosunetuzumab and a research-grade biosimilar. First, the charge heterogeneity of both molecules was analyzed using imaged capillary isoelectric focusing (icIEF) on the MauriceFlex system, followed by the collection of individual charge variant fractions on the same system. Next, the collected fractions were tested for their ability to bind to antigens CD3 and CD20, which are the intended targets of Mosunetuzumab. To accomplish this, surface plasmon resonance (SPR) with the Alto System was used to measure the binding of each fraction and ligand, requiring only 2 µL of each. Alto offers a key advantage over other SPR platforms due to its low sample volume requirements and higher throughput for fractionated samples. The binding data obtained from SPR correlated well with the structural information obtained from LC-MS analysis of the charge variant fractions, revealing a significantly weaker binding of the acidic fraction of the biosimilar to CD20. Overall, this poster highlights the streamlined fractionation and characterization of charge variants in biotherapeutics. This will help identify critical charge species that impact binding potency to inform the control steps needed in the process development and manufacturing of a biotherapeutic.

Uncovering a Mismatched Bispecific Antibody

Bispecific antibodies combine two different ligand binding domains to bring two different targets together. We obtained Mosunetuzumab (Lunsumio®) and a research-grade biosimilar and fractionated each into 9 fractions using MauriceFlex (Figure 3), analyzed at the subunit level by LC-MS (Figure 4), where a mismatched light chain was found (Figure 5).

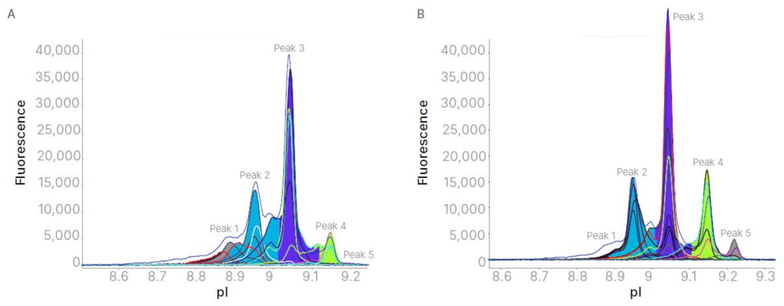


Figure 3. Fractionation of Mosunetuzumab Innovator (Lunsumio®) and a biosimilar. Each BsAb was fractionated using the same method, producing 9 fractions each for the innovator **(A)** and biosimilar **(B)**. The unfractionated molecule (overlaid blue line) is shown to illustrate coverage obtained with fractionation.

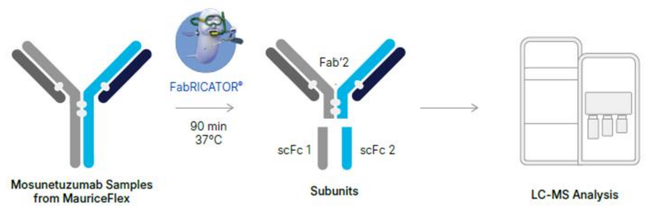


Figure 4. Workflow of subunit analysis for collected charge variant fractions. The fractionated BsAb charge variants were digested using FabRICATOR to generate subunits, followed by LC-MS analysis using a Waters BioAccord LC-MS. This analysis resulted in distinct chromatogram peaks for the subunits Fab'2, scFc1, and scFc2. All digests and LC-MS were kindly performed by Andreas Nägeli, Ph.D., GENOVIS.

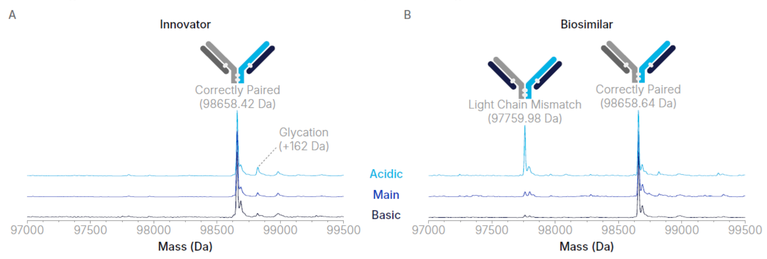


Figure 5. Analysis of the Fab'2 subunit shows critical differences between the innovator and biosimilar. The innovator shows correctly paired BsAbs along with evidence of glycation, especially in its acidic variants (A), while the biosimilar clearly shows the presence of mismatched species in its acidic peaks in addition to the correctly assembled BsAb (B).

icIEF Fractionation

Charge variant fractionation and analysis was performed using a MauriceFlex and cIEF fractionation cartridge (Figure 1). MauriceFlex works by first separating proteins by isoelectric focusing, then chemically mobilizing charge variants into fractions (Figure 2).

