



Live Webinar Q&A Sheet:

Structural characterization of functional polymers via light scattering and differential viscometry

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

SEC-MALS general

Q: Are their specific brands of HPLC that you would recommend with Wyatt instruments?

A: Wyatt Instruments are compatible with a wide variety of HPLC manufacturers. Our instruments are downstream of HPLC systems from almost every major HPLC vendor. For the best user experience, we do offer the option of controlling an Agilent HPLC directly from [ASTRA](#)[®], our light scattering analysis software, or [VISION](#)[™], our FFF control and data acquisition software. I'd encourage you to contact Wyatt for further information.

Q: Are the instruments compatible with UHP-SEC?

A: The advantage of UHPLC technology is the ability to deliver uniform and consistent flow through columns with smaller, more tightly-packed, bead sizes than traditional SEC columns, which requires stronger pumps. Wyatt Technology offers detectors that take advantage of these benefits with low dispersion, high acquisition rates, shorter run times, and higher resolution. Our UHPLC-compatible analytical detectors are [microDAWN](#)[®], [microViscoStar](#)[®], [microOptilab](#)[®].

Q: Could you comment on what low mass recovery% indicates? If a sample only has 50-60% mass recovery, can I still get reasonable results in conjugate analysis?

A: As long as you have the correct dn/dc and extinction coefficient values for your polymer and conjugate, you should still get accurate conjugate analysis results.

However, it is worth investigating the low mass recovery % to find out why sample is lost. If the sample concentration or injection volume you entered were incorrect—due, for example to the sample being wet after Soxhlet extraction or precipitation—this would be a source of error. If much of your polymer is entangled/aggregated, it could be filtered off by the SEC column, which is why AF4 is a good orthogonal technique, it does not remove these large aggregates. If a lot of it is present as a small molecule it might elute in the total included volume peak.



Other causes for low mass recovery are sample sticking to the column, excessive peak dispersion or micro-leaks in the system. Entering incorrect parameters such as flow rate or injection volume is avoided when ASTRA controls the HPLC pumps via HPLC Service™ or HPLC CONNECT™. The best way to check is with a reliable standard that is expected to elute from the current column and mobile phase with high mass recovery.

Q: *Why would the molar mass determined by conventional column calibration be greater than absolute molar mass determined by MALS or triple-detection GPC?*

A: Conventional calibration molar mass is defined by the retention time and how it relates to the retention time of the calibration standard, which elutes according to hydrodynamic size. Your analyte may be more or less dense compared to the standard. If the sample of interest and the standard have different hydrodynamic volumes for the same molar mass, then they elute at different times and only MALS can give you the absolute molar mass.

The distinct advantage of SEC-MALS is that it actually indicates and confirms that conventional calibration has failed. ASTRA has a [calibration curve analysis feature](#) for just this purpose—by comparing the conventional calibration results with MALS, you can make more informed discussions in publications, especially when comparing results to other papers that fail to utilize the distinct advantages of MALS.

Q: *How can you determine the intrinsic viscosity of your functional polymer?*

A: Wyatt's ViscoStar, which you can learn more about in [this white paper](#), calculates the specific viscosity of your functional polymer and uses the concentration to determine the intrinsic viscosity. Using the intrinsic viscosity along with the molar mass from MALS, we can then determine [conformational information](#). All calculations are readily performed in the [ASTRA data analysis software](#).

Q: *Is it possible to determine molecular weight in a solution of unknown concentration by SEC-MALS or AF4-MALS? We generally receive samples in a formulation.*

A: If you know the dn/dc parameter of the sample in the formulation, then you can readily determine its molar mass, even for a sample for which you do not know the concentration. If you do not know its dn/dc , then it must be determined, which is discussed elsewhere.

FFF-MALS general

Q: *What is the minimum molar mass required for FFF?*

A: The lower limit for molar mass in AF4-MALS is typically governed by the molecular weight cutoff (MWCO) of the ultrafiltration membrane. We offer a membrane with a MWCO of 1 kDa, which is



rated for a linear coil polymer. With this cutoff, we might expect—depending on the radius—polymers smaller than 1,000 g/mol to escape through the membrane and cross flow waste, and thus not enter the MALS detector for molar mass characterization. Although smaller cutoffs are possible, too much restriction will also restrict the cross flow and reduce mobile phase permeation through the membrane, which can reduce separation efficiency. It is more common to use cutoffs of 10,000 or 30,000 g/mol.

