A Novel icIEF Fractionation & SPR-Based Workflow

for Correlating the Charge Structure to the Function of a Bispecific Antibody

Abstract

This application note presents a novel and efficient method for determining binding kinetics and affinity based on the charge heterogeneity of biological molecules. The study examined a therapeutic bispecific antibody (BsAb) called 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 ligands CD3 and CD20, which are the 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, 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, the workflow demonstrated in this study offers a straightforward and broadly applicable approach for correlating the charge heterogeneity of a biomolecule to its binding properties. This approach 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.

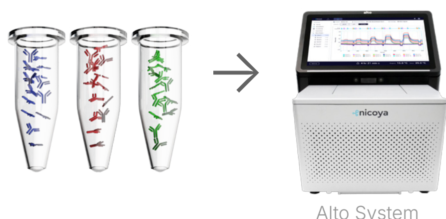
FIGURE // 01

Workflow Illustration

1. Separate and collect charge variants

MauriceFlex System

2. Load individual fractions



Alto System

3. Binding analysis

Biotherapeutic charge heterogeneity analysis and charge variant fraction collection workflow with the MauriceFlex System, followed by binding analysis on the Alto System.

Introduction

icIEF is the gold standard for assessing the charge heterogeneity of biotherapeutics. However, it has long been limited in its ability to isolate individual charge variants for further analysis with mass spectrometry (MS)[1]. For charge variant fraction collection, ion-exchange chromatography has been the method of choice but has drawbacks such as long method development times, labor intensive workflows, and large volumes of waste generation. More recently, advancements in capillary electrophoresis (CE) have sought to address these drawbacks by enabling off-line collection of charge variants followed by the LC-MS characterization.

Certain other CE based solutions such as CE-MS systems are typically compatible only with specific MS platforms. They limit the flexibility of using other downstream characterization methods for analyzing individual charge variants. As a result, users are often forced to resort to the more cumbersome and time-consuming IEX workflows. To address this challenge, the MauriceFlex System was designed to allow icIEF and icIEF-based offline fractionation such that fractions can be analyzed using a wide variety of MS methods. The system's success in enabling LC-MS and peptide mapping has been demonstrated in other studies[2,3], highlighting the high purity of collected fractions that enabled further characterization without much additional sample prep.

MS analysis of charge variants only offers structural information on heterogeneity. Consequently, there has been a growing recognition of the critical role of binding affinity analysis of charge variants, as the charged state of biotherapeutic proteins can affect their structure, stability, binding affinity, and efficacy. Further analysis of these fractions through binding potency assays can offer information on how different charge species impact the function of the molecule. This will help identify critical charge species that need to be monitored during development, and controlled during the manufacturing, of a biotherapeutic. Yet, there are no established methods in literature that accomplish such analysis rapidly using icIEF charge variant fractions.

In this study, fractions collected from the MauriceFlex system were put to test for measuring binding affinity and kinetics. In a separate study, the BsAb Mosunetuzumab and a research-grade biosimilar were fractionated on the MauriceFlex System for subsequent analysis with LC-MS[3]. Critical differences between the innovator and biosimilar were observed, including the presence of incorrectly paired antibodies in the acidic variants of the biosimilar. To evaluate how such modifications influence binding, the fractions were analyzed for binding using low sample volumes on Nicoya's Alto™ Digital SPR™ System.

SPR is a powerful label-free analytical technique for studying interactions between biomolecules. The Alto Digital SPR System from Nicoya Lifesciences is a next-generation SPR instrument that simplifies the collection of kinetics and affinity data by leveraging the power of digital microfluidics (DMF). Alto's DMF technology allows SPR experiments to be miniaturized and automated, allowing for the collection of reliable real-time binding data from only 2 µL of sample. Unlike other SPR systems that are costly to operate and maintain, Alto experiments are run on self-contained, disposable cartridges that integrate all liquid handling with 16 SPR sensors. This approach reduces hands on time, human error, and cross contamination between samples. Combined, these features allow Alto to overcome the challenges of traditional SPR when characterizing biomolecular interactions, and as demonstrated in this application note, making it particularly well suited for the direct analysis of fractions from the MauriceFlex instrument.

