Application Note

Wyatt Technology Corporation

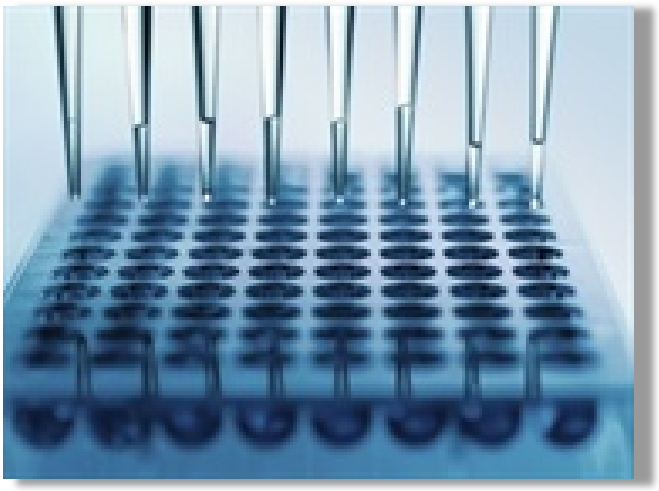
The Diffusion Interaction Parameter (k_D) as an Indicator of Colloidal *and* Thermal Stability

Sophia Kenrick, Ph.D.* and Daniel Some, Ph.D.†

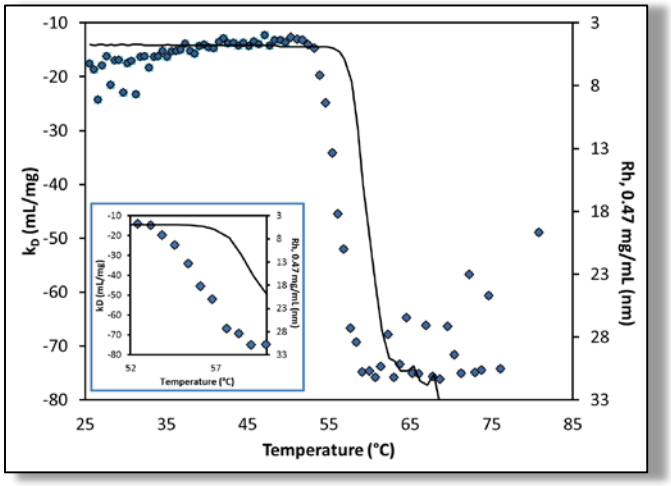
Summary

Stability is a key quality attribute in formulation studies of potential therapeutic biomolecules. In order to minimize time, effort and funds spent on stability studies, researchers rely on high-throughput screening methods that can reliably test hundreds of combinations of candidates, excipients and buffer conditions. Techniques utilized in these screens must determine a variety of stability-indicating parameters (SIPs). Some of the most useful SIPs to date are short term aggregation, thermal stability; and colloidal stability.

The ability to screen protein formulations at the early stages of development enables scientists to concentrate on the most suitable candidates, therefore saving substantial amounts of time, sample and testing equipment. This experiment demonstrates that thermal and colloidal stability of proteins, two indicators of propensity to aggregate, as well as actual aggregation states, are all determined simultaneously during the screening process with dynamic light scattering (DLS) tools in order to rank the effectiveness of candidates and formulation conditions. DLS can also indicate chemical stability and the average molar mass and specific volume of molecules in solution. For these reasons, HTS-DLS provides substantial quantities of information for the rapid screening of candidate molecules, buffer conditions and excipients, allowing the DynaPro® Plate Reader II to maximize productivity in formulation studies.



The DLS Plate Reader enables reliable testing of thousands of combinations of candidate biotherapeutics, excipients and buffer conditions in formulation studies.



The diffusion interaction parameter k_D, measured by dynamic light scattering, identifies the onset of protein unfolding and its impact on colloidal stability well before simplistic analyses of hydrodynamic radius.

*skenrick@wyatt.com †dsome@wyatt.com

I. Introduction

Stability is a key quality attribute of therapeutic biomolecules, critical for establishing drug-like properties and suitability for use in humans. However, establishing the stability of a candidate molecule or formulation can be a long and tedious process. In order to minimize time, effort and funds spent on long-term stability studies, developers of biologics look to high-throughput screening methods that can reliably test and rank hundreds of combinations of candidates, excipients and buffer conditions. Experimental techniques utilized in these screens must determine a variety of stability-indicating parameters (SIPs), since no one parameter has yet proven to be the silver bullet indicative of long-term shelf life or stability under a variety of environmental stresses such as freeze-thaw or elevated temperatures.

