



Live Webinar Q&A Sheet:

## Understanding Monoclonal Antibody Folding and Aggregation

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### ***Understanding stability measurements***

*Q: In some of the measurements of DLS versus temperature, there seems to be a lot of change going on in  $R_h$  and  $k_D$  10 or 20 degrees below  $T_m$ , while in others these changes occur in the vicinity of  $T_m$ . How would you explain these differences in behavior?*

*A: There are at least two primary factors potentially leading to this behavior.*

First, some protein systems exhibit an onset temperature for unfolding by DSC that is well below the melting temperature  $T_m$ , which is actually the midpoint of the transition. Since the onset temperature by DSC/DSF indicates initial stages of protein unfolding, and this is often accompanied by exposure of hydrophobic patches, it makes sense that in the vicinity of the onset temperature for unfolding there will also be an onset of decrease of  $k_D$ . By the same token there may also be a distinct onset of increase in  $R_h$ . It would seem that the  $T_{\text{onsets}}$  for changes in  $k_D$  and  $R_h$  better correspond to  $T_{\text{onset}}$  for unfolding than to the midpoint temperature, and all of these are generally better correlated to stability against aggregation than  $T_m$ .

Second, kinetics may be a factor. The DLS temperature ramps ( $\sim 0.1$  C/sec) in the [DynaPro<sup>®</sup> Plate Reader](#) tend to be much slower than the DSC temperature ramps ( $\sim 1$  C/sec). If the unfolding transition is slow on the time scale of seconds,

a faster ramp will naturally lead to a broad transition in temperature by DSC and a late (i.e., apparently higher-temperature) occurrence of unfolding compared to DLS.

*Q: What, in your opinion, are the relative values, to the formulator, of  $T_m$  from DSC,  $T_m$  from DSF,  $T_{onset}$  from DLS,  $T_{agg}$  from SLS and the  $k_D$  temperature onset point?*

*A:* It is important to consider all of the above in combination, as well as  $k_D$  at storage temperature. Most importantly, one should consider them in the context of the critical degradation pathways of the particular product, i.e. the three C's: conformational, colloidal and chemical stability. The formulator must be aware of the critical pathway and optimize formulation conditions accordingly. For example, if  $T_m$  is relatively low then conformational instability may be the critical pathway and the formulation should first and foremost increase  $T_m$ ; while if  $T_m$  is relatively high but colloidal stability is poor then first improve  $k_D$  at storage or room temperature. Likewise, if deamidation is the primary degradation path, then optimize conditions to prevent this first and only then turn to secondary optimization of the next-most-prevalent instability.

With respect to the various temperatures, if a low-temperature onset is accompanied by a much higher midpoint temperature, the onset temperature will generally be of greater influence in ranking formulations than the midpoint. That is because it is more relevant to storage temperature than the melting temperature.

*Q: You typically measure  $k_D$  at concentrations in the range of 1-10 mg/mL. How well does this measurement predict aggregation behavior at higher concentrations? How would one measure protein-protein interactions at higher concentrations?*

*A:* Measurements of  $k_D$  at moderate concentrations correlate partially, but not perfectly, to behavior at high concentrations. Ideally, in order to understand colloidal interactions at high protein concentration you would make the measurements at the corresponding condition. However **DLS** is poorly understood at high concentrations. More suitable techniques are static light scattering (SLS, **CG-MALS**) and small angle neutron or x-ray scattering (SANS, SAXS), as well as the older method of membrane osmometry.

*Q: Regarding slide #15 in the presentation (variations in  $k_D$  due to pH, ionic strength and excipients): are these buffer effects universal or mAb specific?*

A: For proteins, and mAbs in particular, it would be an overreach to say that any behavior is 'universal', but there are certainly some very common effects. In general,  $k_D$  will be at or near its minimum (most attractive) value at  $pI$  and will increase as the  $pH$  is shifted away from  $pI$ .

When electrostatic interactions dominate protein-protein behavior, the addition of salts will screen the interactions; in some cases the primary electrostatic effect is repulsive (positive  $k_D$ , a result of high molecular net charge) and then increasing ionic strength leads to a less positive value of  $k_D$ ; while in other cases the primary electrostatic effect is attractive (negative  $k_D$ , a result of inhomogeneous charge distributions across the molecule) and then increasing ionic strength leads to less negative values of  $k_D$ .

If hydrophobic interactions dominate (attractive, negative  $k_D$ ) then increasing ionic strength leads to more negative  $k_D$  values. Hence the behavior of  $k_D$  with ionic strength indicates the primary source of protein-protein interaction, which of course depends on the specifics of the protein.

