

*IPQ "In the News," May 11, 2011*

## **FDA May Ask for More Data on Higher Order Protein Structure in Biotech Applications**

As methods and technologies designed to unravel the complexities and intricacies of protein folding, structure and function become more reliable and available, FDA is looking at whether it is time to begin requiring more detailed information on higher order protein structure in biotech product applications.

The importance of understanding higher order protein structure and its relationship to product safety and efficacy was explored by CDER Office of Biotechnology Products (OBP) Biochemistry Laboratory Chief Emily Shacter at the FDA/CASSS CMC Strategy Forum in Barcelona, Spain in late March.

**Shacter explained that the agency is not generally seeing higher order structure evaluations in submissions and is questioning whether and in what context more information should be supplied.**

“Very little of what we know about the higher order structure of proteins is applied in biotechnology submissions to the agency,” Shacter pointed out. “This is not because methods are not available – they are, and some of them are amenable to a QC environment. But we still do not see them very much.”

In turn, FDA is considering whether it is “time to raise the bar” on expectations for this kind of data. And if so, then “in what cases would we do that?” When does it make sense to do this? When will the information be important and meaningful? And when would it not make sense?”

*The full story, normally available only to subscribers, is being made available through a special arrangement with CASSS.*

### **Higher Order Structure Impacts Protein Function**

Shacter emphasized that two “key take home messages” from her presentation are that folding imparts the activity of a protein, and it impacts “what the body sees.”

For therapeutic proteins, she pointed out, some of the “big questions that we have to ask” are how do you know: ● when you have the correct structure? ● when structural variants are present in your protein product? ● if the structure has changed significantly? And ● that a change in structure is clinically important? The last question, she commented, “is what we need to know most.”

Answering these questions requires the use of at least one of the “many methods” for evaluating the higher order structure of a protein. The ones that “we tend to see the most” on applications, she pointed out, are circular dichroism, intrinsic fluorescence, and disulfide mapping. However, “these are really not the most discriminating techniques that are available for evaluating higher order structure.”

In the context of aggregate measurement, the most common methodologies seen in applications include light scattering, analytical ultracentrifugation, field flow fractionation and size exclusion chromatography.

**Shacter emphasized that understanding higher order protein structure is key to understanding the stability and safety of protein molecules.**

Some protein molecules with identical amino acid sequences but folded in different ways have “vastly different biological and clinical activities associated with them.” A “frightening” example, Shacter pointed out, is prion protein, which in its native conformation is present throughout the human body, but in a different conformation causes transmissible spongiform encephalopathy (TSE).

Protein aggregates can also affect the safety of the product through their impact on immunogenicity and receptor binding, and some protein variants have been demonstrated to have different levels of specificity or activity, she noted.

**Current Higher Order Information in Applications is Limited**

Shacter explained that there are instances where the agency requires studies on the higher order structure of a molecule in the investigational new drug application (IND). This includes situations where one of the following is present: • intra- and inter-chain disulfide bonding • aggregates • the potential to form amyloid • proteins for which a higher order structure actually determines the specificity, or • proteins that are associated with a carrier matrix. *[Editor’s Note: Shacter’s complete presentation with more detail on this topic is appended below.]*

The CDER biochemist noted that the agency does sometimes see evaluations for higher order structure in regulatory submissions beyond these specific situations, but “that is rare.”

In general, the analyses seen involve characterization studies from development – in early development where structure-function relationships are being explored, and later in development as part of product evaluation.

Shacter pointed to the agency’s desire to see more higher order structure evaluations as part of comparability analyses. “Although we see some now, we should be seeing a bit more,” she stressed, noting that ICH Q5E recommends such an assessment as part of a comparability analysis. Those submitted to date, in general, have not included “the most discriminating techniques.”

She added that it is “pretty rare” for the agency to see data on higher order structure as part of stability studies.

