Roundtable Session 1 – Table 2 – MS-Based Protein Higher Order Structure Characterization in Biologics Research and Development

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Abstract

MS-based higher-order structure characterization has become a versatile and powerful toolbox to support the discovery and development of biotherapeutics. The techniques of HDX-MS, hydroxyl radical labeling, cross-linking, native MS, etc. have vastly advanced, and widely adopted by researchers. This roundtable discussion will focus on gathering industry perspectives on the application, resource allocation, and strategies for in-sourcing and outsourcing related to MS-based higher-order structure characterization tools, which are crucial for the discovery and development of biotherapeutics.

Discussion Questions:

1. Considering the modalities and development stages you're involved with, how have MS-based methods influenced your approach to studying higher-order structures?

Higher-order structural (HOS) analysis is increasingly being used at various stages of development for common biotherapeutic modalities, including protein and peptide therapeutics. While HOS analysis has traditionally focused on proteins and peptides, there is growing interest in other modalities such as AAVs and RNA/DNA—especially when mass spectrometry (MS) is combined with other technologies.

Antibody and antibody-derived therapeutics currently dominate the HOS characterization landscape. These include monoclonal antibodies (mAbs), bi- and multi-specific antibodies, antibody-drug conjugates (ADCs), and biosimilars. HOS characterization is typically performed during early development to map epitopes or paratopes and to better understand ligand interactions with antibodies. A deeper understanding of payloads and how different drug-to-antibody ratios (DARs) affect the HOS of ADCs is an emerging area of interest, increasingly requested from MS service providers by biopharmaceutical companies.

Biosimilar development is another area where HOS analysis is seeing significant uptake—both in early and later stages. The field is also witnessing increased use of HOS analysis in late-stage development, such as in aggregation studies. Understanding the mechanisms of aggregation, particularly in the presence of different formulations, is of both scientific and developmental interest.

There is a growing demand for HOS analysis in anti-drug antibody (ADA) projects, as ADA characterization is a vital component of immunogenicity assessment. Immunocapture from human plasma followed by hydrogen-deuterium exchange (HDX) is currently the most popular approach, but emerging techniques like oxidative footprinting are also gaining traction.

With the rising popularity of peptide therapeutics—driven by GLP-1 agonists—there is considerable interest in understanding the structural properties of peptide-based drugs. For example, comparing size-exclusion chromatography (SEC) fractions of separated monomers and dimers using HDX-based structural analysis is becoming more common.

Other emerging areas of application include membrane proteins and cross-linked protein–protein interactions. These are challenging samples that require complex data processing, and as a result, such analyses are typically limited to specialized groups within biopharma, CROs/CDMOs, or academia. Many of these projects are early-stage collaborations with academic institutions aimed at better understanding structural features and binding partner interactions.

2. When choosing MS-based HOS techniques, what factors led you to prefer these over other methods like crystallography, cryo-EM, DLS, SEC-MALS, or AI-based structural modeling?

MS-based HOS techniques are preferred because they provide accessible, high-resolution data for decision-making. HDX and oxidative footprinting approaches (such as FPOP) can deliver high-resolution information at the amino acid residue level, making them more attractive tools in early-stage discovery. The use of different radicals offers an opportunity to enhance structural resolution.

Techniques such as SEC-MALS, SPR, and DSC are commonly used in the later stages of development. These methods are simple and useful for assessing batch-to-batch structural changes, especially when the anticipated changes are significant. However, they are limited by their structural resolution and sensitivity, making them less effective for detecting small or subtle changes. When changes observed in later stages are minor, the analytical development team often receives requests to perform more in-depth HOS using higher-resolution MS-based techniques.

CryoEM is a good option for measuring binding, but it takes a long time to obtain data, which can delay timely decision-making during development. Since CryoEM is typically performed by a separate group—often located elsewhere or outsourced to a different CDMO/CRO—it can take months before the data becomes available. Additionally, if the molecule or region of interest is small, CryoEM may not be suitable. X-ray crystallography is always an option, but it presents challenges such as obtaining suitable crystals and long turnaround times.