Figure 1. MauriceFlex and cIEF fractionation cartridge.

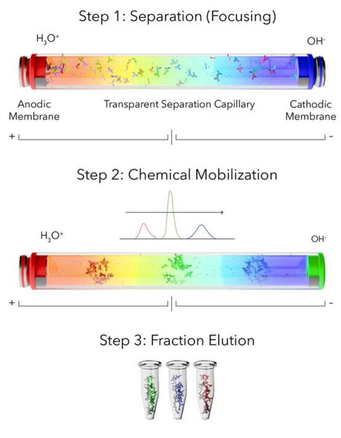
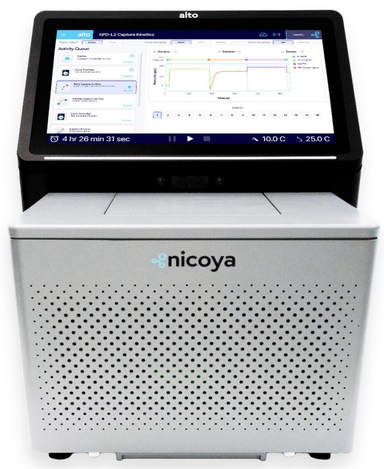


Figure 2. MauriceFlex charge variant fractionation workflow. Proteins are separated by isoelectric focusing (Step 1) before chemical mobilization (Step 2) and collection of individual charge variant fractions (Step 3).

Alto™ Digital SPR: Label-free Binding Analysis in a Droplet

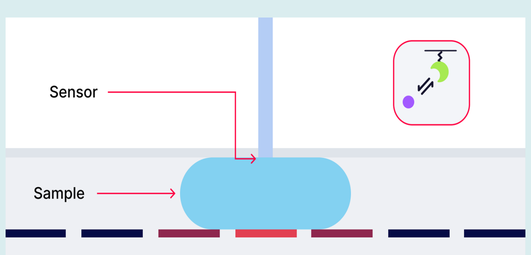
Nicoya's Alto system uses digital microfluidics (DMF) to deliver automatically diluted sample droplets to SPR sensors for effortless real-time characterization of biomolecular interaction analysis including quantitation, screening, epitope binning and binding kinetics.



- 16 independently addressable channels for high throughput.
- Complete kinetics analysis using only 2 µL of crude or pure sample.
- No manual dilutions, tagging, degassing, cleaning, or strenuous assay set up.
- Powerful epitope binning, data visualization and analysis software.

What is DMF? (Digital Microfluidics)

DMF is a liquid-handling technology capable of accurately controlling and manipulating discrete nanoliter-sized droplets across an array of electrodes. The fluidics are contained within a disposable cartridge, allowing Alto to overcome the major limitations associated with increasingly complex fluidic systems present in traditional label-free instruments.



Kinetics Characterization of the Mismatched BsAb

The binding of the BsAb innovator and biosimilar fractions to their CD3 and CD20 antigens were evaluated (Figures 6). Fractions of both samples showed strong binding with the epsilon subunit of the CD3 antigen, but not the delta or gamma subunits. The fractions also showed robust binding only with the CD20 antigen in nanodisc format, and not the individual recombinant extracellular domain proteins, illustrating the need to have a transmembrane protein in its full length and biologically relevant form.

