WYATT TECHNOLOGY

Whitepaper

Screening Developability and Pre-Formulation of Biotherapeutics with High-Throughput Dynamic Light Scattering (HT–DLS)

Daniel Some, Ph.D., Principal Scientist, Wyatt Technology Corp. †

Introduction

Assessing a drug candidate's suitability early in the discovery and development stages is essential for minimizing the risk of costly downstream failure. Multiple aspects of a biotherapeutic molecule's behavior and suitability as a drug candidate may be quantified via biophysical analysis techniques:

- **functionality** (binding to targets/antigens and effectors)
- **homogeneity/purity** (the presence of aggregates or fragments)
- **stability** (the tendency to degrade via changes in higher-order structure as well as chemical modifications).

Viscosity of a concentrated protein solution is another important performance parameter amenable to biophysical analysis; formulations with high viscosity are not suitable for manufacturing or intravenous delivery.

One of the primary technologies for assessing aggregation, stability and viscosity of biotherapeutics, from proteins and peptides to attenuated viruses, is dynamic light scattering (DLS)[1]. DLS measures particle *size and size distributions*, from less then a nanometer and up to several microns, without perturbing the sample. The measurement takes place on time scales of seconds to minutes, and the sample is fully recoverable. This makes DLS a particularly effective technique for assessing aggregation and stability in early as well as late stages. In addition, viscosity can be measured via DLS using probe particles such as common polystyrene latex beads.

HT-DLS Maximizes Productivity

Traditional DLS measurements take place manually, one at a time, in cuvettes, resulting in low throughput and low productivity. Cuvette-based analysis limits the number of candidates and formulations that can be analyzed, restricting the implementation of highly efficient development strategies such as quality by design (QbD) and Design of Experiments (DoE).

Automated, high-throughput dynamic light scattering (HT–DLS) utilizing Wyatt Technology's DynaPro® Plate Reader II (PRII) helps streamline the candidate selection and pre-formulation processes. HT-DLS provides more data, on more samples and more formulations—typically *10x – 30x more measurements* than would be collected using cuvette-based DLS, but with far less effort. The DynaPro PRII can be used as a standalone instrument or as part of a larger automated formulation workflow[2]. Using just a few microliters of solution per sample in standard microwell plates, unattended HT–DLS measures multiple biotherapeutic candidates in a variety of formulation and environmental conditions.

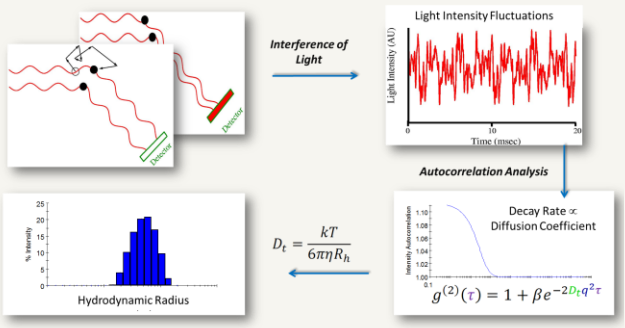
HT-DLS provides rapid, comprehensive studies, resulting in greater confidence in the final selection of candidate or formulation. With the advent of 21CFR11-compliant software for the DynaPro PRII, DYNAMICS® SP, this instrument is suitable for all GLP and GMP labs that need to determine the size, stability and viscosity of biotherapeutics.

This white paper introduces the technology that powers automated HT–DLS and explores its practical applications in candidate and formulation selection.

