

## Characterizing Charge and Size Variants of a Bispecific Antibody Innovator and Biosimilar by icIEF-Fractionation and Mass Photometry

### Highlights

- This workflow enables charge and size variant characterization in a single pipeline, closing a critical gap in BsAb and biosimilar analytics.
- icIEF-based charge variant fractions are directly analyzed for size variants using mass photometry without any buffer exchange.
- The workflow is fast (~4 hr) and has low sample requirements.
- Provides insights into bispecific pairing and stability of the innovator drug and biosimilar.
- Demonstrates that stress conditions amplify charge-dependent size heterogeneity.

### Why This Workflow Matters for Bispecific Antibodies

For BsAbs, correct monomer pairing and molecular stability are CQAs with direct implications for safety and efficacy. Conventional approaches involve ion-exchange chromatography for charge variants and SEC for size variants. Because concentrating IEX fractions for analysis with SEC can be a time-consuming process, combining icIEF fractionation with mass photometry is a faster alternative, enabling both CQAs to be characterized in a single, streamlined pipeline.

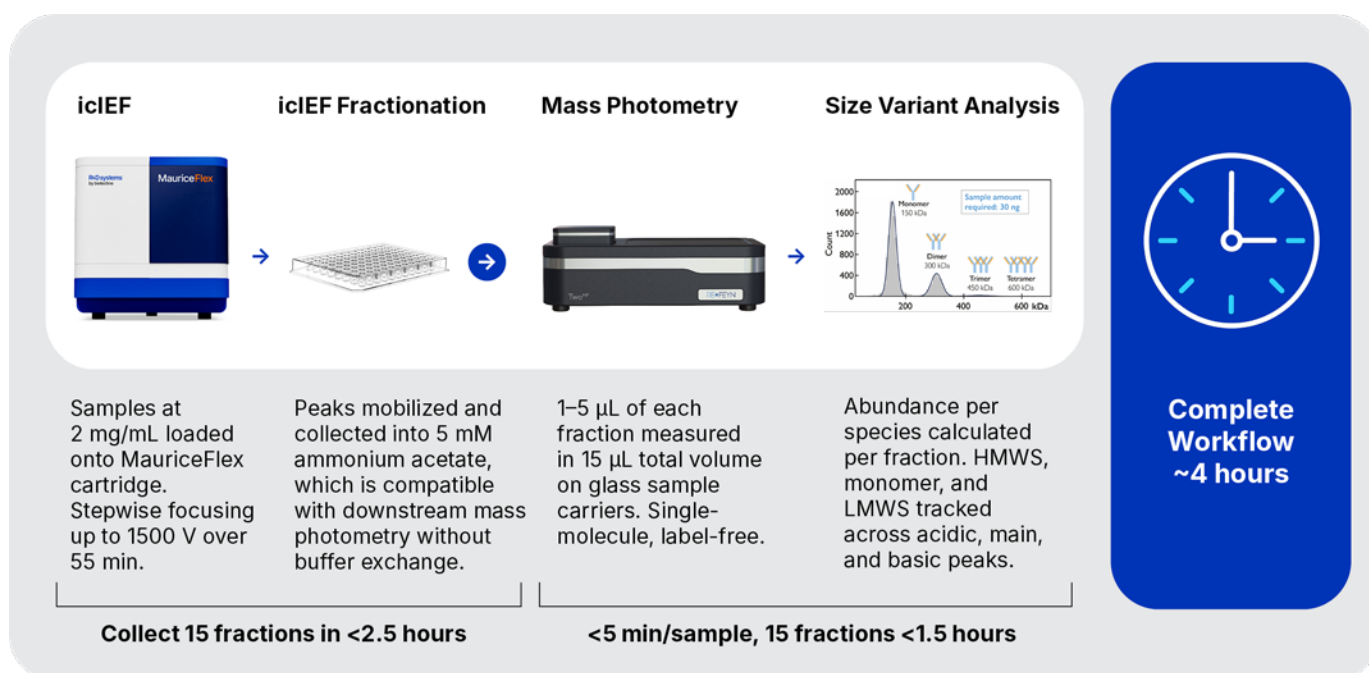


Figure 1. Characterizing Charge and Size Variants with icIEF Fractionation and Mass Photometry. This workflow combines icIEF fractionation using R&D Systems MauriceFlex™ platform and mass photometry using Refeyn's TwoMP instrument.

