

STIMULI TO THE REVISION PROCESS

Stimuli articles do not necessarily reflect the policies
of the USPC or the USP Council of Experts

Higher Order Structure of Proteins in Biopharmaceutical Development

Yves Aubin,^a Michael D Brenowitz,^b Edward Chess,^c Thomas Laue,^d Jasper Lin,^e Donald MacLean,^f John P Marino,^g Linda Narhi,^h William F Weiss IV,ⁱ Qin Zou^{j,k}

ABSTRACT The higher order structure (HOS) of proteins is important to characterize and understand during the process of biologic drug development. Although a wide range of analytical techniques is used for HOS characterization, there are no public guidelines or best practices for the application of physicochemical techniques to characterize specific HOS properties. The timing is now advantageous for developing guidelines or best practices that would facilitate the development and characterization of biologics. These guidelines would inform research and development efforts, promote process understanding, and ensure drug product quality. To this end, in December 2016, U.S. Pharmacopeia (USP) hosted a roundtable attended by invited scientists and regulators with expertise in HOS techniques and applications. As participants in this roundtable, we discussed many practical issues and raised questions for further consideration. We reached consensus on the current applications, strengths, and weaknesses of many of the analytical techniques for HOS characterization ([Table 1](#)). USP, especially through its informational chapters, is uniquely positioned to facilitate these efforts as an independent, scientific, nonprofit standards-setting organization. Comments, suggestions, and opinions are actively sought from all stakeholders on the content expressed here, and on the appropriate next steps as we seek to make the analysis of HOS more consistent in practice and the results more definitive, so that more meaningful comparisons across labs and molecules can occur.

INTRODUCTION

Characterization of higher order structure (HOS) is an important aspect of research and development for biologic drugs. During the development of a new protein-based biopharmaceutical, HOS characterization is a critical step because the protein HOS will play a key role in the function and safety of the drug product. Thus a robust strategy for HOS characterization is needed throughout the process of biologic drug development, from candidate selection and formulation screening through process optimization and product manufacture.

Compared with potency or functional binding assays, most analytical HOS techniques can provide a more direct, and often more sensitive, measure of protein structure. Analytical methods can also reveal whether the structure of a protein therapeutic remains consistent under stressed conditions. In addition, HOS methods are sometimes used in product or process investigations to help understand the root cause.

It is important to note that different amounts of protein present in the native conformation could alter the efficacy of the drug or potentially result in immunogenic effects in patients receiving the drug. In most cases, native conformation is not a single conformation but rather a heterogeneous ensemble of states, thus the measurement of changes in the conformational dynamics and the population of states must be considered. Exposure to different conformations could also lead to increased clipping or aggregate formation, and these species could have different safety and/or efficacy when compared to the intact therapeutic.

Currently, submission of HOS characterization data is expected by regulatory agencies in order to confirm the desired structure. Recent guidance documents issued by the U.S. Food and Drug Administration (FDA) and International Council for Harmonisation (ICH) show increasing expectations for inclusion of quantitative analytical characterization of structures, including HOS, for product

characterization and comparability/biosimilarity studies for both innovative and biosimilar product development.

ROUNDTABLE SCOPE AND PURPOSE

A one-day roundtable meeting on HOS characterization was held on December 1, 2016, at U.S. Pharmacopeia (USP) in Rockville, MD. As participants, we were joined by other invited scientists and regulators with HOS expertise from biopharmaceutical companies, academic institutions, governmental bodies, and regulatory agencies. We discussed current analytical technologies, applications, regulatory expectations, and best practices in HOS characterization. We delineated and addressed questions about the strengths, weaknesses, applications, and future potential of many HOS techniques. The meeting scope was limited to the HOS of proteins, primarily recombinant proteins, and to process and product development. Understanding the impact of changes in HOS on the safety and efficacy of biologic drugs is an important area of research, but was not in scope for this discussion. Some participants presented case studies to illustrate the challenges and problems that can arise during HOS characterization and shared the pros and cons of the techniques used, examples of problem solving, and lessons learned.

The purpose of this *Stimuli* article is to describe the roundtable discussions on current issues and questions in HOS characterization and to actively seek comments and input on those issues from all stakeholders. Input is also sought on the appropriate next steps as we seek to make HOS analysis more consistent in practice across labs and make the results more quantitative, so that meaningful comparisons across labs and molecules can occur.

A key objective of the roundtable was to explore whether a USP general chapter about HOS characterization should be developed. If a general chapter is developed, it is expected to be an informational chapter (numbered over 1000) that would provide guidance to the biopharmaceutical industry for both innovative and biosimilar product development. Information would be provided on the strengths and weaknesses of the different analytical tools available, with suggestions on when to apply these techniques. This would facilitate consistency in the use of best practices and would also stimulate progress in HOS characterization.

