



Live Webinar Q&A Sheet:

## **Crystallized Biotherapeutics: Optimizing mAb Crystallization using Light Scattering**

The recorded webinar may be viewed from the [DLS](#) webinars page. These questions were submitted by live viewers. Additional information on MALS, DLS, and FFF may be found on the Wyatt web [Library](#) under Webinars, Application Notes, Featured Publications and Bibliography, as well as on the corresponding [Product page](#) and [Theory](#) page of our web site.

Please contact [info@wyatt.com](mailto:info@wyatt.com) with any additional questions.

*Q: For crystallized drug product formulations, do you see an increase in soluble and insoluble aggregates upon dissolution of the suspension?*

A: No, we do not. In some cases, we have observed fewer aggregates in the crystallized material than we did the raw material. There may be some purification that happens during crystallization. We are careful to optimize the crystallization and formulation processes so that there is minimal impact to the integrity of the protein after dissolution.

*Q: You mentioned monoclonal antibodies as traditionally difficult proteins to crystallize. Typical antibodies are IgG structures with only 2% carbohydrate. What about more heavily glycosylated proteins? Are these more difficult to crystallize, and can you provide an example of one that was crystallized?*

A: Crystallization is broadly applicable to most proteins. Glycoproteins with up to 18% carbohydrate has been crystallized (Margolin & Navia, 2001 / Yang et al 2003)

Q: *After buffer is chosen, what are the differences in conditions that produced crystallization hits vs. misses?*

A: Each protein is different and will crystallize under different conditions. It is difficult to draw too many sweeping conclusions about the protein buffers or crystallization buffers that produce hits or misses. The first buffer that is chosen is the protein buffer. We select for buffer type, pH and concentration. As mentioned in the talk we use %PD and  $k_D$  to select the buffer. We prefer low concentrations, to minimize the influence on the crystallization buffers. The initial crystallization screen, consists of a wide variety of crystallization buffers (both commercial and custom screens), that in our experience give us a good likelihood of discovering crystallization conditions for mAb's and other proteins. These screens include a variety of salts, buffers, pH's, and precipitants.

Q: *How important is the crystal morphology in the ranking of conditions that produced hits?*

A: It is fairly important, and we rank the conditions based on the more structured particles, 3-D crystals (as opposed to less structured and more gel-like). However, in the first screen, any ordered particle is a good start. Like most crystallographers, we just want to see the protein come together in a structured manner, and we will optimize from there. Additional work focuses on optimizing crystal structure by adjusting concentration, pH, and using additives.

Q: *What about the PK profile? Longer half-life?*

A: For a typical crystalline drug product, we expect a normal PK profile. The crystals typically are fully dissolved within 30 minutes of a subcutaneous injection and detectable in serum within 24 hours. However, we can develop sustained release formulations that dissolve over the course of a week or longer. A sustained release formulation of crystalline recombinant human growth hormone (rhGH) successfully completed Phase II clinical trials and was approved to start Phase III clinical trials. That formulation was released over a week and had minimal irritation at the injection site.

Please see Yang et al 2003:

rhGH crystal clinical trials

<https://clinicaltrials.gov/ct2/show/NCT00297713?cond=rhGH+crystal&rank=1>

Q: *What solution conditions actually promote crystallization of proteins other than the pH, salt, protein concentration?*

A: Each protein crystallizes under unique conditions, with the overall goal of removing the surrounding bulk water from the protein in a controlled fashion. Doing so results in crystals, whereas removing the bulk water too fast results in aggregation. Typically, there is a buffer used to promote protein stability, and sometimes additives like glycerol or salt. Hampton Research sells a nice mix of salts under the name Tascimate. Next would be the precipitants: PEG's, alcohols, or salts. Ammonium sulfate is as close to a universal crystallizer as it gets in the protein crystallization world.

Q: *Any publication on proof of concept?*

A: Yang et al., (2003) "Crystalline monoclonal antibodies for subcutaneous delivery", *PNAS* pp. 6934-6939 (<https://doi.org/10.1073/pnas.1131899100>)

rhGH crystal clinical trials

<https://clinicaltrials.gov/ct2/show/NCT00297713?cond=rhGH+crystal&rank=1>

Q: *How do you actually confirm it's a "crystal hit" other than from simple visual inspection? Many times you get crystals from small molecules or particles that look like crystals but are actually PEGs.*

A: We use UV imaging to compare to the light, and polarized images at the initial screening phase. This is a fairly reliable way to differentiate the salt and protein crystals. Once we move to 15  $\mu$ L, we can also use staining (IZIT Dye) or the poke test to confirm. When we get to the 300  $\mu$ L scale we will measure protein concentration in the batches and optimize for yield. Additional methods to confirm crystallinity can be performed at the 300  $\mu$ L scale, and include secondary harmonic generation (SHG) and x-ray diffraction (XRD).

