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Live Webinar Q&A Sheet:

High-throughput Analytics for Formulation and Process Development of Bio- and Nano-therapeutics

The recorded webinar may be viewed from the Biotherapeutics 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 with any additional questions.

DLS General

Q: *Can I obtain biophysical data concerning intrinsic stability and developability indications with <20ug material?*

A: With the DynaPro Plate Reader II, only 4 µL per sample are required when using 1536 well plates. At a typical concentration of 1 mg/mL, you would have the option of testing aggregation and melting temperature (T_m) for five buffer pH values or ionic strengths.

A more suitable instrument for those with very limited sample quantities would be the DynaPro NanoStar which can use as little as 1.25 µL in the quartz cuvette. Then you could measure T_m at 5 pH values *each* at 3 ionic strengths, or you could measure colloidal stability (k_D) over an abbreviated concentration series comprising 1, 3, and 10 mg/mL as well as Tm for each of those concentrations.

Q: *Can HT-DLS provide consistent low level aggregate detection/characterization, and what is the limit of detection?*

A: The sensitivity of DLS to aggregates is proportional to their molar mass. HT-DLS is not very sensitive to low levels of small oligomers such as dimers, but it is quite sensitive to low levels of large aggregates, e.g. those comprised of hundreds or thousands of monomers. The limit of detection for monomeric IgG is 12.5 µg/mL; the LoD for a thousand-mer (approximately 60-100 nm radius aggregate) would be 12.5 ng/mL.

Q: *Can DLS characterize nanoparticles in a complex matrix, including adsorption of matrix components to the nanoparticle?*

A: Yes, DLS can provide size distributions that differentiate between the nanoparticles and smaller matrix components; DLS is also sensitive enough to resolve changes in particle size of a few percent. This application was recently demonstrated in studying the adsorption of human serum albumin to Au nanoparticles, with and without a PEG coating – see Bhirde et al., *Nanoscale* 2017, **9**, 2291-2300 doi:10.1039/c6nr08579b.

Q: *Can this instrument detect IgG dimer vs monomer (300k Da vs 150k Da)? Would it be possible to establish, in a solution, if there is aggregate and trimer? And can we evaluate concentration of aggregates or just aggregations presence and/or aggregation augmentation?*

A: The hydrodynamic radius of a dimer is ~26% larger than that of the monomer whereas the precision of *average* radii measured by DLS is 1-2%. If you had two solutions, one composed of monomers and one composed of dimers, the DynaPro DLS Plate Reader would definitely distinguish them. However, if you have a mixture of monomers and dimers, the instrument would not resolve them as separate species: it would see a single mode (a single peak in the distribution). If there is an appreciable dimer concentration then the polydispersity, or width of the peak, would be larger than that of a pure monomer, and the average value somewhat increased. However, it would be impossible to say definitively what the dimer concentration is, and if the change in polydispersity is due to dimers, trimers or some mixture of the two.

Similar is true for trimer. Larger aggregates would be resolved as distinct modes if their hydrodynamic radius is at least 3-5x larger than the average radius of the monomer/dimer/trimer mode.

DLS provides an estimate of aggregate concentration as a % of the total mass. However, this result is not highly rigorous and depends on certain assumptions, such as the aggregate having the same density as the monomer or dimer. For an accurate aggregate characterization including rigorous concentrations that do not depend on such assumptions, SEC-MALS (the combination of size exclusion chromatography with multi-angle light scattering) is optimal.

Stability

Q: *What are the best options to measure aggregation propensity of a protein when aggregation propensity is not linked with folding stability?*

A: Generally if aggregation propensity is not linked to folding stability it may very well be closely linked to colloidal stability. Colloidal stability of a protein is quantified by the second virial coefficient (A_2 or B_{22}) using static light scattering, or by the diffusion interaction parameter (k_D or D_1) using dynamic light scattering. Both are measured by means of a concentration series. While A_2 measurements tends to require a fair amount

of protein and is a relatively slow measurement, k_D is amenable to high-throughput screening with much smaller sample quantities in the DynaPro Plate Reader II.

Q: *What is the difference between melting temperatures measured by differential scanning calorimetry (DSC) or fluorimetry (DSF) and those measured by DLS?*

A: Usually these values are very close to each other, but because they rely on different physical phenomena this is not always true. DSC and DSF may be measuring conformational changes that are not associated with any appreciable change in molecular size or aggregation. On the other hand, DLS will not respond to such minor changes in conformation and is only sensitive to changes in size, intermolecular attraction or repulsion, and actual aggregation caused by a thermal transition. DLS also distinguishes between pure unfolding and unfolding associated with aggregation.

Q: *Can HT-DLS replace zeta potential measurements for colloidal stability of particles?*

A: Colloidal stability is impacted by the balance of attractive and repulsive forces between particles. Zeta potential represents one component of those forces, the repulsion due to the particle's net charge. In that sense, zeta potential is not a complete assessment of colloidal stability; concentration-gradient DLS (CG-DLS) carried out in microwell plates provides a more comprehensive measure of colloidal stability since it incorporates all contributions to colloidal stability.