ISSUES AND QUESTIONS ABOUT HOS IN BIOPHARMACEUTICALS

We discussed many issues relevant to HOS characterization at the roundtable, including:

- When to use HOS analysis (stage-tailored; development cycle)?
- What techniques to use (fit for purpose)?
- What defines fit for purpose?
- What are the sensitivities of the methods?
- How to analyze the data (qualitative vs. numerical/quantitative)?
- How to ensure data quality (method qualification needed)?
- How might HOS fit into the pharmaceutical quality system?
- How are meaningful acceptance criteria defined for the molecules or products to be evaluated for comparability by regulators?

The range of techniques can be conceptualized as tools in a toolbox. Different techniques reveal different aspects of protein HOS. If several techniques are used, multivariate data analysis can be applied to combine the results from the different methods, yielding a more comprehensive characterization. However, a distinction needs to be made between orthogonal techniques and redundant techniques. Unnecessary redundancies use up limited resources and also can delay the approval of life-saving medications.

It is important to note that the depth of HOS characterization should be tailored to the specific phase within the product development lifecycle. In some cases, the traditional qualification approach is difficult to apply to HOS techniques, for example, when evaluating accuracy and linearity. However, demonstration of

method repeatability is always expected. The issue of comparability is critical, and questions have been raised by industry stakeholders looking for guidance and clarity on the degree of comparability needed for regulatory approval. During early drug development, HOS measurements can be applied in a limited, fit-for-purpose way. However, when applying HOS measurements to assess comparability, the approach is different; in this scenario, regulators need to decide whether sufficient measurements have been made to determine, with acceptable confidence, that two molecules are HOS comparable.

A starting point for this discussion was a series of articles titled "Technical decision-making with higher order structure data," published during 2015–2016 in the *Journal of Pharmaceutical Sciences* (1–7). This series provides perspectives on how and when to utilize biophysical methods and how to apply the results for decision making in the process of drug development. The articles offer case studies on developing a robust strategy for HOS characterization, including ways to tailor that strategy to a specific biologic.

In the article series, HOS techniques were rated on a scale from "not useful" to "extremely useful" for six different applications: candidate selection, formulation development, process development, elucidation of structure and product characterization, comparability, and biosimilarity. For example, differential scanning calorimetry (DSC) was rated "very useful" for most of the six applications. In contrast, field flow fractionation (FFF) was rated "somewhat useful" to "not useful" in all six applications. Most techniques, such as Fourier transform infrared spectroscopy (FTIR), were very useful for specific applications and not useful for others.

At the roundtable, questions were also raised about regulatory requirements for HOS characterization data. Based on our discussion, it appears that regulators typically will not prescribe specific techniques but rather will focus on whether the techniques selected were fit for purpose. Regulators want to know if the methods the sponsor used are the ones that best measure the HOS critical quality attributes and their changes.

In some cases, HOS changes have no apparent effect on the drug product's efficacy. For example, two lots of the drug might have slightly different HOS, but both pass release testing, including no change in measurements of bioactivity. The question is, if efficacy and safety are not affected, are changes in HOS of concern? One perspective holds that resources should only be used on addressing changes that have relevance to safety and efficacy. But in other cases, an HOS change may render a drug product ineffective, including a significant decrease in, or complete loss of, bioactivity. For these cases, it is clear that assessing the HOS is very useful, and that knowing which technique or techniques to use can save considerable time and resources.

For still other drug products, scientists have limited experience with a particular molecule because only a small number of lots were manufactured during product development; this can occur when source materials are scarce. If HOS characterization tests find small differences, the relevance of these differences is unclear because knowledge of this molecule is incomplete.

At the roundtable, participants reached consensus that overall, the focus should be on measuring HOS as it pertains to clinical efficacy and safety. Manufacturers need to understand their molecule thoroughly, and the regulator cannot give them this understanding. The manufacturer needs to gather comprehensive background information on the molecule of interest. When applicable, historical information from the same structural family [e.g., the same subclass of immunoglobulin G (IgG)] can be leveraged as well.

The application of these HOS studies should also be phase appropriate and should take into consideration factors such as the amount of material available, the lead time before results are needed to inform timely decisions, and the need for quantitation. The HOS techniques appropriate for candidate selection are often not the same techniques used during late-stage comparability studies. Similarly, it is not always feasible to have a quantitative value as the acceptance criterion; this depends on both the technique and the phase of development. During candidate selection, the important thing is the relative ranking of a candidate

molecule, and thus qualitative evaluation is sufficient. For late-stage development, and with more product and method knowledge and experience, numerical or quantitative acceptance criteria might be developed for applications including comparability.