†dsome@wyatt.com

Dynamic light scattering instrumentation

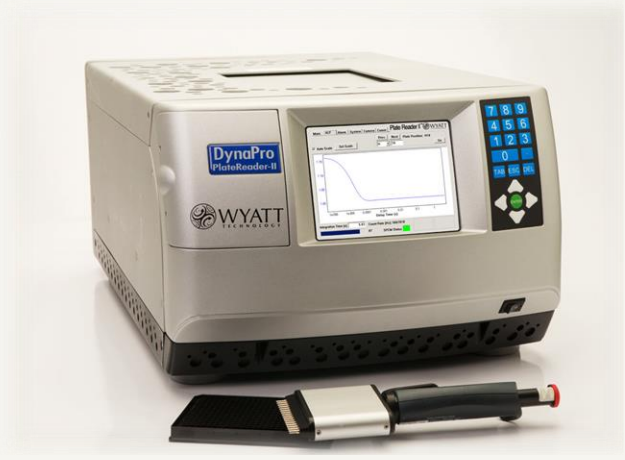
Dynamic light scattering (DLS) uses a sensitive detector to monitor the intensity fluctuation of scattered light that arises from the Brownian motion of particles in solution. DYNAMICS software then analyzes the autocorrelation data to determine the rate of diffusion, which is converted to hydrodynamic radius via the Stokes-Einstein equation, $R_h = k_B T / 6\pi\eta D_t$.



The Stokes-Einstein equation relates light scattering intensity and Brownian motion to hydrodynamic radius.

The DynaPro Plate Reader II carries out high-throughput DLS measurements of the size and interactions of proteins, nanoparticles, and other macromolecules. The instrument automatically self-adjusts its sensitivity by a factor of up to one million in order to accommodate a wide range of particles sizes and concentrations.

Users can program customized temperature ramps in order to assess thermal behavior, and test protein properties such as the onset temperature and the rate of unfolding or aggregation. Since the DLS measurements are completed *in situ* in industry-standard microwell plates, liquid handling is minimized. This capability reduces measurement time and eliminates sample carryover, resulting in a typical scan time of less than one hour for a 96 well plate.



HT-DLS for Developability and Pre-formulation Studies

DLS is a non-invasive, non-perturbative method of measuring protein and particle size distribution in terms of hydrodynamic radius (R_h) across a wide range, covering 0.2 – 2500 nm in cuvette-based instruments and 0.5 – 1000 nm in HT-DLS. The fundamental strength of DLS is its ability to determine changes in protein size, conformation and aggregation/oligomerization state across a range of conditions, allowing users to rigorously investigate a candidate's behavioral profile.

The measurement of DLS does not depend on ambiguous reporter signals like intrinsic or extrinsic fluorescence, or exothermic or endothermic processes, all of which may fail to indicate those conformational changes of actual significance to stability. Rather, DLS directly indicates a true physical change in conformation as well as aggregation state.

DLS delivers essential insight into biopharmaceutical candidates and formulations, including

- Aggregation of nanometer to submicron particulates
  - ✓ before and after applied stress
  - ✓ during accelerated thermal stress
  - ✓ under pH stress
- Stability and aggregation propensity studies
  - ✓ colloidal stability/self-association, via concentration dependence
  - ✓ thermal conformational stability, via temperature ramps
  - ✓ chemical denaturation conformational stability, via denaturant gradients
- Viscosity of high-concentration protein formulations.

Traditional manual, cuvette-based DLS is suitable for testing a handful of samples, but not hundreds. Automation helps to resolve the challenge of analyzing hundreds of samples and conditions; HT-DLS experiments improve statistics, allow for multiple replicates, and are completed in a fraction of the time of manual analyses. With data generated as rapidly as 10 seconds per well, an experiment covering 384 sample wells consisting of a variety of different candidates, pH values, ionic strengths and replicates can be completed in just 90 minutes.

The DynaPro PRII employs industry-standard well plates compatible with other plate-based screening techniques. In practice, this means DLS samples are easily transferable to other instruments for multivariate sample analysis. The ability to rapidly scope a drug product's performance under hundreds of different conditions allows scientists to more readily implement DoE and QbD methods, in order to meet regulatory and corporate productivity expectations.

The following examples illustrate the effective application of automated HT–DLS for a number of essential aggregation and stability investigations.

