Live Webinar Q&A Sheet:

Unraveling the Complexity: Advanced Analysis of Polymeric Nanostructures

The recorded webinar may be viewed from the FFF-MALS webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS, DLS, RT-MALS and CG-MALS 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 with any additional questions.

Questions & Answers

Q: *How suitable is AF4 for characterization of aggregates and for stability studies compared to other techniques, like SEC or DLS?*

A: Compared to size-exclusion chromatography (SEC), which can disrupt aggregates due to shear and filter out aggregates larger than about 100 nm, asymmetric-flow field-flow fractionation (AF4) using an Eclipse™ FFF system has the advantages of low shear and no filtration of large aggregates. Hence field-flow fractionation coupled to light scattering and other detectors (FFF-MALS) detects and measures all aggregates, including those that would not survive the SEC column.

Dynamic light scattering (DLS) has the advantage of being simple to use, low volume (the DynaPro™ NanoStar™ DLS instrument can measure with as little as 2 µL of solution) and is amenable to automation using Wyatt's DynaPro™ Plate Reader. DLS is very sensitive to big aggregates, but since there is no separation prior to detection, scattering from a few large aggregates can overwhelm the signal from small aggregates and unaggregated material, rendering the detector 'blind' to these species. Furthermore, the resolution of DLS is low so the size distribution is only qualitative, whereas FFF-MALS provides a high-resolution size distribution. Perhaps some of the most important benefits of FFF-MALS over DLS are the ability to add additional online detectors for further characterization and the ability to collect size-based fractions for further offline analysis.

Q: *Is the particle concentration measured by MALS comparable to that measured by particle counting technologies like nanoparticle tracking analysis (NTA)?*

A: Yes and no. If the particle is perfectly monodisperse, all techniques will give comparable same results, as long as the concentration is within the measuring range of each instrument. If the sample is polydisperse, however, the results may depend on separation and sensitivity to different sizes. For example, the sample is not separated in NTA, and the presence of strongly-scattering, large particles will decrease the sensitivity to weakly-scattering, small particles. When a highly sensitive, high-dynamic-range detector like a DAWN™ MALS instrument is coupled with a separation method like FFF or SEC, each size fraction is measured independently, overcoming this concern. The limits of the instruments are also different. For MALS the smallest particle for the concentration measurement is ~ 10 nm in radius, while for NTA, the limit is ~ 10 nm for gold but ~ 25 nm for less strong scattering material like polymersomes, lipid nanoparticles or extracellular vesicles.

Q: *Why did you choose to quantify loading by separating and quantifying the free, non-loaded proteins? Wouldn't it be easier to quantify directly with UV measurements of the loaded polymers?*

A: UV measurement of loading in nanoparticles is often incorrect, for several reasons. The primary effect is scattering - when particle sizes are above ~ 40 - 50 nm, the UV signal (which is calculated from the decrease in transmitted light due to both absorption and scattering) is greatly affected by scattering. Since scattering increases as the square of the particle volume while absorption only increases linearly with particle volume, the larger the particle, the more significant the error due to scattering.

Even if scattering is not significant, with certain guest-host combinations, changes in the extinction coefficient occur due to the interactions (e.g. ionic). In addition, depending on the location of the loaded molecules (in shell, membrane or core), changes in the absorption properties or shielding effects can occur.

Therefore, we have chosen to perform the quantification relatively. However, this only works if there are no interactions with the membrane or a stable saturation of the membrane is reached. For this purpose, we inject the pure "guest substance" several times at the beginning. As soon as the peak area stabilizes and the calculated amount of substance matches the injected amount, loading studies can be performed.

Q: *Wyatt's ASTRA software offers a method for directly analyzing LNP payload, that is, the amount of nucleic acid loaded into lipid nanoparticles of a given size, even if the particle scatters a lot of UV light. Is that method also suitable for polymersomes and other types of drug nanocarriers that you study?*

A: Yes it is. The concern for typical drug nanocarriers is that the Nanoconjugate Analysis method requires an accurate absorption coefficient of the loaded molecule, and total absorption needs to be high enough. The method usually works very well for RNA and DNA because of the strong absorption coefficients, but analysis of proteins may be more difficult, especially for low loading of large (i.e., highly scattering) particles.

Q: *How accurate is molar mass determination for complex protein-polymer conjugates by light scattering, given that proteins and polymers have different dn/dc values?*

A: When analyzing protein-polymer and other binary conjugates that are relatively small and do not require the Nanoconjugate Analysis method, it is best to apply ASTRA™'s Protein Conjugate Analysis method that accounts for the differences in dn/dc values and extinction coefficients of the two materials by measuring both dRI and UV absorption along with light scattering. With this method, the accuracy is (as usual for MALS) better than 5 %. The rule of thumb is that each material should constitute at least 3 % of the molar mass in order to be able to quantify it.

Q: *You are studying some very complex particles, and their elution properties depend on conformation - which might change under different solvent conditions. How do you select an appropriate carrier fluid such that the AF4-MALS results are relevant to your research goal? Does the technique limit your choices?*

A: Usually, before AF4 measurements, we always perform batch DLS measurements at the respective conditions. Especially with pH sensitive samples, such as polymersomes, the pH value is extremely important. Small variations can have a big influence on the properties. In addition, especially with psomes, aggregation can occur if the salt concentration is too high. Another case can be that the addition of detergents becomes necessary, because otherwise aggregation or degradation as well as interactions with the membrane can take place. Additional limitations exist in the characterization of temperature sensitive polymers.

Q: *You have emphasized the benefit of AF4 for very gentle separations. Can you provide examples of chromatographic separations causing degradation that was not present with AF4 separation?*

A: In the case of SEC measurements of DNA-modified polymers we observed increased degradation with increased flow velocity. The molar masses are decreased significantly under SEC, while in AF4 no changes happened.

Q: *Can both UV and fluorescence be measured simultaneously and used for analysis in Wyatt's FFF-MALS system?*

A: Yes, that is no problem – the fluorescence signal is just treated like another UV signal. ASTRA can use both signals to identify the molecule, and if there is a known correspondence between fluorescence and concentration, fluorescence can even be used to calculate molar mass or, in the case of small viral vectors, the full capsid ratio Vg/Cp using ASTRA's viral vector analysis.

Q: *Do you need to determine the dn/dc values of these very large and complex structures, like polymersomes, before you are able to calculate their molar masses with MALS? If so, how do you determine these dn/dc values?*

A: Yes, we always determine the dn/dc value if we want to calculate molar masses of unknown chemical structures. To do so, we prepare a stock solution of the respective sample and dilute it in a concentration series with the exact same solvent (e.g. the same batch of buffer). Then we inject the respective concentration directly into the RI detector and via the increase of the refractive index over the increase of the concentration we calculate the dn/dc. The data analysis and calculation are accomplished in ASTRA.

Q: *How can you tell if a peak arises from aggregated polymers or simply from polymers with higher molecular weight?*

A: Just from the elution, it is challenging to differentiate between large polymers or its aggregates. The first option is to change the focus conditions and compare the molar masses. If no changes are observed you can do additional studies (e.g. decrease sample concentration, increase of temperature or change salt composition or pH) and perform further AF4 studies and compare the properties. In the past, we observed different aggregation of sugar-modified dendronized polymers behavior depending sample concentration as well as pH.