



Live Webinar Q&A Sheet:
**Predicting and evaluating the stability of therapeutic protein formulations by
dynamic light scattering and machine learning**

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Q: When you had different populations (aggregated, non-aggregated), how did you determine the R_h ?

A: Analysis of the diffusion interaction parameter k_D , isothermal chemical denaturation, dilution from denaturant and similar assume monomodal size distributions that can be fit to the cumulants model to determine an average R_h . If there are populations of large aggregates such that the sample is multimodal and the cumulants model does not provide a good fit of the ACF, you have three choices: 1) take the average R_h value from the cumulants fit anyway, 2) use the R_h value of the primary peak determined from the regularization fit, or 3) decide that the sample is not suitable for this type of analysis.

Option 1 will lead to erratic plots for k_D or C_m and is not recommended. Option 2 could be viable since the DYNAMICS[®] software tabulates average R_h for different size ranges, so just the nominal monomer size range can be used for k_D/C_m analysis; the plots will be less erratic than option 1 but may still be skewed. Generally I recommend option 3 – the presence of large aggregates means that the sample does not meet the initial criteria for performing these analyses. You should be able to filter the large aggregates from solution with a 20 nm syringe-tip filter before dilution or denaturant titration.

Q: What difference in R_h is considered significant? e.g. for mAb formulations is an increase from 7 nm to 8 nm statistically significant?

A: The sensitivity of the DynaPro[®] Plate Reader DLS/SLS detector is 2-3%. Therefore, an increase of from 7 nm to 8 nm is practically quite significant. As always, the statistical significance of the R_h increase needs to be tested using real replicates if possible, otherwise with analytical replicates.



Q: Industry products often contain more complex solutions, and protein-protein interactions take place in the presence of formulation components such as excipients and lipid emulsions. Can these DLS tools and machine learning be applied to such complex systems containing multiple components?

A: Both the DynaPro Plate Reader and machine learning algorithms can definitively handle such system. DLS has been extensively applied to study formulations with excipients and lipid emulsions. Certain particular cases may be challenging as is usual with all the analytical techniques.

In terms of machine learning, the great advantage of artificial neural networks (ANNs) with respect to classical statistical modeling is that the former can solve highly nonlinear problems, which is often the case in pharmaceutical industry. Excipients would simply be considered as a new factor or input parameter. However, when the complexity of systems is increased, the decision-making procedure of ANNs become increasingly difficult to interpret. Larger data sets will usually be required for the learning process of these more complex cases.

Q: You mentioned measuring sample at concentrations of 3 M of denaturant, but what is the effect of time of exposure to denaturant on these values?

A: As described by Svilenov et al ("A New Approach to Study the Physical Stability of Monoclonal Antibody Formulations—Dilution from a Denaturant." *J. Pharm. Sci.* 2018), after 24 h of incubation with denaturant the samples were rapidly diluted by addition of the respective buffers (or the respective buffer containing an additive) and incubated for an additional 24 h. The incubation periods were monitored to ensure that equilibrium was reached. Moreover, R_h was monitored up to 1 week after dilution from GuHCl and no substantial changes were observed. If this procedure is not followed then the results could be unreliable, since the samples may not have reached their unfolding equilibrium or final aggregation states.

Q: You mentioned that 75% of the data points from the screening procedure were collected by DLS and SEC-MALS. What was the overall impact of DLS in the decision making procedure for the selection of good candidates? What about MALS?

A: As you suggest, it's not only about the amount of information that can be collected but also about the impact that this information has on the decision-making process. In our work the forced degradation studies for characterizing robustness to heat stress were always characterized by DLS and SEC-MALS. Accelerated stress studies are performed to identify the impact of different stresses on the protein before selecting candidate for long term stability studies, to aid in the elucidation of degradation profiles. Therefore, the impact of DLS and SEC-MALS in the selection of candidates was dominant.



Moreover, other protein stability indicator of paramount importance were rapidly available thanks to the DynaPro Plate Reader. Namely, k_D , B_{22} , T_{agg} and solution viscosity can be collected in high-throughput fashion. In other words, if, hypothetically, we had access only to DLS and SEC-MALS for the selection of a good protein candidate with high physical stability, our confidence in our final selection would be very high.

Q: I would like to better understand your new approach for studying the physical stability of mAbs using dilution from denaturant, and how it compares to conventional methods. What is wrong with melting temperature (T_m) and isothermal chemical denaturation (ICD) approaches, and how does the new method overcome the problems? Why does the new method represent a relevant stability-indicating property of the molecule?

A: As described by Svilenov et al., non-isothermal techniques suffer from drawbacks related to the heating of the sample. For example, aggregation during temperature ramps often hinders the thermodynamic evaluation of DSC data and affects the accuracy of the determined protein melting temperatures from both DSC and DSF. Furthermore, non-isothermal techniques suffer from the fact that the properties of many excipients (e.g., the pH of amine buffers like histidine) change during heating, which can affect the obtained stability rankings based on protein melting temperatures.

