

Review of Technologies for Biopharmaceutical Higher Order Structural (HOS) Analysis

Identifying the most robust and state-of-the-art HOS analytical method for biopharmaceutical discovery and development.

Critical Importance of Protein Structure & Function

The efficacy of a biopharmaceutical is directly inherent to its Higher Order Structure (HOS)—the folding and three-dimensional conformation that determines function and stability. Biopharmaceutical formulation further contributes to the activity of the protein as it greatly influences the underlying structure-function relationship.

Incorrect formulations can result in conformers that produce safety and efficacy concerns. Biopharmaceuticals can be adversely impacted in situations where protein aggregation occurs, receptor binding is inhibited, and/or immunogenic epitopes are exposed. Biopharmaceutical HOS anomalies like these are linked to adverse drug reactions (ADR) attributed to aberrant pharmacology and patient immunological response, many manifesting as morbidity, and in some cases death.¹⁻¹¹

Scientists and regulators have become increasingly aware of the critical role that HOS plays in ensuring a drug's stability, safety, and biological function. The FDA, along with the Center for Drug Evaluation and the Center for Research Biologics Evaluation and Research, issued guidelines for the study of protein HOS. The report recommends that drug companies employ state-of-the-art technology for evaluating protein HOS.¹²

Although a variety of traditional HOS analysis techniques and technologies are available, these approaches have shown deficiencies in reliably predicting efficacy and safety, establishing the need for new and improved HOS analyses.¹³

Let us review the available HOS analysis options to determine the most robust, cost-effective, and workflow-friendly approach for your lab.



Comparison of Traditional HOS Techniques

As shown in Figure 1, HOS analysis can be grouped into low-, mid-, and high-resolution techniques.

Low-Resolution Methods

Low-resolution methods provide information that is spatially averaged over the entire protein population. These methods examine a very limited number of specific moieties in the protein structure that have altered biophysical properties dependent upon protein conformation.¹⁴

While more widely available and easiest to use, low-resolution techniques fail to inform on a residue-level, and as such, yield ambiguous and marginally actionable HOS data.

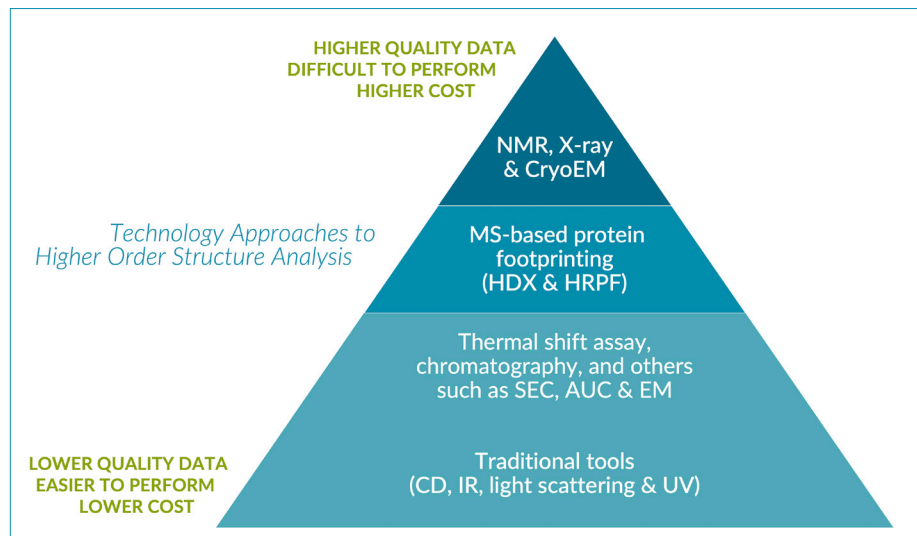


Fig 1: Structural biology HOS analysis technology approaches. Adopted from Professor Michael Gross, University of Washington at St. Louis.

