

Roundtable Session – Table 4 – Approaches Towards Characterization of Multi-specific Antibodies

Facilitator: Fatemeh Tousi, *Sanofi*

Scribe: Mellisa Ly, *Pfizer, Inc.*

Abstract:

Multi-specific antibodies represent one of the most transformative advances in biotherapeutic design, offering the ability to simultaneously engage multiple targets within a single molecule. This enables novel mechanisms of action such as redirecting immune effector cells to tumor targets or blocking multiple disease-driving pathways in parallel unlocking new therapeutic strategies across oncology, immunology, and beyond, positioning them as a cornerstone of next-generation biologics pipelines.

However, their structural complexity introduces substantial challenges throughout research and development, spanning protein expression, purification, and analytical characterization. Conventional techniques such as size exclusion chromatography, capillary electrophoresis, and SDS-PAGE often lack the resolution to characterize critical quality attributes unique to these molecules, such as chain mispairing. Mass spectrometry has emerged as a powerful complementary tool, offering superior molecular-level resolution for intact mass analysis, peptide mapping, and identification of mispaired species and post-translational modifications. As multi-specific antibody pipelines expand, integrating MS-based workflows into characterization strategies is essential for ensuring product quality and supporting regulatory submissions. This round table will explore the analytical challenges unique to multi-specific antibodies and discuss emerging MS-based strategies for their robust characterization.

Discussion Questions and Notes:

1. What unique analytical challenges do you encounter, and which molecular formats are the most difficult to characterize?

- Fusion-protein modalities can be particularly challenging to characterize.
- Assigning unknown cleavage products at the subunit and intact levels is difficult.
- Creating curated lists of unknown masses to support structural assignments helpful
 - For knob-into-hole (KiH) BsmAb molecules, calculators were created with script generators to predict heavy-chain homodimers (H-H).
 - Deglycosylation was recommended to reduce N-glycan and O-glycan heterogeneity.
 - Fusion bispecific domains may be difficult to deglycosylate, and prolonged incubation may induce cleavage.

2. What assays do you use for release testing and extended characterization, and for which sample types?

- Mixed-mode chromatography may provide both size-based and hydrophobicity-based separation of mispaired and correctly-paired species. The method is highly program-specific and gradient based.
- A key challenge is correlating intact-level mispairing with CE-SDS profiles to support clone selection and subsequent studies.
 - Consider non-reducing subunit analysis using FabDello digestion to identify chain mispairing.
 - Light-chain swapping is frequently observed in EE/RR bispecific antibodies.
 - For identity testing, intact mAb analysis may be used in early development; at later stages A non-MS method such as LC-UV separation might be needed.

3. Which separation technologies do you pair with MS to characterize chain mispairing, and what improvements are needed?

- Consider using ion mobility to separate isobaric mispaired species.
- Consider Pro-Ala mutations to reduce the expression of mispaired species.
- Improved LC separations are needed at the subunit level
 - Cost-effectiveness should be considered when selecting chromatography methods and replacing columns or consumables.
- Another challenge is separating and identifying CE-SDS cleavage products by mass spectrometry:
 - Consider online CE-MS. However CE-MS without SDS provides a charge-based separation rather than a size-based separation needed for mispairing screening.
 - For a two-dimensional approach, collect high-molecular-mass species (HMMS) and low-molecular-mass species (LMMS) by SEC, then reinject them for CE-SDS analysis. However, this approach does not separate the intact molecule from the three-quarter-length species.
 - Alternatively, build a CE-SDS reference dataset using purified, MS-identified HHL, H-H, L-L, and related species. Quantify the relative abundance of each species and compare profiles; peaks may then be assigned or estimated using deconvoluted intact masses, with an estimated limit of detection of approximately 1%.

4. What advances would you like to see in MS instrumentation and data-analysis software?

- Instrumentation and software needs vary with laboratory goals, intended use, and sample type. Users must learn multiple vendor platforms, and repeated training across evolving software can be challenging and overwhelming.
- No software platform performs every function optimally, so organizations should support complementary tools such as BioPharma Finder, Genedata for high-throughput workflows, PEAKS, and Protein Metrics Byosphere.

- Cross-functional communication between Discovery and CMC should be maintained throughout the development.
- At late-stage development, all results should undergo manual data verification.

5. Which enzymatic digestion strategies do you use for middle-up MS analysis of chain mispairing?

- A useful option would be a Genovis “nonreducing subunit toolbox” that includes FabDello, FabALACTICA, and IdeS/X.

6. Do you use a high-throughput, MS-based chain-mispairing assay to support process development?

- Recommendations for high-throughput analysis include transferring the assay to a dedicated screening group, automating sample preparation and streamlining data analysis through targeted MS analysis and enabling automated annotation and reporting.