**The CDER official explained that the agency expects the use of state-of-the-art techniques that have as high a resolution power as possible, but understands that developing appropriate techniques can take time.**

In the early 1980s, “capillary electrophoresis was being brought into the fore of protein analysis,” but it took 25 years – until 2006 – “to actually come into being a routine QC method that is used widely by the biotechnology industry.”

The biotech regulator advised firms “to get started now. It is going to take a long time for you to actually get whatever techniques you find to be useful into the QC environment so that they can be validated as being suitable for their intended purpose.”

“We recognize that techniques are being developed now. There are many techniques that are along the way and we would like to see them coming forward.”

**Q&A Focuses on Risk Assessment in Higher Order Structure Determinations**

In the Q&A after Shacter's presentation, an audience member asked, "what are the appropriate methods to use, and when? And what would you do with the information?" The questioner noted that in the case of a replacement therapy for a low-level endogenous protein, immunogenic response to a new product variant could be "potentially catastrophic for the patient," whereas in other cases the information is of less importance.

Shacter responded, "I think you are raising a very important question – when do we care? I think you are right to try to apply a risk assessment to the importance of the data that you might get." Although there are few good ways to predict immunogenic response, she pointed out, "there are other techniques that can be applied" – for example, immunological techniques such as protein surface mapping.

"What I come back to," the OBP official emphasized, is "what does the body see?" The immune system is "exquisitely sensitive to seeing changes in proteins – probably still better than any technique that we have available." However, there are other aspects that also need to be considered – for example, cellular uptake of a protein and the intrinsic activity of the molecule.

"So you put it all together, as you are doing, and you evaluate 'what is the importance of what I am looking at,'" Shacter explained. "I think it is an iterative process. The first step is to know your molecule, then to know how changes in your molecule might impact its clinical activity."

**Another audience member asked for clarification of what the agency expects regarding analysis of higher order structure in early clinical phases. For example, she asked, "if there is very limited information or almost none, would you consider that safe?"**

Shacter replied that there is no immediate safety concern. However, she commented, "it helps you greatly, in my opinion, to know as much about your molecule as you can as early...as you can, because that allows you to understand through a controlled clinical trial that some change that may result to a molecule from improving your process is not going to impact clinical activity."

The CDER biochemist acknowledged that the situation presents "a little bit of a Catch-22." On the one hand, "you want to have some consistency during the course of the clinical trials, because the Phase I clinical data will tell you how to proceed through Phases II and III." On the other hand, "you would kind of like to know if a change to the molecule has impacted its clinical activity. The best way to know that is through a controlled clinical trial."

How a change might impact the product molecule will not be discovered "if you are not looking," Shacter stressed. "I would recommend that the time to start looking, at least for information purposes, is early on."

Addressing how the agency would proceed in the absence of higher order structure analysis in an IND submission, the OBP official stated that, in general, the agency would not stop the IND from going forward. However, the presence of aggregates would cause "an exception."

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## **CDER'S EMILY SHACTER ON HIGHER ORDER PROTEIN STRUCTURE**

*At the CASSS-sponsored European CMC Strategy Forum in Barcelona in late March, CDER Office of Biotechnology Products Biochemistry Lab Chief Emily Shacter gave a presentation on "Regulatory Perspectives on Higher Order Structure Evaluations for Protein Products." She provided a definition of higher order structure and explained why it is important, how it is measured, and the current regulatory thinking for addressing the issue in product submissions.*

Our group at the FDA regulates a lot of the novel proteins that are coming in for clinical investigation, not including monoclonal antibodies.

During this talk I am going to discuss and give examples on: What is higher order structure? Why is it important? How is it measured? And what are regulatory expectations for measurements of higher order structure?

