# Live Webinar Q&A Sheet:

# Analysis of viral proteins, monoclonal antibodies and their interactions with light scattering

The recorded webinar may be viewed from the Biotherapeutics webinars page. These questions were submitted by live viewers. Additional information on SEC-MALS and CG-MALS 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 light scattering questions

Q: *What is the resolution of light scattering? Can I also detect small changes e.g. 5 kDa of a 150 kDa protein? What is the minimum amount of glycosylation that can be detected via SEC-MALS and can the system distinguish between different types of glycosylation?*

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 difficulty.

*Q: What do you need a refractive index detector for? Is it only for conjugate analysis? Does it have any advantages in comparison to an UV-detector?*

A: Conjugate analysis is certainly one important use of a refractive index detector. It offers several other benefits:

1. It is a universal concentration detector that works with samples that have no chromophores or fluorophores and thus are “invisible” to a UV or fluorescence detector.

2. For the vast majority of pure, unconjugated proteins, the refractive index response (dn/dc) is the same to within 1-2%. That means you can analyze unidentified proteins or those for which the absorbance coefficient is unknown.

3. Taking advantage of #2, it is straightforward to combine UV detector and Optilab refractive index detectors in order to determine empirically the UV extinction coefficient of a sample. ASTRA software includes a method for doing so.

Q: *How can the refractive index dn/dc be the same for all proteins?*

A: The dn/dc values are the same to within 1-2% for about 99% of all proteins. This is simply the result of proteins consisting of a large number of amino acids with quasi-random distribution: each amino acid has its own dn/dc value, between 0.165 and 0.277, and the protein's dn/dc is simply the weight average of these. However, if a specific protein has a predominance of amino acids with high dn/dc (tyrosines, tryptophan, phenylalinine) then it will have a higher dn/dc value, and if it consists of a predominance of low-dn/dc amino acids (proline, alanine) it will have a lower value. Please see Zhao et al, *Biophys. J.* 100:2309-2317 (http://dx.doi.org/10.1016/j.bpj.2011.03.004) for more details.

Please note that dn/dc depends on the refractive index of the buffer. High concentrations of excipients such as sucrose or arginine will typically reduce dn/dc relative to water or PBS.

Q: *Are these techniques only suitable for crystalline substances, since we need refractive index of the substance in the equation?*

A: No, in fact they are primarily suitable for fully dissolved substances. The equation requires the refractive index of the solvent, and also *dn/dc*, the specific refractive index increment of the solute, which relates changes on the refractive index of the solution to the solute concentration.

Q: *Can the method be used to measure a fluorescently-labeled protein in plasma? Can the instrument tolerate a dirty biomatrix?*

A: CG-MALS is never appropriate for plasma or dirty biomatrices because all the components scatter light, and the scattering signal from the protein will be buried in the noise; no separation takes place to remove the protein from the other species present. The instruments will typically be fouled by such solutions.

SEC-MALS is generally not appropriate for plasma or dirty biomatrices, which will foul the column.

Wyatt offers a different separation technique, field-flow fractionation, which could be amenable to plasma or dirty biomatrices since it does not have a packed stationary phase. It is also possible to combine MALS and the other detectors with ion-exchange columns for separation, which can tolerate dirty solutions. Then the question is, if the detectors can handle the matrix. Plasma should be ok, crude lysate is not recommended.

The MALS detector will be fine with a fluorescently-labeled protein if the label is not excited by the 658 nm laser incorporated in this instrument. If the label is excited by this wavelength, Wyatt can offer several solutions to eliminate the fluorescent signal in the MALS detector.

Q: *How does SEC-MALS cope with PEGlyated proteins where the products are of mixed mass, centered about a mean?*

A: SEC separates by hydrodynamic size, which for these samples is dictated primarily by the PEG. Upon separation you will see a broad peak, with each eluting fraction consisting of molecules within a small range of sizes. The outcome of the analysis will indicate how the molar mass and (average) protein fraction varies across the peak, and can be converted to a quantitative distribution of molar masses. It can also be converted to different moments, i.e. different types of average molar mass: weight-averaged, number-averaged or z-averaged.

Q: *When you measure conjugates, do you need to know individual (protein and modifier) dn/dc values or dn/dc of the whole conjugate?*

A: In order to perform a "conjugate analysis", which provides the molar mass of each component, the individual values – *dn/dc* and extinction coefficient - must be known for each component, and you need MALS + UV + RI detectors. The conjugates overall dn/dc is actually calculated in the course of this procedure and provided in the report.

Alternatively, if you just wanted the overall molar mass of the molecule, you could do so with MALS + RI and knowledge of the conjugate's overall *dn/dc*.

Q: *Might mAbs aggregate inside the column during SEC-MALS run?*

A: This is, in part, dependent on the choice of mobile phase and column packing, but usually the probability of aggregation is low. Mostly the opposite is of concern: aggregates might be filtered out by a frit or interact with column material, or dissociate due to shear or dilution. Therefore columns sometimes remove aggregates from a solution.

