Live Webinar Q&A Sheet:

Analysis of viral proteins, nucleic acids and biomolecular interactions with light scattering

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 FFF may be found in the Wyatt website Resources section under Webinars, Application Notes, 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.

Q: *What is the molar mass resolution of light scattering in general? How does it compare to mass spectrometry?*

A: Light scattering usually provides accuracy of 3-5% and precision within 2%, so the molar mass of an antibody with a 5 kDa modifier should be distinguishable from a clean antibody without modifier. Different types of glycosylation cannot be identified with SEC-MALS, but it can be used for pre-screening to choose which samples are further analyzed via mass spectrometry. More typically SEC-MALS is used to analyze heavily glycosylated or otherwise modified proteins where mass spectrometry actually has difficulties.

Q: *What is the minimum amount of glycosylation that can be detected via SEC-MALS? Are those limits also valid for other types of modifications e.g. when labeling a protein with dyes or other modifiers?*

A: This also ties into the answer of the last question. The degree of glycosylation should also be at least in the range of 3-5% if SEC-MALS is used. Different types of glycosylation cannot be identified with the system. However, it provides an easy first-pass analysis of molecules and can be used for pre-screening to choose which samples are further analyzed via mass spectrometry. The same is true for other types of modifications. If a modified protein is analyzed with the ASTRA conjugate analysis, the overall change in the molar mass should be equal or exceed 3-5% of the protein molar mass.

Q: *There was an example shown for SEC-MALS but can the method CG-MALS also be used for nucleic acids e.g. mRNA or Protein-DNA complexes?*

A: Yes, it can. CG-MALS only needs the concentration of the stock solutions in mg/ml and the dn/dc values of the sample components. Like most macromolecules, the dn/dc for DNA and RNA is constant for a given solvent, wavelength of light, and above a certain threshold molar mass

(typically a few thousand g/mol). The dn/dc used for all the DNA data in this presentation was 0.17 mL/g. The concentration needs to be known and can be either entered manually or each plateau can be measured by including e.g. a RI or UV-detector to the set-up. From that point RNA or DNA behaves just as normal molecule that scatters light and it can therefore be analyzed. A protein-DNA complex can be analyzed just as a protein/protein complex.

Q: *What happens when the conjugate analysis is used for protein-polymer complexes, where the polymer shows a certain size distribution?*

A: What can be expected for the modifier is a molar mass distribution across the peak from large molar masses to lower molar masses, depending on the quality of separation, similar to measuring a broadly distributed polymer. The molar mass distribution itself depends on the degree of modification and also on the polydispersity of the modifier. SEC-MALS does very well with such samples, provided that the molar mass differences between products are not too small and at least exceed 3-5% of the total molar mass. ASTRA also does provide several tools to better judge and compare such samples e.g. by distribution plots, that display cumulative or differential weight /number fractions. It might also help to look at Mn, Mw, Mz to better judge differences between polydisperse samples or batch-to-batch variations

Q: *Why do you recommend a refractive index detector? Is it only required for conjugate analysis? Usually we only use UV-detection for our proteins. Does it have any other advantages when used as concentration detector?*

A: The applications for a dRI-detector are quite broad. e.g. glycans on a glycoprotein, DNA-protein complexes, the DNA-load in AAVs, membrane proteins, PEG and other components that might be conjugated to a protein. Even if a dRI is just used as alternative concentration detector, it provides the concentration even for unknown proteins, in contrast to a UV-detector where the UV-extinction coefficient of the protein has to be known. Additionally, with a dRI detector, UV-extinction coefficients and dn/dc values can be experimentally determined with ASTRA.

Q: *How much sample consumption do I have to expect for CG-MALS experiments and what is the sensitivity when compared to SEC-MALS?*

A: This depends on the type and strength of the binding events. If you look at specific interactions with high affinities (nM range), the required protein concentration is quite low with a few hundred micrograms of protein. If you try to analyze non-specific binding with weak affinities, significantly more protein might be required, since interactions often cannot be readily observed at very low protein concentrations. The sensitivity is comparable to a SEC-MALS set-up. However,

since no separation is performed in batch experiments, a molar mass change of at least 5-10% is recommended for the analysis by CG-MALS.

Q: *Does CG-MALS also work for very high protein concentrations and /or the interaction of the sample with smaller excipients?*

A: Very high protein concentrations of hundreds of mg/ml were analyzed previously with CG-MALS for the analysis of non-specific interactions within the sample that often cannot be seen after diluting the injected sample on a SEC-column.
It is also possible to measure excipient interactions if the excipient is large enough to cause a significant molar mass change upon interaction (e.g. if poloxamer). The total molar mass change should be at least 5%, better 10%. As long as these requirements are met the analysis would work.