## Understanding Protein Structure and Folding

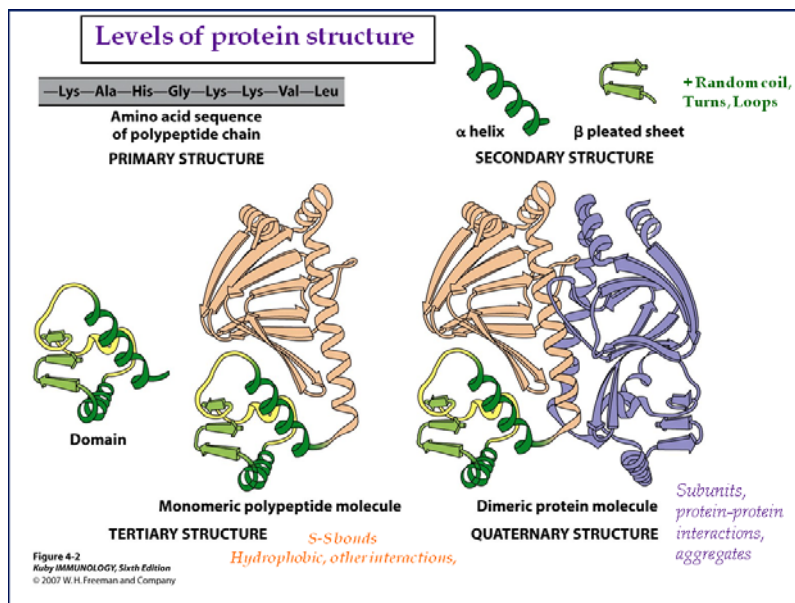
The first regulatory challenge that we face is that we would like to understand a little bit better how proteins fold and how they develop and maintain their three-dimensional [structure]:

- How do proteins adopt their shapes – and is knowing the amino acid sequence enough?
- What is the impact of variations in the three-dimensional structure of proteins?
- How does that impact their safety and effectiveness and the ability to have a consistent protein product?

From a regulatory perspective these are all important, with the last being the most important.

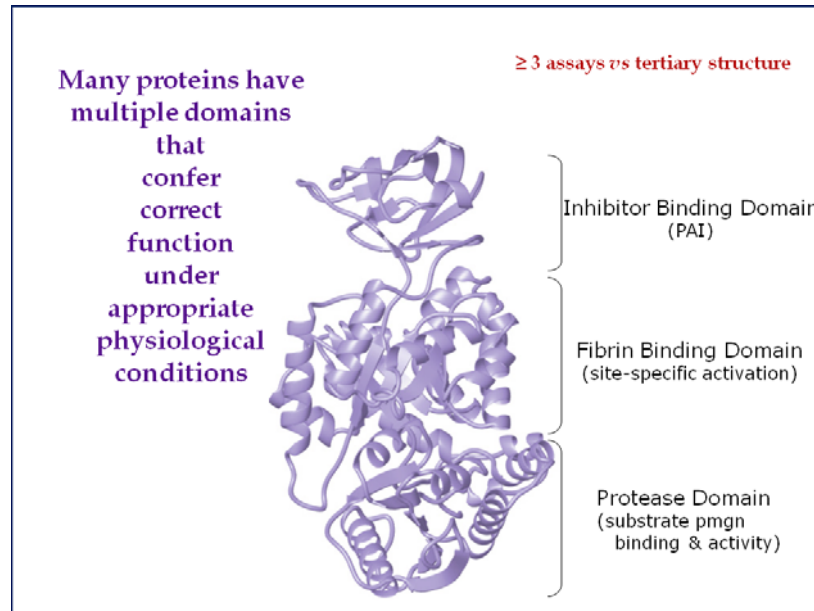
I want to make sure that we are all on the same page about **terminologies**.

- ◇ The amino acid sequence is the primary protein structure. This could also involve post-translational modifications.
- ◇ The secondary structure is the ordered linear structure that proteins adopt resulting from their amino acid sequence and from hydrophobic interactions: the alpha helix, beta sheets, coils, turns and loops.
- ◇ Then proteins fold into their tertiary structure. This includes their disulfide bonding and how they fold on top of each other. This example is of a domain that is a functional unit of this folded protein.
- ◇ Finally we have the quaternary structure, which involves protein-protein interactions. Where these are normal, they might be subunits of a protein. Where these are abnormal, these might be aggregates. Both types are lumped into the quaternary structure definition.



