



## Live Webinar Q&A Sheet:

### Characterization of Nanotherapeutics by Dynamic Light Scattering

The recorded webinar may be viewed from the [DLS](#) webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, CG-MALS, 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 [Solutions](#) page of our web site.

Please contact [info@wyatt.com](mailto:info@wyatt.com) with any additional questions.

#### Application-specific

*Q: Can micro-RNA be detected?*

A: As long as the sample falls into the size-range specified above, it can be measured. Typically, microRNA consists of at least 21 nucleotides, i.e. approximately 7 kDa. This can be measured quite readily for both size and molar mass.

*Q: Is DLS an approved method by regulatory agencies for detecting aggregation of virus drugs (i.e. AAV)?*

A: In general, DLS is in use by certain producers of drugs based on bionanoparticles such as VLPs for quality control, and has been used in successful regulatory filings, and therefore has met regulatory approval. As always, it is incumbent on the pharmaceutical manufacturer to demonstrate to the regulatory agency that the measurement is fit for purpose and well validated.

*Q: Prof. Guebitz: Were you able to detect a change in the size of the nanocapsules as a function of pH, corresponding to the release of drug?*

A: A clear pH dependent release of model drugs was seen, most likely due to increased porosity of the nanocapsules.

*Q: Prof. Guebitz: Why does the silk fibroin stabilize the DLS measurement?*

A: We speculate that silk fibroin at higher concentrations has a positive effect on beta-sheet formation and enhances crosslinking. This allows better separation.



*Q: Prof. Guebitz: Can DLS be used to characterize coupling of monoclonal antibodies onto the HSA nanocapsules?*

*A:* We have successfully shown that DLS can be used to characterise nanocapsules based on HSA and other proteins. It will especially be interesting whether coupling of antibodies onto nanocapsules will have an impact on size.

*Q: Dr. Krivitzky: What was your target for the size and zeta potential of the delivery particle, and why those values? Did you consider the final results to be within the target range?*

*A:* The preferred range of diameters for our nanoparticles is between 10 to 150 nm. We want the nanoparticles to be large enough to be retained in the blood stream by a low rate of renal elimination, and at the same time small enough to permeate through the nanometric pores of the impaired blood vessels at the tumor site. We consider a nanoparticle size of 100 nm to be ideal to exploit these properties.

The requirements for the surface charge of our nanoparticles are more complex, and sometimes even contradictory. On the one hand, dense positive charge is crucial to the colloidal stability of our solutions and enhances cellular internalization via interactions with negatively charge membrane proteins. On the other hand, excess charge may cause aggregation with blood components and activate the immune system. Fine tuning is required between the ability to electrostatically bind the RNAi, colloidal stability and cellular internalization versus toxicity and immunogenicity. Each of our formulations is evaluated in vivo for its maximum tolerated dose prior to use to validate that we do not reach toxic concentration.

*Q: Dr. Krivitzky: the electron micrograph in the Summary slide shows dimers and trimers of the polyplex particle. Do those also appear when the particles are in solution, or is it an artifact of cryo-EM? Does their presence carry any adverse effects?*

*A:* The dimers and trimers shown in the SEM image in the summary slide are artifacts of the method and did not appear by DLS measurements. We prepare the sample for SEM imaging using a dried droplet on the surface, so crystallization effects occurred, and the nanoparticles appeared to be much more concentrated than they would be in the solution state. In this respect, the DLS measurement is more reliable and loyal to the solution that is actually used.

*Q: Dr. Krivitzky: How much sample was needed for the DLS and PALS measurements?*

*A:* The DLS and PALS measurements were performed simultaneously using the Mobius dip cell, so the overall amount needed was quite low – about 50  $\mu$ L per measurement.



