# The Role of Higher-Order Structure in Defining Biopharmaceutical Quality

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Cosponsored by CASSS (an International Separation Science Society) and the US FDA, the 17th CMC Strategy Forum was designed to explore the relationships between higher-order molecular structure and quality of therapeutic proteins and peptides, vaccines, and blood-derived products. Understanding those relationships is important to defining and controlling the critical quality attributes (CQAs) of biopharmaceutical products. The forum program highlighted the current state of the art for analytical tools used to monitor higher-order structure. Case studies demonstrating the effects of changes to higher-order structure on biological function illustrated approaches to defining correlations. Presentations by experts from regulatory agencies, academia, and industry were followed by discussions focused on correlating data derived from analytical tools to biological functions of molecules. A predefined set of questions helped focus the discussions (see "Structure of the Forum").

**Product Focus:** Protein biologics

**Process Focus:** Manufacturing

**Who Should Read:** Product and process development, analytical, formulations, and QA/QC personnel

**Keywords:** Characterization, analytical methods, biotinylation, protein folding,

**Level:** Intermediate

## Regulatory Perspectives

Proteins are complex, three-dimensional (3D) structures capable of considerably changing their conformation in response to their environments. Although methods are available to characterize their three-dimensional structures, such methods are not applied routinely to biotechnology products. Without this characterization, how do we know that a given protein has the "correct" structure, whether a formulation contains variants, and whether those variants (if present) could affect the safety/efficacy of a drug product?

Characterizing the higher-order structure of a protein increases product knowledge, as required by the US FDA's quality by design (QbD) initiative. This includes understanding batch-to-batch consistency, stability, and whether variants or aggregates can be linked to safety and efficacy. But what is required to integrate higher-order structure into existing biopharmaceutical processes? Will doing so prove worth the cost, time, and effort?

Among monoclonal antibodies (MAbs), intrachain disulfide differences can affect IgG2 affinities, and different intrachain bonds can cause formation of chain swapping (IgG4), half molecules, or tetramers, all of which can affect the activity of the protein. Regulatory agencies require that we understand intra- and interchain disulfide bonding, aggregation, and polypeptide structure in general. These are particularly important if the higher-order structure of a protein determines its specificity. Moreover, understanding of combination products attached to matrices includes whether and how binding affects protein structure and function. And of course, higher-order structure may prove vital to approval

**Structural analysis is more commonly a tool of drug discovery research, as shown here at Novartis AG in Switzerland.** (WWW.NOVARTIS.COM)

of follow-on biologics, for which a different process could create subtle differences in the three-dimensional structure of an expressed protein.

At present, assays for higher-order structure are not generally used for lot release or stability, but they are used for early and late characterization and comparability studies. Their use in the latter is stipulated by ICH Q5(E): "The nature and the level of knowledge of the product — product complexity, including heterogeneity and higher order structure — physicochemical and in vitro biological assays might not be able to detect all differences in structure and/or function" (**1**). Because biotechnology products are heterogeneous, a sponsor must understand whether its analytical methods can detect average changes to the population of molecules that comprise a product, and whether they can evaluate a single molecular entity. The word *sensitivity* should be used with caution. It may mean detecting a very small change in all molecules of a batch, or it may mean detecting a change in a small percentage of them — two entirely different goals that require different approaches.

Techniques to evaluate higher-order structure are also important for evaluating products with large structural modifications such as PEGylation (hydrodynamic radius, polydispersity), glycosylation (we commonly analyze linear components, but not the 3D structure), and other large conjugates.

Tests for secondary and tertiary structure do not replace bioassays. And bioassays are not a complete substitute for characterization of higher-order structure. They provide a good measure of whether changes in such structure affect intrinsic activity, but bioassays do not predict bioavailability or immunogenicity and may be insensitive to differences in activity attributable to low-level variants present in the protein mixture. For products with multiple activities, higher-order structure can influence one activity more than another, so multiple bioassays could be required to correlate data and get a clear picture of the relationship between structure and activity. A comprehensive characterization of higher-order structure includes both physicochemical and biological assays.