I want to talk about domains for a moment because this is somewhat at the heart of what protein folding gives us – it gives us **functional domains**. In most cases, a linear amino acid sequence does not confer function unless the protein is folded into the correct domains.

If you take one example of a protein, such as tissue plasminogen activator (TPA), it may have three domains:



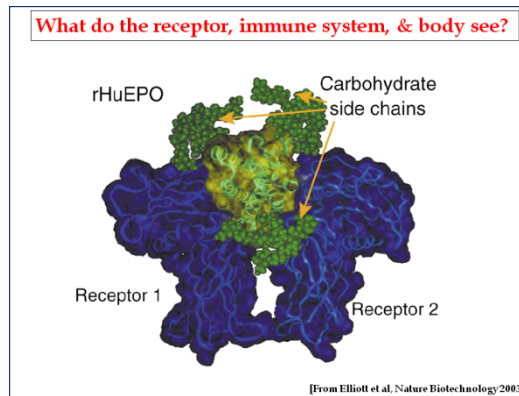
One is the protease domain. This is the enzymatic activity of the protein that converts plasminogen to plasmin in the body. This, however, does not really fully control the activity of a TPA molecule.

You also have a fibrin binding domain. This directs the molecule in the body to go to the right place so that it only acts where it is supposed to act, which is on the fibrin clot.

You might also have an inhibitory domain, such as a binding region for tissue plasminogen activator inhibitor (PAI). This domain regulates the activity of TPA in the body, but in this case through the actions of physiological modulators that may be in the plasma.

You have to actually know about all three of these domains in order to fully understand the biological and clinical activity of the molecule. One way to assess this is to have three different bioassays for protein structure – but that might not tell you everything that you need to know.

Two key take home messages during this talk: ● One is that folding imparts the activity or the many activities of a protein. ● The other is that folding impacts what the body sees. It is not something that we talk about as much. The example shown here is rHuEPO binding to its receptor in the body.



What does the immune system see? What do other elements of the body see that might impact, for example, the pharmacokinetics of the protein? These are some of the things to consider when trying to understand the impact of folding on a therapeutic protein.

**How do proteins fold?** In the early days, through the work of Chris Anfinsen, it was thought that amino acid sequence is enough to direct correct protein folding. But actually there is a very complex path that leads to protein folding, including free energy states of the protein and chaperone proteins in the cell, which differ between prokaryotes and eukaryotes. The rate of synthesis impacts protein folding in the host cell in which it is made. These are just a few of the elements that can impact protein folding.

The work that Chris Anfinsen did to earn the Nobel Prize was to determine that the amino acid sequence actually did determine the folding of the model protein on which he worked, which was ribonuclease A (RNase). He showed that you can unfold the molecule, and just by having the correct amino acid sequence it would take on its active three-dimensional structure.

He was not looking at all of the things that we look at when we look at therapeutic proteins, but his finding was correct – at least in terms of the *in vitro* activity of RNase.

Over the ensuing years we have learned that amino acid sequence is not enough to determine the higher order structure of a protein.

This is a paper that was published in 2007 [Kimchi-Sarfaty, et al. A 'Silent' Polymorphism in the MDR1 Gene Changes Substrate Specificity. Science. Vol 315. 26, Jan 2007] in which they found that making a silent mutation in the gene sequence – in a single nucleotide in the gene sequence – impacted the substrate binding and the activities of P-glycoprotein.

In this case, they did not change the amino acid sequence at all. They hypothesized that their observations had to do with co-translational folding of the molecule, in which a change in one of the nucleotides changed the rate of folding. I am not sure if that hypothesis is what has held up or whether there are others, but the point is that you can make a silent mutation in the gene sequence and still change the folding and activity of your protein.

### Important Questions and Observations

For therapeutic proteins, some of the big questions that we have to ask are: ● How you know when you have the correct structure? ● How do you know when structural variants are present in your protein product? ● How do you know if the structure has changed significantly? ● Most importantly, how do you know that a change in structure is clinically important? Because at the end of the day, that is what we need to know most.