## The Advantages Offered by Each Method

### icIEF and icIEF Fractionation:

- Separates and isolates acidic, main, and basic charge variants with high resolution
- Enables collection of distinct charge isoforms with >70% purity
- Collected fractions are compatible with downstream analytical systems

### Mass Photometry (MP):

- Identifies and quantifies size variants based on molecular mass at the single-molecule level
- Detects low-abundance fragments and aggregates that are difficult to resolve by SEC
- Requires minimal sample with no buffer exchange or method development

## Case Study: Comparing Charge and Size Variants of Mosunetuzumab and a Biosimilar

The combined icIEF and MP workflow was implemented to analyze charge and size variants of the innovator bsAb Mosunetuzumab-axgb (MOS) and a research-grade biosimilar (BSM).

The bispecific antibodies were analyzed in the following conditions:

- ✓ In the native state (control)
- ✓ After heat stress (45 °C for 7 days)
- ✓ After forced aggregation induced with BS3 cross-linker

Details on the materials and methods used for icIEF fractionation are provided on **Appendix 1**.

Following icIEF fractionation, fractions were selected for further analysis by MP to assess size variant distributions across the charge variant landscape. Fractions demonstrating >70% purity from a single icIEF peak were taken as representative of that peak and selected for further analysis with MP. More details on the fractions selected are provided in **Appendix 2**.

## Results

**icIEF separation reveals higher degradation of the BSM charge variants than the innovator drug's corresponding peaks under stress conditions and an additional basic peak under native (control) and stressed conditions.**

FIGURE 2A

### Mosunetuzumab

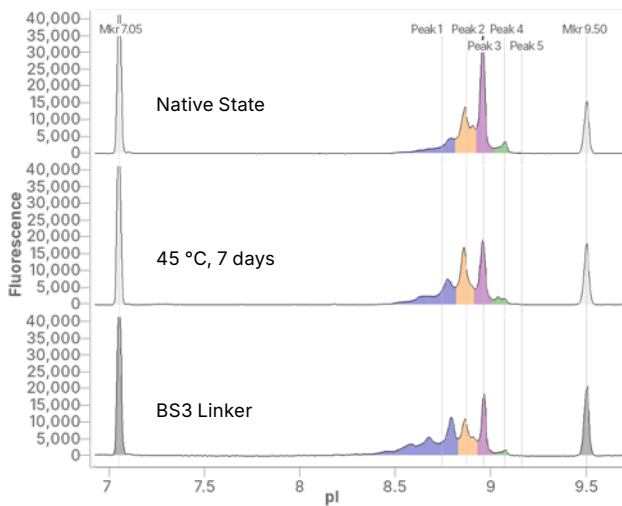
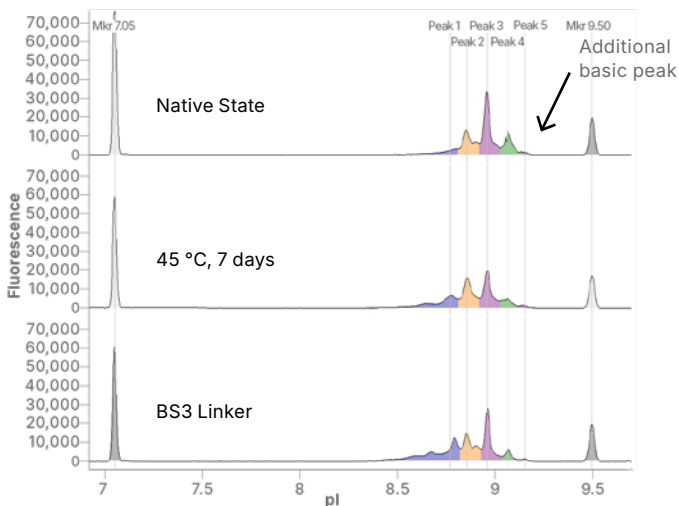


FIGURE 2B

### Biosimilar



**Figure 2.** icIEF analysis of Mosunetuzumab innovator and biosimilar under native and stress conditions. Representative imaged capillary isoelectric focusing (icIEF) electropherograms of Mosunetuzumab innovator (A) and a research-grade biosimilar (B) analyzed under native conditions, heat stress (45 °C for 7 days), and BS3 cross-linker-induced stress. The innovator shows a dominant main peak with minor acidic and basic variants across all conditions. In contrast, the biosimilar exhibits an additional basic peak under native conditions that intensifies under stress, indicating differences in charge heterogeneity between the two molecules.