Q: *If you separate your sample using a different type of chromatography (reverse phase instead of SEC) will the MALS calculations still be accurate?*

A: MALS- detectors can be used in any chromatographic set-up e.g. SEC-MALS, IEX-MALS, HIC-MALS and also RP-MALS. Usually we use a UV-detector as concentration source in case of gradient runs. But if a dRI-detector has to be used (e.g. for non UV-active samples), ASTRA also offers the means to correct the dRI-baseline with a baseline subtraction method to compensate for occurring gradient effects. For the correction, a blank run is subtracted from the experiment which will compensate for an increasing baseline, which results from the change of the refractive index of the solvent during a gradient, while the reference cell of the dRI-detector is still filled with the starting solvent. This correction is often not necessary for a SEC-MALS-UV set-up.

Q: *With so many methods gives different values which do you report to regulatory? All of them?*

A: This question probably refers to the introduction slide to SEC-MALS, where the results of different methods were compared. What has to be reported, depends of course on your type of sample, how it will be used etc. If you have more specific questions about this topic, feel free to contact us for more insight.

Q: *What does the trailing indicate on an SEC-MALS chromatogram?*

A: Trailing of the signal can have various reasons. The column might be old or compromised, the sample might interact with column material and slowly elute over the duration of the run, aggregates might be present and “smear” over the column or the sample might stick to the respective flow-cell. Getting rid of trailing is part of method development but not a MALS-specific occurrence. However, using light scattering will help to evaluate the reason for the trailing e.g. you can make the distinction between a co-elution of aggregates and simple column-interactions where the sample is still monodisperse and unchanged.

Q: *What is the suggested sample mass to inject for MALS detection? For example, for a 10 kDa sample.*

A: This depends on the type of HPLC, the column and the size of the sample. Light scattering is not only related to the concentration of the sample but also its size. If an antibody of 150 kDa is analyzed, we require less material than for a peptide. If a 10 kDa sample is analyzed with a UHPLC-set-up, it should be sufficient to work with 5-10 µg injections, since the columns dilute the sample to a lesser degree. If a standard HPLC-setup is used, I would recommend starting with 10 µg injections and increase the amount if necessary. Another consideration can be how clean and well-maintained the system is in order to achieve the highest possible signal-to-noise ratio. But injection amounts for peptides or small samples should rarely exceed 100 µg, even under difficult conditions. Usually, the larger injection amounts are only necessary to detect oligomers, which naturally are less abundant than the main species. We are always ready to evaluate if SEC-MALS works well for your samples by doing test-measurements or instrument demonstrations to show the performance of our detectors.

Q: *How are experimental MW values shown in plots processed to report a single value?*

A: ASTRA will report different molar mass moments. The two most common statistical approaches for describing the molar mass are the number-average and the weight-average, followed by the z-average. When applied to a molar mass distribution, these respective molar mass moments are Mn, Mw, and Mz. Those are differently weighted towards lower or higher molar masses and this is mostly of importance in characterizing broadly distributed polymers. If the molar mass shows a distribution, the polydispersity can also be reported by ASTRA. If working with proteins, which are monodisperse (mostly), those numbers should all yield a similar result, since there is no distribution within the peak.
If a peak region is defined in ASTRA (which will be visualized with a molar mass plot over the peak area), the average molar mass of the selected region will be calculated and reported. ASTRA determines for each data slice the concentration and the molar mass of the sample from the light scattering intensity and the concentration and calculates MW as follows:

$$\bar{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} = \frac{\sum c_i M_i}{\sum c_i}$$

Q: *Does the CG-MALS can detect binding between a protein and a small molecule?*

A: As long as the total amount of small molecules bound exceeds 5-10% of the protein molar mass, CG-MALS can be used. If the system is extremely well maintained, even changes of 3% might be detectable.

Q: What is the minimum concentration required for affinity measurements? And what is the range of KDs that can be resolved?

A: This depends on the affinity between the binding partners. If the binding is strong (nm-range) a few hundred µg of sample material might be sufficient to see an increase of the average molar mass of the sample. If the binding is weaker (e.g. for non-specific interactions) it might be necessary to increase concentrations significantly to see a consistent binding event and 10-100 mg of material might be required. Generally, the range of measurable affinities goes from ~100 pM to 1 mM for typical 100 kDa proteins.

Q: When comparing associated versus unassociated states of virons and antibodies, etc. do you have to account for changes in the dn/dc?

A: The aggregation state does not have an influence on the dn/dc. The values that are used are the same for associated samples and unassociated samples. If a sample consists of different components (e.g. a pegylated protein) a conjugate analysis has to be performed (e.g.with the known dn/dcs of PEG and the protein) or a combined dn/dc has to be entered. If such a protein is to be used in a CG-MALS experiment to investigate binding to another protein, a combined dn/dc would also have to be entered for molecule A and standard values can be used for the interacting protein, which would be molecule B.