We then ask ourselves, ‘to what extent can physicochemical and biological methods answer these very important questions?’

At the FDA, one of the observations that we have made is that there is vast scientific knowledge about the complexities and intricacies of protein folding, three-dimensional structure, and function. There is also a lot that we do not know about structure-function relationships. I think that this is an area that can be explored a lot more through accumulating information on the higher order structure of proteins.

We also know that very little of what we know about the higher order structure of proteins is applied in biotechnology submissions to the agency. This is not because methods are not available – they are, and some of them are amenable to a QC environment – but we still do not see them very much.

One question that we are asking is, ‘is it a time to **raise the bar** on expectations for this, and in what cases would we do that?’ When does it make sense to do this? When will the information be important and meaningful? And when would it not make sense? This is part of the dialogue that we are having now.

As I said, there are many methods available for evaluating the higher order structure of a protein. The ones that we tend to see the most in regulatory submissions are circular dichroism, intrinsic fluorescence, and disulfide mapping. I will talk about that a little bit more later. However, these are really not the most discriminating techniques that are available for evaluating higher order structure.

Because looking at aggregates is important, we do see some data on quaternary structure. The techniques that have been used the most for that are light scattering, analytical ultracentrifugation, field flow fractionation and size exclusion chromatography. We see these most in the context of aggregate measurements.

### **Why do all this?**

One reason all of this is necessary is that we need to understand the product. You need to control the consistency of your product. This is very important if you make a manufacturing change, for comparability analyses, and it may also be very helpful in the context of product development using quality-by-design approaches. For example, how can what you know about the higher order structure of your molecule help you with your design space? I am not going to talk about that, but I will put it out there as something for you to consider.

You need to know about higher order structure in order to understand the **stability and safety** of your molecule.

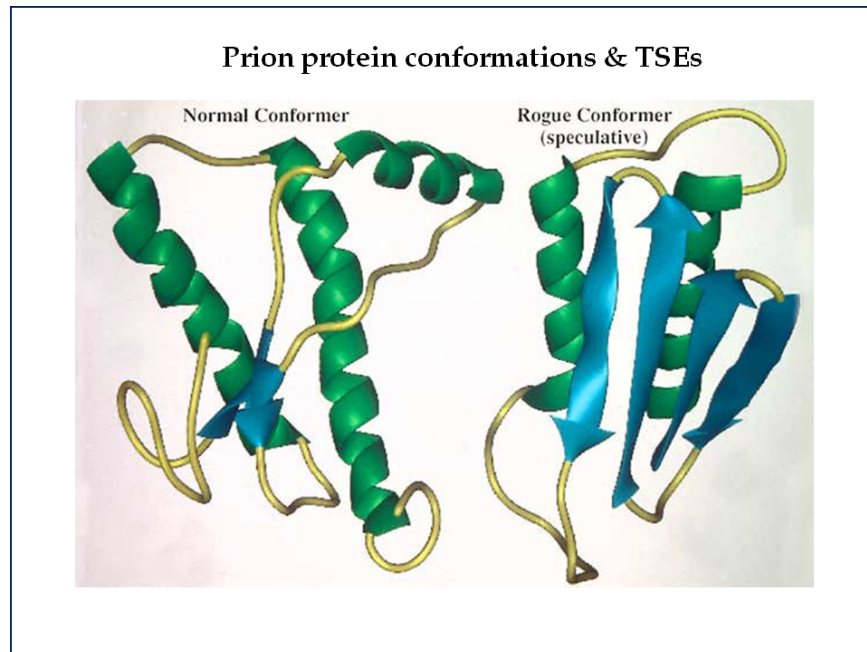
We also need to understand about aggregates. In this context we talk most about **immunogenicity**, but that is actually not the only area where aggregates can be important. You can have aggregates of a protein that actually might increase receptor binding in certain cases, but the issue that we talk about most is immunogenicity.

We can also have variants that have different levels of specificity or activity.