Measuring gross aggregation

Protein aggregation is a major concern during biopharmaceutical development. Unchecked aggregation reduces the efficacy of a biotherapeutic, affects its manufacturability, and may present a serious immunogenic risk to a patient.

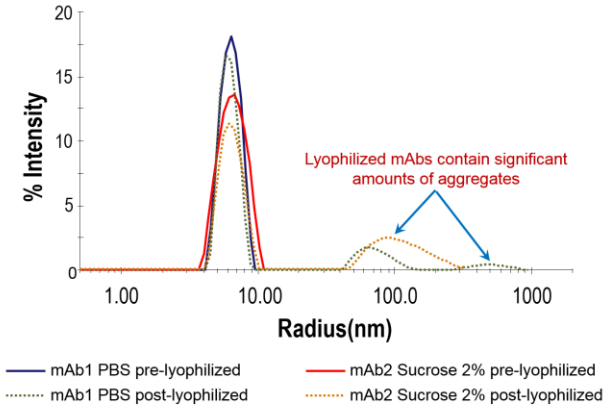


Figure 1: HT–DLS quantifies gross aggregation of a protein sample under a range of conditions. Following lyophilization and reconstitution, a monoclonal antibody (mAb) solution containing PBS is shown to aggregate in the submicron size region.

By monitoring changes in particle size distribution with DLS, formulators are able to map out protein aggregation as a function of pH or excipient concentration. Alternatively, aggregation may be tracked through a dynamic temperature range, or followed during key waypoints in the formulation process. For example, Figure 1 shows the particle size distribution of two monoclonal antibody (mAb) formulations with and without sucrose, before and after lyophilization, measured using the DynaPro PRII. Lyophilized samples were reconstituted to the same concentration as pre-lyophilized solutions.

Before lyophilization, the size distributions of both samples were monomodal. The pre-lyophilized solution containing sucrose (Figure 1, solid red line) has a marginally wider distribution peak, which suggests that the small sucrose molecules impart some polydispersity. It is clear that the lyophilized mAbs contain large aggregates, with the PBS sample in particular forming extremely large species post-lyophilization (Figure 1, green dotted line).

Despite the intensity of the post-lyophilized aggregate peaks between 50-200 nm, the number of these aggregates is actually very small. Converting these data to percent mass via DYNAMICS software reveals that although the lyophilized antibody's primary peak between 4-10 nm has an integrated intensity of only 80%, its total percentage mass was 99.6%. Aggregates in the 50-200 nm range, therefore, account for only 0.4% of the overall protein population. Whether this percentage falls within the limits of suitability depends on the candidate; the role of HT–DLS is to quickly provide the information required for formulators to make this decision.

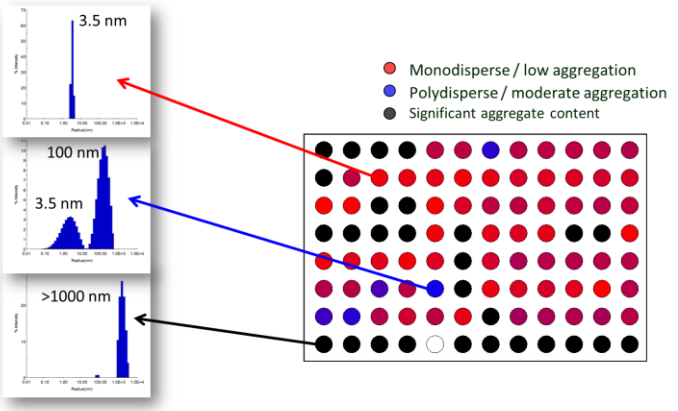


Figure 2: The heat map display in DYNAMICS may be programmed to differentiate size distributions and visualize at a glance different degrees of aggregation. Data courtesy Sabin Vaccine Institute.