AI approaches are increasingly assisting with HOS. AI training models for HDX data, as well as tools like AlphaFold for protein modeling, are helping to advance the field.

3. Which data elements would you correlate with HOS studies to achieve the most significant and impactful outcomes?

Understanding structural and interaction information at high resolution requires MS-based HOS analysis. MS-based HOS techniques are powerful because they provide detailed molecular-level structural information as well as highly localized, residue-specific insights. Together, these capabilities offer an opportunity to deeply understand both the structure and the interactions occurring at the amino acid residue level—such as post-translational modifications and, in certain cases, hydrogen bonding interactions.

To obtain meaningful results from HOS experiments, it is helpful to consider both the building blocks—like the sequence and shape of molecules—and the environmental conditions, such as temperature or pH. Using AI-based approaches to identify patterns can improve the accuracy of predictions and enhance their usefulness for translational research.

4. How do you optimize resource utilization and streamline processes when using MS-based methods for HOS studies, what's your consideration when to outsource or establish the capabilities in-house?

The utilization of MS-based HOS techniques is both need-based and volume-driven. These techniques are relatively specialized and require significant expertise for successful in-house implementation—capital and personnel investments are substantial, and a strong business case along with institutional commitment is essential. Even with institutional support and investment, establishing a successful platform remains challenging due to the current state of the technology.

Specifically for HDX, several issues need to be addressed:

1) Sample preparation is consistently challenging and time-consuming.

2) The HDX solutions available in the market are typically multi-vendor systems, often affected by inter-instrument communication issues, which can take a long time to resolve. An end-to-end solution from a single vendor is much needed to enable broader adoption of this technology along with better software solution.

3) Automation enables the generation of data from over 100 epitope mapping experiments on newer, faster MS systems. However, data analysis remains a bottleneck. Large file sizes make both data processing and management difficult.

Due to the high investment required, outsourcing HDX projects is often a straightforward business decision when project volume is low. However, as volume increases, it becomes easier to justify the investment needed to build in-house capabilities.

The automation of MS-based HOS techniques is making their implementation more attractive, as complex automation systems offer significant flexibility to establish diverse workflows in the laboratory. However, complex automation also means more involved troubleshooting when systems fail, which can unintentionally become a new bottleneck.

Sample preparation in MS-based HOS experiments is complex and time-consuming, and experimental design is critically important. Key considerations include:

· De-glycosylation prior to HDX

· Measures to control cysteine scrambling

· Ensuring good coverage with overlapping peptides

· Use of alternative fragmentation techniques to improve coverage and resolution

A high level of technical expertise is certainly one of the barriers to bringing this technology in-house. Experts are needed for sample preparation, executing experiments with strict protocols and controls, interpreting data accurately, and recognizing advanced changes such as cysteine scrambling.

Advances in software solutions for data processing are key to accelerating these techniques—from data acquisition to decision-making—and promoting broader utilization and adoption.

5. Could you share any recent advancements in MS-based HOS techniques that have impacted your work?

HDX and oxidative foot-printing are the dominant MS based HOS characterization tools. Newer techniques are emerging, and it is encouraging to see increased adoption of

other techniques. Native MS based workflows are starting to be adopted in biopharma and are being offered as service by CDMO/CRO. Even in this conference we saw a good representation of native MS workflows. Native top-down or collision-induced-unfolding (CIU) are very capable techniques that can provide structural and stability information. These techniques in the past have primarily been used in academia for research, but now we are starting to see the workflow becoming more robust and resulting in increased industry adoption. Still, the software solutions and workflow automaton need work, but the value of the information provided by native MS is being recognized.

Other techniques and application areas that have been subject of HOS discussion:

1) DIA based approach—can we have the software to use DIA based approach to generate richer data faster

2) Soft landing—combining native MS with CryoEM. This is exciting area and has great potential.

3) Accessing and studying membrane proteins, Intrinsically Disordered Proteins (IDPs)

4) Structural proteomics

5) AI based modeling using large MS based HOS data sets

6) Training programs for specialized workflow such as native MS, CIU etc. Community working together with vendors to prepare next generation of scientists