Live Webinar Q&A Sheet:

Applications of Analytical Light Scattering in a Biophysics Core Facility

The recorded webinar may be viewed from the SEC-MALS 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.

General SEC-MALS questions

Q: *What, in your opinion, is the primary benefit of SEC-MALS?*

A: The real benefit is the ability to determine, in solution, accurate molecular weights for various macromolecules, including proteins, protein-protein complexes, protein-nucleic acid complexes, PEG-ylated proteins, membrane proteins that are isolated as complexes with detergents and lipids etc. regardless of the shape of the assembly. SEC/MALS can characterize oligomers that are not amenable to analysis by standard SEC or native PAGE because of the non-globular shape, interactions with the column media, modifications that affect shape, etc. Another important benefit is the dynamic range of an analytical SEC/MALS system that allows measurements over four orders of magnitude in concentration and molar mass.

Q: *What is the minimum mass difference can you reliably detect?*

A: Usually a difference of 5% is quite reliable for non-modified polymers, like proteins that are pure polypeptides; or ~10% for modified proteins- like glycosylated proteins or proteins in complex with detergents or lipids. If two assemblies are differentiated well by extinction coefficient (i.e., by ratio of UV to RI signals) then we can say with confidence that they are distinct species, even if they have very similar molar masses.

Q: *Have you analyzed temperature sensitive samples (Proteins that are stable at 4C and unstable at RT)?*

A: The system, including HPLC autosampler, column and instruments can all be stabilized at 4 °C if necessary.

Q: *How much protein do you use in a SEC-MALS experiment?*

A: Typically, 50 - 100 µg, though when necessary as little as 10 µg, assuming the protein elutes well off the column. Experiments aimed at testing self-association will often go to relatively high concentration and use more sample.

Wyatt now offers a MALS instrument for UHPLC, the µDAWN®, which (when used with UHPLC) reduces the protein quantity by about 10x.

Q: *Can you measure small peptides in SEC-MALS or large protein assemblies such as virus-like particles?*

A: Yes, I have measured peptides that were 1 kDa and less, and protein assemblies up to 100 MDa as long as the radius is below 500 nm. The concentration for small peptides needs to be increased relative to proteins in order to obtain sufficient signal.

If the assembly is too large for the SEC column then I can separate them on an Eclipse® field-flow fractionation (FFF) system upstream of the detectors.

Experimental details

Q: *What type of columns do you usually use?*

A: For proteins, I prefer Superdex or Superose columns that allow separation of peptides (Superdex peptide columns) to megaDalton assemblies (Superose 6 columns). For very large assemblies, I use TSKgel G6000PWXL column; on occasion, I use a silica-based column such as TSK3000, but only if it is specifically requested by the user. If customers have developed a separation method on their own columns I prefer to use the customer's column.

Q: *You use 2 columns in series in your articles, is the separation better?*

A: Yes, two columns provides better separation – at a cost. The potential disadvantages are longer equilibration and run times, higher loss of protein to the column and lower final concentration, hence lower final signal-to-noise ratio. In some instances, the costs are justified.

Q: *Do you need to check protein quality before running it on SEC-MALS? Are there any solutions you would not run, or do you inject any kind of proteins?*

A: Usually I ask for fractions directly from the final SEC purification which tend to be of high quality and usually indicate that the protein elutes well from SEC column. If these are not available, then at least fractions after a two-step purification such as nickel followed by another chromatographic step, like for example ion exchange. I prefer that the submitting lab verifies that the sample does elute reasonably well from the sizing column, otherwise there may be nothing to characterize.

Over the years I have analyzed a very diverse range of samples.

It is generally a good idea to check protein quality by one or more methods prior to running on SEC-MALS, but typically final SEC step in combination with SDS-PAGE is a good indicator of sample purity, especially if the sample was re-tested on SEC after it has been stored for a few days.

Analysis details

Q: *How were the actual calculations were done - in Wyatt software or offline?*

A: All calculations except K_d analysis are done in Wyatt's ASTRA® software. K_d analysis is done offline, using light scattering and concentration values obtained from ASTRA.

Q: *When you test heavily glycosylated protein, do you use the modifier approach by Wyatt? what dn/dc do you use for glycans?*

A: I use the "three detector approach" as described by Wen J, Arakawa T, Philo JS. (Anal Biochem. 1996 Sep 5;240(2):155-66) and compute the amount of modifier in off-line computations. I use this approach rather than Protein Conjugate calculation in Wyatt's ASTRA software because performing the computations off-line accounts for any systematic errors of the SEC/MALS system like, for example, slight shifts in wavelength due to mis-calibration of diode array detector etc. The dn/dc parameter of glycans is taken from the literature: 0.14 mL/g.

Q: *How do you determine f/f_0 (frictional ratio) in DLS experiment?*

A: f_0 is calculated from the diffusion coefficient of a globular protein with the same molar mass as that measured by MALS using specific volume of 0.74 ml/g; *f* is determined from the measured diffusion coefficient. I actually utilize the calculator feature in DYNAMICS software (software that I use for the DLS detector), which computes frictional ratio for a given molar mass and hydrodynamic radius..

Protein self-association studies

Q. *When you determined the dimerization constant from SEC-MALS, were you worried about the dimerization constant may be underestimated due to the fact that protein will be diluted in SEC?*

A. The concentrations used in the analysis are the actual sample concentrations measured by the detectors at the apex of the eluting peak, so dilution by SEC is accounted for in the calculation. There may still be a residual dilution between the MALS and the concentration detectors, but I work with 10/30 columns, where the eluting peaks are rather large (~ 1.5 ml in volume), so such dilution is minimal because the band broadening is relatively small. The effect of this small discrepancy on the final K_d value is minimal.

Q: *We would like to know if the concentration of the samples mentioned in the presentation refers to the concentration of the injected sample or the local concentration at the peak apex?*

A: The concentrations are those measured by the RI or UV detector at the apex of the eluting peak.

Q: *K_d of dimer could only be determine for fast reversible equilibrium complexes. It's not the case of all complexes, is there a way to determine K_d for other complexes by SEC-MALS?*

A: If it is a very slow equilibrium (i.e., the dissociation rate is slow compared to the time scale of the SEC separation) then upon separation you will be able to determine the total amount of monomer and total amount of dimer for a given starting concentration (i.e. of the injected sample). You can estimate K_d by comparing the ratio of dimer concentration to monomer concentration for a series of injected concentrations, which have been equilibrated over a sufficient time prior to injection.

Monomer-dimer systems that equilibrate on intermediate time scales are not suitable for K_d analysis by SEC-MALS. For such interactions, as well as more complex interactions including general self- and heteroassociation, CG-MALS is the technology of choice. CG-MALS uses the same MALS detector in combination with the Calypso® II stop-flow instrument (rather than SEC) to analyze protein-protein interactions.