Rapid visualization of the aggregation behavior of dozens or hundreds of samples in a plate is accomplished via heat maps such as the one shown in Figure 2. Color coding was applied to differentiate between unaggregated, lightly aggregated and heavily aggregated samples (red, blue and black, respectively). This visualization approach allows users to quickly obtain an overview of the results and pick out regions of the plate corresponding to specific excipients, pH conditions or candidate molecules that appear promising for further study.

Imaging contaminants and precipitates

The DynaPro PRII is equipped with a camera that can be programmed to acquire images of each well after DLS measurement. One of the benefits of the camera images is the ability to discern precipitation and crystallization as particulates sink to the bottom of the well, shown in Figure 3.

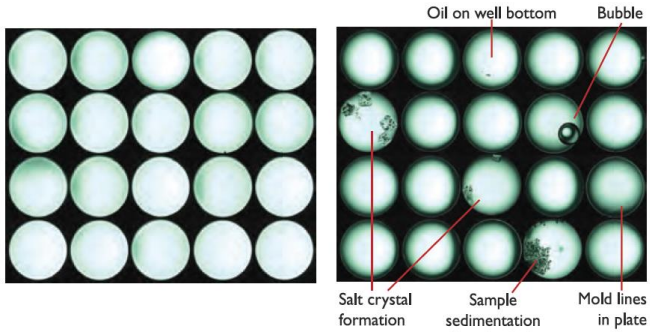


Figure 3: Tiled images from the DynaPro PRII's onboard camera. Left: wells containing no precipitates or other contaminants. Right: wells containing a variety of contaminants.

Measuring thermal conformational stability

DLS allows formulators to probe other aspects of protein behavior, such as conformational changes across a dynamic temperature range. Figure 4 shows a temperature scan of lysozyme in an acetate buffer of pH 4. Lysozyme proteins fold and unfold readily without aggregation. In this case, the molecular size of the sample began increasing around 65°C before reaching a plateau around 90°C. T_m indicates the midpoint for the transition, and when the transition does not allow for setting a midpoint, T_{onset} is used to indicate the onset temperature.

When lysozyme was held at 90°C for multiple readings, the size remained constant, indicating unfolding but not aggregation. This behavior is typical of single-domain proteins but not of multi-domain proteins.

Moreover, DLS systems inherently incorporate relative static light scattering (SLS) measurements in the form of the DLS count rate, enabling users to differentiate between folding and aggregation behavior. SLS is proportional to molar mass, a property that increases in value during aggregation but remains unchanged during purely conformational changes. In the case of lysozyme, the change in size is not accompanied by a change in SLS (not shown), supporting the assumption that the protein is unfolding rather than aggregating.

Instruments that measure intrinsic fluorescence and SLS simultaneously utilize UV illumination to assess aggregation. However, UV is known to actually induce protein aggregation[3], making this approach to aggregation analysis highly suspect. The wavelength of the DynaPro, 830 nm, is not capable of inducing aggregation, guaranteeing a reliable measurement.

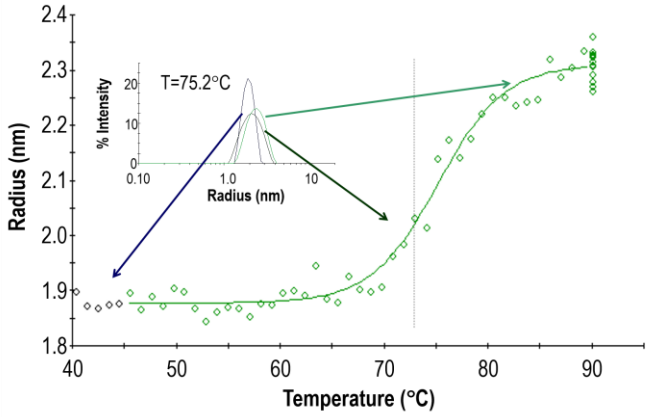


Figure 4: Dynamic light scattering (DLS) is used to measure conformational changes in structure, such as the unfolding of lysozyme across a dynamic temperature range.