Some of the most useful SIPs to date are: short term aggregation (the formation of, usually, small aggregates); thermal stability (the tendency of a protein to unfold and/or aggregate with temperature, usually as a consequence of exposure of the hydrophobic core); and colloidal stability (the tendency of molecules to associate due to weak, attractive forces related to surface charges, hydrophobic surface residues and similar moieties). These SIPs are not entirely independent of each other. For example, even though colloidal stability generally pertains to reversible association, the enhanced proximity under self-attractive conditions can enhance irreversible aggregation rates. Conversely, increased surface charge may reduce colloidal attraction and improve colloidal stability, yet degrade thermal stability as the charges destabilize the protein's tertiary structure.

A variety of techniques such as differential scanning calorimetry, intrinsic and extrinsic fluorescence, circular dichroism, infrared or Raman spectroscopy and static light scattering have been applied to assess SIPs. One technique in particular stands out for its great versatility: dynamic light scattering (DLS). DLS provides quantitative insight into a broad range of phenomena related to stability, as it can *simultaneously* quantify aggregation and distribution of aggregate sizes; thermal stability, discriminating between pure unfolding and aggregation through a temperature transition; and colloidal stability, via the concentration dependence of diffusion. The same data may be analysed to determine changes in average molar mass and specific volume.

The stability of a biomolecule is not a wholly intrinsic property, but depends on buffer composition and the concentration at which the protein is formulated. Protein stability must be quantified as a function of pH, ionic strength, specific ion type and excipient profile for an optimal and successful formulation. Fortunately, DLS is amenable to high-throughput, low-volume screening of hundreds of conditions per hour by means of a plate reader utilizing industry-standard microwell plates. High-throughput screening by dynamic light scattering (HTS-DLS) is accomplished by means of the DynaPro Plate Reader II (Wyatt Technology, Santa Barbara, CA) which accommodates 96, 384 or 1536-well plates, performing temperature scans of all samples *in parallel* from 4°C - 85°C. The multiplexed approach provided by HTS-DLS can be extended to a variety of other formulation conditions for rapid characterization of protein behaviour.

The *simultaneous* measurement of thermal and colloidal stability offers qualitatively novel information: the direct interaction between thermal and colloidal stability mechanisms, reflected in the temperature dependence of the colloidal interaction parameter in the vicinity of a thermal transition. This article demonstrates HTS-DLS measurements revealing the impact of thermally-induced protein unfolding on colloidal interactions, yet another quantitative metric for rapid ranking of protein formulations.

The Interaction Parameter

DLS directly measures fluctuations in scattering intensity due to Brownian motion, which are analysed to determine the translational diffusion coefficient D_t and hence an effective measure of molecular size, the hydrodynamic radius R_h. DLS can also provide a rough measure of size distributions in order to assess populations of monomers and aggregates[1]. Though not as rigorous as a separation technique such as size exclusion chromatography coupled to light scattering detectors (SEC-MALS), this is often sufficient for screening purposes and will even indicate the presence of size populations that differ by 3-5x in radius.

As a consequence of non-specific protein-protein interactions arising primarily from charged and hydrophobic residues, D_t is a function of concentration, c. Analysis of D_t vs. c leads to the first-order diffusion interaction parameter k_D (not to be confused with the equilibrium dissociation constant k_D), per equation 1:

$$D=D_0\ (1+\ k_Dc+\ ...) \qquad \text{Eq. 1}$$

Positive values for k_D are indicative of repulsive intermolecular interactions while negative values indicate attraction. The diffusion interaction parameter is directly related to the second virial coefficient A_2, a commonly-accepted thermodynamic measure of colloidal stability and propensity for aggregation[2,3]. A_2 is generally more difficult to measure with low volume and high throughput, so k_D serves as a convenient proxy. As a result, k_D can be utilized to rapidly compare different protein formulations and guide the selection or engineering of more stable biomolecules[4,5,6].

II. Materials and Methods

A DynaPro Plate Reader II ran HTS-DLS measurements for simultaneous thermal, colloidal and mixed stability analyses, also assessing the degree and size distribution of aggregation. A significant benefit of this instrument is measuring the sample entirely *in situ* in the well, eliminating concerns of carry-over common to microfluidic platforms while boosting throughput.