FIGURE 3A

### Mosunetuzumab (%PA)

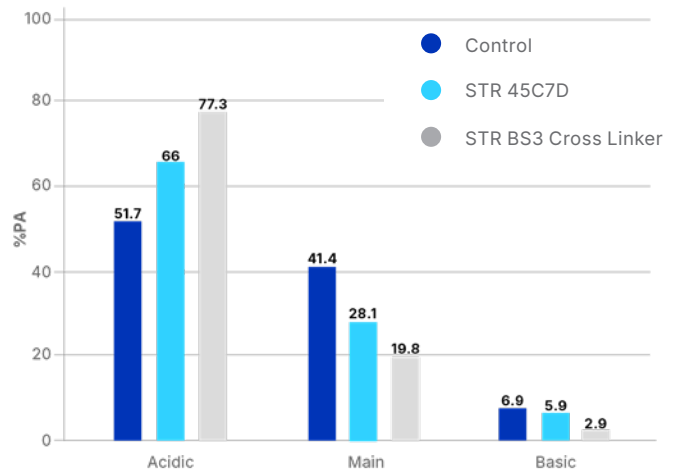
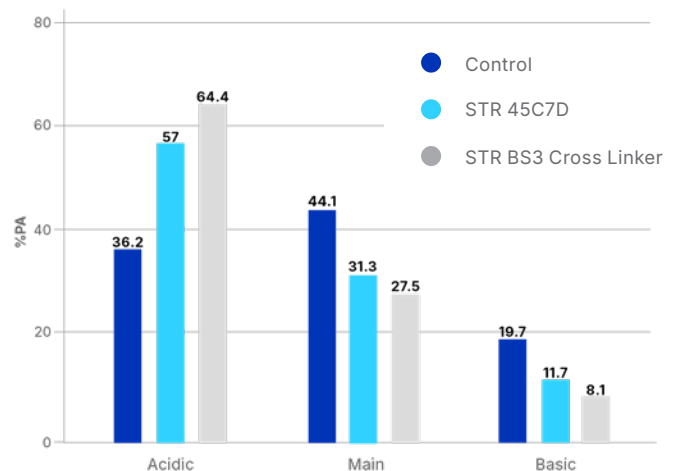


FIGURE 3B

### Biosimilar



**Figure 3.** Charge variant distribution of innovator and biosimilar across stress conditions. Quantification of acidic, main, and basic charge variant populations for Mosunetuzumab innovator (A) and biosimilar (B) under native, heat-stressed, and BS3 cross-linked conditions. The bar graphs represent icIEF peak area percentages. The biosimilar displays a higher relative abundance of basic variants compared to the innovator across all conditions, consistent with the appearance of an additional basic peak observed in the electropherograms.