A multi-domain protein, IgG, exhibits both unfolding and aggregation as shown in Figure 5. While the large changes in both size (DLS) and count rate (SLS) above 60°C are clearly associated with aggregation, a closer look in the vicinity of the transition temperature clarifies the different onset temperatures for unfolding (58°C) and aggregation (59°C). The temperature ramp rate was 0.1°C/min, so the temperature lag is probably not a kinetic effect associated with the time lag.

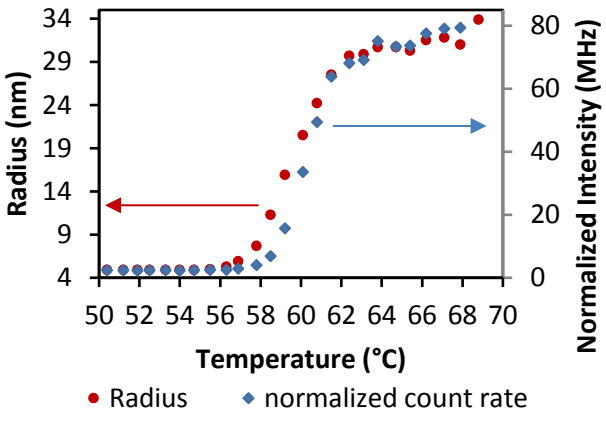


Figure 5: Unfolding vs. aggregation in 0.47 mg/mL monoclonal IgG, identified by DLS and SLS (i.e., the DLS count rate). Unfolding is seen to precede aggregation by about 1°C, exhibited in the delayed onset of SLS intensity increase relative to the increase in radius.

Holding a series of formulations at a constant temperature and measuring the rate of aggregation as expressed in the changes in particle size (radius) and molecular weight (via count rate) is another effective way to assess stability and rank-order formulations. This type of stability assay should generally be performed at a temperature well below the temperature of unfolding. HT-DLS affords parallel aggregation-rate measurements of large numbers of candidates and/or formulation conditions.

Utilizing both temperature ramps and accelerated aggregation at a fixed temperature, the DynaPro PRII provides invaluable insight into the nature of protein interactions in solution and the transition temperature at which conformational changes give way to aggregation.

Measuring chemical conformational stability

An additional means for quantifying a molecule's stability against conformational changes is chemical denaturation gradients. A denaturant such as urea or guanidine HCl is titrated into the protein solution in order to determine the denaturant concentration at which the molecule is present in equal amounts as folded and unfolded species, and hence the Gibbs free energy of unfolding.

The common method for assessing unfolding in a chemical denaturation measurement is intrinsic fluorescence. However, DLS is a superior quantifier of unfolding since it does not rely on the presence of fluorophores, does not lead to photon-induced damage, and is a positive, reliable indicator of unfolding via changes in actual size. Chemical denaturation screening of a multi-domain protein has been demonstrated in the DynaPro PRII by Yu *et al.*[4]

HT-DLS offers key advantages over other techniques used to study thermal and chemical conformational stability, such as intrinsic fluorescence or differential calorimetry:

- By directly reporting changes in size, there is no guesswork involved in determining whether the signal does in fact relate to a conformational change or is a spurious result of changes in the chemical environment.
- Unfolding without aggregation is directly discriminated from aggregation.
- The nature and size distribution of aggregates that may form, whether reversible or irreversible, is determined.
- Intrinsic fluorescence is excited by short-wavelength UV which is known to induce protein aggregation even without thermally-induced denaturation. This phenomenon is particularly pronounced in antibody-drug conjugates[3].

Measuring aggregation propensity

HT–DLS also measures aggregation propensity (colloidal stability), enabling users to better predict stability at early phases of analysis or to secure more in-depth information of protein behavior around unfolding and aggregation transition points.