### **Data interpretation**

Q: *On slide 15, the diffusion coefficients converge to one value at zero concentrations. My experience is that when I measure diffusion coefficient as a function of protein concentration in different formulations I rarely get nice curves where all converge to the same diffusion coefficient. How do you interpret the data if you get different diffusion coefficients when extrapolating to  $c=0$ ?*

A: If significantly off, one should consider two possible sources.

First, check the goodness-of-fit  $R^2$ . If  $R^2$  does not decrease significantly when you force the fitted lines to have the same intercept then in all likelihood they do in fact converge.

Second, the presence of differing amounts of irreversible aggregates or foreign particles will naturally cause the zero-concentration intercepts to differ. Because DLS give the z-averaged diffusion coefficient (weighted by the square of the molecular mass), it is strongly affected by aggregates; check for the presence of large particulates and aggregates using DLS regularization analysis and for the presence of small aggregates with SEC or SEC-MALS. If either of the latter is the cause then you can improve your results by filtering the samples with Anotop 0.020  $\mu m$  filters.

Q: *Can DLS discriminate between reversible and irreversible interactions?*

A: Yes, by applying experimental probes that challenge the reversibility. Some examples of such probes are: dilution, pH change, ionic strength change, and temperature increase/decrease.

Q: *Can you obtain antibody folding/unfolding information with SEC-MALS, without DLS?*

A: In an ideal system, elution time is indicative of hydrodynamic size, so the combination of elution time and molecular mass by MALS should give you information about conformation and hence the degree of unfolding. However, elution time is also influenced by non-ideal interactions between the protein and the column, so this is not really a reliable means of obtaining such information.

### ***Experimental Details***

Q: *How much protein is needed for a  $k_D$  measurement, and does it require any special handling such as dialysis or filtration?*

A: Dialysis and filtration are, in most cases, needed to ensure that the measurements represent the conditions we think they do. Only if the protein stock comes at very high concentration, e.g. approx. 100 g/L, you may perform a dilution series into the relevant formulations without too large of an error. Filtration is very important to guarantee clean samples; one should use filters with small pore size of 0.02  $\mu\text{m}$  and minimal dead volume. We can perform a complete  $k_D$  measurement with less than 200  $\mu\text{g}$ .

Q: *How many different formulation conditions can you evaluate per week, day or hour using the DynaPro Plate Reader?*

A: Assuming an acquisition time of 3 seconds with 5 measurements per well and 3 replicates of each sample, one sample measurement takes about 1 minute so you can perform hundreds of samples per day, or dozens of formulations if  $k_D$  is measured for each one. In the end pipetting and data evaluation actually take longer than the measurement itself.

Considering a ramp rate of 0.1  $^{\circ}\text{C}/\text{min}$  from 25  $^{\circ}\text{C}$  to 85  $^{\circ}\text{C}$ , a temperature ramp series will take 10 hours and provide measurements of 60 samples every 1 $^{\circ}\text{C}$  or 30 samples every 0.5  $^{\circ}\text{C}$ .

Q: *Where is the information for  $T_{agg}$  coming from?*

A: Aggregation is indicated by static light scattering. Therefore,  $T_{agg}$  is obtained from SLS measurements in a temperature ramp. For the DynaPro Plate Reader III, SLS is provided directly as molar mass while for the DynaPro Plate Reader II the information is contained in the 'normalized count rate'.

Q: *How do you perform the temperature ramping studies in Wyatt's DynaPro Plate Reader DLS system? Since the whole plate is heated up, do you heat stepwise and measure all wells before the next temperature step? What temperature ramping speed do you recommend (in degrees per minute)? Do you normally see any significant impact of the ramp rate on the results?*

A: We generally ramp continuously at 0.1 °C/min as the instrument scans across the wells, rather than a stepwise temperature ramp. There will be small differences in the temperature at each well but the actual temperature is recorded with the data so we are still accurate in plotting diffusion coefficient and SLS vs. temperature. It is also possible to do stepwise ramps but these take more time and do not provide real additional benefit when you are looking for transition temperatures.

We have not studied different ramp rates, but in theory you should see an effect when the interaction is irreversible and/or rate-limited; if you go much faster than this you will also have an error in the temperature difference between the measured plate temperatures and the actual temperatures at each well.

We usually cover the wells with silicone oil to prevent evaporation. Transparent sealing tape can also be used.