CG-DLS is particularly appropriate to complex particles such as proteins or inhomogeneous particles where hydrophobic interactions and inhomogeneous charge distributions lead to significant short-range attractive forces that are not quantified by zeta potential. On the other hand, zeta potential is more appropriate for assessing the colloidal stability of large, homogeneous particles, in part because of concerns of multiple scattering phenomena which can bias CG-DLS.

Q: *What is the best way to measure self-association - concentration studies by DLS or CG-MALS?*

A: DLS is best for screening multiple conditions in order to identify the presence of self-association and obtain a rough idea of its magnitude, but DLS cannot definitively quantify self-association (affinity and oligomers formed) across a wide range of concentrations. CG-MALS is a slower technique that requires more sample, but provides absolute characterization including the type or types of oligomers formed and the binding affinity of each. CG-MALS is roughly 100x more sensitive to measure high affinity (low K_d) samples and, thanks to established light scattering theory for non-ideal solutions, can extend to roughly 100x the concentration to quantify self-association of low-affinity samples.

HT-DLS Operation

Q: *How can I perform data mining on HT-DLS data?*

A: All data generated by DYNAMICS (the software that runs the DynaPro HT-DLS instrument) may be exported to .csv files for import into MS Access, MS Excel or other database software. The exports may be defined to include all relevant sample information which has been imported into DYNAMICS such as sample names, buffer conditions, etc. for comprehensive data mining.

Q: *Can you tell me what kind of robotics (liquid handler) system this DLS is working with. How do you automate measurements with the DLS System?*

Can it be integrated with Hamilton, or Tecan or Freeslate liquid handler?

A: The primary automation means entails the measurement of DLS in plates, where the optical read head moves from well to well to measure the contents of each, automatically. The results are stored and analyzed at once (the data are also available for manual analysis).

The DynaPro uses industry-standard well plates that can be loaded and transferred using standard liquid handlers for plates, and standard plate handling systems, as a higher-order means of automation. Wyatt offers an application programming interface (API) that allows external control of the instrument, software and results for more extensive control.

While Wyatt provides the software interface to integrate the DynaPro DLS Plate Reader with those liquid handlers, we do not supply end-user integration. Freeslate has integrated Wyatt's HT-DLS instrument into their Biologics Formulation System and provides a complete solution. For other liquid handling systems, the user must take care of integration.

Sample Issues

Q: *Can you comment on the effect of high and low concentration of a virus-like particle (VLP) sample on the DLS readout?*

A: VLPs tend to have molar masses in the millions of Da, so DLS will generally be quite sensitive to them, accurately quantifying sizes at concentrations of a few µg/mL and below. The primary concern at low concentrations is the sample vanishing due to, e.g., sticking to the container walls, but no direct issue with DLS readout.

At very high concentration, e.g. 100 mg/mL, there may be some concerns of multiple scattering which does impact DLS accuracy. The degree of multiple scattering is a function of particle size; monomeric IgG does not produce multiple scattering even at

hundreds of mg/mL, but particles in the size range of 1 micron can do so at a few % v/v. VLPs are intermediate between these cases.

Q: *Would it be workable for optimizing crystallization?*

A: HT-DLS is in fact used in various labs to optimize protein crystallization conditions. The primary approach is to screen for conditions that result in the lowest polydispersity, since aggregation is detrimental to formation of well-ordered crystals. HT-DLS can also be used with concentration series, in a secondary step, to identify buffers that provide just the right degree of moderate self-attractive conditions that promote orderly packing.

Q: *How do you separate protein aggregates vs impurities from the sucrose and other sugar impurities from the manufacturing process?*

A: If the sucrose impurities are similar in size to protein aggregates, there is no good way of distinguishing them via DLS. If there is a concern over sugar impurities it is best to carefully characterize the sugar itself prior to use in the manufacturing process (by DLS!) and throughout manufacturing, to verify that the source material is of high quality and that manufacturing steps do not cause particle formation.

Q: *How to make sure excipients do not disturb the DLS measurements?*

A: This depends on the excipient. Very small excipients such as salts generally do not disturb the DLS measurements as long as they are well-dissolved. Sucrose (r_h ~ 0.2 nm) and other sugars might, if the concentration is high and the sample itself is small such as lysozyme (r_h = 1.9 nm), but larger samples such as IgG (r_h > 5 nm) are generally not impacted much. Sucrose impurities, e.g. cross-linked nanocrystals, are larger and it is very important for analytical purposes to ensure that these are not present in the sample, by filtering the sucrose prior to use or by purchasing high-quality sucrose which has been verified to not contain such impurities.

Perhaps the most difficult common excipients from the point of view of DLS are micelle-forming surfactants. The micelles can be comparable in size to the biotherapeutic of interest or even larger, and there is no good way of eliminating the signal from micelles in the DLS data.