Q: Which carrier solvents are compatible with AF4? Can you run AF4 using DMSO?

A: The [Eclipse AF4 separation systems](#) are broadly compatible with aqueous buffers and some organic mobile phases. With AF4, we've had success with organic solvents such as THF, chloroform, toluene, and methylene chloride.

Compatibility is mostly dictated by the semipermeable membrane used in the separation channel. Wyatt currently only offers membranes made from polyether sulfone and regenerated cellulose. However, it is possible to cut your own membranes from other materials such as PTFE ultrafiltration membranes, which would expand the range of compatible solvents.

DMSO is one of the more challenging organic solvents to utilize with AF4-MALS; Regenerated cellulose (RC) and polyether sulfone (PES) which dissolve in DMSO. Even finding an alternative membrane material that performs really well can be challenging. DMSO is also a fairly viscous solvent, which affects fluid flux through the membrane, often necessitating higher operating temperatures (putting the separation channel in an oven) to reduce the viscosity. I won't say it is impossible but there are some challenges to overcome.

Q: When can we expect new generation of AF4?

A: Wyatt Technology prides itself on continuously developing and improving its technology. Some recent examples are the release of the [NEON line of analytical detectors](#), our [solution for process analytical technology](#), and the upcoming release of ASTRA 8 and HPLC CONNECT 2.0. Our R&D team is working on improving the electrical/asymmetric flow field flow fractionation (EAF4) technology, which you can learn more about by watching our [webinar](#). Although we cannot comment on any plans right now, you can learn about upcoming product releases and events by staying connected and [signing up for our newsletter](#).

Q: Is it possible to use in silico methods to confirm or drive experiments?

A: Our [VISION®](#) suite of software tools for the [Eclipse FFF separation system](#) provides an *in silico* method development module that enables you—from scratch—to simulate the outcome of an AF4 flow profile for the hydrodynamic sizes that you estimate to be present in your sample. This module uses basic FFF theory with patented algorithms to predict and simulate fractionation, where you can virtually try out different membranes, spacer heights and cross flow profiles.



After running the method in the lab, you can import the measured fractogram and further optimize your method to improve separation. The software helps you figure out where to begin your AF4 method, and then tweak and optimize. Usually you will have an optimal method by the second physical run.

Q: *Why does AF4 take a longer time than SEC, up to an hour per sample?*

A: SEC has more theoretical plates, and so the separation is more efficient and can take less time; however, it has lower selectivity, so the required time can vary. If you can achieve your SEC separation with one column, then maybe SEC will be shorter than AF4. If you need several columns for good SEC separation, then the run time is comparable and possibly longer. A key advantage of AF4 is its flexibility in dynamic separation range without having to go through many different columns.

Once an AF4 method is optimized, runs times can be tweaked by adjusting the rate of cross flow change, so the cross flow can be dropped to allow the sample to immediately elute from the channel once sufficient separation has occurred. On the other hand, you cannot easily shorten an SEC method without changing the column.

Q: *Can we separate different size nanoparticles with AF4-MALS (quantitatively)?*

A: Absolutely! Although MALS has an upper limit for size characterization of about 1 micron with a shape model, AF4 can separate particles even up to 10 microns. We have several examples of nanoparticle size distributions via AF4-MALS in [the application note section of the Wyatt website](#). Please also check out [this webinar on nanoparticle size distributions](#). Our customers have had success separating everything from [gold nanoparticles](#) to viruses and [virus-like particles](#) to [lipid nanoparticles](#) with AF4-MALS. In addition to separation and high-resolution sizing, AF4-MALS also provides particle concentrations.

ASTRA analysis

Q: *How do I perform the branching analysis in ASTRA? Do you have a tutorial or application note?*

A: We do have some application notes (such as [Analysis of Polymer Branching](#)) that describe the results from a branching analysis but for a detailed exploration of the step-by-step procedure for analysis data and getting branching analysis results, I would recommend taking a look at two technical notes available on the [Wyatt Support Center](#), TN1001 - SEC-MALS Branching Analysis using the Radius Method and/or TN1002 - SEC-MALS Branching Analysis Using the Viscosity Method which describe using your MALS detector only or your MALS/Differential Viscometry combination, respectively. The Support Center is freely accessible to all Wyatt customers whose instruments are no more than 10 years old.

