



## Live Webinar Q&A Sheet:

### Absolute Molar Mass Analysis of Medical Device and Pharmaceutical Polymers

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, and ELS 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](mailto:info@wyatt.com) with any additional questions.

#### SEC-MALS

- Q: Triple-detection SEC—combining light scattering, RI and viscometry—is sometimes used for polymer analysis. Can you do that with this system, and what would be the benefit?*
- A: This system can be coupled with a [ViscoStar® viscometer](#); this is useful for branching or molecular conformation analysis, especially pertaining to polymers with  $R_g < 10$  nm.
- Q: Why is there such a large difference in molar mass values between MALS and column calibration for the polypeptide samples?*
- A: This conventional column calibration analysis is strictly relative to standards of known molar mass, which actually elute according to hydrodynamic size rather than molar mass. Because the standard used for calibration will have a different molecular density or conformation, that is, a different relationship between hydrodynamic radius and molar mass than that of the unknown sample, there can be an extreme difference between the molar mass of polymer A that elutes at X minutes and the molar mass of polymer B that elutes at X minutes.
- Q: I do not have MALS, but rely on GPC with viscometry for analysis of my polymers. Do you think I am getting reliable results?*
- A: There is a general trend that polymers increase in viscosity with increasing molar mass, but this is not always the case for all polymers in all molar mass ranges. If all necessary parameters are known for universal calibration, this is an accurate measurement, but only if the separation is based solely on size, the sample is a random coil, and there are no other interactions affecting the elution of the analyte molecules. MALS analysis is able to accurately measure weight average molar mass even if separation is not ideal and without Mark-Houwink parameters.



*Q: Can SEC-MALS be used for separation, identification and characterization of highly branched polysaccharides? Or do they have to be linear bio-polymers or homo-polymers?*

A: SEC-MALS is excellent for highly branched polysaccharides because the characterization is independent of viscosity or of molecular density.

*Q: How do you and your customers use the information on  $M_n$ ,  $M_w$  and  $M_z$  as opposed to just one average molar mass?*

A: When it comes to polymers, all molar masses are averages due to the fact that not every single molecule will have the same number of repeat units. There are many ways to calculate an average, and each has a role to play. The number average,  $M_n$ , is the simple arithmetical average of the molar mass of molecules. At first, this sounds like the obvious choice for the information you want to receive about your polymer. However, this means that a small molecule and a large molecule have the same influence over the average, even though the small molecule represents a smaller portion of the sample mass. Thus,  $M_n$  is more dependent on the smaller molecules in a polymeric sample than the larger ones.  $M_n$  is an important value to monitor if you're concerned about the degradation of larger molecules into smaller ones, as it will be the most sensitive to this change. This sensitivity can be a problem, though, as low molar mass portions of sample peaks sometimes tail out, and the tail is subject to effects from noise and low signal, creating less reliable results.

The weight average molar mass,  $M_w$ , is directly measured from MALS analysis. This is the weighted average molar mass based on the portion of sample mass represented by each molecule (or, in technicality, each slice of the chromatographic peak). This value is more stable, and largely corresponds to the middle of the peak. We recommend that this average be the main focus of characterization, as it is less susceptible to change due to noise, chromatographic issues, and differences in processing between analysts.

The z-average molar mass averages the molar mass of molecules giving even more weight based on the mass portion represented by each molecule. Because of this, very large molecules have the strongest effect on this statistical average.

Though we recommend that  $M_w$  be the focus of sample characterization, looking at all three averages helps you to understand the characteristics and polydispersity ( $M_w/M_n$ ) of the sample.

*Q: Can the radius of gyration be extracted from the analysis? How can you measure  $R_g$  of macromolecules below 10 nm in size?*

A: The radius of gyration  $R_g$  is determined from the angular dependence of the scattered light, and is calculated at each point in the chromatogram by [ASTRA®](#) software. random-coil conformation,



The general cutoff for a molecule to exhibit an angular dependence of scattered light at the MALS laser wavelength is approximately 10 nm, corresponding to a 50 kDa random coil. The radius of gyration can be measured for anything larger than this.

Below 10 nm is too low for MALS detectors to accurately determine the angular dependence and therefore  $R_g$  analysis is marginal or unreliable. It can be measured by small-angle x-ray scattering (SAXS). However, a Wyatt MALS instrument can be combined with [online dynamic light scattering \(DLS\)](#) or differential viscometry in order to measure the hydrodynamic radius ( $R_h$ ) down to 0.5 nm and below.