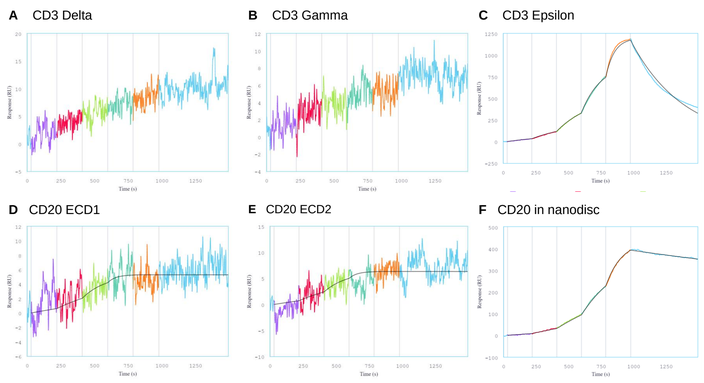


Figure 6. Binding of Mosunetuzumab innovator unfractionated sample. Binding to (A) CD3 delta, (B) CD3 gamma, and (C) CD3 epsilon subunits, and (D) CD20 ECD1, (E) CD20 ECD2, and (F) CD20 full length in nanodisc. Results show strong binding with the CD3 epsilon and CD20 nanodisc subunits.

We asked if the mismatching impacts antigen binding using SPR (Figure 7). Kinetics of CD3 and CD20 binding to the fractions were determined. A significant decrease in binding affinity (higher K_D) was observed for the fraction with the enriched mismatched biosimilar (acidic fraction).

Alto offers powerful time, labor and reagent savings for running traditional multi-cycle (MCK) or single-cycle kinetics (SCK) on 48 unique interactions per cartridge. For each analyte, Alto measures five (5) unique concentrations by automatically performing 3X serial dilutions. Experiments with SCK and MCK binding kinetics confirmed that the kinetics are effectively the same using either format (Figure 7) hence the SCK format data are presented in the rest of the study.

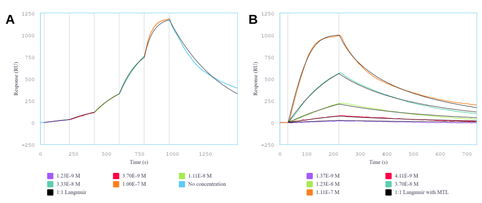
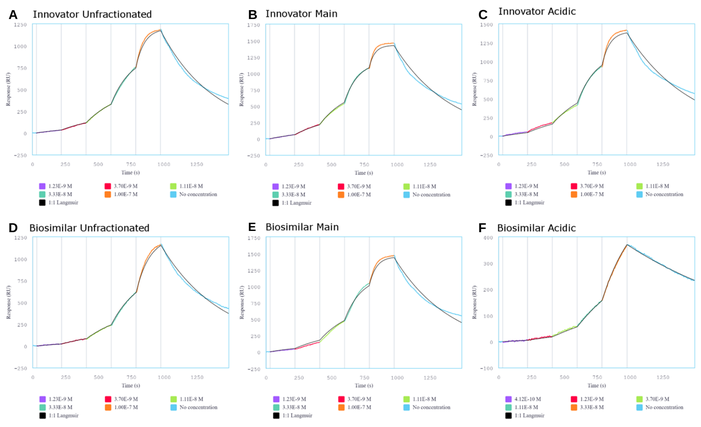


Figure 7. A comparison of SCK (A) and MCK (B) binding kinetics of the unfractionated BsAb innovator with CD3 epsilon

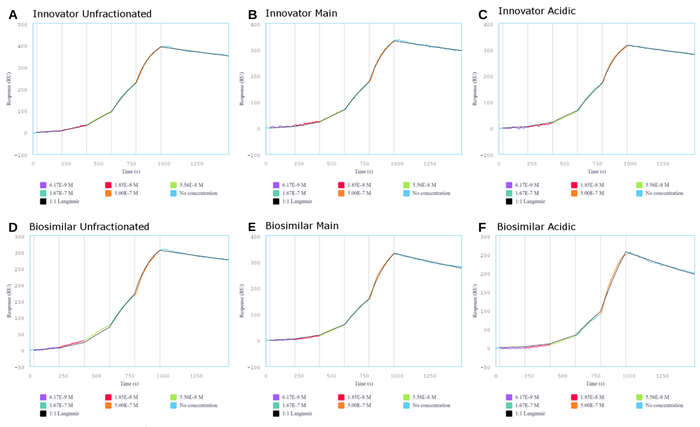
Table 1. Summary of binding affinity and kinetics for various charge variants with CD3 Epsilon and CD20 nanodisc. Acidic, main, and unfractionated samples are indicated by A, M, and U, respectively.