Q: *Is it possible to make a Mark-Houwink-Sakurada plot with DLS data?*

A: You can make a conformation plot—but not strictly a Mark-Houwink-Sakurada (MHS) plot—with DLS data. The MHS plot graphs intrinsic viscosity vs. molar mass, which requires viscometry, and the interpretation of slopes would be different for a conformation plot.

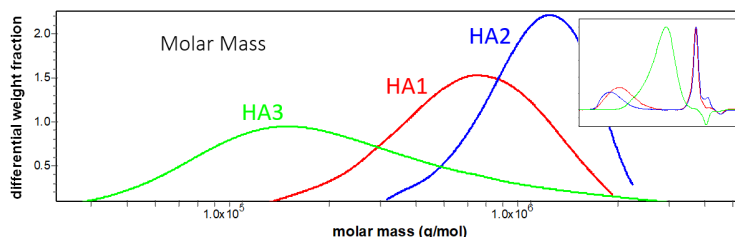
A combined online MALS-DLS measurement for a DLS conformation plot can be made a [WyattQELS™](#) module embedded in the MALS detector, or with a [DynaPro® NanoStar®](#) or [Mobius™](#) optically connected to the MALS flow cell with the DLS Compatibility Kit. This gives you the hydrodynamic radius, which ASTRA can plot against molar mass to generate a conformation plot. The trends you might expect between size and molar mass are similar but the technique is different. For more information, check out our [Conformation](#) webpage.

Q: *Are the molar mass vs volume plots referenced to elution volume? Or is that related to the sample by concentration and density?*

A: In this webinar, the molar mass vs. volume plots refer to the elution volume, which makes comparing chromatograms under different flow rates a little easier (compared to retention time, which is flow rate dependent). Information on molecular density can be inferred from the relationship between molar mass and size or intrinsic viscosity.

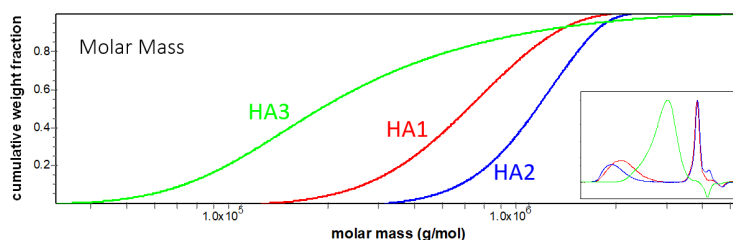
Q: *Can you explain a little in detail the difference between differential weight fraction and cumulative weight fraction plot?*

A: Each point in the **differential** weight fraction is defined as the fraction of the total mass with X halfway between the previous and current value, and halfway between the current and next x-scale value. For example, looking at a molar mass distribution, if the adjacent points have x values of 104,000, 105,000 and 106,000, then the values of a point located on the x-axis at 105,000 g/mol would represent the entire fraction with molar mass between 104,500 g/mol and 105,500 g/mol. The y-values, shown in units of 1/(g/mol) are normalized so that if you sum up the product of $y * \Delta x$ (where Δx is the distance between adjacent x-axis points), it comes out to unity.



Each point in the **cumulative** weight fraction represents the fraction of the total mass with X less than or equal to the current x-axis value. In the same example, the value of a point at 105,000

g/mol represent the total fraction with molar mass from zero to 105,000 g/mol. Basically it's doing that same integral on the differential weight fraction mentioned in the previous paragraph, taking each point from zero to x, multiplying by Δx and adding. That's why the point in the cumulative distribution with the highest x-axis value always has a y value of unity.



MALS technology

Q: How does SEC-MALS compare to mass spectrometry?