A monoclonal antibody (mAb1) was dissolved in 50 mM bis-tris-propane buffer (BTP) at pH values of 6.5, 7.5, 8.5 and 9.5 to a final concentration of 15 mg/mL. This stock solution was filtered to 0.1 µm and then diluted and arrayed in a 384-well microtiter plate (Aurora) at five replicates of six different protein concentrations between 0.47 mg/mL and 15 mg/mL, for each of the four pH values, loading 20 µL of solution into each well. The plate was centrifuged at 400*g* for 1 minute and each well was then capped with 1-2 drops of paraffin oil to prevent evaporation. Prior to measurement, the plate was centrifuged again at 400*g* for 1 minute.

Initial measurements at 25°C included control samples of bovine serum albumin and lysozyme in the same plate, not discussed here (though the measurements appear in the some figures). An extended series of measurements was then conducted as a function of temperature, ramping from 25°C to 85°C at a rate of 0.1°C/min. During the ramp the mAb1 solutions were measured sequentially, completing five 2-second acquisitions for three replicate wells at each concentration and pH, every 0.5°C.

Instrument control, data acquisition and analysis were carried out via the DYNAMICS software (Wyatt) and Microsoft Excel. D_t and R_h were determined from autocorrelation analysis and k_D was calculated from the linear regression of D_t vs. c. Aggregation onset temperature T_{onset} was determined per concentration and pH by fitting the plot of R_h vs. temperature to an appropriate model.

III. Results and Discussion

Interaction parameter as a function of pH

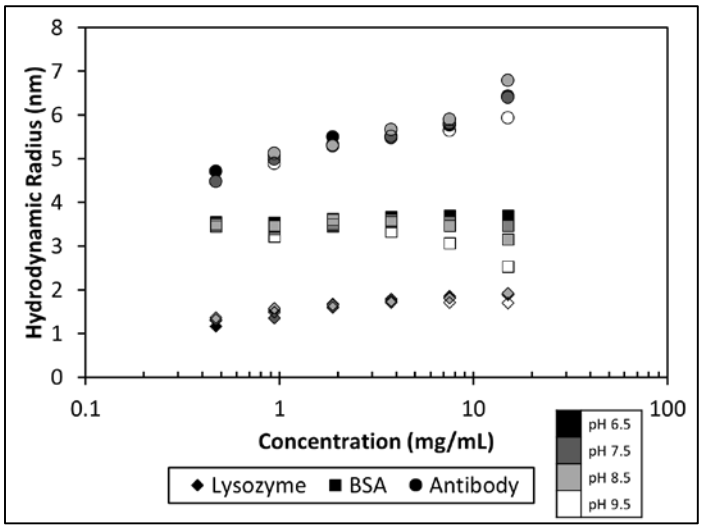


Figure 1: Measured hydrodynamic radius as a function of concentration and pH for three proteins.

MAb1 exhibited increasing R_h (decreasing D_t) as a function of concentration (Figure 1), corresponding to a negative k_D and hence protein-protein attraction. This antibody exhibits $k_D < 0$ for all pH values tested (Figure 2), indicating a predisposition to assemble into oligomeric species.

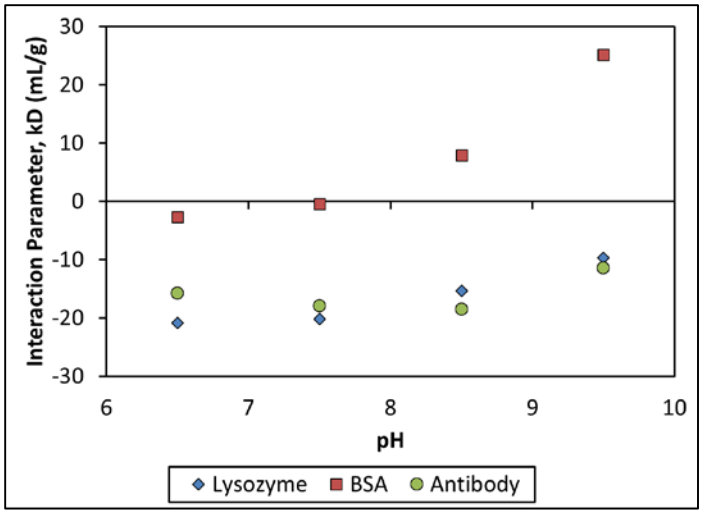


Figure 2: Interaction parameter, k_D, as a function of pH for three proteins.