## MP analysis of charge variants reveals assembly-related impurities enriched in basic biosimilar fractions

FIGURE 4A

MOS

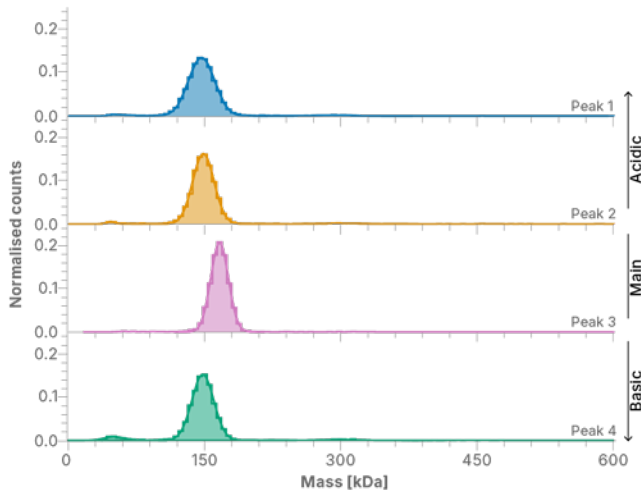


FIGURE 4B

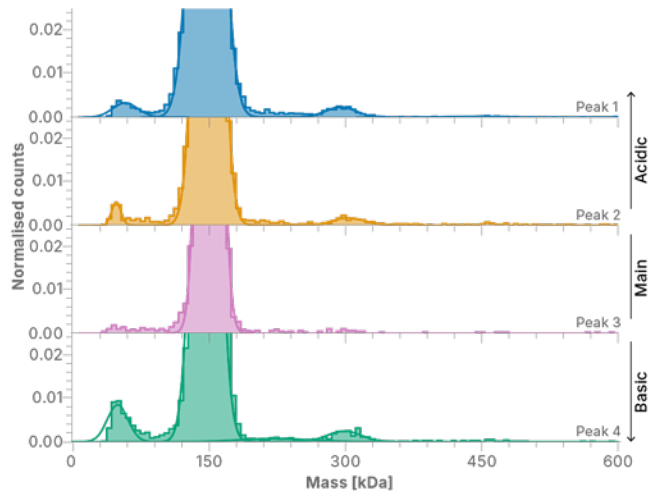


FIGURE 4C

BSM

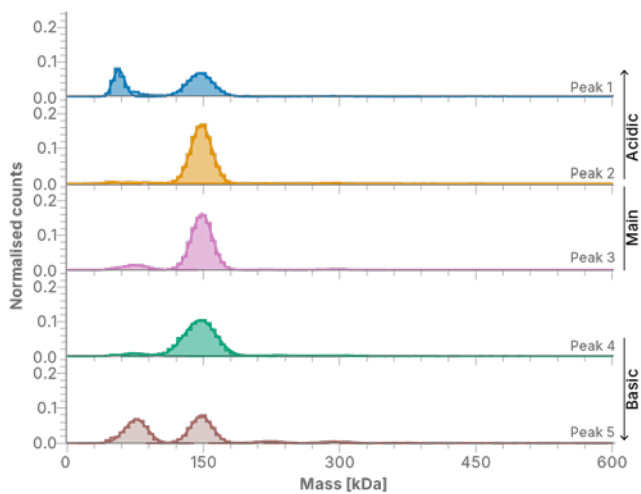
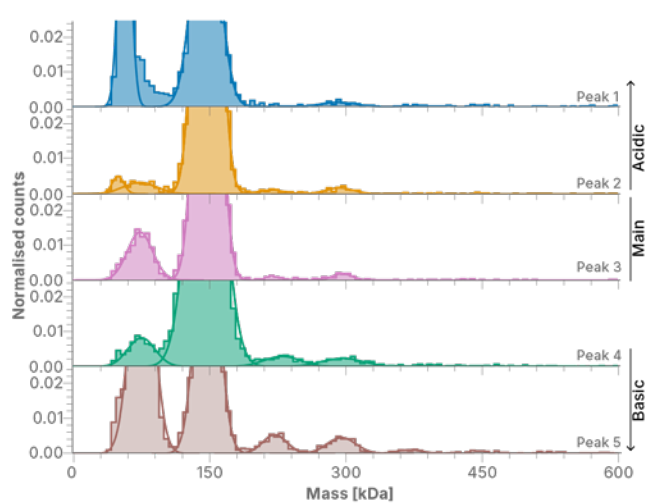


FIGURE 4D

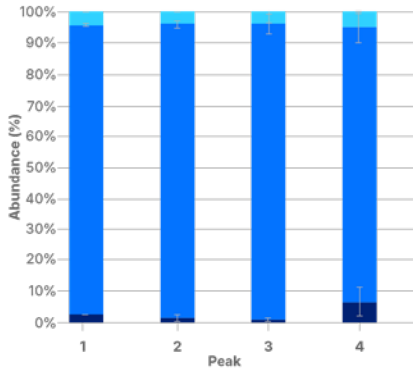


**Figure 4:** MP analysis of icIEF charge variant fractions from control (native state) samples reveals half-antibody impurities enriched in basic fractions of the biosimilar. Selected icIEF fractions were analyzed by MP. Mass histograms show the relative abundance of species of different mass in fractions of the innovator (MOS) and biosimilar (BSM) (A, B). In control (native state) samples, mass histograms of all the innovator (MOS) fractions showed a dominant population consistent with the expected full bsAb mass (~150 kDa), with no significant impurity species. In contrast, novel ~75 kDa (half-antibody) and ~225 kDa (1.5-antibody) species (arrows) were observed in the BSM 45 and appeared enriched in the basic fractions. Zoomed-in mass histograms (C, D) highlight the half-antibody species (asterisks).