An additional factor to consider is the implementation of HOS characterization in the laboratory, including instrument parameters, analyst training, and sample preparations. All of these aspects can be critical to the outcome. Therefore, the procedure and training should be tightly controlled, similar to other techniques that are well established in typical controlled systems.

TECHNIQUES FOR HOS CHARACTERIZATION

Various techniques are currently used to characterize the HOS of proteins. The roundtable participants discussed the available techniques and created the chart below ([Table 1](#)), which shows the most commonly used techniques and their applications, strengths, and weaknesses/limitations. When deciding which technique(s) are suitable, one must consider the molecule being assessed and also the stage of development. This is because the selection of HOS techniques is product specific as well as phase specific.

In addition, we noted that there are many “right answers” when selecting techniques to achieve the desired depth of HOS characterization. If the characterization objective can be met with one technique, then that technique is fit for purpose. In other cases (i.e., with different molecules), multiple techniques will be needed in order to meet the objective.

Table 1. Analytical Techniques Toolbox for HOS Characterization

Technique	HOS Applications	Strengths	Weaknesses/Limitations
Far-UV circular dichroism (CD)	Secondary structure (α -helix, β -sheet, random coil); elucidation of structure, investigations support	Absolute secondary structure, and can be deconvoluted—"this is your molecule." May be used for chemical stability studies. Combine with tertiary method to determine if molten globular state. Comparability can be quantitated.	Low sensitivity. Many/most formulations interfere to some degree. Relegated to <2 mg/mL because smaller path lengths lead to surface characterization rather than bulk properties.
Near-UV circular dichroism	Tertiary structure; comparability, investigations support	One of the more sensitive HOS methods for tertiary structure fingerprinting. Very well-established in the field. Fairly routine analysis that is amenable to moderate/high sample throughput. Comparisons can be quantitated.	Gives a "fingerprint" rather than an absolute measurement. Can have inconsistent definition of change.
Fourier transform infrared spectroscopy (FTIR)	Secondary structure; elucidation of structure, investigations support; especially good for β -sheet, and transitions to intermolecular aggregate	Absolute secondary structure fingerprint—"this is your molecule." Relatively less interference from formulation excipients compared to Far-UV CD. Can be done on solids as well as liquids.	Low sensitivity. The main difficulty with FTIR is water signal subtraction.
Intrinsic fluorescence	Tertiary structure; comparability, stability	Simple, inexpensive. Thermal transition stability. Determines if unfolding is reversible. Multistep unfolding. Can be a very good technique but needs to be combined with a stressing technique such as temperature ramp or oxidation. The fewer markers in the molecule, the better this method becomes.	Low resolution/data content in many cases. Becomes less useful the more fluorophores present in the protein.

Technique	HOS Applications	Strengths	Weaknesses/Limitations
Extrinsic fluorescence	Tertiary structure; interactions and mechanisms	Works with small quantities of material. Useful for estimating hydrophobic patch content [8-anilino-naphthalene-1-sulfonic acid (ANS) dyes] and β -sheet (thioflavin T). May be used for stability studies, detecting binding, indirect probe for thermal stability. High-throughput relative thermal stability can be determined by exploiting this approach.	Used phenomenologically: useful for specific cases but low resolution/data content in many cases. A correct dye-to-molecule ratio must be determined.
UV-Vis spectroscopy	Tertiary structure	Simple, inexpensive	Lack of sensitivity when trying to interpret very small shifts in 2nd or 4th derivative. Compared to near-UV CD, results can be sensitive to the details of the experimental procedure and data processing, requiring interpolation/smoothing.
Raman spectroscopy	Secondary and tertiary structure	Less interference from water environment than with FTIR. Small volume required. Can be done on solids as well as liquid formulations.	Interpretation of results and potential impact of excipients can be challenging. Requires concentrations above 10 mg/mL.
Dynamic light scattering	Aggregates; measures size and some shape; screening for soluble aggregates	Fast, sensitive to large aggregates. Can be used for detection and characterization of reversible self-association.	No separation, resolution is limited—e.g., no resolution of small levels of dimer from monomer (would be a shift in peak rather than a separate peak).
Analytical ultracentrifugation	Aggregates; purity; minor aggregates and fragments quantitation and sizing	First-principle, matrix-free separation, orthogonal to SEC. Gentle, allowing sample to be recovered for further use. Best method for detection and characterization of reversible self-association.	Throughput is low; limit of quantitation (LOQ) is poor; variability at low levels of aggregates is high. Small changes in S-value (conformation) hard to interpret for routine testing in terms of instrument variation (alignment, temperature, radial calibration, etc.)
Size exclusion chromatography (SEC) with multi-angle light scattering (MALS) detection	Aggregates; purity; minor aggregates and fragments quantitation and sizing	Routine method, good for moderate- or high-throughput work. Highly automated. Can be used for detection and characterization of reversible self-association.	Smaller size range. Recovery yield and sample profile could be impacted by column or frit adsorption, change in solution in mobile phase, or interactions with column.