Materials and Methods

TABLE // 01

Materials and Kits Used

Material	Vendor	Catalog #
Mosunetuzumab innovator	Genentech	
Mosunetuzumab biosimilar	Ichorbio	ICH5026
MauriceFlex System	Bio-Techne	090-158
Maurice cIEF Cartridge	Bio-Techne	PS-MC02-C
Maurice cIEF Method Development Kit	Bio-Techne	PS-MDK01-C
MauriceFlex cIEF Fractionation Cartridge	Bio-Techne	PS-MC02-F
MauriceFlex cIEF Fractionation Method Development Kit	Bio-Techne	046-432
Nicoya Alto™ 16-Channel Instrument	Nicoya	ALTO16
Alto 16-Channel Carboxyl Cartridge	Nicoya	KIN-CART-CBX-16
Alto running Buffer: PBS-T (0.1% Tween 20), pH 7.4	Nicoya	ALTO-R-PBST
Alto CBX Surfacing Kit: cleaning, normalization, activation	Nicoya	ALTO-R-CBX-SURF
Alto regeneration Buffer: Gly-HCl pH 1.5	Nicoya	ALTO-RGLYHCl-1.5
Alto™ immobilization Buffer: Sodium Acetate pH 4.5 & 5.5	Nicoya	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)	Acro Biosystems	CD0-H52H1-20ug
R&D Systems Recombinant Human CD3 epsilon Fc Chimera Protein	Bio-Techne	9850-CD

icIEF Method

The innovator and biosimilar samples were prepared at a final concentration of 0.1 mg/mL in an ampholyte solution containing 4% Pharmalytes (3:1 8-10.5:3-10), 5 mM arginine, 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 minute at 1500 V, then 12 minutes at 3000 V.

Fractionation Method

Samples were prepared at a final concentration of 2 mg/mL in an ampholyte solution containing 4% Pharmalytes (3:1 8-10.5:3-10), 30 mM arginine, 33% SimpleSol, pI markers 7.05 and 9.50. 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 mobilized for 25 min at 1000 V, followed by fraction collection for 45 sec at 1000 V. Fractions were collected in 40 µL ammonium acetate (5 mM); then pooled and concentrated via speed vac. All data were analyzed using Compass for iCE software. For this study, four fractionation runs of each sample were pooled for analysis with SPR. Although multiple pools were provided to facilitate assay development it should be noted only a single plate of fractions is needed for full kinetics analysis.

SPR Method

The label-free SPR assay was performed using the Alto System after optimizing conditions using Nicoya Lifesciences' binding kinetics eBook. Binding kinetics analysis for the CD3 antigen against the biosimilar and innovator antibody fractions was performed via direct immobilization of the CD3 antigens. Three CD3 antigens were tested to determine which subunit the fractions bound to: the Human CD3 epsilon Fc Chimera Protein, Human CD3 delta/CD3d Fc Avi-tag Protein, and Human CD3 gamma Fc Chimera Protein (Bio-Techne/R&D Systems). The sensors of an Alto cartridge were cleaned with 10 mM HCl for 60 sec, followed by a 5 min activation with 25 mM EDC and 25 mM NHS. 2 µL of 2 µg/mL CD3 antigen in sodium acetate pH 4.5 was passed over each even channel sensor for 5 min. All sensors were then blocked with 1 M Ethanolamine for 5 min. For the biosimilar and innovator fractions, 2 µL of each was loaded on the cartridge at a concentration of 300 nM, except for the biosimilar's acidic fraction, which was loaded at 100 nM. Five 3-fold serial dilutions (1.2, 3.7, 11, 33, and 100 nM) were then automatically prepared by the Alto System in PBSTE (0.01 M Phosphate buffer, 0.14 M NaCl, 3 mM KCl, 0.1% T20, and 10 mM EDTA at pH 7.4). For kinetic experiments, both single-cycle kinetics (SCK) and multi-cycle kinetics (MCK) formats were run. For the SCK format, 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. For the multi-cycle kinetics (MCK) format, the analyte was introduced from lowest to highest concentration for 180 sec each, with a 600 sec dissociation step and 60 sec regeneration step, with 10 M glycine HCl pH 1.5 following each concentration.