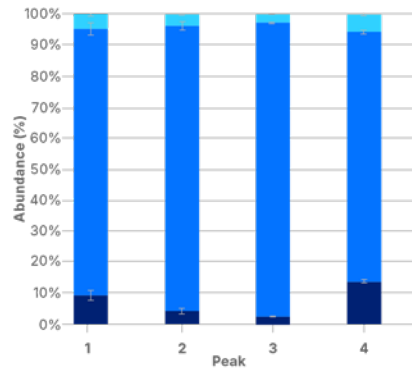
## MP analysis of charge variants revealed greater accumulation of LMWS and HMWS in the biosimilar, under stress conditions

FIGURE 5

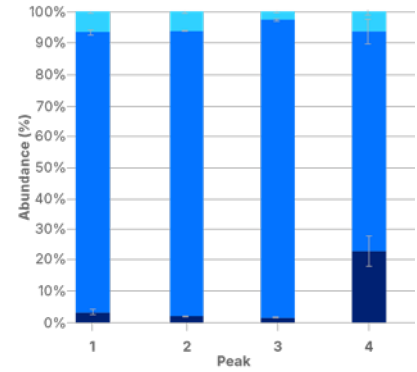
### MOS Control



### MOS 45°C/7D



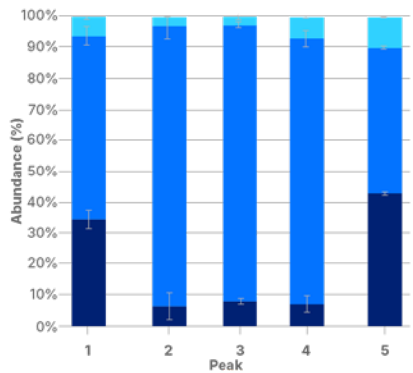
### MOS + BS3



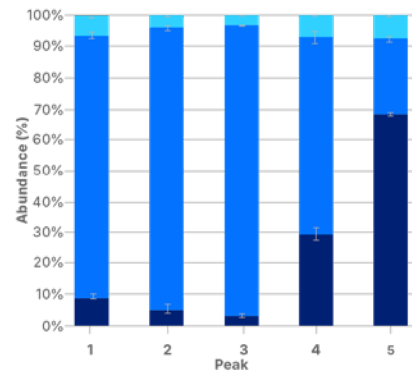
#### KEY

● Total HMWS    ● Monomer    ● Total LMWS

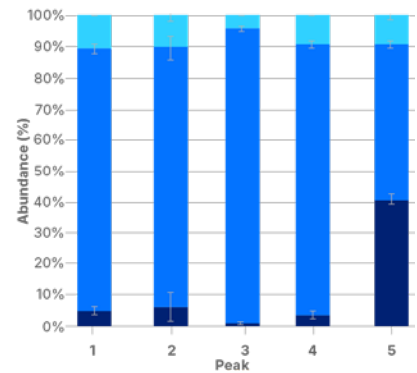
### BSM Control



### BSM 45°C/7D



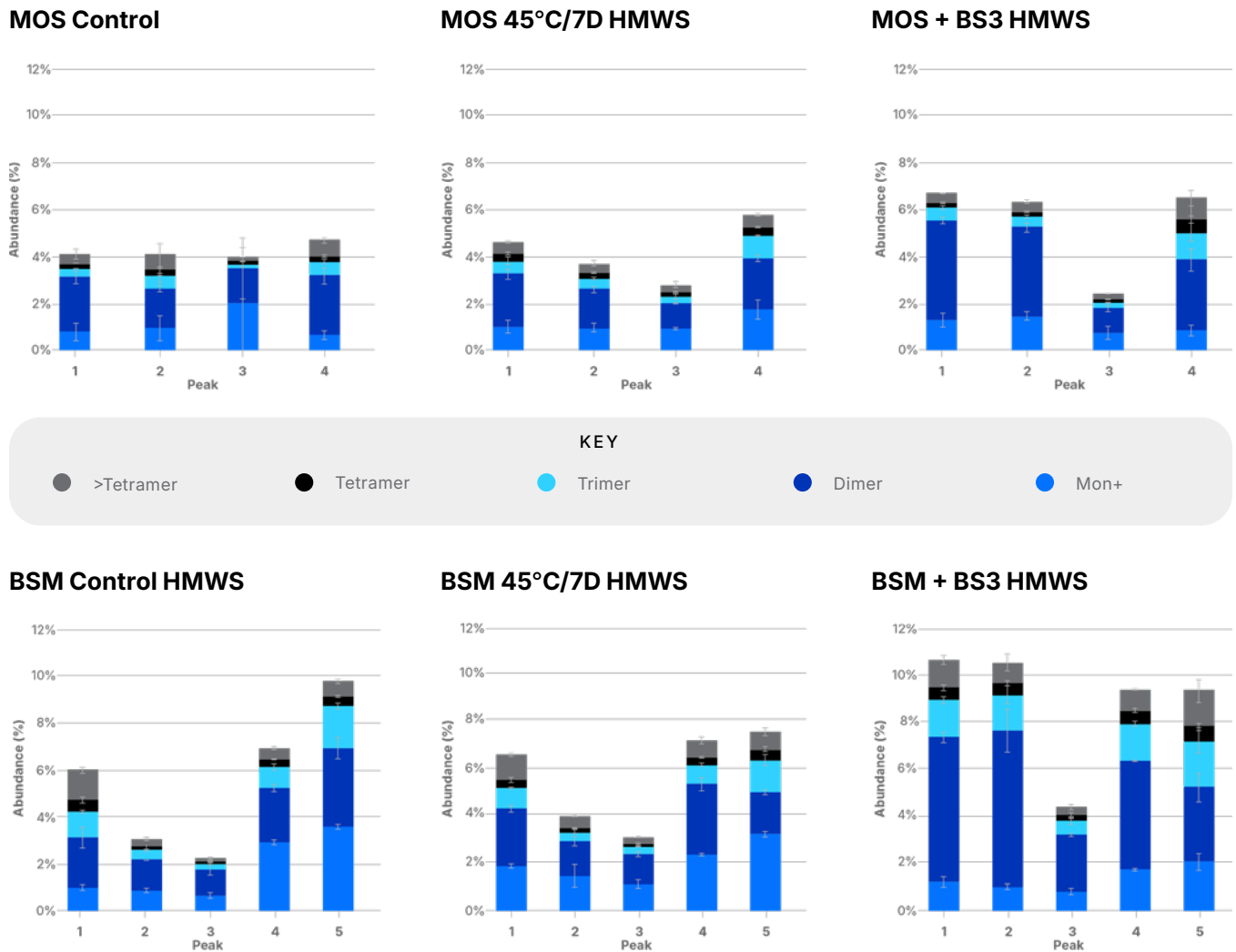
### BSM + BS3



**Figure 5.** Quantification of monomers, LMWS and HMWS reveals greater stability of MOS relative to BSM under native and stress conditions. MP was used to assess selected icIEF fractions from the samples exposed to heat stress (7 days at 45 °C) and crosslinking (with BS3). For the innovator (MOS), MP profiles were broadly consistent across control and both stress conditions, with only small increases in low-molecular weight species (LMWS) and high-molecular weight species (HMWS) observed across all the charge variant fractions (top). Heat stress resulted mainly in small increases in LMWS for MOS, while cross-linking resulted in some increased aggregation (HMWS) across fractions, and increased fragmentation (LMWS) for Peak 4. This largely stable distribution across conditions is consistent with the profile expected of a clinically approved biotherapeutic. The biosimilar (BSM), in contrast, showed notably greater variability than MOS. The quantification confirmed the observation (from Fig. 4) that BSM exhibited more fragmentation and aggregation in the unstressed control. BSM also showed increased fragmentation and aggregation under both stress conditions relative to MOS (bottom), with specific charge variants (e.g. Peaks 1 and 5) appearing most likely to degrade or aggregate.

## MP analysis resolves and quantifies distinct aggregate species in innovator and biosimilar fractions

FIGURE 6