## CG-MALS

Q: *What is the sensitivity of the Calypso system? How much sample consumption do I have to expect for CG-MALS experiments?*

A: This depends on the strength of the binding events as well as the molar mass of the proteins. For specific interactions with high affinities (0.1 – 100 nM range), for example antibody-antigen binding, the required quantity is typically 100 micrograms of each protein. Weaker interactions require higher concentration and hence more material; a 1 µM interaction between proteins on the order of 25 kDa might require a few micrograms of each.

Q: *Is there a difference to reported values of other methods to measure intermolecular interactions e.g. Biacore or fluorescence-based methods?*

A: CG-MALS is has been shown to be quite equivalent to other first-principles, label-free, solution-based techniques like sedimentation equilibrium (SE-AUC) or isothermal titration calorimetry

(ITC) for standard interactions in terms of the reported $K_d$ values. ITC often cannot correctly analyze complex interactions such as multi-valent interactions or simultaneous self-and hetero-association, which can be analyzed by CG-MALS, and so discrepancies in reported results will arise for such interactions.

CG-MALS has also been shown to provide very similar $K_d$ results as surface plasmon resonance (SPR/Biacore) when immobilization does not affect the binding properties of the proteins and avidity effects related to multi-valent analytes do not occur. These are easy to check since using different SPR chips or immobilization schemes, or reversing the ligand and analyte, will provide different results when such phenomena do pop up. Of course they are not an issue for solution-based techniques. In general, SPR is only suitable for analysis of 1:1 binding, whereas CG-MALS covers self-association and multivalent hetero-association.

Q: *Can CG-MALS also determine the stoichiometry of more complicated protein complexes? What happens if I have a heterodimer that binds another protein?*

A: The CALYPSO CG-MALS software only analyzes reversible self-interaction or formation of complexes between two interaction partners. If the hetero-dimeric complex is irreversible or at least stable at the concentrations required to measure its interaction with the third protein, it can be treated as a single entity, and the analysis can be performed. However, if the complex dissociates at these concentrations, quantitative analysis cannot be performed with CALYPSO. However, the data may be exported for analysis by custom software e.g. a Matlab routine written specifically for this experiment.

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

A: Yes, CG-MALS can be used at 100 mg/mL and higher, typically for analysis of weak self-interaction or interactions with excipients. The software can take into account thermodynamic non-ideality related to the protein's excluded volume.

Wyatt offers the Optilab HC, which measures protein concentrations up to 180 mg/mL, and the DAWN MALS detector can typically measure these concentrations as well. However, if the solutions have viscosity above ~ 5 cP, the Calypso composition-gradient system will not provide good mixing and sample delivery to the flow cell. In such cases the measurements are carried out in a cuvette with a manually (or otherwise externally) prepared concentration series.

In order to measure protein-excipient interactions, or interactions between any species that differ significantly in molar mass, the change in molar mass between the large component (protein) and complex must be at least 5%, preferably 10%. For example, binding of a single 5 kDa poloxamer to a 150 kDa IgG is insufficient, but binding of two or more poloxamer molecules will provide a large enough change.

*Q: Are the detectors used for CG-MALS the same as for operation with a HPLC? Is it possible to upgrade an existing system with CG-MALS?*

A: Yes, the detectors are the same; if you have a SEC-MALS setup, you can create a CG-MALS system simply by disconnecting the tubing at the output of the HPLC column and connecting it to the output of a Calypso composition-gradient system. While a three-detector system is preferred for SEC-MALS analysis of proteins and other biomolecules, CG-MALS uses MALS plus either a UV or Optilab refractive index detector, depending on the application.

*Q: Are there any limits for CG-MALS? Could it be used for DNA-polymer complexes?*

A: CG-MALS can also be used to analyze the stoichiometry and equilibrium dissociation coefficient of a DNA-polymer complex. The primary limits are the range of $K_d$ values, which depends on molar mass of the constituents; the relative molar masses (see previous question regarding excipients); and the number of molecules in the complex (a few is OK but the analysis may not be satisfactory for more than a couple of dozen).

*Q: Can you look at temperature dependent associations / interactions? For example, looking at filament formation that is temperature dependent.*

A: Yes, but with some caveats. Typically in CG-MALS only the DAWN flow cell's temperature can be controlled, but not the stock solutions in the Calypso or the tubing between the Calypso and MALS detector. Hence temperature control only begins once the sample reaches the flow cell. If the filament formation is dependent on the history between the time of mixing solutions in the Calypso and reaching the flow cell (usually on the order of 5 - 10 seconds), this might not be an appropriate method. If it is a fully reversible interaction and independent of that history, CG-MALS could be appropriate.

CALYPSO software does not control the MALS temperature and the DAWN is not an ideal instrument for variable-temperature measurements. If the idea is to vary the temperature in the course of the measurement, then a better option would be the DynaPro NanoStar, which can perform temperature ramps quite readily. Both dynamic and static light scattering are measured by the NanoStar.