Binding kinetics for the CD20 antigen against biosimilar and innovator antibody fractions was performed via capture by Nano-CaptureLigand® human IgG/rabbit IgG, Fc-specific VHH (Chromotek/Proteintech). The sensors of a CBX cartridge were cleaned with 10 mM HCl for 60 sec, followed by a 5 min activation with 25 mM EDC and 25 mM NHS. 2 µL of 20 µg/mL human IgG VHH in sodium acetate pH 5.5 was passed over each 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. For the analytes Human CD20, Nanodisc antigen (Acro Biosystems) and CD20 Recombinant Protein Antigen (Bio-Techne/Novus Biologicals), the creation of five 3-fold serial dilutions (6.2, 18.5, 55.6, 167, and 500 nM) were automatically prepared by Alto in PBSTE. Binding kinetics were performed in the SCK format. 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.

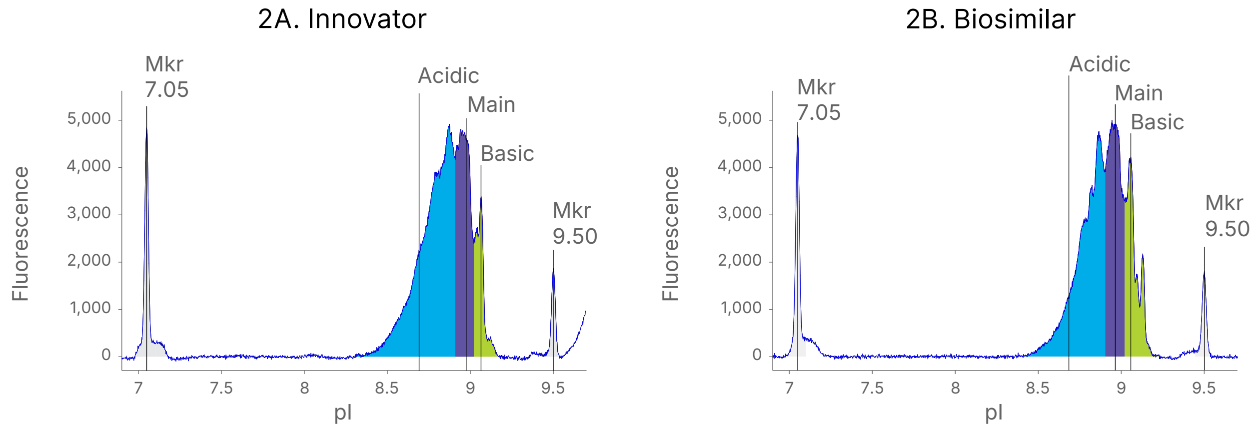
Results

icIEF Fractionation

The Mosunetuzumab innovator and biosimilar molecules were first analyzed for charge heterogeneity with the Maurice cIEF cartridge, resulting in clear acidic, main, and basic peaks for each molecule (data not shown, see our previously published study for charge profiles with the cIEF cartridge[3]). Prior to fraction collection, charge heterogeneity was assessed again using the MauriceFlex Fractionation Cartridge, which also resulted in the expected acidic, main, and basic peaks for the innovator and biosimilar, as shown in Figures 2A and 2B respectively. Percent peak area values are provided in Table 2. The detected peaks were mobilized for fractionation and verified with an unfractionated sample, as shown in Figures 3A and B.

FIGURE // 02

Fractionation Cartridge Charge Profiles



Charge profiles of the BsAb innovator Mosunetuzumab (A) and a research grade biosimilar (B) with the MauriceFlex Fractionation Cartridge. Acidic, main, and basic peaks were clearly detected for both samples.