**Figure 6.** Analysis of the HMWS in the MP data reveals abundance of distinct MOS and BSM aggregate species under native and stressed conditions. MP makes it possible to resolve the individual species formed, so that distinct populations – fragments (LMWS), monomers, and aggregates (HMWS) – can be identified and quantified across each charge variant fractions. The innovator (MOS, top) was broadly stable against aggregation compared with the biosimilar (BSM, bottom), with dimers as the dominant HMWS across most fractions and conditions. The BSM showed more evenly distributed abundance across species, from 1.5-antibody species up to aggregates larger than tetramers, with greater overall HMWS abundance across fractions and stress conditions. 'mon+' refers to species with mass equivalent to 1.5 antibodies; '>tetramer' refers to all species with mass greater than four antibodies. This level of detail provides a granular picture of how the two molecules differ in their stress responses.

## Takeaways

- ✓ Combining icIEF fractionation on MauriceFlex with size variant analysis on TwoMP enables the characterization of charge variants in ~4 hours without any buffer exchange or method optimization, all the while reducing hands-on effort and sample consumption.
- ✓ In this study, the workflow provided critical insights on innovator and biosimilar bispecific antibodies with respect to the abundance and arrangement of monomers, aggregates, and other species.
- ✓ Biosimilar charge variants contain higher levels of both low molecular weight species (LMWS) and high molecular weight species (HMWS) compared to corresponding innovator fractions, indicating increased heterogeneity.
- ✓ MP analysis of basic biosimilar fractions shows impurities consistent with half antibody loss or gain, suggesting pairing or assembly related discrepancies.
- ✓ Stress conditions amplify charge dependent size heterogeneity, with BS3 cross linking increasing HMWS (notably dimers and higher order species) and heat stress primarily increasing LMWS.
- ✓ This integrated workflow closes a critical gap in bispecific antibody characterization, enabling assessment of charge and size variant CQAs in a single pipeline - to support faster development and earlier identification of quality issues.