Regulatory attendees confirmed that their agencies have not been requiring advanced higher-order structure studies for most investigational new drug (IND) submissions, unless they are necessary to establish comparability. It was acknowledged that some of the latest available technologies may not yet be amenable for measuring higher-order structure in a quality control (QC) setting. In line with QbD, higher-order structure analysis will increasingly become an expectation. To date, however, regulatory agencies have not yet received extensive data from sponsors using the latest analytical techniques that could be

## Structure of the Forum

The 17th CMC Strategy Forum took place on 24 January 2010 at the Renaissance Mayflower Hotel in Washington, DC.

**Plenary Session One:** Methodologies for Evaluating Higher-Order Structure was chaired by Roman Drews of the FDA's Center for Biologics Evaluation and Research (CBER) and Muppalla Sukumar of Eli Lilly and Company. This session included the following presentations: "Regulatory Perspectives on Higher-Order Structure Evaluation for Protein Products," by Emily Shacter of CDER; "Structures of Larger Proteins by NMR," by Marius Clore of NIH; and "Microcalorimetry: Application for Evaluation of the Structural Stability and Integrity of Biotherapeutic Products," by Yuri Griko of NASA.

**Plenary Session Two:** Advanced Technologies for Higher-Order Structural Determination was chaired by Keith Webber of the FDA's Center for Drug Evaluation and Research (CDER) and Ziping Wei of MedImmune. Presenters and topics were as follows: "Advanced Fluorescence Methodologies for Protein Detection, Quantization, and Structural Determination," by Chris Geddes of the University of Maryland Biotechnology Institute; "Opportunities and Challenges in Using Hydrogen/Deuterium Exchange with Mass Spectrometry (H/DX-MS) Detection in the Biopharmaceutical Industry," by Steven Berkowitz of Biogen Idec; and "Single-Molecule Analytical Characterization: A Window into Biomolecular Heterogeneity," by José Casas-Finet of MedImmune.

**Plenary Session Three:** Real-Life Applications — Case Studies was chaired by Patricia Cash of MedImmune and Muppalla Sukumar of Eli Lilly and Company. Presenters and their talks included the following: "Establishing Relationship Between Higher-Order Structure and Product Quality During Product Development — Monoclonal Antibody Case Studies," by Ziping Wei of MedImmune; "The Role of Protein Optimization in Improving the Pharmaceutical Properties of Biotherapeutic Candidates," by John Beals of Eli Lilly and Company; and "Biochemical and Biophysical Characterization of Influenza Virus-Like Particle Vaccines Produced in Insect Cells," by Steve Pincus of Novavax.

Following all sessions, the panel discussion was moderated by John Dougherty of Eli Lilly and Company and Mark Schenerman of MedImmune. Panel members were Yves Aubin of Health Canada, Steven Berkowitz of Biogen Idec, Brigitte Brake of BfArM in Germany, Roman Drews of CBER, Chris Geddes of UMBI, Tom Patapoff of Genentech, Emily Shacter of CDER, and Keith Webber of CDER.

### Questions Posed at the Forum

What analytical tools are available to determine higher-order structure of biopharmaceutical products? What are the discriminating capabilities and sensitivity limits of these methods?

What strategies are being used to correlate higher-order structure with biological function? Are other experimental approaches worth trying?

Functional assays are often used as confirmation of higher-order structure. How might this paradigm be applied to various product classes (e.g., MAbs, enzymes, cytokines, and coagulation factors)?

What process steps or conditions contribute to changes in higher-order structure?

How should assessment of higher-order structure be included in comparability exercises and stability studies?

used. The agencies will be having discussions with sponsors and examining their developing strategies for analyzing higher-order structure. Currently, some assays (most commonly near- and far-ultraviolet circular dichroism and intrinsic fluorescence) are used for characterization and comparability evaluations — and in the European Union, as part of biosimilarity assessments. Data are provided in regulatory filings case by case. FDA attendees stated that more studies and data are needed from sponsors so that important information from assays for higher-order structure can be applied to understanding and controlling product quality.