TABLE // 02

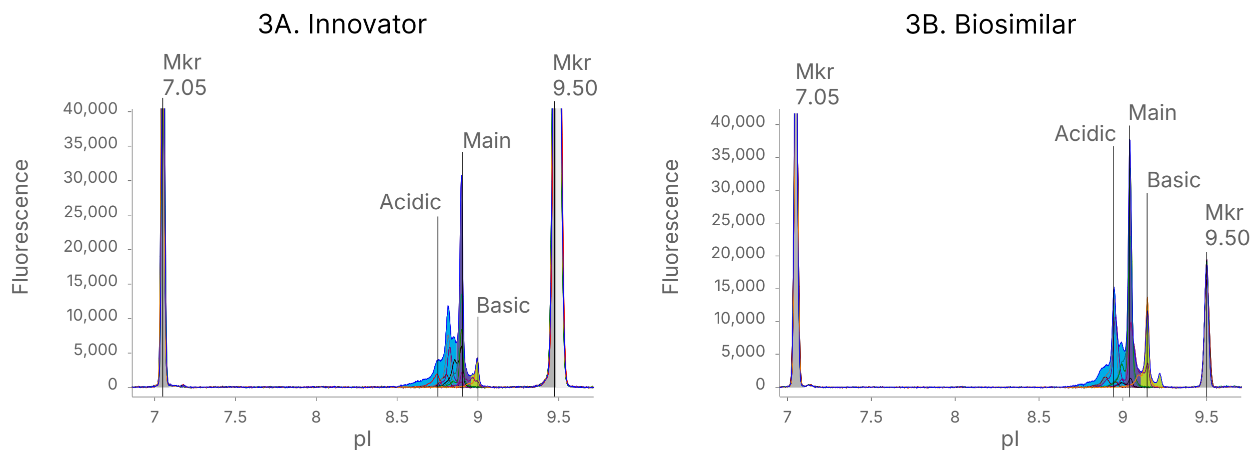
Percent Peak Area

Sample	Acidic	Main	Basic
Innovator	60.7	28.0	11.3
Biosimilar	47.6	32.1	20.2

Percent peak area values for the innovator and biosimilar samples with icIEF analysis.

FIGURE // 03

Fractionation of Charge Variants



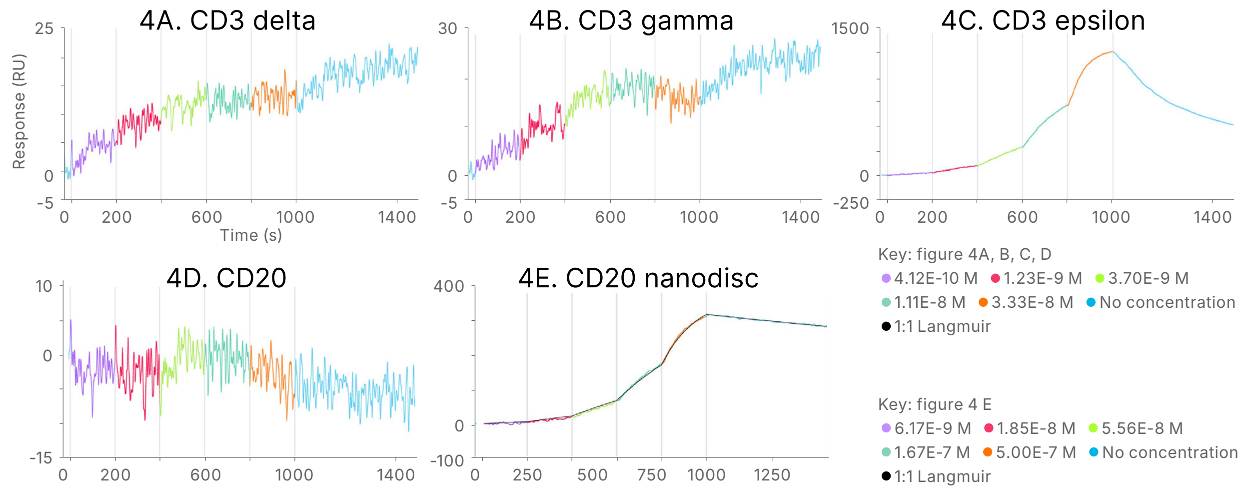
Fractionation of charge variants on the MauriceFlex system. The innovator molecule resulted in 11 fractions, and the biosimilar in 8 fractions, with unfractionated samples overlaid in blue. Pooled fractions from four runs were used for SPR analysis.