Learn more about the MauriceFlex system



Learn more about the TwoMP system

## Appendix 1

TABLE 1

### Materials and methods used in this study.

Material	Vendor	Catalog #
Mosunetuzumab innovator	Genentech	
Mosunetuzumab biosimilar	Ichorbio	ICH5026
MauriceFlex System		<a href="#">090-158</a>
Maurice cIEF Cartridge		<a href="#">PS-MC02-C</a>
Maurice cIEF Method Development Kit	R&D Systems	<a href="#">PS-MDK01-C</a>
MauriceFlex cIEF Fractionation Cartridge		<a href="#">PS-MC02-F</a>
MauriceFlex cIEF Fractionation Method Development Kit		<a href="#">PS-MDK01-C</a>
TwoMP System	Refeyn	<a href="#">MP-PRO-00001</a>
MassGlass UC Sample Prep Kit	Refeyn	<a href="#">MP-CON-21022</a>

### 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, or; 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. Each fraction was collected for 45 Sec at 1000V. All data were analyzed using Compass for iCE software.

### Mass Photometry Method

Samples were analyzed on the TwoMP system using the standard materials and methods as described by the manufacturer. All dilutions and measurements were performed in 5 mM ammonium acetate. Sample wells, carriers, and cassettes were provided by Refeyn. All samples were measured on a TwoMP (Refeyn) using the large field of view, default acquisition settings, and an acquisition duration of 60 sec. Unfractionated MOS and BSM samples were diluted to 15 ng/μL in 5 mM ammonium acetate at room temperature and measured as a 15 ng droplet after focusing. For fractionated samples, 1 μL of each fraction was measured in a 14 μL droplet of 5 mM ammonium acetate after finding focus. The number of binding counts/μL in each fraction was used to estimate the volume to be measured to provide approximately 15,000 counts. Fractions showing more than 15,000 counts/μL were diluted in 5 mM ammonium acetate to achieve this target and were kept at room temperature after thawing. AcquireMP v2024.2.0 and DiscoverMP v2025.1.0 (Refeyn) were used for acquisition and analysis, respectively.

## Appendix B

Tables 2A-2C list the %PA of the fractions collected from the innovator (MOS), based on which select fractions were analyzed with MP. Similarly, Tables 2D-2F list the %PA for the biosimilar fractions collected. Across all tables, %PA values highlighted in yellow were selected for further analysis with MP.

TABLE 2

### 2A

Innovator Control (%Peak Area)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Control	17.2	34.5	41.4	6.7	0.2
Fr E5	5	0	14.9	0	80.1
Fr E4	3.1	0	2.1	94.9	0
Fr E3	0.3	0	0.7	98.1	0.9
Fr E2	0.7	4.1	67.4	27.6	0.3
Fr E1	0.2	13.3	85.9	0.7	0
Fr F1	0.7	20.5	78.7	0.1	0
Fr F2	2.3	57	40.4	0.1	0.1
Fr F3	12.5	85.3	2	0.1	0
Fr F4	17.9	81.9	0.2	0	0.1
Fr F5	69.2	30.3	0.2	0.2	0.1
Fr F6	99.9	0	0.1	0.1	0
Fr F7	99	0.2	0.4	0	0.4
Fr F8	97	0	0.2	0.7	2.1
Fr F9	96.2	0	0	1.9	1.9
Fr F10	94.8	0.5	1.6	3.2	0

### 2B

Innovator Stressed: 45°C/7Days (% Peak Area)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Control	33.9	32.1	28.1	5.6	0.3
Fr E12	0	8.9	0	0	91.1
Fr E11	0.1	0	12.9	85.2	1.7
Fr E10	0.3	0	24.7	75	0
Fr E9	0.1	11.5	83.5	4.9	0
Fr E8		12.1	87.8	0.1	0
Fr E7	0.4	46	53.5	0.1	0.1
Fr E6	1.7	95.2	3.1	0	0.1
Fr E5	4.9	95.1	0	0	0
Fr E4	61.4	38.4	0.2	0.1	0
Fr E3	93.2	6.7	0	0	0.1
Fr E2	99.4	0.1	0	0.2	0.3
Fr E1	99.3	0.1	0.1	0.1	0.5
Fr F1	99.4	0	0	0.4	0.2
Fr F2	98.9	0.1	0	0.8	0.2
Fr F3	97.5	0.5	0.9	1	0.1
Fr F4	100	0	0	0	0