Finally, the better you can understand your higher order structure, the more this will help you with your formulation development.

I want to give one example of **conformational issues**. You can have diseases that come strictly by a change in the conformation of the molecule. Even though they have the same amino acid sequence, they have vastly different biological and clinical activities associated with them. Shown here is prion protein in the normal conformer and then in the conformer that causes transmissible spongiform encephalopathies (TSEs). This is

the most frightening type of example. And we do have some proteins coming in that are amyloid-like proteins, so this is not just about prions, because nobody is developing that as a therapeutic protein – it is there by example.



### Evaluations in Regulatory Submissions

With respect to **monoclonal antibodies**, we know that the disulfide bonding is extremely important. We know that both intra- and inter-chain disulfides are important. Intra-chain disulfides can impact the ligand affinity. In the case of IgG4, we know that the disulfide bonding can impact the proclivity of the molecule to form half molecules and to undergo chain swapping. With respect to IgG2, we know the tetramers can also form. All of these can result in altered activity and specificity *in vivo*.

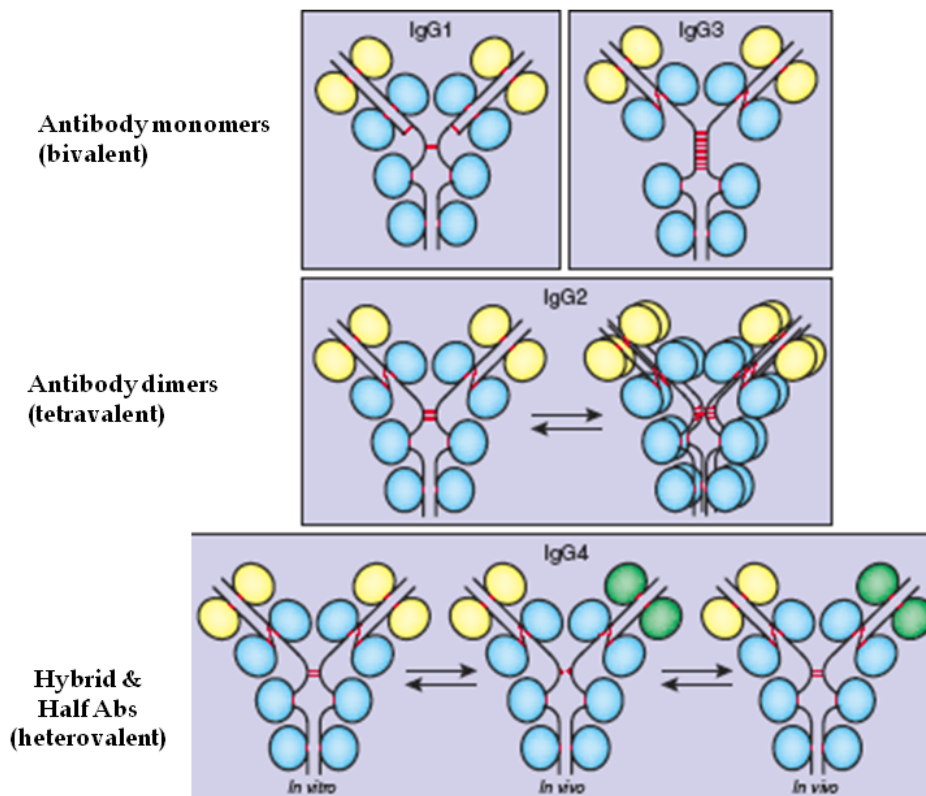
Here is an example with monoclonal antibodies to show some of the different structures that you can get. There is the bivalent monomer. Even though it has four subunit chains in it, this is referred to as the monomeric form of IgG.

You can also have dimers forming between two monomers, which would join two IgG molecules. You would then have a tetravalent molecule.

You can also get hybrid molecules. For example, for those of you who are using IgG4 to form your Fc conjugates, we are interested in knowing what the proclivity of the molecule is to form half molecules and to possibly swap with other IgG4s in the body.