Information regarding higher-order structure is valuable in protein design because early knowledge will help with formulation work, stability assessment, and process development. To understand whether a structural, manufacturing, or formulation change effects a difference in higher-order structure, you have to analyze as many batches as it takes to thoroughly understand the process and method variability.

## Analytical Methods

A number of tools and techniques for analyzing higher-order function were discussed. Others are listed in the "Other Technologies" box.

**Nuclear magnetic resonance imaging (NMR)** uses the ability of magnetic nuclei to absorb energy and radiate it back when exposed to electromagnetic pulse or pulses. The reaction is influenced by the environment of those nuclei and their interactions with others. It is possible to label different protein chains with different isotopic labels and analyze the atomic interactions. However, the labeling process can affect conformation, which must be taken into account when analyzing results. Labeling is not always required for NMR, but sensitivity drops, and time to develop a profile increases without it.

**Advantages:** NMR is not affected by weak complexes for quaternary structure analysis, and it works in solution. Its sensitivity depends on the ability to enrich a solution with magnetic nuclides (200 μM/0.3 mL). The time required for analysis depends on protein concentration, but elucidating a protein's structure using NMR is generally slow. Once you're finished, however, you have a well-defined profile. NMR is very sensitive to change. It may not enlighten you about the meaning or cause of a change, however, or whether that change will affect protein activity.

A shorthand version of NMR known as *fingerprinting* can be useful and is much less time consuming; it can be done in a matter of hours. If all resonances in two fingerprints are the same, the two molecules share amino acid sequence and conformation. It isn't clear at present, however, exactly what it means if the two fingerprints are different.

**Microcalorimetry** illustrates the structure of a protein from a thermodynamic perspective and can illustrate how protein structure reacts to different environments. It requires comparison with a reference and measures molar heat capacity (absorption/release) over temperature increases. This technique uses only microgram quantities of material (0.2 μM–10 mM), but results are concentration dependent. Denaturation can occur at both high and low temperatures. Microcalorimetry is useful for comparing stability when a molecule is exposed to different stresses caused by different excipients or conditions such as pH. This rapid assay is sensitive to change. This rapid test is sensitive to changes in the total population of molecules in a given batch.

**Metal-enhanced fluorescence (MEF) or chemiluminescence (MEC)** dramatically increases sensitivity of fluorescence-based or chemiluminescence-based methods. It uses glass or plastic with metal coatings (silver or zinc, for example) that have enhanced fluorescence or luminescence, potentially up to 38,000-fold. This technology has potential for higher-order structure analysis applications that can be limited due to weak intrinsic fluorescence signals or lack of selectivity for methods involving binding of extrinsic fluorophores.

**Hydrogen/deuterium exchange with mass spectrometry** uses the exchange of atoms over time to illustrate conformational dynamic changes. Labeling is stopped at various time points, the protein is digested, and the peptides are analyzed. This requires only small amounts of material (10 pmol), and provides high spatial resolution, which can be increased further with electron transfer disassociation to one amino acid. This high-throughput technique gives nearly complete protein sequence coverage and is robust and reproducible. Its challenges include difficult data analysis and presentation, interference effects (from detergents, for example), and day-to-day drift.

**Electron tomography** involves adding colloidal gold to a protein solution, placing it on a grid, flash-freezing it, and then acquiring multiple images at different angles. Collected images are analyzed to obtain a 3D structure of individual protein molecules for size, shape, and core density. This requires very little

### Other Technologies

Several other analytical methods have possible application to analyzing 3D higher-order structure of protein molecules:

- Analytical ultracentrifugation
- Anilinonaphthalene sulfonate (ANS) binding
- Chromatography using interactive resins
- Circular dichroism
- Cryoelectron microscopy
- Fieldflow fractionation
- Fourier-transform infrared spectroscopy
- Free-electron laser scattering
- Mass spectrometry
- Raman spectroscopy
- Single-cell sensing
- Single-molecule fluorescence spectroscopy
- Size-exclusion chromatography
- Static light scattering
- UV/fluorescence spectroscopy
- X-ray crystallography

material, provides resolution down to 20 Å, and visualizes individual molecules including membrane proteins. The technique can be used in situ on intact cells or in vitro on purified proteins. It can be made quantitative using the frequency of certain conformers to provide a profile of polymorphisms and/or aggregates.