Technique	HOS Applications	Strengths	Weaknesses/Limitations
Field flow fractionation (FFF)-MALS	Aggregates; size primarily with some shape and molecular weight; purity; minor aggregates and fragments quantitation and sizing for investigation support	Unique separation method useful for some samples (highly aggregated or particle-laden samples, viruses, vaccines). Can be used for detection and characterization of reversible self-association.	Method robustness/reliability may limit wider technique implementation. Recovery yield and sample profile could be impacted by focus step (concentration of sample), change in solution in mobile phase, or interactions with membrane. Multiple mechanisms involved in separation.
Nuclear magnetic resonance (NMR)	Primary, secondary, tertiary, and quaternary structure; 1D NMR as a screening technique for changes in HOS, comparability assessments; 2D-NMR experiments provide primary, secondary, and tertiary structure in one readout.	1D NMR is rapid; it requires no labeling and fewer data manipulations than 2D NMR. Flexible, multinuclear approach to assigning average dynamic structure in solution phase. Especially useful for H, C, N, and P. Very sensitive to the smallest structural changes.	Expensive; specialists are required to develop methods and interpret complex data. Relatively low sensitivity, especially for non-enriched samples. Long acquisition times for some experiments. The impact of protein size is still a factor for high-quality spectra.
Structural mass spectrometry	Stoichiometry (especially for conjugates), structure, flexibility [hydrogen deuterium exchange (HDX), and other]	Specific and sensitive structural information	Expensive; specialists are required to develop methods and interpret complex data.
Differential scanning calorimetry	Conformational stability or thermodynamic stability if reversible; screening, comparability, investigations support	One of the more sensitive HOS methods as it can detect domain stability and therefore changes to both secondary and tertiary structure. Automation possible. Very well-established in the field. Easy to interpret data in many cases. Fairly routine analysis that is amenable to moderate/high sample loading. Can determine if unfolding is reversible. Low and high concentrations are possible.	Gives "domain" level information, so it is useful for detecting change but not pinpointing it. Endothermic and exothermic transitions can cancel the signal. Changes may depend on the formulation and may not reflect the changes in native ensemble.

Technique	HOS Applications	Strengths	Weaknesses/Limitations
X-ray crystallography	Detailed structure of crystalline phase; complete structure elucidation of solid crystal form	All HOS revealed. Crystals are "proof" of sample purity.	Expensive equipment. Specialist required for operation/ interpretation. Difficult to make crystals. Dynamic regions of structure may be missed in the crystal structure, and these are often the active regions for which comparison in HOS is important. May be difficult to use for the purpose of comparability/biosimilarity due to potential for multiple crystal forms.
Cryo-electron microscopy (Cryo-EM)	Tertiary structure, morphology, dynamics	All HOS revealed. Samples prepared in vitrified ice so well-formed crystals are not required.	Unknown sensitivity to HOS changes
Small-angle X-ray scattering (SAXS)	Tertiary structure, morphology, dynamics	High sensitivity. Can be applied to fully formulated samples at high concentration.	Sensitive to aggregates, requires expensive equipment and expertise. Interpretation of data may be difficult.
Small-angle neutron scattering (SANS)	Tertiary structure, morphology, dynamics	Can be applied to fully formulated samples at high concentrations. Contrast matching can be used to "highlight" subcomponents of the formulation mixtures.	Expensive equipment/expertise. Need national lab access.

FUTURE DIRECTIONS AND NEXT STEPS

A major theme that we discussed at the roundtable is the need for collaboration among stakeholders—including industry, academia, and regulators—in the evaluation, selection, and use of HOS characterization methods. Effective collaboration will be critical as stakeholders work towards productive, consistent use of HOS characterization throughout the biopharmaceutical industry as well as an understanding of the lab-to-lab variability of the techniques.

