

Roundtable Session - Table 6 - MS-Based Protein Higher Order Structure Characterization in Biologics Research and Development

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Abstract

Maintaining the correct higher-order structure (HOS) is critical for ensuring the safety, efficacy, and quality of therapeutic proteins, peptides, and complex biologics. Even minor conformational deviations might alter a product's potency or expose neo-epitopes that trigger unwanted immunogenic responses. While traditional biophysical methods have historically driven HOS analysis, advanced mass spectrometry (MS) techniques, such as hydrogen-deuterium exchange MS (HDX-MS), ion mobility mass spectrometry (IM-MS), and collision-induced unfolding (CIU) under native MS conditions, have rapidly emerged as indispensable tools for evaluating HOS at different levels of detail. However, as the integration of these sophisticated, multi-dimensional MS datasets into regulatory filings expands, both industry and regulators face ongoing challenges regarding data standardization, multimodal integration, and the validation of these advanced analytical methods for routine Quality Control (QC) environments.

Discussion Questions:

- Experiences with the submission and regulatory assessment of MS-based HOS data (e.g., HDX-MS, native MS, IM-MS, and CIU) in recent regulatory filings for proteins, peptides, conjugates and biosimilars.
- What are the specific opportunities and analytical challenges in utilizing HDX-MS and IM-MS to assess conformational stability, domain unfolding, and structural comparability?
- Opportunities and challenges in standardizing the presentation of complex, multi-dimensional MS data to facilitate efficient and consistent regulatory assessment.
- Strategies and hurdles for establishing robust validation criteria (e.g., reproducibility, limits of detection, assay variability) necessary to transition MS-based HOS assays from characterization tools into GMP/QC environments.

Notes:

The discussion opened with participants introducing themselves and their experiences with HOS techniques. Most participants were experienced users of HOS techniques while a few were participating to learn from the discussion.

What is the drive to use HOS in biopharma?

Participants responded as follows:

BLA filing questions need to be supported with structure function studies and HOS as a technique is a useful tool. In addition, HOS techniques such as HDX can be used for epitope

mapping. The targeting can be for the same antigen; however, the interactions can vary in different molecules and formulations impacting the mechanism of action (MOA).? Looking at BLAs from 2000–2020-time frame, BLAs with HDX data have seen an increase between 2016–2020. This is perhaps due to the evolution of HDX as a technique and availability of tools and dissemination of tools that became best practices in industry.

Regarding best practices, a publication in Nature Methods was mentioned by a participant <https://www.nature.com/articles/s41592-026-03083-0>

Participants indicate one of the major barriers towards broad application of FPOP in industry is the data reproducibility across lab and standardized workflow.

FPOP technology became reproducible across labs due to the introduction of dosimetry. Dosimetry looks at the radical load, which must be maintained as a constant for meaningful determination in changes of protection / exposure.. There are blind spots in footprinting. The highly reactive sites are cysteine, aromatic residues and aliphatic residues. CF3 salts react with H2O2 to produce *CF3 (radicals) that can label silent residues. Albeit, recent studies have indicated that much of what was initially reported as missed residues, can be addressed by improved LC-MS dynamic range and DIA analysis. A participants opinion was that carbene footprinting causes the protein structure to perturb and this bias will affect structure prediction. Labeling can cause artifacts. However, if the labeling is faster compared to the unfolding of the protein, then you can capture the structural state with the covalent label that can be detected by MS. This is the key principle in fast photochemical reactions.

The mass spec acquisition mode of HRF data was discussed-DDA vs DIA better for coverage?

The opinion by a subject matter expert was that DIA shows more coverage of footprinting data.

What additional techniques for HOS?

Discussion started with participants discussing structure perturbations for structure function studies. Aggregation analysis, covalent labeling and cross-linking techniques were discussed as additional methods.

What is one of the most challenging problems that you solved using HOS methods?

A participant was able to illustrate the utility of combining orthogonal methods to understand aggregation problems arising from stapled scFv molecules. The aggregation propensity due to domain swapping mechanism was systematically evaluated by comparing a monomer and dimer pairs of the antibody, by HRP technique to elucidate the binding interface and the data was complemented with disulfide mapping and SEC-MS and MD simulations/Insilco modeling. The mechanistic insights lead to single amino acid change of the molecule to resolve aggregation issues. The consensus was that multiple orthogonal techniques are essential to understand and validate a structural problem.

A participant also discussed the application of HOS methods in high-concentration formulations, with lysine used as excipients to reduce aggregation and viscosity. There are four FDA-approved protein-based biologics currently incorporate Lysine in their formulations.

HOS throughput was a concern. All participants conferred that for speed to decisions, none of the HOS methods/techniques were fast enough.

limited availability of aggregated species restricts broader HOS application. Enrichment may be needed for aggregates and other high-molecular-weight species (HMWS). Investment in state-of-the-art HOS instrumentation, however, requires strong scientific rationale and clearly defined, high-impact applications.

A participant also brought up the "The electronic common technical document (eCTD)" modules 1 and 2 and ICH M4Q, which is the specific international guideline that defines how pharmaceutical quality CMC data must be structured and presented within Module 2.3 emphasizing a dossier to add more development data.

The key take away was that the FDA encourages additional supportive data on how the molecule is developed- story telling of an issue and what problems were present and how they were addressed and resolved.