Binding Analysis

The binding of the BsAb innovator and biosimilar fractions to their CD3 and CD20 antigens were evaluated, as shown in Figures 4 and 5. Fractions of both samples showed strong binding with the epsilon subunit of the CD3 antigen, and not the delta or gamma subunits. The fractions also showed good binding only with the CD20 antigen in nanodisc format, and not the individual recombinant extracellular domain proteins. The CD20 results illustrate the need to have a transmembrane protein in its full length and biologically relevant form. Consequently, the CD3 epsilon and CD20 nanodisc subunits were used for further SPR analysis in this study.

FIGURE // 04

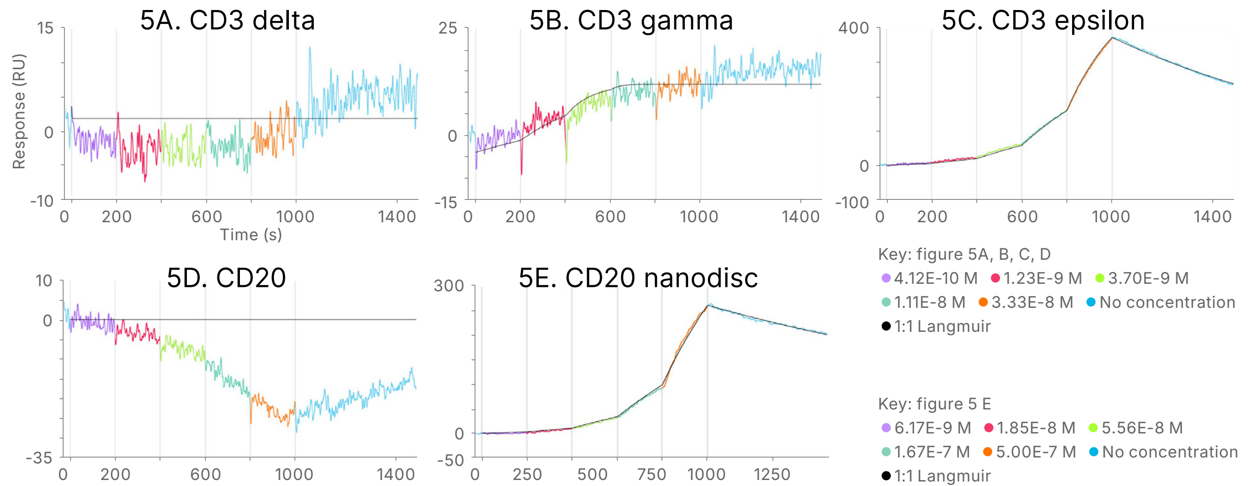
Binding of BsAb Innovator



Binding of Mosunetuzumab innovator unfractionated sample with CD3 and CD20 subunits. Concentrations of the 5 analyte sample dilutions assessed are shown in the key in the figure. Results show strong binding with the CD3 epsilon and CD20 nanodisc subunits.

