Live Webinar Q&A Sheet:

Experimental Methodology in a Core Facility: Light Scattering as Preliminary Experiments and Final Answers

The recorded webinar may be viewed from the Protein webinar page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, CG-MALS, and FFF, may be found in the Wyatt website Resources section under Webinars, Application Notes, and Bibliography, as well as on the corresponding Product page and Solutions page of our website.

Please contact info@wyatt.com with any additional questions.

Dynamic light scattering (DLS)

Q: *Can DLS measurements be automated?*

A: Absolutely! Integrating our DynaPro® Plate Reader with liquid handling robotics allows for automated plate loading and data collection. Industry-standard 96, 384, or 1536 well plates are compatible with the Plate Reader, allowing for analysis of the same sample using other plate-based techniques

Q: *Can DLS distinguish proteins that have different 3D conformations?*

A: A given protein can take on different conformations depending on temperature, solution conditions, photo-excitation, degradation, etc. These changes in conformation may lead to different hydrodynamic size, though this is not always the case. If changes in the 3D conformation of a protein lead to average hydrodynamic radii of the ensemble that differ by at least a few percent, DLS can monitor the change.

It is certainly well within the capabilities of DLS to monitor unfolding due to, e.g., chemical or thermal denaturation. Subtle transitions such as cis-trans might not be detectable via the change in hydrodynamic size, though in some cases, the effect of such a transition on protein-protein interactions is detectable by DLS via measurement of k_D, the diffusion interaction parameter.

Q: *When can I use the Cumulants fit?*

A: The cumulants algorithm fits monomodal monodisperse or polydisperse (PD) samples. However, when the percent polydispersity (%PD) is greater than 57 %, DYNAMICS® DLS software identifies it as a multimodal sample, and the Cumulants fit becomes invalid. DYNAMICS will let you know if the sample is monomodal or multimodal. In such instances, we recommend you use the Regularization analysis, which provides a more detailed size distribution than is possible with the method of cumulants.

Q. *Why would DLS measurements vary between different instrument vendors?*

Various software-selectable parameters may influence the size measurements, especially if the sample is multimodal, including the autocorrelation range selected for fitting, the specific fitting algorithm, and the resolution parameter chosen for the regularization analysis. The default values generally differ between vendors, so if you are comparing results, be sure to set the same parameters and use the same algorithms. Additionally, if the distribution is multi-modal, hardware configuration such as scattering angle may influence the results.

Multi-angle light scattering (MALS)

Q: *Are there any restrictions on the types of columns and mobile phases that can be used?*

A: Wyatt's MALS detectors such as DAWN®, miniDAWN®, or microDAWN® have been used with a wide variety of size exclusion (SEC) and gel permeation (GPC) columns. You can choose the column material, pore size, and dimensions best suited for your application. For aqueous protein applications, we recommend our Wyatt SEC columns for the best resolution and optimal light scattering performance. Our detectors are compatible with a wide range of aqueous and organic solvents, but if you expect to use a particularly aggressive mobile phase, please check the specification on our website to view a list of wetted materials or contact support@wyatt.com.

Q: *How can we use MALS to characterize samples with protein and its degraded forms?*

A: MALS is commonly used with a size-based separation technique like size-exclusion chromatography (SEC) of field-flow fractionation (FFF) to analyze protein samples containing degradants and impurities. The various species must have sufficiently different hydrodynamic radii in order to be separated by the column, and if they meet this requirement, MALS can determine their molar masses. MALS can also determine their size if the diameter is above ~ 25 nm, while DLS can be integrated with the MALS detector to measure smaller sizes down to 1 nm.

More subtle degradants like deamidized proteins require gradient chromatographic techniques such as reverse phase or ion-exchange chromatography (RPC or IEX) for separation. The peaks are collected for identification by mass spectroscopy. While MALS cannot generally provide sufficient resolution to identify such chemical degradants or charge variants, it can still be used with IEX or, in certain conditions, with RPC in order to confirm that a peak corresponds to a monomer rather than to a fragment or aggregate, saving unnecessary mass spec measurements. We encourage you to check our white paper WP1615: SEC-MALS for absolute biophysical characterization to learn more.

Q: *What is the range of radii that FFF-MALS can effectively separate and characterize in contrast to SEC-MALS?*

A: Wyatt's FFF-MALS technology combines an Eclipse™ FFF instrument with a DAWN MALS instrument for effectively separating and characterizing molecules with radii ranging from 1 nm to 1 µm. A single channel and instrument can cover the entire range, though for optimal performance in a specific range, a membrane of suitable pore size can be selected.

Separation in the case of SEC-MALS depends on the specific SEC column's separation range. The maximum range for SEC columns is about 50 nm in radius while the minimum could be less than 1 nm, and columns with different pore sizes can be combined to cover a larger range than any individual column can provide. Additionally, it may be possible to elute larger particles such as viruses from ion-exchange or mixed-mode columns, which may also be coupled to a MALS instrument for characterization.

Q: *How does the resolution of FFF compare to SEC?*

A: SEC tends to have higher theoretical plates and is a more efficient separation technique, particularly with UHPLC columns. FFF shines with its flexibility and higher selectivity, especially for samples larger than 30 - 50 nm. Although FFF may give rise to broader peaks, with MALS, these peaks can be characterized regardless of peak width. In general, due to the nature of the separation mechanisms and the inverted elution orders, SEC provides better resolution for smaller molecules or particles, while FFF provides better resolution for larger molecules or particles. Hence these techniques can be considered complementary.