## Criticality of disulfide bonds in Antibody products



From J.G. Salfed  
Nat. Biotech.  
Dec. 2007

There are some areas where we **require studies** for higher order structure in an IND:

- Prior to licensure, you need to know the intra- and inter-chain disulfide bonding of the molecule. This would be required for all products.
- We require studies for aggregates for all protein products. This would be an aberrant quaternary structure.
- We have had occasion to require an NMR analysis as part of the development of a generic polypeptide hormone.
- We would need to know about higher order structure for those proteins that have the potential to form amyloid. This is not just theoretical. This is a real requirement.
- Proteins for which a higher order structure actually determines the specificity – we have some designer proteins that are formed, for example, by phage display, which is all about binding affinity. These molecules are developed in some cases just to replace monoclonal antibodies through their binding specificity. So it would be important to know their higher order structure.
- Finally, proteins that are associated with a carrier matrix – for example, combination products where a protein is bound to a matrix going into the bone. You might know the structure of your protein before you mix it with the matrix, but we also need to know the structure of the protein after it is eluted from the

matrix in the body. It is not going to work if it does not come out of the matrix, so what does that protein look like in that case?

When do we see evaluations for higher order structure in the **regulatory submissions** that we get?

- It is very rare that we see them for release except for the case of aggregates – and we do always expect to see them in that case.
- We often see such studies in early characterization, which is good. This is when you are studying the molecule and you are trying to understand structure-functional relationships. These are usually done as characterization studies.
- We also see them later in development as process development is going forward, as part of the evaluation of how you are developing the product.
- Although we see some now, we should be seeing a bit more as part of comparability analyses. Again, we typically do not see the most discriminating techniques. But I will remind you that ICH Q5E says that the determination of comparability should involve an assessment of higher order structure.
- For stability, it is pretty rare for us to see studies on higher order structure.

### ICH Q5E Expectations for Higher Order Structure Evaluation for Process Changes

Following a manufacturing process change, manufacturers should attempt to determine that higher order structure (secondary, tertiary, and quaternary structure) is maintained in the product. If the appropriate *higher order structural information* cannot be obtained, a relevant biological activity assay...could indicate a correct conformational structure.

### Guiding Principles

A guiding principle for how to think about the role of higher order structure and characterizing therapeutic proteins is to remember that proteins are dynamic, breathing molecules. You need to understand that when you are applying any analytical technique to understanding the higher order structure.

The 3-D structure of a protein can be affected at any stage in the product lifetime – from manufacture to storage and stability to development and changing the formulation and so on. Every part of manufacturing can impact the higher order structure of a protein.

**Variants** can impact function and clinical outcomes. You can have a low-level variant of a protein in your heterogeneous sample and it can have a tremendous impact on the safety and efficacy of your product.

An example would be myozyme, with the presence of phosphorylated mannose moieties as part of the glycosylation. Levels of mannose bis-phosphate significantly impact cellular uptake and clinical activity. And then there is Eprex – everybody’s favorite example – where probably a low level of certain variants were responsible for very serious adverse events.

Finally, I think it is important to look at **protein modifications** that are large and that impact the protein product in a very big way – like glycosylation, PEGylation and proteolysis. These can all have a great impact on what the body sees with respect to the molecule....

I thought I would bring out some work that we published in *Nature Cell Biology* a year and a half ago, where we were looking at the impact of protein oxidation on the induction of apoptosis in cells. We were trying to discover if proteins were oxidized, and if so, which proteins and what was the impact? How would that impact the ability of a cell to die in the context of oxidative stress?

We discovered one protein, cofilin, which underwent oxidative modification in response to oxidative stress. We characterized the structural changes in the molecule and found that there was a gain of function in the protein. So a protein that usually regulates actin polymerization was now also regulating apoptosis induction in the cell.

In the Office of Biotechnology Products (OBP) we have active scientists, like myself. I run a research laboratory. We stay up-to-date with scientific research and can understand most of the data that you bring to us. It does not mean that we know everything – we have a lot to learn. However, we can understand the data. We do the work. We walk the walk. I know that there is so much that we do not know, but that is life....