High-Resolution Methods

High-resolution techniques such as x-ray crystallography, multi-dimensional nuclear magnetic resonance (NMR), and cryo-electron microscopy (CryoEM), demand the deep expertise and very expensive equipment typically found only in dedicated core labs. Beyond the complexities associated with these very advanced HOS approaches, each also has idiosyncratic technical challenges that limit their value for HOS studies as follows.

X-Ray Crystallography

The adoption of x-ray crystallography in HOS studies has been limited by the scarcity of electron beam sources, which is aggravated by difficulties in crystallizing large bio-therapeutics such as monoclonal antibodies.

Multi-Dimensional Nuclear Magnetic Resonance

NMR is arguably the most sensitive method for fingerprinting the HOS of a protein in solution. Yet, two-dimensional NMR can yield poor selectivity which is problematic for unambiguous fingerprinting of biological macromolecules. And this method is quite laborious and costly, consuming considerable time and sample to properly assign multi-dimensional spectra.¹⁵

Cryo-Electron Microscopy

The utility of Cryo-EM is predominantly limited to the study of large proteins (>100 kDa) and demands substantial sample preparation and purification to ensure the absence of impurities.¹⁶

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Mid-Resolution Methods

Intermediate resolution techniques include mass spectrometry (MS) combined with protein footprinting using surface chemical modification and/or amide Hydrogen-Deuterium Exchange (HDX).

Hydrogen-Deuterium Exchange

Because integrated commercial packages for data acquisition and analysis are available, HDX-MS has been widely used in biopharmaceutical research.¹⁷⁻²³

In HDX studies, electrospray ionization MS detects mass increases from exchanged hydrogen with deuterium. The back-exchange of deuterium to hydrogen occurs at substantial rates, requiring the reaction to be quenched rapidly by cooling the sample to 1°C while suspended in

acidic buffer (pH 2.7). The quenching must be promptly followed by peptic digestion and then LC-MS analysis.

While HDX can provide insight into solvent addressable backbone amide hydrogens, its time-course incubation often results in generating products from both rapid and slow exchange processes. Rapid exchange is associated with solvent addressable residues with unbound amide hydrogens, while slower reactions occur with internal, titratable, backbone amide protons

involved in hydrogen bonds that make up secondary structural elements, whose exchange rates are driven by conformational change.³³⁻³⁵ The net result is an ambiguous understanding of HOS and secondary structure stability.

Performing HDX HOS analysis is a complex and burdensome process. Perhaps this point is best exemplified by Professor Mark Chance of Case Western Reserve University when he remarked, *"I invented HRPF because HDX is just too hard to do."*³⁶

Hydroxyl Radical Protein Footprinting (HRPF)

HRPF is an intermediate level technique that utilizes hydroxyl radicals ($\cdot\text{OH}$) to covalently modify solvent-exposed amino acid side chains (Figure 2). The $\cdot\text{OH}$ modification is very stable, allowing ample time to denature and digest the protein for bottom-up proteomics after labeling.

The average peptide and residue oxidation is calculated using the chromatographic peak areas of the unmodified and modified peptides. When solvent accessibility changes due to alterations in protein structure or interactions, $\cdot\text{OH}$ modification concordantly changes.³⁷⁻³⁹ Researchers have used HRPF to successfully:

- detect defects in protein HOS and function,⁴⁰
- expose monoclonal antibody (mAb) production problems,⁴¹⁻⁴⁴
- demonstrate the interplay of mAb HOS and drug function,⁴³⁻⁴⁴
- illustrate biosimilar failure and storage-induced defects for innovator products,⁴⁵
- characterize membrane protein targets,⁴⁶⁻⁴⁷
- determine protein conformational change and binding Kd upon ligand complexation,⁴⁸
- facilitate the study of allostery,⁴⁹ and
- enable in vivo labeling of cellular proteins and whole organisms.⁴⁹⁻⁵²