Isothermal chemical denaturation (ICD) is a valuable technique that avoids the above-mentioned drawbacks of DSC and DSF. However, the accurate thermodynamic evaluation of ICD data assumes that the protein unfolding process is fully reversible, the system is in equilibrium, and the denaturation graph fits a known model (e.g., two-state, three-state unfolding, etc.). It has been shown that neither reversibility nor equilibration times in an ICD experiment are trivial. In addition, multidomain proteins may also exhibit multiple, (partially) overlapping transitions, introducing a large error to the parameters derived from the fit.

Last but not least, high-throughput DSF and ICD methods are usually based on intrinsic protein fluorescence measurements, relying on a change in the tryptophan exposure after protein unfolding. This creates complications when no tryptophan is present or when the tryptophan in a certain protein domain is already solvent exposed in the native protein conformation.

Instead, our approach entails a unique combination: i) it is isothermal ii) it simultaneously probes chemically-induced unfolding and colloidal stability iii) no complex data evaluation or fitting is necessary iv) it is label-free and does not rely on intrinsic fluorescence v) it can distinguish between overlapping ICD curves based on differences in colloidal stability.

Q. Among the various artificial neural network models you showed, the one for the prediction of the melting temperature T_m seems the most accurate. Do you have any



explanation for this? It is really possible to achieve this quality of prediction from the primary sequence only?

- A. It is certainly difficult to assess exactly why artificial neural networks can predict T_m in such an accurate way. If we look at the raw data, T_m presents an “easy to predict” behavior for a human mind as well as ANN. Most of the mAbs exhibited lower thermal stability at lower pH, reaching a plateau at higher pH values. This is not the case for T_{agg} which presents more complex behavior, mostly different for each protein. It is worth noting that the prediction of T_{agg} is still very accurate, in most cases with an R^2 of 0.9 and a RMSE of around 2. In the case of the sign of k_D , in most cases predictions were 100% accurate due to the fact that such binary classification problem are usually easier to assess.

We have shown that it is possible to obtain such predictions from the primary sequence, which would allow the selection of candidates with good T_m , T_{agg} , k_D and—in certain cases--monomer loss, even before expression. At this stage, the approach cannot exclude all poor constructs/formulations. However, the approach can still help to reduce the number of protein candidates to be expressed or formulations to be tested. It can thus facilitate the drug development process. Moreover, it is a novel concept; the publication of these results should constitute a stimulus for scientists to investigate further, adding more parameters and molecular descriptors into ANNs to refine and improve the approach. It is worth noting that the access to public database, as will be provided for the PIPPI database for protein formulation, will greatly increase the possibility of building useful machine learning algorithms.

Q: You showed aggregation temperatures (T_{agg}) measured by the DynaPro Plate Reader and NanoTemper’s nanoDSF instrument. How do the two values correlate?

- A: I guess that this is mostly a terminology issue. I will refer to the T_{agg} based on back-reflection, implemented in the nanoDSF instrument, as T_b . Due to the high sensitivity of SLS, the onset temperature for protein is usually determined at the real onset of aggregation, when molecular weight increases by 5-10%. On the other hand, T_b is based on the intensity of the back-reflected light which will start increasing when big aggregates are present, e.g. 40 nm or higher.

Notably, T_b is an important quality control parameter of the T_m measurement, since aggregation may affect the accuracy of determined protein melting temperatures. For our dataset we used T_b as a go/no-go parameter to assess T_m accuracy.



Q: Let's say we only have high-throughput DLS and a low throughput differential scanning calorimetry (DSC) instrument. What kind of workflow would you suggest for protein formulation development?

A: Supposing that you are a lucky owner of a DynaPro Plate Reader III, your DLS/SLS screening based on a full factorial design of experiment would give you the chance to obtain multiple indicators: i) R_h , PDI, and M_w , as a function of the applied stress; ii) k_D and B_{22} at room temperature and/or as a function of the temperature during a temperature ramp; iii) T_{agg} , the onset temperature of aggregation; and iv) the solution viscosity. This is plenty of information for a first-pass selection of promising candidates.

Before making a final decision, you need to make sure to select candidates that exhibit high T_m . The first step would be to use models to predict T_m (e.g. artificial neural networks or your own platforms). Based on this prediction you would build a suitable design of experiment, e.g. fractional design, to measure T_m by DSC, focusing on the reduced sample space defined by earlier DLS screening. After measuring the selected conditions, you could double check that your results overlay with your prediction; if not, it may be necessary to collect more data. Given the DSC results you will be able to reach a final formulation selection.