Colloidal stability analysis with DLS allows formulators to determine the magnitude of attraction between molecules in solution and is indicative of native-state protein-

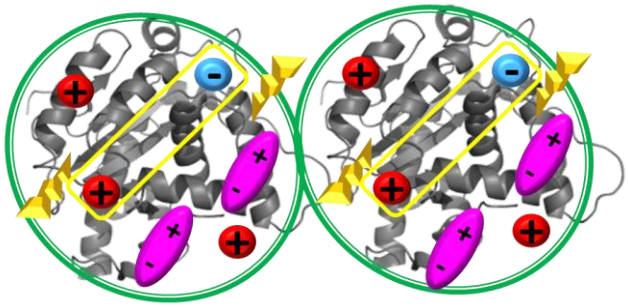


Figure 6: Colloidal instability is a result of the combined effects of surface charge distributions, local dipoles, hydrophobic patches, and other physical contributions to protein-protein interactions.

protein interactions such as those shown as a cartoon in Figure 6. This is accomplished by measuring the change in a protein's diffusion coefficient as a function of concentration. If the diffusion coefficient decreases with a decrease in concentration, then larger particulates are forming and self-association occurs. If the coefficient increases with concentration, then molecules are likely repelling each other and are stable in solution.

These measurements are described by the diffusion interaction parameter k_D, the slope of the diffusion coefficient vs. concentration. The magnitude of k_D directly relates to aggregation propensity. When k_D is greater than 0, repulsive forces dominate and the solution is more likely to be stable. When k_D is less than 0, attractive forces dominate and the sample is likely to aggregate. The diffusion interaction parameter k_D has been shown to be an important stability-indicating parameter, perhaps even more useful to formulators than T_m[5].

Figure 7 shows the measurements of R_h as a function of concentration for three proteins in buffers with the indicated pH. The change in diffusion coefficient as a function

of concentration, which is used to determine k_D, can also be viewed as a change in apparent hydrodynamic radius A series of concentrations are prepared for each sample and each buffer condition, and these are loaded into microwell plates for DLS analysis. The set of 24 conditions per protein sample, each with 5 replicates, takes about 20-30 minutes to complete in the DynaPro PRII. Figure 8 plots the k_D values vs. pH for each protein. Lysozyme and antibody are self-associative at all pH values, whereas BSA is stable above pH 7.

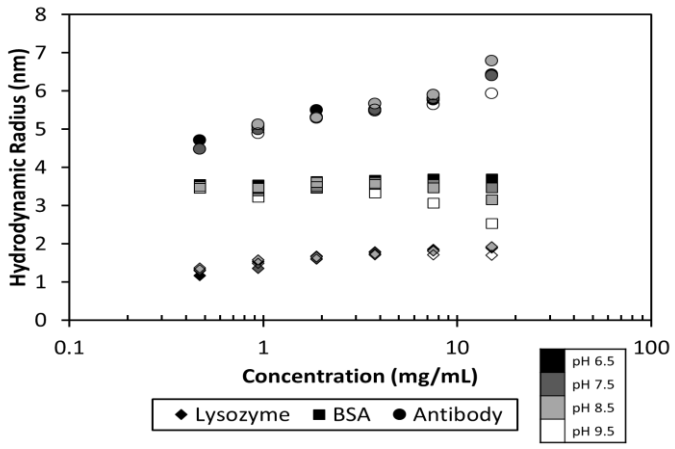


Figure 7: Measured hydrodynamic radius as a function of concentration and pH for three proteins, from which k_D may be determined.

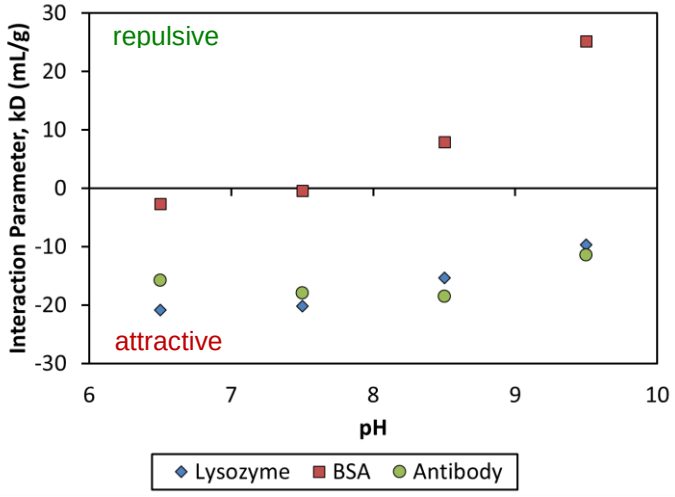


Figure 8: Interaction parameter k_D as a function of pH for three proteins.