**Dynamic light scattering (DLS)** uses the ability of higher-order structures to scatter light detected by a photomultiplier. The technique is extremely sensitive to larger aggregates and can be used directly on a liquid sample over a broad range of protein concentrations. However, some buffer components or excipients (e.g., polysorbate 80) can interfere. DLS does not work well for polydisperse and/or highly concentrated samples. Standardization may make this technology more useful.

**Low-voltage electron microscopy** provides higher image contrast and resolution than light microscopy without the need to fix samples, and it can work with liquid-phase solutions. This technique offers an increased scale range (from nano to submilli) and can image individual elements within a given sample.

Atomic force microscopy (AFM) uses a cantilever/probe to detect specimen height. It covers a very broad range (from angstroms to subvisible) and requires low-nanogram, small-volume samples. The method is fast and easy, allowing users to visualize a sample with excellent resolution. It works well for characterization and would be possible to move into quality control. The technique can be used as an identity test with a derivatized tip and can be quantitative. AFM force spectroscopy can measure binding affinities of MAbs by pulling away bound molecules and measuring the rupture force.

## Questions and Answers

**What strategies are used to correlate higher-order structure with biological function? Are other experimental approaches worth trying?** In general, it appears that comparing different higher-order structure profiles or creating specific mutants and linking them to read-outs in potency assays (including animal models if appropriate) would be the most common strategies for correlating higher-order structure with biological function. We cannot expect a single bioassay to correlate with all parts of the molecular structure of a protein. Data should be collected during process development to determine whether additional tests could be of value in making that correlation. We know that bioassays don't predict pharmacokinetics, bioavailability, or immunogenicity. Another approach could be immunological assays, probing with conformation-specific antibodies. As more technologies and more sensitive tests become available, it remains to be seen how much uncertainty will be acceptable.

**Functional assays are often used to confirm higher-order structure. How might this paradigm be applied to different product classes?**

**MAbs:** Large proteins such as MAbs that have four chains can complicate results provided by most techniques because small changes in a single chain may not be detected. It is possible to correlate bioactivity with different levels of protein modification, such as tryptophan oxidation and methionine oxidation or Fc mutations. That can illustrate changes in thermal stability or the exposure of certain amino acids. Binding and Fc functions need to be considered with this approach.

**Vaccines:** It is possible to use ion-exchange (IEX) chromatography, Malvern Instruments' Zetasizer instrument, and field-flow fractionation to analyze higher-order structure in vaccine molecules. Cryoelectron microscopy can be used to visualize virus-like particles (VLPs). Liquid chromatography/mass spectrometry (LC/MS) can identify proteins within VLPs, then reverse-phase high-throughput liquid chromatography (RP-HPLC) quantitates them. It would be necessary to correlate those results with immune response.

**Coagulation Factors:** Very large proteins present limitations for most technologies. However, coagulation cascades allow for multiple functional assays, which can probe the intricacies of such molecules. It is especially valuable to compare recombinant proteins with serum-derived products.

**Enzymes** have multiple domains for control or for cellular uptake. What you need to test depends on the enzyme and on the substrate used as well as the molecule's interactions with natural inhibitors.

**What kinds of process steps or conditions can contribute to changes in higher-order structure?** From cell culture through purification, formulation, fill–finish, and transportation, most steps in the development and manufacturing process of therapeutic proteins and other protein drugs have the potential to affect protein higher-order structure. Changes can be effected through pH and salt levels, solvent and surface interactions, shear forces, container–closure interactions, temperature and freeze–thaw cycles, moisture (especially with lyophilized formulations), light, pressure, and protein concentration, just to name a few factors.