Q: *In an FFF experiment, what type of method development must be carried out?*

A: The method development for a typical FFF separation experiment consists of channel height selection and designing and optimizing a series of steps to inject, focus, and separate a sample. These steps are always the same; only the duration and flow rates must be modified for separate samples.

FFF method development by the standard 'trial-and-error' technique can be time-consuming and tedious. VISION™ DESIGN software performs sophisticated simulations of FFF separations according to user-defined parameters like channel flow, crossflow, and gradient parameters to calculate elution behavior and optimize separations without actually running the instruments. This software is only available with Eclipse FFF systems.

Q: *What is the main limitation of FFF?*

A: One of the fundamental limitations is the lower resolution compared to separation techniques with higher theoretical plates like SEC, which results in broader peaks. However, with MALS, you can characterize these peaks regardless of peak width. To improve peak efficiency, you can

steeply drop the cross flow to promote elution once the sample is separated. The more time sample spends in the system, the broader the peaks become, but the advantage of FFF is how tunable the system is.

Another limitation is separation of strong cations, which tend to stick to the membrane. This can be ameliorated somewhat by judicious choice of membrane material and carrier solution, but not always fully overcome.

Multi-detector

Q: *How do Wyatt's DLS instruments compare to other nephelometer systems for investigating the solubility of small molecules or small molecule/protein aggregation?*

A: Commonly used nephelometer systems have drawbacks, including a lack of temperature control and the requirement for relatively high sample volumes. Wyatt's DynaPro NanoStar® provides dual 90° angle SLS and DLS measurements. This is the requisite angular configuration for making nephelometric turbidity measurements. The NanoStar facilitates a wide range of temperature-controlled measurements and requires only a few µL sample volume. A comprehensive study comparing different nephelometric techniques can be found in the publication Opalescence Measurements: Improvements in Fundamental Knowledge, Identifying Sources of Analytical Biases, and Advanced Applications for the Development of Therapeutic Proteins.

Q: *How can Wyatt's light scattering instruments be used to characterize biomolecular interactions and dynamic equilibria?*

A: SEC-MALS or FFF-MALS can help determine the absolute stoichiometry of tightly bound complexes that do not dissociate upon chromatographic dilution or column shear. Complete characterization of interactions using such fractionation techniques is only feasible either under very rapid or prolonged equilibrium conditions (on the time scale of the separation). AN1605: Identification of insulin oligomeric states using SEC-MALS describes the characterization of interactions arising from the self-association of proteins with fractionation.

A more comprehensive characterization under true equilibrium conditions can be conducted using CG-MALS. CG-MALS technology employs a Calypso® composition-gradient system and a MALS detector to determine the magnitude of interactions and absolute molecular stoichiometry of reversible complexes.

DLS is another technique that allows for the characterization of biomolecular interactions under well-defined kinetic states. While not as sensitive as CG-MALS, DLS offers microwell-plate-based, high-throughput screening. If you are interested in learning more about how light scattering is used for characterizing biomolecular interactions. Please refer to https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3641300/.

Q: *Is DLS/MALS suitable for fibrillar or fibrillar/globular objects? Any examples of fibrillar supramolecular biological analysis?*

A: Yes, both DLS and FFF-MALS have been used to analyze fibrillar molecules. Please refer to https://link.springer.com/article/10.1007/s00216-009-2899-1, which discusses the development of an FFF-MALS method for time-dependent characterization of aggregation patterns of fibrillar. FFF-MALS allows the separation and characterization of both early-forming soluble aggregates and larger prefibrillar and fibrillar proteins. Biophysical characterization of amyloids by FFF-MALS and batch DLS has also been described here https://febs.onlinelibrary.wiley.com/doi/full/10.1002/1873-3468.13428.

Physical Biochemistry core facility

Q: *How do you make decisions at the Physical Biochemistry core facility on which instruments to run samples on?*

A: The final decision is always left to the primary researcher. Usually, they will have an instrument in mind, and we will advise on whether it will answer their questions or if they should look for an alternative method. We do not restrict access to any of the instruments but make recommendations if we do not see the experiment being successful on a particular instrument or if another instrument would be easier/faster/etc. Still, the final decision is always left to the investigator.

Q: *How do you decide at the Physical Biochemistry core on which instruments to acquire for your facility?*

A: There are two ways. If I sometimes hear of an instrument that could help our research community, I canvas them and see if it appeals to enough faculty to be worth starting searching for funding. The more systematic way is when a faculty member approaches me about an instrument that would help them. I then gather support from other faculty and start trying to procure funding. Suppose it is an instrument that can be useful across multiple disciplines (chemistry, biology, physics, etc.). In that case, it is a lot easier to access funding than if it is simply for one department.

Q: *How much time do you spend optimizing run conditions for new samples? Can you outline your general thought process when you begin optimization?*

Optimization is relatively quick. The light scattering instruments do not usually require specialized buffers, so I recommend using whatever buffer their sample is most stable in and starting at around 2 mg/ml (which is a concentration I've found to work well on the MALS). After running a sample, we then tinker as needed to improve the signal and possibly change some experimental designs, but I find it best to run a sample as soon as possible and then optimize as you have a starting point.