Q: *After ranking the formulations by high-throughput dynamic light scattering, you select only one to go forward into crystallization trials. Are you missing a potentially much better formulation by picking only one, or do you expect fairly similar performance from the top-ranked formulations according to DLS?*

A: I would expect fairly similar performance. Often our top-ranked protein buffers have very close %PD and  $k_D$  values.

Q: *What are your typical DLS data collection parameters?*

A: We use 10 scans per well, with 10 s each on the 384-well plate. (so that would be 1.6 hours for 96 wells). Some preliminary studies have shown similar data with 10 s / scan and 1 s / scan, which would reduce a 384-well plate to ~ 2 hours. We may move forward with reducing the scan duration but want to collect more data before committing to the change.

Q: *Would it be feasible to run the DLS screen with a regular cuvette-based DLS instrument?*

A: I would say no. The number of samples needed to screen the pH (which is critical) and protein concentration (to get  $k_D$ ) would make it time consuming to run one sample at a time. Even without replicates, there would be 128 samples. Additionally, use of the Pre-Set methods in the [DYNAMICS®](#) makes setting up the instrument and analyzing the data simple and fast. We sometimes export the data to JMP for further statistical analysis, but find that DYNAMICS does most of the hard work.

Q: *You mentioned that hydrodynamic radius and melting temperature,  $T_m$ , are also of interest in picking the best formulations to go forward. Have you taken these into account in the case studies? Do you measure  $T_m$  with the DLS plate reader, or another method?*

A: For soluble protein we use the DLS to measure viscosity,  $T_m$ , %PD,  $k_D$  and  $R_h$ . Since we are developing the use of the DLS plate reader to quickly determine formulations to move forward into crystallization trials, a methodology that includes  $T_m$  and  $R_h$  was not used in the current case studies.

$T_m$  of the crystals are also of interest. However, we can't use the DLS for crystalline protein because the particle size, 10-100  $\mu\text{m}$ , is too large for DLS analysis. So, to get the  $T_m$  of the crystals and crystalline slurries we use differential scanning calorimetry (DSC).

Q: Are there any downsides to crystallized high-concentration biologics, whether physiological concerns or product efficacy, shelf life or manufacturability? Is there any concern that subcutaneous injection of crystals causes cellular damage or rupture?

A: I don't see any significant downsides to developing crystallized high-concentration biologics, except for the additional time it takes to develop the crystallization and formulation conditions (~1 year). There have been studies in animals and humans that show that the cellular damage is similar to soluble therapeutics (Herceptin histopathology, see below). The crystals dissolve in 30 minutes or less once injected and have been shown to have the same efficacy as solutions (Remicade PD data). Once crystallized, the proteins should be more stable, as they have fewer available charges for degradation.

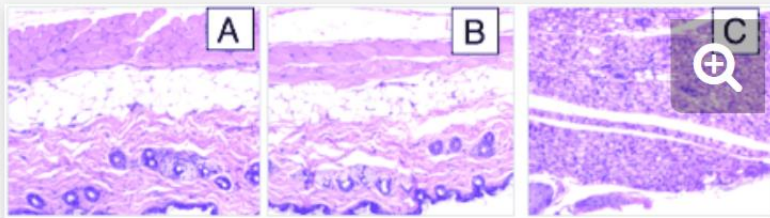


Fig. 5.

[Download figure](#) | [Open in new tab](#) | [Download powerpoint](#)

s.c. injection site analysis for trastuzumab. Injection sites were isolated at necropsy 12 h after the injection of 0.1 ml of 200 mg/ml trastuzumab crystals in a formulation buffer, which is different from the mother liquor, containing 10% ethanol and 10% PEG 3350 (800 mg/kg of body weight; A), 0.1 ml of a 7.5 mg/ml soluble commercial formulation of trastuzumab (30 mg/kg; B), or lipopolysaccharides (LPS; positive control; C). A portion of the skin at the site of injection was fixed in neutral buffered formalin oriented with the internal surface on a cassette-size square of paper, embedded in paraffin, and sectioned for histology. Paraffin sections were processed through hematoxylin/eosin stain. (All micrographs shown are  $\times 40$ .)

(MX Yang et al, 2003)

Q: It sounds like you have a lot of data to analyze after an overnight DLS run. How much of that is automated and how much net time do you spend processing and analyzing the data? Do you use any tools outside of the software that comes with the [DynaPro<sup>®</sup> Plate Reader](#)?

A: Inspection of the decay correlations (looking for wells that had a fiber or bubble etc) is probably the biggest time consumer (~ 2 hours for a 384-well plate). Past that, if you use the pre-sets, and have your plate information already loaded into the method, then the analysis is really a matter of minutes. I would say the data could be interpreted in less than 3 hours.

Q: *Clearly DLS gives you a major advantage over the antibody's formulation buffer. Would you recommend the HT-DLS screen for general protein crystallization research?*

A: Yes, I think the HT-DLS is a great tool for understanding the protein in different buffers, which is applicable to protein crystallization in general.