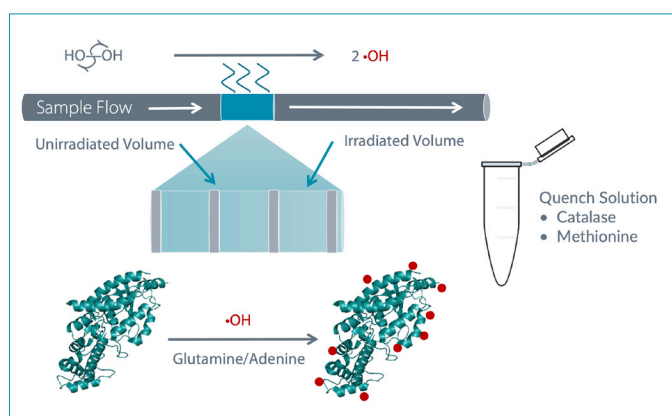


Fig 2: HRPF Schematic. Proteins are exposed to a pulse of diffusing $\cdot\text{OH}$ generated by the flash photolysis of H_2O_2 . Radical attack modifies solvent exposed side chains. With bottom-up proteomics, changes in the solvent accessible surface area are identified at the peptide to amino acid level.

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HDX Versus Traditional HRPF

As shown in Table 1, traditional HRPF has several inherent advantages when compared to HDX, arguably making it a first method of choice for HOS studies.

Feature	HDX	Traditional HRPF
Workflow	Laborious time course study	Simple and straightforward experiment
Label Stability	Unstable: must act fast	Stable: can take your time
Post-Labeling Demands	Imperative to immediately quench, digest, and perform LC-MS at 4° C	No need to immediately proceed, convenient and flexible workflow
Label Location	Backbone amide groups	19 out of 20 amino acids' side chains
Digestion	Acid protease at 4° C	Various protease at room temperature
Label Biases	Stability of protein's hydrogen bonding network and chemical properties of amino acid sequence	Residue-reactivity differences towards ·OH
Data Interpretation	Change in isotopic distribution following deuterium uptake overtime	XIC of modified (+16 Da) peptide vs unmodified peptide
Limitations	Limited LC gradient and challenges with MS2 acquisition	Complex data analysis and expense of excimer laser

Table 1: Comparison of HDX and HRPF attributes.

Factors Impeding the Adoption of Traditional HRPF in HOS Studies



While the attributes of traditional HRPF are impressive, its adoption within pharma has been slow. HRPF work can be found primarily in commercial laboratories that collaborate with or hire from HRPF academic groups.

A primary reason for this lack of adoption is that in the past, the only means to quickly generate ·OH required scarcely-available, elaborate x-ray synchrotron beamlines or expensive, dangerous, and hard to maintain excimer lasers using hazardous KrF gas.⁵³

Additionally, ·OH can react with non-analyte components in the sample, such as buffer and excipients. Variability in the rate of background scavenging is associated with irreproducibility, which has limited comparative studies.⁵⁴

Overall, older HRPF approaches have shown obvious advantages over low resolution techniques, while generating results compatible with high resolution approaches. However, the battles using these HRPF methods are hard won. With the introduction of the world's first commercially available HRPF instrument and software platform, GenNext Technologies has changed all that.

Flash Oxidation (Fox™) Protein Footprinting System for HRPF Studies

The Fox System is the world's only benchtop platform available for HRPF studies. From bottom to top, the system is comprised of:

- **Fluidics and Control Module:** provides microfluidic sample introduction for HRPF processing
- **Flash Photolysis Module:** high energy pulsed plasma lamp system that supplants the use of synchrotron radiation or lasers
- **Radical Dosimeter:** an in-line, capillary photometric absorbance detector that monitors change in reactant/product UV absorbance
- **Product Collector:** an automated carousel that selectively collects properly labeled protein