FIGURE // 05

Binding of BsAb Biosimilar

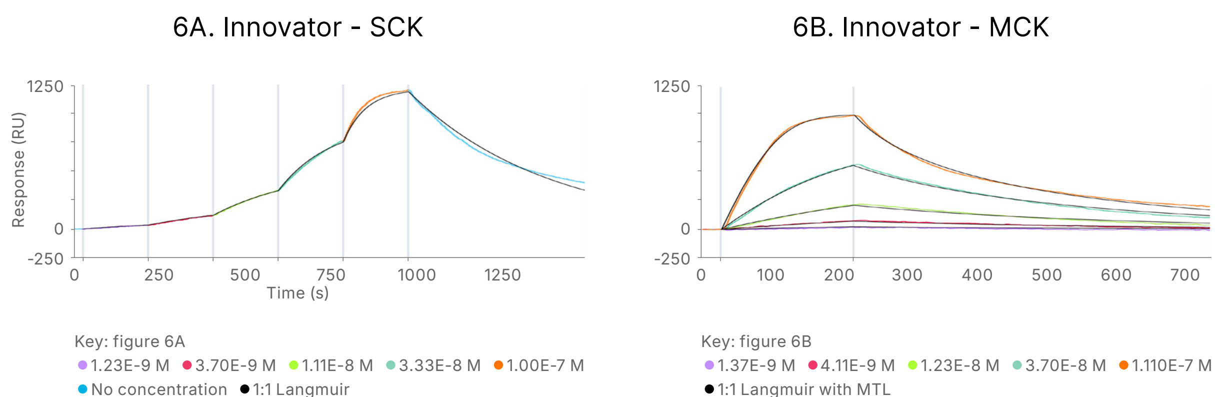


Binding of the BsAb biosimilar unfractionated sample with CD3 and CD20 subunits. Concentrations of the 5 analyte sample dilutions assessed are shown in the key in the figure. Similar to the innovator peaks, the results here show strong binding with the CD3 epsilon and CD20 nanodisc subunits.

Experiments with SCK and MCK binding kinetics confirmed that the kinetics are effectively the same using either format, as illustrated in Figure 6, hence the SCK format data is presented in the rest of the study. Furthermore, mismatched species have been identified in acidic peaks before[3], therefore this study focused on the acidic and main peaks of both samples. Binding data for innovator BsAb peaks with CD3 epsilon are shown in Figure 7, where A, M, and U refer to acidic, main, and unfractionated peaks respectively. Similarly, binding data for the biosimilar are shown in Figure 8. In both samples, all fractions fall within the range of comparable association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium dissociation constant (K_D) values when binding to the CD3 antigen, as summarized in Table 3.

FIGURE // 06

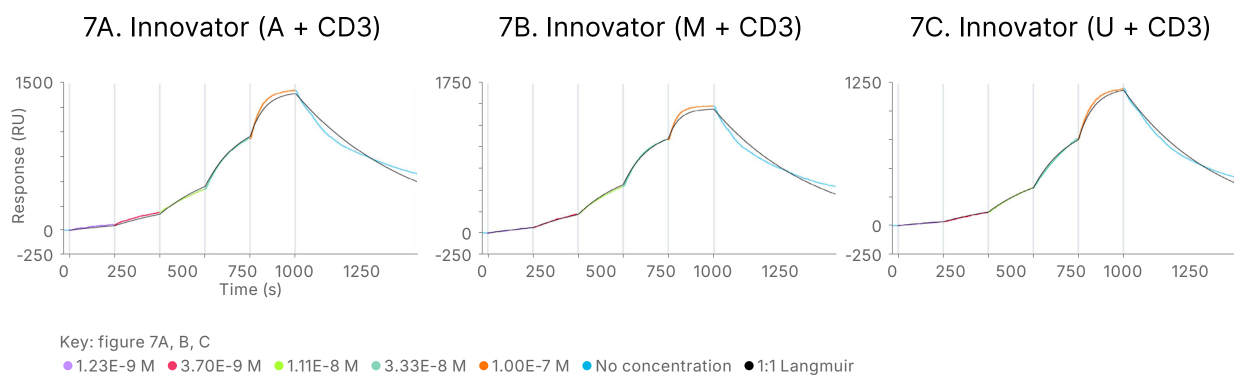
Comparison of SCK and MCK



A comparison of SCK (A) and MCK (B) binding kinetics of the unfractionated BsAb innovator with CD3 epsilon, where the kinetics were found to be comparable.