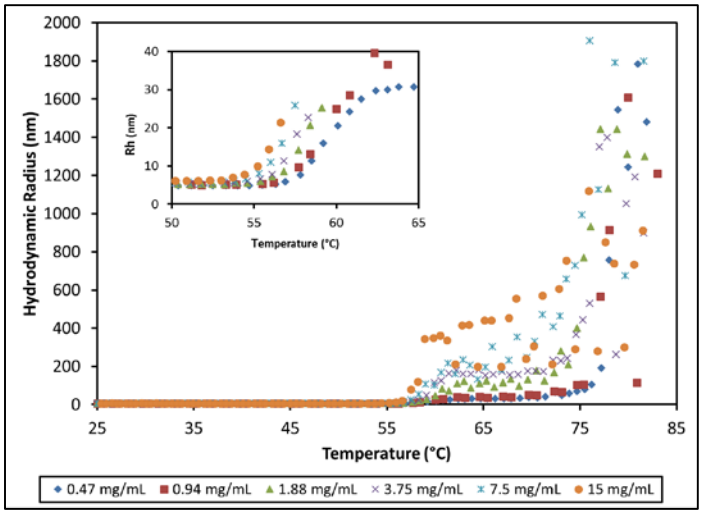


Figure 3: Hydrodynamic radius as a function of temperature and concentration for an antibody formulation at pH 8.5. High order aggregate formation is evident for temperatures >56 °C.

Thermally-induced aggregation

At pH 8.5 mAb1 rapidly aggregates into large complexes beyond the thermal transition around 55°C, with R_h at 75°C ranging from 80 nm to 800 nm (Figure 3). T_{onset} decreases with concentration, ranging from 55.0 to 56.8°C (Figure 3, inset). The final aggregation state is highly dependent on the concentration, varying by over two orders of magnitude in average particle size between the lowest and highest concentrations. A second transition occurs at 70-75°C, possibly related to the unfolding of another IgG domain, that results in large-scale aggregation into particles >1 µm. The high degree of aggregation suggests that the observed concentration dependence past the thermal transition is primarily a consequence of higher molecular collision rates. This can be confirmed by varying the temperature ramp rate.

In contrast, at pH 9.5, mAb1 exhibits a shift from R_h = ~4.8 nm, typical of IgG, to a stable value between 15 and 22 nm, depending on concentration, for temperatures above 62°C (Figure 4). The small size and stability of the aggregates at this pH suggest reversible oligomerization. This can be confirmed by reversing the temperature ramp and/or varying the ramp rate. Around 75°C, the antibody appears to enter a second unfolding transition, similar to pH 8.5 though with much smaller magnitude of aggregation and little effect at the lower concentrations.

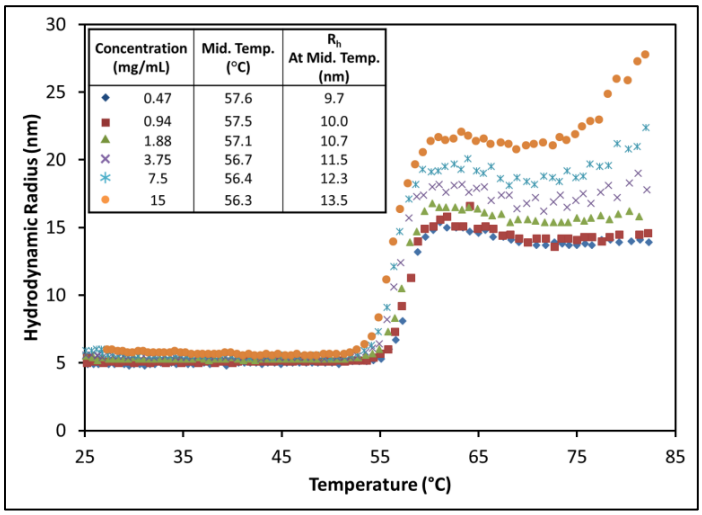


Figure 4: The hydrodynamic radius exhibits a sigmoid relationship as a function of temperature for all antibody concentrations at pH 9.5, showing little change in the midpoint with concentration.