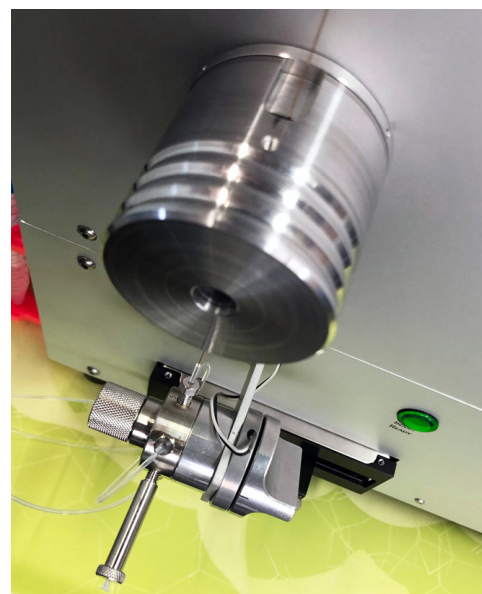
In Fox-based HRPF, protein pre-mixed with H_2O_2 and adenine is introduced into the photolysis module by pumping sample through a fused-silica capillary. Adenine serves as an internal standard whose change in photometric absorbance reflects effective $\bullet OH$ yield.

At the photolysis module, photo-irradiation generates $\bullet OH$ that attack the protein and adenine.

The sample continues to flow upwards and into the detection zone of the radical dosimeter, where the change in UV absorbance is measured. During labeling, the dosimetry module assesses the effective $\bullet OH$ load, enabling the user to adjust flash intensity in response to changes in background scavenging. This unique, real-time feed-back loop vastly improves labeling reproducibility, providing confidence in differential studies.

At the product collector, labeled protein is ultimately deposited in microtubes containing a quench solution of catalase and methionine.

The Fox Protein Footprinting System can easily perform all the HRPF-based HOS studies that previously required complicated, expensive, and dangerous laser or beamline sources.



With its simple workflow and integrated FoxWare™ Protein Footprinting Software, the results from the Fox-based platform are on par with the most advanced, difficult, and expensive HOS techniques.

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Applications of Fox™-Based HRPF

As shown in Table 2, the Fox Protein Footprinting System provides actionable results for both biopharmaceutical drug discovery and drug development.

The applications of this new HRPF technology in biopharmaceutical discovery include evaluating Rx Binding, mAb therapeutics, conformational analysis, and biosimilar assessment. In the drug development phase, this technology can be used to study expression/harvest optimization, aggregation, thermal stability, and formulation.

Biopharmaceutical Discovery	Biopharmaceutical Development
Rx Binding Validation <ul style="list-style-type: none"> Orthosteric Allosteric Conformational Change GPCR cascade 	Expression/Harvest Optimization <ul style="list-style-type: none"> HOS Integrity at Peptide/Residue Levels Differential Process Analysis
mAb Therapeutics <ul style="list-style-type: none"> Epitope Mapping Paratope Mapping Affinity Determination 	Therapeutic Aggregation Studies <ul style="list-style-type: none"> Interactive Domains/Residues Excipient/Amino Acid Effects
Conformational Analysis <ul style="list-style-type: none"> Conformer Detection Discrete Functional Analysis 	Thermal Stability Studies <ul style="list-style-type: none"> Thermal-Induced Changes in Conformation
Biosimilar Assessment <ul style="list-style-type: none"> Candidate validation via HOS 	Therapeutic Formulation <ul style="list-style-type: none"> Concentration Excipients Delivery System

Table 2: Applications of Fox-based HRPF technology.

Conclusion

The discovery and development of successful biotherapeutics is dependent on understanding critical HOS factors. The Fox Protein Footprinting System is an effective and easy-to-use platform that can be leveraged to advance your biotherapeutic discovery and development. To learn more about how our solutions can benefit your biotherapeutic business, contact the experts at GenNext Technologies.

GNTPNRO0621

Discover the Benefits of Protein Footprinting

Contact us for products and services to investigate biopharmaceutical structure, interactions, folding, aggregation, formulation, and delivery.

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