FIGURE // 07

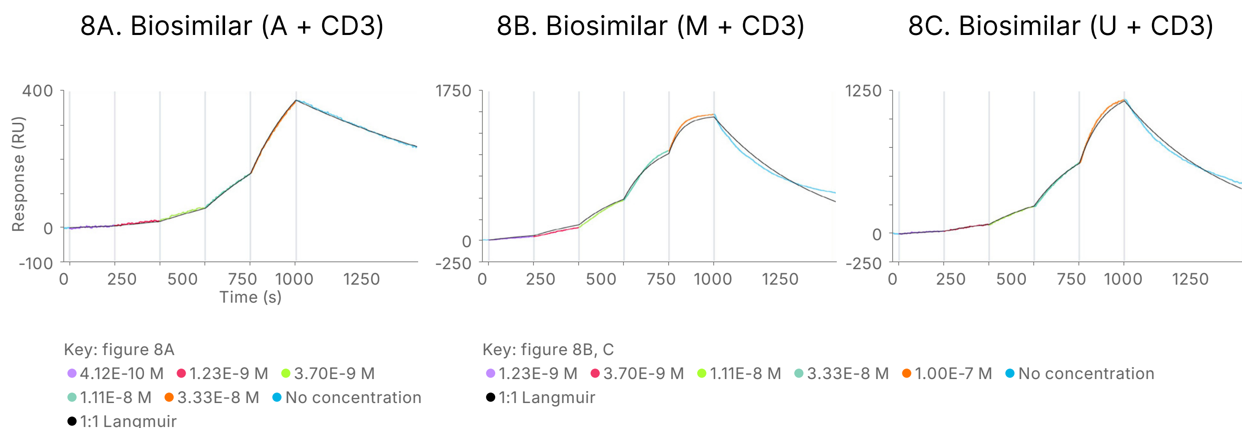
Binding Affinity and Kinetics of Innovator Fractions with CD3



Binding affinity and kinetics measured for different innovator peaks with CD3 epsilon, using SPR analysis on the Alto System. Data are shown for the acidic (A), main (B), and unfractionated samples (C), all showing relatively strong binding with the ligand.

FIGURE // 08

Binding Affinity and Kinetics of Biosimilar Fractions with CD3

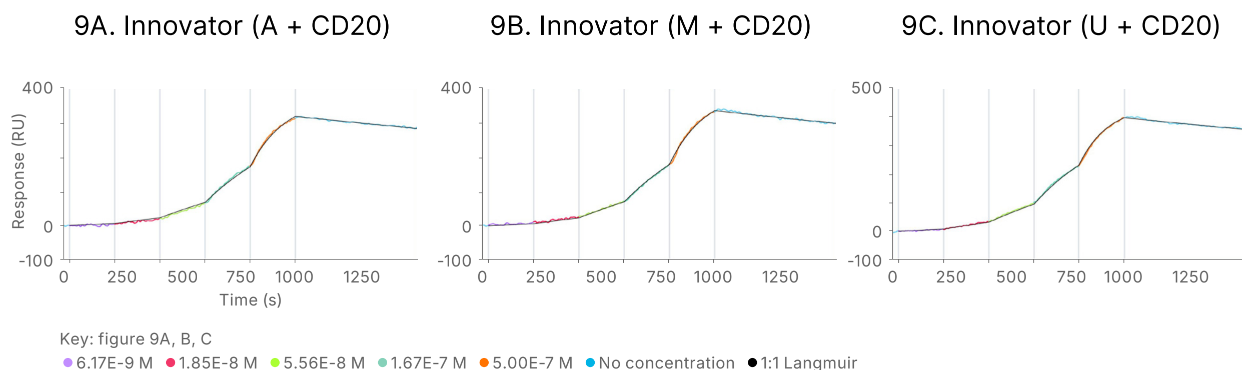


Binding affinity and kinetics measured for different biosimilar peaks with CD3 epsilon. The figure displays the data for the acidic (A), main (B), and unfractionated samples (C), all indicating strong binding with the ligand.

Binding data for the innovator and biosimilar peaks with CD20 nanodisc are shown in Figures 9 and 10. Notably, binding data of the biosimilar's acidic fractions displayed different kinetics, while the rest of the peaks provided results within an expected range (Table 4). The high KD value for the binding between the biosimilar's acidic peak and CD20 indicates low affinity, which corresponds to the LC-MS results in which the same peak was found to have incorrectly assembled BsAb[3]. Binding kinetics of charge variants with CD20 are summarized in Table 3, and a comparison of binding affinities of different innovator and biosimilar fractions with ligands CD3 and CD20 is summarized in Figure 11.