The qualitative difference in aggregation processes the two conditions are further elucidated in the size distributions obtained with DLS regularization analysis. At 80°C, the pH 8.5 sample with 1.88 mg/mL concentration exhibits a bimodal distribution with populations of 30-100 nm and 300-3000 nm, while the pH 9.5 sample at the same concentration presents a single distribution at 80-300 nm, as shown in Figure 5.

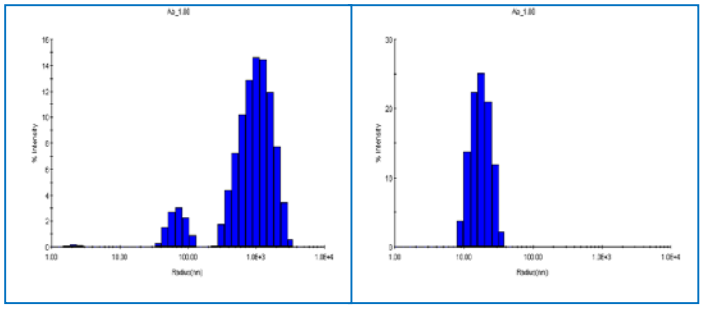


Figure 1. Size distributions obtained at 80°C via regularization. Left: pH 8.5; right: pH 9.5.

Interaction parameter through the thermal transition

Below the thermal transition, k_D is negative and approximately constant with temperature increase for both pH values. The magnitude of k_D at pH 8.5 is about twice that of pH 9.5, indicating stronger intermolecular attraction, which correlates to the vastly different aggregation behavior. In the vicinity of the folding-unfolding transition and onset of aggregation, k_D exhibits distinct transition behavior versus pH.

At pH 8.5, k_D undergoes a dramatic step-change from between 53°C and 59°C (Figure 6 and inset). Strikingly, the shift begins several degrees before any appreciable aggregation appears and is suggestive of increased protein-protein attraction due to pure unfolding. Beyond 59°C, k_D is constant (though noisy due to the numerical difficulty of ascribing a single average radius when the population is bimodal). As seen in Figure 3, the degree of aggregation depends on concentration, but this would appear to be due primarily to higher collision rates and therefore the measured value of k_D beyond ~56°C probably is not indicative of a true thermodynamic interaction, but rather of the history and kinetics of the aggregation process.

A similar change in k_D signals the unfolding transition at pH 9.5: once again k_D becomes more negative (more attractive) several degrees prior to aggregation. Instead of a step-change, however, we now observe a local *minimum* occurring just as the measured hydrodynamic radius begins to indicate aggregation (Figure 7). Upon aggregation, the magnitude of k_D decreases to become less negative. This trend in k_D indicates once more that attractive interactions increased during the first unfolding transition as the hydrophobic core is exposed. However in this condition, once a stable structure has been achieved, these interactions are partially mitigated as the exposed regions are now hidden from the solution. A secondary unfolding transition around 75°C is clearly reflected in k_D.

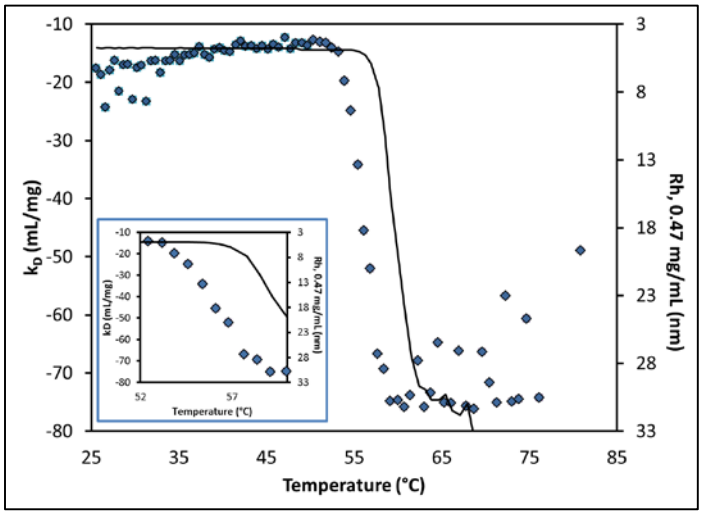


Figure 2: Diffusion interaction parameter (symbols, left axis) and radius (solid line, right inverted axis) at lowest concentration as a function of temperature at pH 8.5. Inset: same, highlighting the transition region.