### 2C

Innovator Stressed: B53 Cross Linker-Induced (% Peak Area)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Control	54.4	22.8	19.8	2.8	0.1
Fr E8	1.1	1.3	2.1	86.3	9.1
Fr E7	0.9	0		95.2	4
Fr E6	0	10.6	83.2	6	0.1
Fr E5	0.4	17.7	80.7	0.5	0.7
Fr E4	0	62.8	37.1	0	0
Fr E3	0	91.1	8.9	0	0
Fr E2	0.7	99.3	0	0	0
Fr E1	97.1	1.8	0.2	0.2	0.7
Fr F1	98.6	0.1	0.2	0.4	0.8
Fr F2	98.2	0.1	0.2	0.6	0.9
Fr F3	100	0	0	0	0
Fr F4	98	0.1	0.1	0.8	0.9
Fr F5	99.9	0	0	0.1	0
Fr F6	99.1	0	0	0.7	0.2
Fr F7	99.7	0	0	0.3	0
Fr F8	99.1	0	0.6	0.3	0

### 2D

BioSimilar Control (% Peak Area)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Control	10.8	27.6	43.8	15.9	2
Fr E11	0.6	0	0	0.9	98.6
Fr E10	0.4	0	0	8.1	91.5
Fr E9	0.1	0.6	18.6	65.6	15.1
Fr E8	0		33.5	66.2	0.3
Fr E7	0.1	1	89.5	9.3	0.1
Fr E6		14	85		0
Fr E5		18.2	81.6	0.2	0
Fr E4		33.7	66.3	0	0
Fr E3		67.3	32.5	0	0.3
Fr E2	14	85.5	0.5	0	0
Fr E1	21.3	78.4	0	0.1	0.1
Fr F1	57.9	41.5	0.5	0	0.1
Fr F2	88.5	11	0.1	0.3	0.1

### 2E

BioSimilar Stressed 45°C/7Days (%Peak Area)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Control	26.9	30.1	31.3	9.6	2.1
Fr E10	0	5.1	10.3	6.3	78.2
Fr E9	18.3	0	0.4	10	71.4
Fr E8	5.3	2.2	6.9	85.7	0
Fr E7	0.7	1.6	14.5	80.4	2.8
Fr E6	1	0	6.9	90.4	1.7
Fr E5	1.5	1.4	90.5	5.4	1.1
Fr E4	0	15.5	83.7	0.4	0.3
Fr E3	1.2	23.3	72.9	0.8	1.8
Fr E2	9.8	76.8	11.1	0.9	1.3
Fr E1	2.3	94.7	1.4	1.3	0.3
Fr F1	2.3	95.1	0.7	1.1	0.8
Fr F2	70.3	23	1.8	3	1.8
Fr F3	91.7	0	1.7	3.8	2.7
Fr F4	88.5	0	4.9	3.5	3
Fr F5	75.8	4.7	9.5	9	0.9

### 2F

BioSimilar Stressed: B53 Cross Linker-Induced (%Peak Area)					
Injection	Peak1	Peak2	Peak3	Peak4	Peak5
Control	37.4	27	27.5	7.1	1
Fr E6	2.5	15.7	0	10.9	70.9
Fr E5		2.9	1	1.1	95
Fr E4	0	1.2	0	67.9	30.9
Fr E3	0.2	0.3	19.8	79.5	0.1
Fr E2	0	0.8	51.4	47.8	0
Fr E1	0	0.6	92.9	6.1	0.4
Fr F1		18.7	81.3		0
Fr F2	0.3	52	47.4	0.3	0.1
Fr F3		86.9	12.9	0.1	0.1
Fr F4	33.7	66	0.3	0	0.1
Fr F5	72.3	27.6	0	0.1	0
Fr F6	90.6	8.8	0.4	0.1	0.1
Fr F7	98.5	1	0	0	0.5
Fr F8	99.2	0.3	0	0	0.6
Fr F9	99.7		0	0.1	0.1
Fr F10	99.6	0	0.1	0	0.3