Binding Kinetics of Charge Variants with CD3 Epsilon and CD20 Nanodisc							
		CD3 Epsilon			CD20 Nanodisc		
Sample	Peak	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_D (nM)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_D (nM)
Innovator	A	1.30E+05	2.03E-03	16.3	1.31E+04	2.31E-04	17.6
	M	1.98E+05	2.32E-03	11.9	1.05E+04	3.09E-04	31.1
	U	7.82E+04	2.24E-03	28.6	1.63E+04	2.09E-04	12.9
Biosimilar	A	8.09E+04	1.03E-03	12.9	3.20E+03	5.20E-04	**162**
	M	1.50E+05	2.19E-03	14.7	1.23E+04	2.70E-04	22.4
	U	1.09E+05	2.45E-03	22.8	1.46E+04	1.96E-04	13.4

CD3



CD20



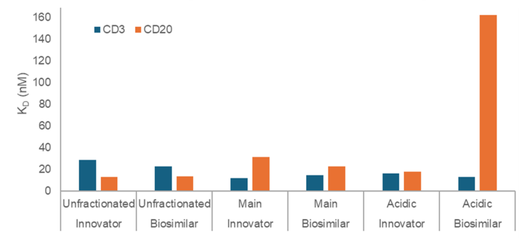


Figure 7. Ligand binding assays using the Alto SPR platform correlates mismatching with reduced ligand binding.
Binding affinity and kinetics measured for different fractions with CD3 epsilon (left panel) and with CD20 nanodisc (right panel). The figure displays the data for the Mosunetuzumab innovator acidic (A), main (B), and unfractionated peaks (C) and biosimilar acidic (D), main (E), and unfractionated peaks (F). A clear reduction in affinity (increase in K_D) is observed in the acidic fractions of only the mismatched biosimilar for CD20 (orange), but not CD3 (blue).

Material	Vendor	Catalog #
Mosunetuzumab innovator	Genentech	
Mosunetuzumab biosimilar	Ichorbio	ICH5026
MauriceFlex System	Bio-Techne	090-158
Maurice cIEF Cartridge		PS-MC02-C
Maurice cIEF Method Development Kit		PS-MDK01-C
MauriceFlex cIEF Fractionation Cartridge		
MauriceFlex cIEF Fractionation Method Development Kit		046-432
Nicoya Alto™ 16-Channel Instrument	Nicoya	ALTO16
Alto 16-Channel Carboxyl Cartridge		KIN-CART-CBX-16
Alto running Buffer: PBS-T (0.1% Tween 20), pH 7.4		ALTO-R-PBST
Alto CBX Surfacing Kit: cleaning, normalization, activation		ALTO-R-CBX-SURF
Alto regeneration Buffer: Gly-HCl pH 1.5		ALTO-RGLYHCl-1.5
Alto™ immobilization Buffer: Sodium Acetate pH 4.5 & 5.5		ALTO-R-IMB-4.5/5.5
Capture molecule: ChromoTek Nano-CaptureLigand® human IgG/rabbit IgG, Fc-specific VHH, biotinylated	Proteintech	shurbGB-1
Antigens: Human CD20 / MS4A1 Full Length Protein, His Tag (Nanodisc):	ACROBiosystems	CD0-H52H1-20ug
R&D Systems Recombinant Human CD3 epsilon Fc Chimera Protein	Bio-Techne	9850-CD

Conclusions

Certain charge species in antibodies can negatively impact binding affinity, and by extension, potency, as has been documented in other studies. It is imperative, therefore, that individual charge variants of biotherapeutic molecules are thoroughly characterized to ensure product quality. In this study, a novel workflow that leverages the combination of icIEF-based fractionation and SPR analysis, offering a robust and efficient approach to evaluating charge variants is illustrated. High-purity fractions, of both the innovator drug Mosunetuzumab and a research grade biosimilar, were obtained from the MauriceFlex system and subjected to SPR analysis on the Alto System for binding studies with ligands CD3 and CD20. The analysis revealed notable differences in binding affinity, particularly between the biosimilar's acidic peak and ligand CD20. These findings underscore the importance of advanced analytical techniques in the development and quality control of biotherapeutics, ensuring optimal product quality and consistency.

Learn more about the MauriceFlex System

Learn more about the Alto System