



Live Webinar Q&A Sheet:

Separation and Characterization of Macromolecules with AF4-MALS

The recorded webinar may be viewed from the [FFF](#) 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 [Theory](#) page of our web site.

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

AF4-MALS analysis of polyelectrolytes

Q: In the polyvinylimidazolium fractograms, the light scattering and dRI signals overlap nicely, but in the PEGMEMA fractograms the LS and dRI signals have different shapes. Why does this happen?

A: LS and dRI signals are determined by different parameters of the sample.

dRI is solely dependent on the concentration and refractive index increment (dn/dc) of the sample in the detector, while for LS, the scattered light intensity is related to the product of the concentration and the molecular weight. Consequently, we see a mismatch between the dRI peak and the LS peak, with a shift of LS peak towards the high MW domain (right for AF4 and left for SEC).

This mismatch of dRI and LS is exacerbated for polydispersed samples, which is the case for the PEGMEMA sample, while for the polyvinylimidasolium sample, the polydispersity is small, and hence results in close overlapping of the two signal peaks.

Q: Prof. Thormann mentioned injecting samples at different concentrations to measure dn/dc . Which instrument was used for the measurement - light scattering, refractive index or both?

A: We used the Optilab (dRI) detector. The dn/dc value is simply measured with a batch mode, where a series of sample solutions with specific concentrations were injected directly into the detector, and the dRI values obtained were plotted and fitted linearly through the origin. The slope is recorded as the dn/dc value. All the data processing and fitting are performed with an dn/dc measurement method in ASTRA.



Q: The polyelectrolytes require a lot of method development for SEC separation. Is the same AF4 method used for all the polyelectrolyte samples? If not, how much method development is required for different samples?

A: It didn't take us much effort to develop the method. As you may see, the method we used is a typical method that is frequently used for many polymer systems.

AF4 is more versatile for polyelectrolyte measurement than SEC, although it might also require some method design and adjustment.

Q: Which membrane material and pore size (MWCO) did you use? Which buffer system did you use?

A: For our polyelectrolyte characterization, we used a RC (regenerated cellulose) membrane with a MWCO of 5 kDa, an FI channel with a W350 spacer. The eluent is a pH 7.4 phosphate buffer. Details regarding GLP-1 analogue separation method will be provided in a journal publication.

Q: For Esben: why does the exponential decay cross flow provide the best separation profile? Is that a universal trend?

A: When designing the method, we aim for good separation, taking advantage of the options available in the software. The cross flow can either be constant, with a linear slope, or with an exponential decay, depending on the nature of the sample. Sometimes it requires several trial-and-error cycles before one can find an optimized method. [SCOUT DPS™](#) facilitates method development by simulating the flow dynamics for any set of assumed particle sizes.

Q: Do polyelectrolytes with different structures—for example, linear or star-shaped—but the same molecular weight give different MALS signal intensity?

A: Sometimes. The molecular weight is determined from the MALS intensity *extrapolated to angle 0°*, so polymers with different conformation but the same molecular weight and concentration would have the same 0° scattering signal, but not necessarily the same at other angles. If they are larger than about 10 nm in R_g these two polymers would exhibit different scattering patterns due to the conformation difference, so at angles other than 0 the signals would differ. If the polymers are small, i.e. both have $R_g < 10$ nm, then they scatter equally in all directions and so would have the same MALS intensity.

AF4-MALS of GLP-1

Q: The molecular mass of GLP-1 is just 3.3 kiloDaltons. Which pore size membrane is used for such small molecules, in other words, what is the molecular weight cutoff?

A: For the acylated GLP-1 analogues, we used a membrane with 1 kDa cutoff. However, due to the low cutoff, we experienced some issues with maintaining the crossflow pressure - this turned out to stem from a faulty membrane batch. So changing the membrane to one from another batch



was an easy solution to this problem. Luckily, it is rather easy to quickly spot those faulty membranes: If the crossflow is too low after about 10 min in elution mode, or if the channel is leaking within the same timeframe, it is most likely due to a faulty membrane.

Q: Why do GLP-1 analogues elute at very different times in the fractogram when the salt concentration or pH are changed? Could you analyze these peaks without MALS?

A: Since the oligomers increase in size, the retention time will also increase. We would have to investigate further to give a more elaborate answer, but one could do complimentary SAXS experiments.

SEC-MALS analysis of proteins

Q: The SEC-MALS data suggest a monomer-dimer equilibrium for that the tryptophan hydroxylase in the presence of tryptophan, and possibly a dimer of dimers. Can these interactions be quantified, by injecting different concentrations of protein?

A: In principle this can be done. However, we have not looked into this as we have problems with the monomer aggregating.

General AF4-MALS and SEC-MALS questions

Q: What is the unit for the y axis?

A: In most of the fractograms presented, the y axis is a relative scale. For the dRI detector you can of course get the dRI value. There are also figures where the y axis is molecular weight or R_g .

Q: AF4-MALS can be used to separate the polymers with different molecular weights. Is it possible to quantify the separated species? if yes, what method will be recommended?

A: The separated species are quantified directly online using MALS and dRI, using [ASTRA®](#) software. ASTRA calculates the concentration in each fraction and the corresponding molecular weight and size, producing complete distributions of MW and R_g . With integration it also provides information about total recovered mass and moments of the MW and R_g such as weight average, number average and z average.

It is also possible to collect fractions and apply additional means of quantification, for example mass spectrometry.

Q: Which fit model do you use to correlate your data and obtain MW?

A: We used a 1st-order Zimm model. This provides a good estimation for small particles such as, in our case, single polymer chains with MW below 100 kDa.



In other measurements, we have used a 2nd-order Berry model to determine R_g of nanoparticles (~150 nm). In this case, since R_g is large, the 1st-order Zimm analysis tends to underestimate the R_g value.

Q: *What are the molar mass and size limits for MALS analysis?*

A: Wyatt's DAWN[®] MALS instrument can determine molar masses from 200 g/mol to 10⁹ g/mol, and R_g from 10 nm to 500 nm.

Q: *On the slide 23, after stopping the cross flow there is a signal in MALS spectra. What you would say that signal corresponds to?*

A: That signal corresponds to some minor impurities with large particle size. When the crossflow decreases to 0, they are flushed out of the channel.

This kind of impurity peak typically has a relatively large LS signal, yet little dRI signal. LS is correlated to both size and concentration, while dRI is dependent solely on concentration. So we can draw a conclusion that, these particles have a very low concentration (very little signal from dRI) and very large particle size (strong LS signal).

Q: *Is it possible to have two peaks in an AF4 fractogram that give the same size in nm, but one has higher MW than the other?*

A: Yes, this is an entirely valid possibility, assuming that the size you measure is the rms radius, or radius of gyration R_g , using MALS angular data. The elution volume is determined by the diffusion coefficient of the particle, which is linked to hydrodynamic radius (R_h). The relationship between R_h and R_g depends on conformation, so peaks with different elution volumes but the same R_g have different conformations and it makes sense that they would also have different molar masses for a given R_g .

Q: *Can we use MALS detector in Waters HPLC system for determination of molecular mass/molecular weight of polysaccharide - protein conjugate?*

A: Yes, Wyatt's MALS instruments interoperate with most major HPLC and FPLC systems. Polysaccharide-protein conjugates are usually characterized using a three-detector arrangement: MALS, UV and dRI. This enables the determination of the conjugate's protein molar mass and polysaccharide molar mass, as well as the total molar mass, at each elution volume.