FIGURE // 09

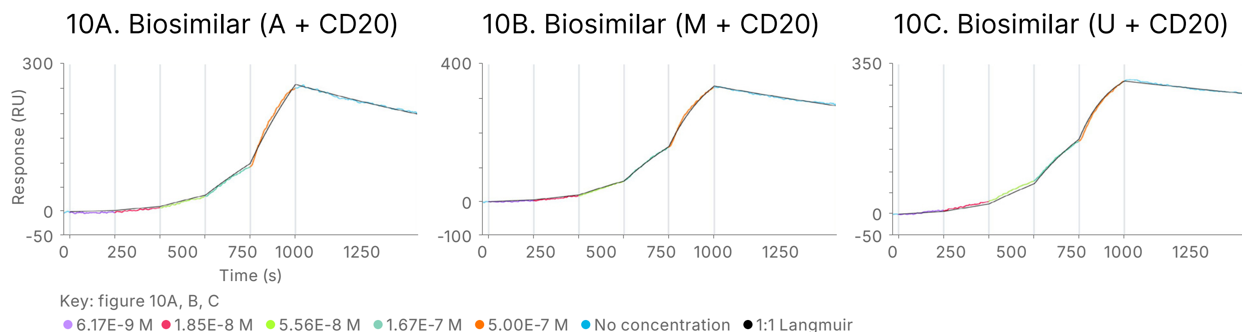
Binding Affinity and Kinetics of Innovator Fractions with CD20 in Nanodisc



Binding affinity and kinetics of the innovator fractions with CD20 nanodisc, where data are shown for the acidic (A), main (B), and unfractionated peaks (C). All three peaks showed a high degree of binding with the ligand.

FIGURE // 10

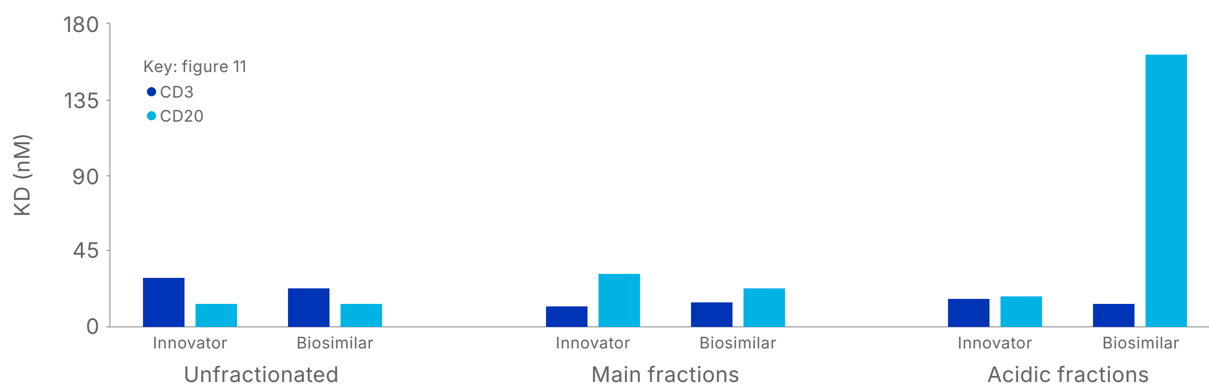
Binding Affinity and Kinetics of Biosimilar Fractions with CD20 in Nanodisc



Binding affinity and kinetics for different biosimilar fractions with CD20 nanodisc, with data shown for acidic (A), main (B), and unfractionated peaks (C). The acidic peak showed a much lower affinity for the ligand, while the main peak and unfractionated sample shower comparable binding to that of the innovator peaks.

FIGURE // 11

Comparative Analysis of Binding Kinetics



A comparative analysis of binding kinetics (KD) between different charge variant fractions and the CD3 epsilon and CD20 antigens. Data are shown for unfractionated, main fractions, and acidic fractions of innovator and biosimilar samples binding with each antigen.

TABLE // 03

Binding Kinetics of Charge Variants with CD3 Epsilon and CD20 Nanodisc

Sample	Peak	CD3 Epsilon			CD20 Nanodisc		
		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	31.3
	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

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. The comparatively low binding affinity of the biosimilar's acidic peak with CD20 is highlighted.

Conclusion

Certain charge species in antibodies can negatively impact binding affinity, and by extension, potency, as has been documented in other studies[4,5]. 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.

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