*Q: What is the lowest molecular weight detectable by light scattering? Is it sensitive to detect MWD < 50,000 g/mol?*

A: We commonly analyze down to 1,000 g/mol, and can go lower with the right concentration and column selection. If the concentration is high enough, molecules in the 100's of g/mol can be evaluated by MALS analysis.

*Q: Is it a common practice to extrapolate the light scattering data to estimate MWD < 50,000 g/mol?*

A: Extrapolation is used in the leading and trailing edges of the molecular weight distribution where either the light scattering or concentration signals are too low for good analysis. There is nothing special about molar masses below 50,000 g/mol in this respect, though the lower the molar mass, the higher the concentration that is needed for good analysis.

*Q: How many of the MALS detectors are really useful for molar mass distribution; noise versus accuracy?*

A: For an [18-angle DAWN® MALS instrument](#), typically 10 – 18 of the angular detectors will be useful, depending on the solvent and quality of chromatography. For example, in clean aqueous systems 16-17 detectors will be viable, whereas if the column is not well maintained and not properly equilibrated, only 10 might be useful while the others are too noisy. Organic solvents such as THF and toluene tend to be cleaner than aqueous so typically 17 – 18 angles will be useful with low noise and high accuracy.

*Q: Do you also have dynamic light scattering capability?*

A: We do not perform dynamic light scattering in house, but Wyatt supplies the equipment necessary for this analysis: the [WyattQELS™ embedded DLS module](#) for MALS instruments.

*Q: Is there a minimum  $dn/dc$  value that is useful to measure a polymer by MALS?*

A: It is possible to create a polymer-solvent system with  $dn/dc = 0$ , in which case the sample will not scatter any light and therefore MALS analysis is impossible. However, a typical value of 0.1 – 0.2



mL/g provides a robust scattering signal at typical SEC concentrations. Good measurements have been made with lower  $dn/dc$  e.g. 0.05 mL/g, though this changes the concentration of polymer necessary to achieve adequate RI response since the scattered intensity varies as  $(dn/dc)^2$ .

*Q: How do we do molar mass determination of polymer blends? For example- PCL/PLA?*

A: If the polymers have the same  $dn/dc$  value then there is no problem using SEC-MALS to analyze molar mass of the blend.

If the polymers have different  $dn/dc$  values, the molar mass distribution cannot accurately be determined by MALS from a single peak containing both polymers. If these are truly blends, not copolymers, and they have different  $dn/dc$  but the distributions of the two types do not overlap, then they may be separated based on size, and each polymer characterized as separate peaks in the same chromatogram. Alternatively, you could attempt to selectively dissolve the polymers and analyze each in separate SEC experiments.

*Q: Since light scattering intensity depends on refractive index increment and other factors such as shape factor both of which depend on molecular weight in separate ways, isn't it natural to have RI and LS signals that may not overlap?*

A: First, molar mass analysis by multi-angle light scattering does not depend on molecular shape. While the specific form of the angular dependence may differ for different sizes and shapes, molar mass is determined via extrapolation to zero angle which (if done correctly) is independent of shape and size.

The RI and LS signals will always partially overlap, but for polydisperse samples they will, by definition, not be aligned: The LS signal will be relatively higher at the leading edge, where MW is higher, and the RI signal will be relatively higher at the trailing edge, where MW is lower; the peak of each signal will be shifted correspondingly. For monodisperse samples, signals should perfectly overlay after applying band broadening and interdetector delay corrections.

*Q: Is there any molecular weight range for deciding model and degree used in ASTRA software and what is its impact on molecular weight determination?*

A: Molecules with  $R_g$  below 10 nm (roughly 50 kDa for random coils and 1 MDa for globular molecules) are considered isotropic scatterers, so no model or degree are necessary (degree can be set to zero). Above that, there are recommendations for selecting the model from Zimm, Berry or Debye if the conformation is unknown; ASTRA also incorporates conformation-specific models such as random coil, sphere or rod for better accuracy when the conformation is known.

Specifying an inappropriate model will result in inaccurate molar mass determination, so it is useful to review the goodness of fit, especially when  $R_g$  is relatively high, e.g. > 100 nm. Retaining



lower angles while deselecting the higher angles also reduces dependence on the specific model selected.

*Q: How reliable is LS measurement of MW for complicated architecture homopolymers/copolymers?*

A: The shape, conformation, branching, etc. of a molecule will have little to no effect on the accuracy of MALS analysis results for molar mass determination (with the above caveat about goodness of fit for large molecules).

For copolymers, the measurements are quite reliable as long as the comonomer ratios are consistent across the molar mass distribution. If this is not true, the polymer  $dn/dc$  may be different across the molar mass distribution and introduce error into molar mass calculations. However, if at least one of the building blocks of the copolymer has UV absorbance, the three-detector conjugate analysis method can be used to determine the copolymer ratio, and hence the correct average  $dn/dc$ , at each slice of the chromatogram in order to provide accurate MW.