As a result of the above discussion, several areas for future exploration as well as opportunities to reach consensus were identified:

- A high-priority clinical issue is the connection between changes in HOS and potential changes in immunogenicity. Can HOS characterization help reduce immunogenicity?
- More interlaboratory comparison studies are needed to evaluate the reproducibility of outcomes from different labs, and to assess the sensitivity of the different techniques to changes in protein conformation.
- Comparisons are also needed regarding the use of different instruments and different analytical methods to measure the same parameter (e.g., comparing CD and FTIR for percent composition of secondary structure elements). Which method is most sensitive to what types of changes? How does one know which tool to choose for a specific question?
- Future round robin studies should involve the instrument manufacturers because rapid advances are occurring in instrumentation, and also in some cases the analytical software packages; manufacturers could provide useful input.

- For analytics, data from each HOS technique are currently analyzed separately. In the future, it would be useful if all the HOS data could be combined, as in chemometrics, to yield more valuable findings.
- It would be beneficial to develop the ability to analyze HOS directly in the solid state, rather than the current practice of analysis after the drug product is put into the liquid state (the exceptions are FTIR/Raman, DSC, and fluorescence).
- In the clinical setting, biopharmaceuticals are often mishandled by health care personnel who have not been trained on proper handling of these sensitive protein-based products. Training is urgently needed so these drugs do not lose their therapeutic benefit before administration to patients.

Perhaps most importantly, much work is needed to delineate how HOS characterization can aid in early-stage development, licensing application, and commercial manufacturing of protein therapeutics. What are the roles that HOS characterization can play, and what advantages do these biophysical techniques offer over other approaches? Conversely, what are the drawbacks and limitations of the HOS techniques? Based on the discussions and data shared at this roundtable, participants reached consensus that it would be helpful to have an informational general chapter on HOS characterization for use during biopharmaceutical development.

Comments, suggestions, and opinions on the content of this *Stimuli* article and the proposal for an informational chapter are actively sought from all stakeholders.

ACKNOWLEDGEMENTS

The authors, a subset of the roundtable participants, acknowledge the helpful discussions by the other participants at the roundtable. The authors also thank USP staff members Christina Chase, Michael Huang, and Maura Kibbey for their help in drafting and editing the article.

REFERENCES

1. Gabrielson JP, Weiss WF 4th. Technical decision-making with higher order structure data: starting a new dialogue. *J Pharm Sci.* 2015;104(4):1240–1245.
2. Jiang Y, Li C, Li J, Gabrielson JP, Wen J. Technical decision-making with higher order structure data: higher order structural characterization during protein therapeutic candidate screening. *J Pharm Sci.* 2015;104(4):1533–1538.
3. Gruia F, Du J, Santacroce PV, Remmele RL Jr, Bee JS. Technical decision-making with higher order structure data: impact of a formulation change on the higher order structure and stability of a mAb. *J Pharm Sci.* 2015;104(4):1539–1542.
4. Budyak IL, Doyle BL, Weiss WF 4th. Technical decision-making with higher order structure data: specific binding of a nonionic detergent perturbs higher order structure of a therapeutic monoclonal antibody. *J Pharm Sci.* 2015;104(4):1543–1547.
5. Arthur KK, Dinh N, Gabrielson JP. Technical decision-making with higher order structure data: utilization of differential scanning calorimetry to elucidate critical protein structural changes resulting from oxidation. *J Pharm Sci.* 2015;104(4):1548–1554.
6. Wei JY, Bou-Assaf GM, Houde D, Weiskopf A. Technical decision-making with higher order structure data: detecting reversible concentration-dependent self-association in a monoclonal antibody and a preliminary investigation to eliminate it. *J Pharm Sci.* 2015;104(11):3984–3989.
7. Weiss WF 4th, Gabrielson JP, Al-Azzam W, Chen G, Davis DL, Das TK, et al. Technical decision-making with higher order structure data: perspectives on higher order structure characterization from the biopharmaceutical industry. *J Pharm Sci.* 2016;105(12):3465–3470.

^a Health Canada, Ottawa, Ontario.

^b Albert Einstein College of Medicine, New York, NY.

^c BioPhia Consulting Inc., Lake Forest, IL.

^d University of New Hampshire, Durham, NH.

^e Genentech, South San Francisco, CA.

^f Bayer Pharmaceuticals, Berkeley, CA.

^g National Institute of Standards and Technology, Gaithersburg, MD.

^h Amgen Inc., Thousand Oaks, CA.

ⁱ Eli Lilly and Company, Indianapolis, IN.

^j Pfizer Inc., St. Louis, MO.

^k Correspondence should be addressed to: Michael Huang, Ph.D., Global Biologics, US Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852-1790; tel: 1.301.692.3348; email: BMH@usp.org.