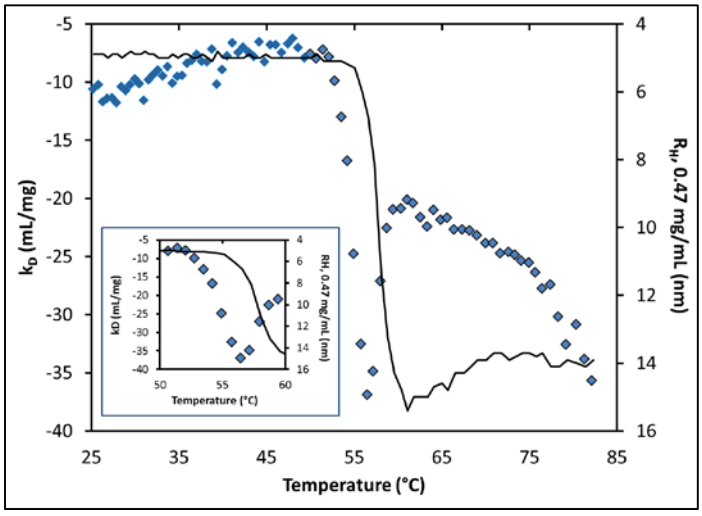


Figure 3: Diffusion interaction parameter (symbols, left axis) and radius (solid line, right inverted axis) at lowest concentration as a function of temperature at pH 9.5. Inset: same, highlighting the transition region.

IV. Conclusions

The ability to screen protein formulations at the early stages of development enables scientists to concentrate on the most suitable candidates and so save substantial amounts of time, sample and testing equipment. This experiment demonstrates that thermal and colloidal stability of proteins, two indicators of propensity to aggregate, as well as actual aggregation states, are all determined simultaneously during the screening process with the DynaPro Plate Reader II in order to rank the effectiveness of candidates and formulation conditions.

Thermal stability is quantified as T_{onset} and colloidal stability as k_D. The temperature dependence of k_D provides unique insight into the effect of unfolding on colloidal interactions, as the unfolding process reveals moieties previously 'hidden' from buffer and other proteins. Not discussed here, DLS can also indicate chemical stability and the average molar mass and specific volume of molecules in solution as a function of temperature, as well as solution viscosity[7] which is another important factor in formulating high-concentration biotherapeutics. Therefore, HTS-DLS provides substantial quantities of information for the rapid screening of candidate molecules, buffer conditions and excipients in order to drive higher productivity.

V. References

1. Jocks, T., & Roessner, D. Performing Automated Dynamic Light Scattering Using Plate Reader Technology. *IPI* **2**(1), 22-25 (2009).

2. Some, D., Hitchner, E. & Ferullo, J., Characterizing Protein-Protein Interactions via Static Light Scattering: Nonspecific Interactions. *American Biotechnology Laboratory* **27** (2), 16-20 (2009).

3. Kuehner, D. E. et al., Interactions of lysozyme in concentrated electrolyte solutions from dynamic light-scattering measurements. *Biophysical Journal* **73**, 3211-3224 (1997).

4. Lehermayr C., Mahler H.-C., Mader K., Fischer S. Assessment of Net Charge and Protein-Protein Interactions of Different Monoclonal Antibodies. *J. Pharm. Sci.* **100**(7), 2551-2562 (2011).

5. He F., Woods C. E., Becker G. W., Narhi L. O., Razinkov V. I. High-Throughput Assessment of Thermal and Colloidal Stability Parameters for Monoclonal Antibody Formulations. *J. Pharm. Sci.* **100**(12), 5126-5141 (2011).

6. Saito S., Hasegawa J., Kobayashi N., Tomitsuka T., Uchiyama S., Fukui K. Effects of Ionic Strength and Sugars on the Aggregation Propensity of Monoclonal Antibodies: Influence of Colloidal and Conformational Stabilities. *Pharm. Res.* **30**(5), 1263-1280 (2013).

7. He F., Becker G. W., Litowski J. R., Narhi L. O., Brems D. N., Razinkov V. I. High-throughput dynamic light scattering method for measuring viscosity of concentrated protein solutions. *Anal. Biochem.* **399**(1), 141-143 (2010).

The DynaPro Plate Reader II enables high-throughput DLS studies of aggregation and stability-indicating parameters, increasing productivity in formulation studies.