A: Mass spectrometry can give higher accuracy and precision than SEC-MALS; however, you are often limited by how a polymer ionizes and if it can “fly” so-to-speak. You will also get a very complex mass spectrum for anything but monomers and low oligomers. Although MALS accuracy is about $\pm 5\%$ and so not nearly as good as mass spectrometry, it typically has a much larger molar mass range and a simplified analysis (needing only intensity of scattered light, concentration, and dn/dc).

When comparing oligomer molar masses from mass spectrometry with oligomer molar masses from MALS, we have seen good agreement. Where MALS further shines is the ability to acquire detailed structural analysis by comparing the simultaneous absolute molar mass and intrinsic viscosity results, or molar mass and size measurements (where size is obtained from MALS or DLS).

Q: What is the lowest molecular weight detectable by light scattering?

A: Light scattering intensity is proportional to molar mass, concentration, and $(dn/dc)^2$. Depending on the dn/dc value, some lower molar mass samples will be easier to study than others. We have had success measuring down to 200 g/mol with sufficiently high concentration and dn/dc values. More typically, we regularly analyze polymers down to 1,000 g/mol with higher concentrations.

Q: Depending on particle size to wavelength ratio the scattering mode will gradually switch from Rayleigh to Mie scattering. What is the approximate magnitude of the bias in size/related parameters resulting from this transition for the higher sizes of real-life systems like broadly distributed polymers?

A: Mie scattering is relevant to uniform spheres, but such polymers are rarely spherical and so it is likely that the Lorenz-Mie analysis is not appropriate. The Rayleigh-Gans scattering model loses



validity when $|n_{\text{sample}}/n_{\text{solvent}} - 1| * \sin^2(\theta/2) * R_g / \lambda$ is no longer $\ll 1$, and clearly this happens faster for higher angles than for lower angles. As you get to larger sizes approaching the wavelength, it is best to use the Berry model and rely primarily on the lower angles, disabling the higher angles. If this is done right then the molar mass result, which is calculated from the extrapolation to zero angle, should not be impacted much.

Q: You mentioned that 10 nm is the lower limit for R_g measurements by MALS. Recent technological in-line MALS innovations have gone down to 2 nm, specifically for the Lens₃ instrument. Can you comment on this?

A: From a theoretical standpoint, it is possible to measure down to 2 – 4 nm with MALS; however, there are some caveats and tradeoffs to consider. MALS instruments that measure R_g well below 10 nm of necessity use shorter laser wavelengths and very low scattering angles compared to the DAWN or miniDAWN.

Shorter laser wavelength means higher susceptibility to erroneous molar mass results due to absorption and/or fluorescence by the analyte. Wyatt's MALS instruments use a red laser at 660 nm, which has low probability of absorption or fluorescence, whereas the instruments that have demonstrated lower R_g use a green laser at 514 nm. In addition, our optical design allows for placement of the forward monitor detector, which can be used to correct for absorption by the sample, and this correction is implemented in ASTRA. The novel optical geometry allowing for much lower scattering angles makes incorporation of a forward monitor much more difficult, precluding this correction—which will be needed more often when the laser wavelength is shorter. Wyatt also offers fluorescence-blocking filters for those cases where fluorescence is excited by the MALS laser.

Other important considerations are signal-to-noise and data quality. At lower scattering angles, you are significantly more vulnerable to noisy data because particulates produce copious forward scattering, and SEC columns—including even the lowest-shedding columns—generally contribute particles. The hero data we have seen for the new instrument indicates that the measured rms radius values for known standards are only within a 10 – 20% error range. Wyatt specifies an accuracy of 5%, so if you are willing to accept less accurate results, you could measure R_g values below 10 nm in the DAWN or miniDAWN.

What's more, Wyatt's specified range does not require incorporation of a post-column filter, which might adsorb sample or add dispersion, or even clog and damage the column. The normalization method required by the Lens₃ seems quite cumbersome and involved compared to standard MALS normalization with a single sample injection.

Another tradeoff is the upper limit for R_g . The same modifications that reduce the lower limit—shorter wavelength and larger angular range covered by only three detectors—also reduce the



upper R_g limit of 50 nm (150 nm with a known shape model) measured by the miniDAWN, not to mention the upper limit of 500 nm measured by the DAWN.