- Q: Dr. Krivitzky: Was there any characterization performed to verify the amount of free drug versus unencapsulated drug?*
- A: We used a qualitative electrophoretic mobility shift assay (EMSA) to validate that there is no free siRNA in the formulation. The EMSA is presented in Slide No.10 entitled “steps of formulation development”, and demonstrates that both N/P ratios of 1.2 and 3 used in this presentation were able to retard the migration of siRNA.
- Q: Dr. Krivitzky: What are the sizes of APA and RNAi separately?*
- A: The size of APA was characterized by MALS to yield about 28,000 g/mol. The RNAi is commercially available at the molecular weight of 14,883 g/mol. We did not perform DLS analysis of their sizes.
- Q: Dr. Krivitzky: Which conditions were used to measure zeta potential? Is it possible to measure zeta potential in phosphate buffer saline?*
- A: The same conditions were used to measure both the DLS and the Zeta potential. Hence, in each step of the development, we measured both. Initially, we prepared the complexes in DDW, and then in PBS or glucose solution. The PALS enables measurement of zeta potential in PBS. DLS general
- Q: What is the principle of the DynaPro NanoStar?*
- A: The NanoStar measures the static light scattering and dynamic light scattering signal simultaneously, using the same laser beam to probe the sample. The total light scattering intensity measured in static light scattering allows the determination of the molecular weight up to 1 MDa. The intensity fluctuations caused by Brownian motion are measured with a single-photon-counting module (SPCM) and enable the determination of the translational diffusion coefficient. Using the Stokes-Einstein equation, the hydrodynamic radius is calculated based on the diffusion coefficient taking into consideration the temperature and solvent viscosity.
- Q: What is the difference of radii that can be separated/resolution?*
- A: Two species need to have a difference in size of the factor of 3-5 in order to be resolved in regularization (NNLS) analysis.
- Q: What is the scattering angle? Is there any angle dependence for sizing results?*
- A: The scattering angle is 90° for the DynaPro NanoStar and 169° for the DynaPro Plate Reader III. Both instrument terms take the angle into consideration and hence yield the same result for monomodal samples. For mixtures containing different particle sizes, the larger scattering angle is beneficial in detecting small particles in the presence of large particles while the smaller scattering angle is beneficial in highlighting a small amount of large particles in the presence of a large concentration of small particles.



*Q: What's the purity requirement to obtain a meaningful measurement using DLS?*

A: If the impurities are comparable in size to the particle of interest, for example dimers and trimers relative to the monomer, the measurements will be meaningful regardless of these impurities (an average size will be measured). If the impurities are much smaller than the analyte, even a purity level of 20% could yield good results. Conversely, if the impurities are much larger than the particle of interest, even a very small amount, 1% and less, can skew the results. Hence it is important to filter the sample in order to remove large impurities such as dust particles.

*Q: Are there any limitations on the material composition of the nanoparticles that are analyzed?*

A: There are, in principle, no limitations concerning the material – it could for example be proteins, liposomes or inorganic particles. The only prerequisite is that they actually scatter light, which means that the refractive index differs from the solvent and they do not absorb heavily at the laser wavelength. In case the samples fluoresce upon excitation at the laser wavelength, a fluorescence-blocking filter may be installed (Mobius) or an instrument operating at a different laser wavelength can be used.

*Q: Is concentration important to consider when determining the size distribution? Is the sensitivity of the DynaPro NanoStar concentration-dependent, e.g. do samples require dilution?*

A: Concentration is important in DLS for several reasons:

1) **Lower limit** - for proteins and similar small particles, the lower limit is size-dependent and is captured in the sensitivity specification of 0.1 mg/mL of 14.4 kDa lysozyme for the NanoStar and Mobius, 0.125 mg/mL of lysozyme for the Plate Reader. For other molecules, the limit of sensitivity is inversely proportional to molar mass, e.g. for 144 kDa IgG, the lower limit of the NanoStar is 0.01 mg/mL. When the particles are much larger, with radii on the order of 100 nm and more, the lower limit is typically set by number fluctuations, which are systematic errors that arise when a small number of particles are present in the measurement volume.

2) **Upper limit** - the signal may saturate or multiple scattering affects the results. For proteins that do not aggregate, typically neither saturation nor multiple scattering will occur, but the result is affected by intermolecular interactions (see #3). For larger particles, typically in the range of tens of nanometers and above, either saturation or multiple scattering may occur, depending on the optical properties and the specific instrument. Multiple scattering effects, which are easily diagnosed by visual inspection for a hazy or cloudy sample, often occur at lower concentrations than are needed for detector saturation. If either of these effects occur, the sample can be diluted for more accurate measurements.

3) **Concentration dependence** - for proteins and other macromolecules, aggregates may occur in a concentration-dependent manner, leading to actual physical changes in the sample and the corresponding size distribution. Even if aggregation does not occur, macromolecules such as



proteins exhibit intermolecular interactions when the concentration exceeds a few mg/mL (less if the ionic strength is very low), which will impact the measured size distribution.