As an indicator of protein-protein interactions, it can be especially informative to utilize k_D to understand the phenomena occurring upon unfolding. Figure 9 shows one example of how k_D changes with temperature around the unfolding transition of a mAb sample. At lower temperatures, k_D and R_h are fairly constant, approximately -10 mL/g and 5 nm, respectively. When the temperature exceeds 55°C, however, R_h increases significantly as aggregation begins. Several degrees before the point of aggregation, the value of R_h decreases sharply. This observation can be rationalized by considering the unfolding behavior of the protein around this point. As proteins unfold, their hydrophobic cores expose and the molecules become self-attractive. The magnitude of k_D increases and the value becomes more negative at the onset of aggregation, indicative of the increase in attractive interactions. However, as aggregation progresses, the hydrophobic cores become hidden from the other molecules in solution and k_D again becomes less negative.

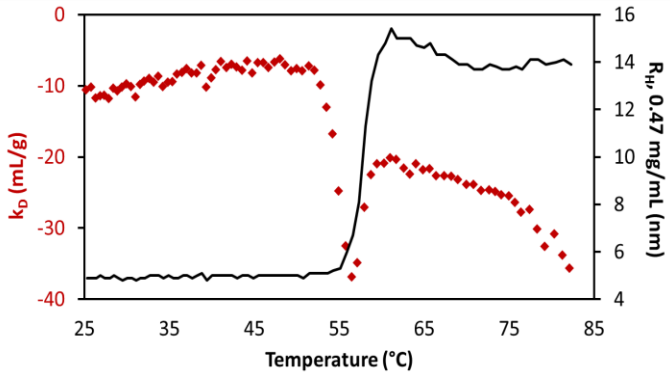


Figure 9: The diffusion interaction parameter, k_D, decreases prior to the point of aggregation as protein molecules begin to unfold.

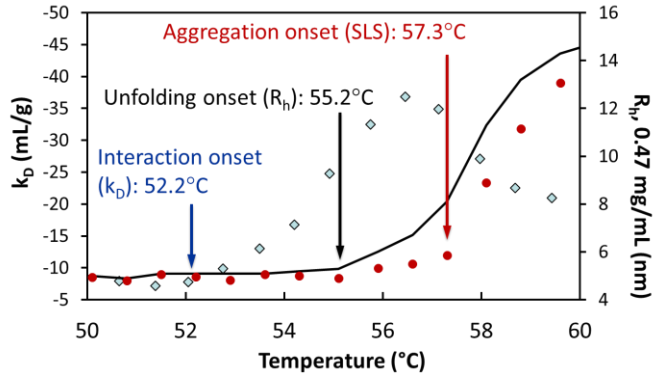


Figure 10: The data of Figure 9, blown up in the vicinity of the thermal transition, with the addition of SLS data. Blue diamonds: k_D values; black line: R_h values; red circles: SLS values. Three distinct transition temperatures are evident. The k_D scale has been inverted for clarity.

A closer look at the vicinity of the transition region with the addition of SLS data (Figure 10) brings out three distinct transition temperatures: the onset of protein-protein interactions, at 52.2°C; the onset of unfolding, at 55.2°C; and the onset of aggregation at 57.3°C. The onset of interactions is most likely the result of a minor conformational change that creates intermolecular attraction but is insufficient to cause aggregation.