A final point is the lack of use of MALS angular data for determining the most important property of your macromolecules: molar mass. The material we have seen for the Lens₃ indicates that the molar mass is not calculated using the traditional, highly reliable MALS method of extrapolating multi-angular scattering data to angle zero. Instead, you are asked to choose which result you prefer – the molar mass determined at low, medium or high scattering angle. In my opinion, absolute measurements don't work that way.

I encourage you to reach out to us or your local team to learn about all the advantages of Wyatt Technology's software, hardware, customer support, and over three decades of experience in the field. We do want you to be successful and we want you to get the best data possible—and that governs a lot of our specifications.

Refractometry & dn/dc

Q: How can dn/dc be measured?

A: The specific refractive index increment, dn/dc , is a property of the sample and refers to the change in refractive index with respect to concentration. One way to determine it is to prepare a few accurate concentrations of the sample and measure the refractive index in an [Optilab® differential refractive index detector](#), then plot the differential refractive index vs. concentration. The slope of this plot, which is created and analyzed in ASTRA, gives dn/dc .

We can also integrate an RI peak area, with or without chromatography, in the [ASTRA](#) software. Here, dn/dc is determined by comparing the peak area to the amount of analyte injected. You need to enter the correct flow rate, the sample concentration and the injected volume. The calculation assumes that 100% of the mass that was injected goes through the dRI detector. If the sample goes through an SEC column then it is properly dialyzed against the solvent, but there is a possibility of losing some of the sample to the column and the result may not be accurate. If you have concerns about near-100% recovery then it is best to bypass the column and MALS detector, injecting directly into the Optilab.

Q: Is the dn/dc value the same or different for the same polymer with different molecular weight?

A: Refractive index is a function of chemical composition, and polymers may have end groups that differ chemically from the repeating motif. When the overall contribution of the end groups is minimal, the refractive index can be considered constant, but this might not be true for oligomers and smaller polymers. Hence dn/dc is typically constant for polymers above a certain molar mass, for example a 30,000 g/mol polymer would have the same dn/dc in a given solvent as its much larger 1,000,000 g/mol version.



Similarly, many polymer properties, including dn/dc , do not plateau until the polymer is roughly 10,000 g/mol, though they might only vary by a small amount below that. As an example, for 10,000 g/mol polyethylene glycol (PEG) in water dn/dc is around 0.142 mL/g whereas for a 1,000 g/mol PEG it is around 0.136 mL/g.

Q: *Is dn/dc of a mixture of components (95% PLGA, 5% small molecule) much different than for PLGA alone?*

A: If it is purely a mixture, then we would expect in either SEC or AF4 that the polymer will separate from the small molecule. If you find two separate peaks, then apply just the PLGA's dn/dc to the PLGA peak. If there is a covalent bond and these components elute together, then dn/dc of the composite is the weight average of the respective constituent dn/dc values, so 5% small molecule won't cause it to vary much from that of PLGA alone.

Polymer-specific applications

Q: *How to differentiate between degradation and just disaggregation?*

A: Understanding whether your polymer is degrading or disaggregating may require control studies. For example, in the webinar we discussed shear degradation (SEC/GPC-induced degradation) and how you can use variable flow rates to evaluate if shear degradation is occurring and how AF4 can overcome shear degradation. Other degradation modes depend on the polymer structure—solvent, temperature, light, air, etc. Comparing the results after synthesis, [after exposure to degradation conditions](#), and after time (stored dry vs. stored in solution) can reveal polymer characteristics.

SEC- and AF4-MALS are relatively quick experiments but aliquots can also be measured in batch experiments by MALS or with batch DLS instruments such as the DynaPro Plate Reader or NanoStar. These types of batch experiments are better suited for long-term studies to monitor the gradual change in a polymer over time or temperature. For additional information about DLS instruments, [read our online overview](#).

Q: *Why does the a -value (from MHS-plot) of anionic polysaccharides decrease with increasing molar mass, although there are definitely linear?*

A: I think a great example of this is sodium hyaluronate, which for years was thought to be a purely linear polysaccharide until detailed characterization with Wyatt detectors proved for the first time that there are both linear and branched polymers present as a result of unsuspected side reactions. You can read more in [Podzimek, et al. *Journal of Applied Polymer Science*, Vol. 116, 3013–3020 \(2010\)](#). Many polymers contain highly linear but also highly branched content, which we revealed for several of the polysaccharides in this webinar.