*Q: What is the significance of % uncertainty in ASTRA result and is there any limit for it?*

A: The stated % uncertainty in ASTRA results is indicative of the precision of the measurement, and is calculated according to the baseline noise and variation of the signal across the detection angles and the selected peak region. In many cases this uncertainty can be a very small value, but it should not be interpreted as the accuracy or repeatability of the result – those depend on various external factors that need to be entered accurately into the software, such as sample  $dn/dc$  and instrument calibration constants.

*Q. You mentioned stereoregularity of PLA. Does this system separate or detect D- and L- versions of lactic acid?*

A. SEC-MALS is not able to separate or detect the difference between D- and L- lactides, as ideal SEC separates bases solely on molecular size.

*Q: Could be possible to analyze polymers in PET bottled water?*

A: This would theoretically be possible, but because PET is not remotely soluble in water, it would be wise to first measure any residue present after evaporating the water to see if anything is present at a significant concentration, and if so, try to identify the residue, perhaps by IR spectroscopy, before assuming that the water contains a significant amount of polymer in solution.



*Q: In the HA and polypeptide analyses, why do you include in the analysis, regions of the peak that have such low signals that the molar mass calculations are obviously incorrect? Can (or should) Results Fitting be applied to these?*

A: Results Fitting can allow the usage of noisy/erroneous data in the tails of the peak where the light scattering or concentration responses are very low, if you have confidence in the extrapolation. The word 'extrapolation', however, indicates that this is estimation, not measurement, and some clients do not want estimates.

Processing SEC-MALS data requires judgement about where and how to set baselines and integration limits, as setting limits where only robust data is produced may exclude a significant portion of the chromatographic peak area. If the entire peak is included, noisy/erroneous data may influence the results. It is examples like this where monitoring the  $M_w$  rather than  $M_n$  is vitally important, as the  $M_n$  and  $M_z$  values can change drastically depending on where you set your limits.

Additionally, it is important to remember that sometimes elution does not follow the expected pattern, due to anchoring or column interactions, and the molar mass of eluting species may truly rise in the tail of a peak as larger material elutes later than it would ideally.

## Sample prep and chromatography

*Q: 2x MIXED-H columns can cover HA MW range? Why not use MIXED-M?*

A: According to Agilent, the Mixed-M series covers a range of 1,000-500,000 g/mol, and HA regularly exceeds this limit.

*Q: How do you prevent aggregation?*

A: Aggregation is prevented largely based on what the suspected cause is. Preventing molecular aggregation begins with the mobile phase. It is largely prevented by increasing salt concentrations to act as an electrostatic shield, or by adjusting the pH to prevent ionization (or cause ionization to prevent hydrophobic interactions).

*Q: Are the small peaks in the polypeptide chromatogram evidence of aggregation?*

A: These small peaks also appeared in standard injections and seemed to represent some particles which were shedding from the columns due to the pressure pulse caused by injection.

*Q: Have you used mix of aqueous and polar organic to analyze the polymer?*

A: We have used this technique, but avoid it if possible. SEC analysis sequences can be long, and mobile phase solvent compositions can change if one of the mobile phase components is evaporating more quickly.



- Q: Regarding filtering versus centrifugation of polymers with MW > 1 million, is that a general rule for your lab or only applied to the hyaluronic acid work?*
- A: This is a general rule. Polymers over 1 million Da are subject to shear forces. That is not to say that any filtration of such large polymers will cause degradation, but this should be investigated if filtration is to be employed.
- Q: Which precautions should I take for heparin MW measurement?*
- A: For proper heparin analysis, the moisture content and cation content should be measured and accounted for. Heparin is commonly analyzed in aqueous ammonium acetate, and there are some good articles regarding this analysis in the literature.
- Q: Would the use of a small amount of linear alcohol along with salt help in better dissolution of materials like hyaluronic acid?*
- A: There aren't typically any issues with the dissolution of hyaluronic acid, due to its extremely hydrophilic nature, unless the hyaluronic acid has been crosslinked, in which case only degrading the molecule will aid in dissolution.
- Q: Would you recommend acidifying THF for the analysis of unknown polymer mixtures? Would you also suggest the same for salt and organic modifier use in aqueous systems?*
- A: You may want to be careful about adding an acidic modifier if you do not know whether the sample is susceptible to hydrolysis. We typically only modify a mobile phase if we expect that aggregation or column interaction is a possibility based on foreknowledge of the sample.