**How should assessment of higher-order structure be included in**

### The CMC Strategy Forum Series

The CMC Strategy Forum series provides a venue for biotechnology/biological product discussion. These meetings focus on relevant chemistry, manufacturing, and controls (CMC) issues throughout the lifecycle of such products and thereby foster collaborative technical and regulatory interaction. The forum committee strives to share information with regulatory agencies to assist them in merging good scientific and regulatory practices. Outcomes of the forum meetings are published in this peer-reviewed journal with the hope that they will help assure that biopharmaceutical products manufactured in a regulated environment will continue to be safe and efficacious. The CMC Strategy Forum is organized by CASSS, an International Separation Science Society (formerly the California Separation Science Society), and is cosponsored by the US Food and Drug Administration (FDA).

**comparability exercises and stability studies?** For comparability, analysis is useful in determining whether differences in higher-order structure occur before and/or after process changes. However, several issues arise:

• Are observed differences "real" or a result of sample, assay, and/or process variability? Having adequate reference standards should help you address this question.

• As techniques become more sensitive and produce more complex results, acceptance criteria may need to be updated. This requires an iterative process approached with flexibility and patience.

• What subtle differences are occurring? Higher-order structural analysis tends to tell you that "thermal stability has changed" or "a protein is less stable," or "the position of amino acids on the surface has changed," without revealing underlying cause(s). You may need to use orthogonal techniques to find out more.

• How do you correlate changes in structure with function and link that information to something meaningful in clinical results? Bioassays may not be sensitive enough to detect changes illustrated by these techniques, so would you have to go back to animal studies — or even clinical studies? Again, how much uncertainty is acceptable?

• Most analytical techniques mentioned here detect gross changes. Differences could be missed if they "balance out" or are diluted by larger and/or multiple signals. Sensitivity is low to small percentages of change among molecules, although a certain percentage of complete unfolding can be detected.

• You have to understand the effects of storage and/or freezing on samples used for comparability.

• For formulation changes, the presence of detergents can limit the technologies you can use for characterization. However, formulation greatly affects higher-order structure, so it must be addressed in some way.

Techniques are available for looking at small populations: Single-molecule spectroscopy, for example is feasible with fluorescence labeling and sensitive detection. However, the molecular effects of labeling also must be considered. Fluorescence spectroscopy can provide a quick answer, but interpreting its results can be a challenge. Immunological techniques against structural epitopes can be useful, especially for vaccines. Preparation techniques such as IEX or affinity chromatography can be used, depending on the type of structural change involved. The choice depends on the molecule of interest.

## Permanent Advisory Committee for These Forums

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## Useful Information

Structural alterations have been seen in some cases before other changes were detected, so they are a good indicator of overall change. However, they often seem to be associated with some form of chemical change such as oxidation or deamidation, or a difference in formulation or solvent environment. Orthogonal assays — such as peptide mapping, IEX, isoelectric focusing, and bioassays — are thus more likely to be useful indicators of change in a stability study once degradation pathways (or the causes of structural change) are understood.

The biotechnology industry is only now beginning to implement advanced techniques for determining higher-order structure of proteins, and regulatory agencies are hoping to learn along with the industry as investigational new drug (IND) and license applications are filed reporting use of these techniques. At present, available technologies are perceived to pose challenges for cost-effectively obtaining timely, reliable profiles. However, companies are experimenting with a number of methods and will no doubt find ways to improve or better use those technologies as experience is amassed and data are evaluated. Among the questions yet to be answered is just how valuable the additional data will prove to be. It is generally agreed that a better understanding of higher-order structure will lead to safer and more effective biopharmaceutical products.

## References


1 ICH Q5E: Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process. US *Fed. Reg.* 70(125) 2005: 37861–37862; www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5E/Step4/Q5E_Guideline.pdf.



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