Q: *When dealing with oligomeric content how do you account for differences in dn/dc to determine absolute molar mass to use in conjunction with the intrinsic viscosity?*

A: It's true that dn/dc can vary with molar mass for low oligomers and even smaller polymers. Typically dn/dc becomes effectively constant above 10,000 g/mol, since the end groups that have different properties than the bulk of the polymer then represent a tiny fraction of the total molecule, but in some cases it is relatively constant between 1000 and 10,000 g/mol as well.

In order to perform optimal analysis, you would have to know how dn/dc varies with molar mass.

- You could do a first-pass analysis by using a constant dn/dc value to estimate the molar masses across the chromatogram.
- Then you could select multiple 'peaks', i.e. regions of the chromatogram that roughly correspond to different dn/dc values, and enter the corresponding dn/dc in each selected range to get a more accurate molar mass.
- You might iterate this one more time to tighten up the results.

Q: *Is R_g a function of chemical composition of polyethylene?*

A: The rms radius or radius of gyration is a shape- and conformation-independent value. It measures the average distance of mass from the center of gravity for a polymer. It is actually calculated from the slope of the angular dependence relationship and does not strictly require dn/dc information for accurate determination. So chemically-modified polyethylene—provided it has a minimum rms radius of 10 nm—can be accurately sized. That does not rule out the possibility that chemical modifications affect the molecular conformation and hence R_g vs. molar mass.

Q: *Using AF4-MALS, could we determine or gather some information about the polymer network (crosslinked with reversible covalent bonds)? And, subsequently, could we monitor the alteration in the molecular weight of the polymers at a specific temperature at which the crosslink breaks (due to reversibility)?*

A: This is an interesting application and one that we may be able to further discuss further. It is possible that these types of studies are best executed in batch experiments—where we can measure the change in the overall weight-average molar mass of the sample as a function of the temperature. While we can run our instruments at varying temperatures and the channel can be placed in an oven, if the sample is dynamically changing molar mass then separation in real-time becomes more challenging. What is feasible would be to apply a temperature stress to a sample and then immediately inject the sample and measure the molar mass. In this capacity, we can



monitor the effect of temperature on the polymer under the assumption that no further change happens during the sample elution.

Polymer GPC general

Q: Can you mix solvents to analyze a polymer?

A: You can mix miscible solvents together. Commonly, additives like salts or acids are added to help prevent aggregation or mitigate column interactions, but co-solvents can also be used for improved solubility. Please keep in mind that as you change the refractive index of your mobile phase, you alter the sample's dn/dc value (which is solvent dependent) and the normalization coefficients, so those values will need to be updated. The dn/dc value can be readily determined by injecting a series of known concentrations into the [Optilab® differential refractive index detector](#) or by using one of ASTRA's [online \$dn/dc\$ determination methods](#).

For SEC/GPC with co-solvents, it's always best to pre-mix your mobile phase and stir it to keep the mobile phase homogenous, which will afford you the most stable RI baseline. Take precautions to minimize evaporation of one co-solvent over others, which can cause the RI and dn/dc to drift as the solvent composition changes.

For IEX- or RPC-MALS, we've had some success with solvent gradients. If you the sample elutes in a region of relatively constant refractive index, you can use the appropriate dn/dc and normalization coefficients for that solvent composition. It may be possible to create an isorefractive gradient with judicious addition of inert excipients or co-solvents. More information about IEX- or RPC-MALS can be found in our blog post [Next steps in biophysical characterization by MALS: ion-exchange and reversed-phase chromatography](#).

Q: How do you prevent polymer aggregation?

A: Strategies for minimizing polymer aggregation depend somewhat on what the cause is. Preventing molecular aggregation can sometimes be done with the mobile phase—using salts or adjusting the pH can minimize electrostatic or hydrophobic interactions, respectively. If you are referring to polymer solubility, sometimes longer dissolution time (several days) or dissolution under elevated temperatures can disaggregate polymers and solubilize them. If you think aggregation or chain entanglement is occurring during the focus step in AF4, you try different focusing parameters or can use a specialized channel called the frit-inlet channel that excludes the focusing step.