Q: What concentrations and volumes are needed for CG-MALS and how many different concentrations?

A: Usually a typical CG-MALS experiment requires a few milliliters of sample if performed with the Calypso hardware, since the flow cells of all downstream detectors have to be filled. Alternatively, if not enough volume is available, a batch DLS/SLS-instrument (DynaPro NanoStar) can be connected to the Calypso software and the experiment can be performed in calibrated quartz cuvettes which requires much less volume (1.5 µl minimum).
The required concentrations depend on the strength of the binding, which was already answered above.

Q: Is necessary use standard molecular weight particles to calibrate the size distribution range?

A: Wyatt MALS-detectors do not require molecular weight standards for the determination of the molar mass. A Wyatt MALS-detector is stably calibrated with toluene, a strongly scattering solvent. This circumvents issues with typical molar mass standards and is the most unbiased way to generate a calibration constant. This calibration constant, which is saved on the instrument, is stable for at least one year and is usually renewed during technical service. However, the

calibration is a simple software-guided procedure that can also be easily performed by the user.

Q: *What about other HPLC methods like Ion-exchange? Does the salt gradient interfere with the MALS measurement?*

A: The salt gradient in IEX-MALS typically does not interfere strongly with measurements, since neither UV nor light scattering are very sensitive towards changes in the ionic strength of the buffer. However, if a dRI-detector like the Optilab is used as concentration source, the salt gradient will interfere with the baseline, since the content of the reference cell cannot be "updated" during the gradient. For such cases, ASTRA contains a baseline subtraction method, which allows subtracting a blank run from the collected data. This procedure corrects for any gradient effects in the raw data.

Q: *Is it possible to characterize inorganic molecules and similar interactions this method?*

A: The hard- and software is quite versatile and can handle almost any samples, which fall into the size range that is stated in the specifications. SEC-MALS and CG-MALS can also be used for polymers with organic solvents e.g. THF, HFIP or toluene as mobile phase. The only prerequisite for an analysis is that the dn/dc of the sample is known. If polymers are the sample of interest, a SEC-MALS system can also be upgraded with a viscometer to gain additional information e.g. about branching. The ASTRA conjugate analysis can also be used for the analysis of co-polymers in a similar way that was shown for glycosylated proteins. CG-MALS can also be used for polymers and organic solvents.

Q: *What are the required or minimal "knowns" or inputs needed to perform aggregate analysis?*

A: This depends on the system set-up. If a combination of HPLC, UV and MALS is used, the minimal required "knowns" for a molar mass analysis (e.g. dimers and oligomers) are:

1. The UV-extinction coefficient of the protein to determine the concentration if an UV-detector is used. This value depends on the protein sequence.
2. The dn/dc, which is universal for protein samples with a value of 0.185. If the concentration of the sample is measured with a dRI-detector instead of a UV-detector, the only required known

input for the analysis is the dn/dc of the sample, which is universal and can be used for most standard conditions.

If large aggregates (e.g. 100 nm) elute early and do not give a concentration signal, their size (rms radius) can still be analyzed just with the MALS-detector. So, in the very least, the user still can extract a size range for those large aggregates or particles.

Q: *How accurate is the DLS measured molar mass for oligo aggregates compare to the Mass spectrum or other method?*

A: Dynamic light scattering is a diffusion-based method and does not output the molar mass of a sample but the translational diffusion coefficient Dt. The hydrodynamic radius is then derived from Dt via the Stokes-Einstein equation. For SLS measurements with a MALS-detector, light scattering usually provides accuracy of 3-5% and a precision within 2%.

Q: *Do you think that data obtained with circular dichroism would be useful for comparison with your results?*

A: Since circular dichroism provides information about the secondary structure of proteins it can certainly be useful to shed some additional light on observations in CG-MALS or SEC-MALS.

Q: *Are the vGP and trimeric spike proteins essentially tetrameric or dimeric or trimeric when they interact with surface receptors (ACE -glycoprotein and can it be determined by CG-MALS/UV/RI in case of SARS COV- spike proteins as well?*

A: Such interactions can be investigated via CG-MALS, if the interaction partners are available in a purified form. The Calypso-software provides a variety of binding models to cover a large variety of interactions. The main consideration is that the concentrations of the binding partners and the dn/dc values must be known to calculate accurate molecular weights.
The dn/dc value of a heavily glycosylated protein ranges somewhere between the dn/dc of the protein and the dn/dc of the glycans. Often that value can be experimentally determined with a dRI-detector or estimated, if the degree of glycosylation is known. If the degree of glycosylation is not known, a conjugate analysis would provide that data.