As I alluded to earlier, protein products are usually mixtures of molecules. It is very rare that we have a homogeneous protein product.

### **Methods and Measurement**

Most often we see spectroscopic methods used to measure protein structure. But these methods only look at averages in protein solutions. They are not sensitive to looking at low levels of **variants** in your product. They are also probably not particularly useful for monoclonal antibodies because they are structurally similar in so many ways.

It is important to tease out which of the techniques can actually look at changes in higher order structure for a monoclonal antibody. Fortunately, there are some out there, and we saw some pretty elegant techniques at the strategy forum in Washington last year.

These methods look at averages. But you want to know what you have in the way of variants in your sample. So you will probably have to separate out the variants from the larger population. For this you would need to use some high-resolution separation techniques and then couple those with the 3-D assays that you are doing.

Which techniques are best? For example, is chromatography adequate? Should you be using a flow-based method? We do not know – that is for you to figure out....

Some of the assays can see individual variants in the molecular population, like atomic force microscopy. You do not necessarily have to separate out the molecules. But in most cases, especially for using the more common techniques, that is likely what you will end up doing.

How do you know if the method that you are using is sensitive to changes in higher order structure? In most cases you will probably stress the molecule. You need to have ways to verify that the technique that you are using can actually do the job that it is intended to do. We expect the use of state-of-the-art techniques that have as high a resolution power as possible.

This brings us to the question of **sensitivity**. What is sensitivity in this context? Is it your ability to see a change in a small subset of the molecules, or is it your ability to detect a small change in the larger population of molecules? Those have two very different meanings, and the word sensitivity really applies to both.

In the early 1980s, capillary electrophoresis was being brought into the fore of protein analysis. It took 25 years – until 2006 – to actually come into being a routine QC method that is used widely by the biotechnology industry.

My advice then would be to get started now. It is going to take a long time for you to actually get whatever techniques you find to be useful into the QC environment so that they can be validated as being suitable for their intended purpose.

We recognize that it takes a long time. We recognize that techniques are being developed now. There are many techniques that are along the way and we would like to see them coming forward.

I want to make the point that **large modifications** to a protein impact the size of the molecule. They have various different impacts on what the body sees. They may also be impacting the protein moiety to which they are attached. I think it is important to look at both what they are doing in and of themselves and how they are impacting protein structure and function.

In the case of glycoproteins, for example, we talk about linear structures of sugars. But should we be looking at their higher order structure as well?

### **Bioassays**

I want to talk for a moment about bioassays, because bioassays are an extremely important part of evaluating the structure and function of the molecule – especially function, if you have a relevant bioassay. Bioassays are not going away with the introduction of higher order structure techniques.

Bioassays are extremely valuable for evaluating protein quality and function, but they do not tell you everything that you need to know about a molecule. They tell you a lot about activity, but they do not tell you certain aspects of **clinical activity**. For example, they do not tell you about:

- Biodistribution, site of tissue binding, and how the protein gets around the body. They do not necessarily tell you about pharmacokinetics.
- Physiological modulators, that will bind to activation or inhibitory domains. For example, unless you are assaying plasminogen activator inhibitor in the case of TPA, then you are not going to know the structure or the function of that part of the molecule.
- Activity of low-level variants, because bioassays also look at averages in your population.
- Potential immunogenicity of your product – unless you have completely lost your correct folding and hence bioactivity, in which case your protein is likely to be immunogenic. But we are not talking about that kind of extreme.

So I want to reiterate the point – what does the body see? This is one of the more important reasons why we need to look at higher order structure of proteins beyond the role of bioassays.

Some proteins have multiple bioactivities. So in those cases, multiple bioassays might be helpful – also if you are looking at various domains. Even still, we need to have other evidence of correct and consistent structure.

The bottom line is that tests for secondary and tertiary structure are not substitutes for functional bioassays. At the same time, bioassays are not complete substitutes for the evaluation of higher order structure.

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