Please contact Wyatt support to discuss the specifics of your analysis with an application expert.

*Q: What types of information are reported about the size distributions?*

A: The software reports %Intensity, %Mass (similar to %Volume) and %Number and all can be displayed depending on the application. %Intensity is always the closest representation to the original data and requires the least assumptions.

D10/50/90 measurements can also be reported. These are the radii for which 10, 50 and 90% of the sample is equal to or smaller than that radius. For example, if D50 = 65 nm, then 50% of the sample has a hydrodynamic radius smaller or equal to 65 nm.

By changing the display on the regularization graph to %Intensity, %Mass or %Number, the D10/50/90 calculations also change accordingly to represent 10%/50%/90% of scattered intensity, mass or number of particles.

*Q: Which weighting did you use for distributions: intensity, number, or volume weight? What will be the basis of this selection?*

A: %Intensity is closest to the original data and usually inspected first. Especially when the relative amount of individual species is to be visualized, the %Mass representation is helpful (this is the same as volume-weighted distribution).

*Q: DLS in batch has certain limitations, in particular for broadly distributed samples or samples with aggregates. Please comment on the use of the data filter in the DYNAMICS software for processing DLS time-, pH, or else-dependent measurement series: is it feasible to eliminate bias or "artificial beautification" of DLS data series?*

A: It is tempting to "cherry pick" DLS data so as to eliminate outliers and "beautify" the results. While it is certainly valid to eliminate outliers, which most typically are the results of dust and other foreign particulates, it is important that this is done in a manner that is objective and unbiased in order to avoid "artificial beautification". DYNAMICS software offers multiple data filters, which can – with care – be set to produce unbiased reduction of outliers. For example, if your sample is a protein with  $R_h$  of 5 nm which generally exhibits little to no aggregation, but a few samples also present particles with size of 500 nm, it is reasonable to exclude them from the data set.

In DYNAMICS data filtration can be set up, prior to actually taking any data, according to specific objective criteria such as minimum or maximum ACF amplitude, baseline quality, noise, etc. Then, when acquiring a large data set such as a time or temperature series, application of the data



filters is fully automated with no subjective “cherry picking”. This results in elimination of outliers which is not only unbiased, but also requires no additional effort on the part of the researcher to review individual acquisitions. Technical note *TN2009 - Utilizing Data Filters in DYNAMICS*, which explains the use of data filters and recommends default settings, available for Wyatt customers on the Wyatt Support Center at [www.wyatt.com/Support](http://www.wyatt.com/Support).

*Q: Should I be performing DLS in a DynaPro Plate Reader with the plate lid on or off?*

A: The lid needs to be removed. If you need to seal the plate to prevent evaporation and are using the Plate Reader III, the plate can be sealed with a specific transparent sealing tape.

*Q: You mentioned that asphericity changes the friction coefficient and the corresponding diffusivity. If my particles have an aspect ratio of 3:1 and the software reports an  $R_h$  of 50 nm, what can I infer about the actual dimensions?*

A: The volume of an ellipsoid is approximately the volume of a sphere with the measured hydrodynamic radius, though higher asphericity results in larger deviations from that approximation. When the axial ratio is 3:1, the volume of the ellipsoid is about 10% smaller than the measured hydrodynamic volume.

### Comparison of instruments

*Q: Given the choice, which would you recommend for particle concentration (particles per mL), the DynaPro Plate Reader or cuvette-based DynaPro NanoStar? High throughput not needed, just the most reliable data.*

A: Both instruments have been tested extensively and are equally well suited. The maximum radius for particle concentration is slightly higher for the NanoStar (175 nm) than the DynaPro Plate Reader (160 nm). The NanoStar offers the flexibility of quartz cuvettes for a larger range of solvents, while the Plate Reader offers the convenience and lower cost of consumables when multiple measurements are needed.

*Q: How do the results from the cuvette (NanoStar) and plate-based (DynaPro Plate Reader) systems correlate to each other?*

A: The results correlate very well. The biggest difference is the maximum sample hydrodynamic radius, which is 1  $\mu\text{m}$  for the Plate Reader and 2.5  $\mu\text{m}$  for the NanoStar.

*Q: What is smallest/largest hydrodynamic radius that DLS can measure?*

A: The Plate Reader allows measurements of  $R_h$  from 0.5 nm to 1000 nm, the NanoStar allows measurements from 0.2 up to 2500 nm.