One of the key advantages of DLS over other stability-indicating techniques is the ability to interrogate the types of aggregates that are formed, in addition to the degree of aggregation. Figure 11 presents the quite different aggregates created at high temperature under two different pH conditions.

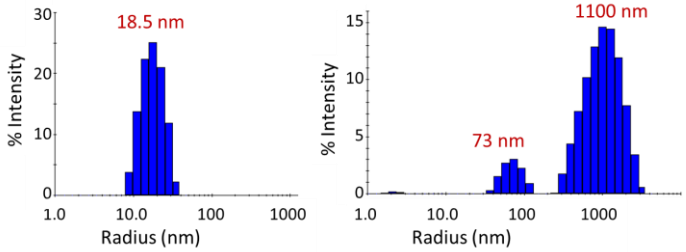


Figure 11: Size distributions obtained at 80°C via regularization. Left: pH 9.5, indicating aggregate species of ~ 18 nm; right: pH 8.5, indicating species with sizes of ~70 nm and ~ 1000 nm.

Using HT–DLS to determine viscosity

Finally, DLS can be used to assess formulation viscosity to help determine the long-term stability and deliverability of a biotherapeutic. DLS measures viscosity by spiking sample wells with polystyrene beads of a known diameter and radius, as shown in Figure 12. The Stokes-Einstein equation is manipulated to determine viscosity. The results from DLS have been shown to correspond well to traditional rheological techniques, such as ball-and-cone

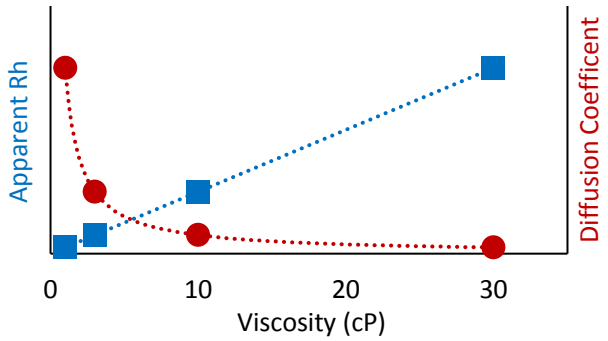
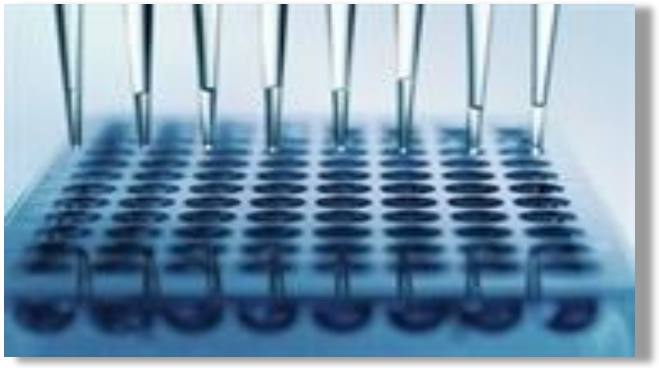


Figure 12: In DLS, increasing solution viscosity associated with higher protein concentration causes decreased diffusion and an apparent increase of radius of a probe particle of known size. The diffusion coefficient is analyzed to yield the solution viscosity.

viscometry. The advantage of employing automated HT–DLS for viscosity measurements is that low-volume samples are assessed rapidly, in the same plates and at the same time as a variety of other features, adding a further layer of detail to scientists' understanding of their formulations.

Identifying the best candidates as early as possible

As the pharmaceutical industry targets increasingly complex biologic candidates, automated formulation and analytical workflows play an important role in streamlining the development process. Capable of performing 96 runs in under 45 minutes and 384 wells in 1.5 – 2 hours, with completely unattended operation, high performance HT–DLS accelerates analysis time, reduces the need for expert operator supervision, and improves confidence in data quality. This level of automation provides developers with the depth of data and resources necessary to implement highly efficient development strategies for better performing, safer biotherapeutics.



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

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