



**WYATT**  
TECHNOLOGY

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Live Webinar Q&A Sheet:

## Optimizing Protein Biotherapeutic Formulations with the Light Scattering Toolkit

The recorded webinar may be viewed from the [Biotherapeutics webinars page](#). These questions were submitted by live viewers. Additional information on MALS, DLS, 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](mailto:info@wyatt.com) with any additional questions.

### Polymer Applications

Q: *Which light scattering method is appropriate to analyze biopolymers?*

A: The most common light scattering method for general biopolymer analysis is SEC-MALS with a DAWN HELEOS MALS detector and Optilab T-rEX dRI detector, for determination of absolute molar mass and size distributions as well as conformation and branching. Additional analyses may require extended methods. For example, FFF-MALS extends MALS analysis to biopolymers that are not amenable to SEC. The addition of a ViscoStar differential viscometer is useful in analyzing conformation and branching of smaller biopolymers with  $R_g < 10$  nm.

Batch DLS using a DynaPro NanoStar or Plate Reader is also useful for biopolymer characterization, especially if the conformation is known to be globular or random coil. However, batch DLS will only provide an average value or semi-quantitative distribution.

Q: *How can I monitor polymer synthesis?*

A: If the reaction is relatively slow then the best approach is to periodically extract aliquots and run them through SEC-MALS in order to determine the molar mass distribution. As the reaction approaches its end point you will see an appropriate increase in molar mass, and can determine the optimal distribution in terms of  $M_n$ ,  $M_w$  and/or  $M_z$  for quenching the reaction. For faster reactions you might prefer the faster separations afforded by APC using a  $\mu$ DAWN and Optilab UT-rEX.

For much faster reactions requiring monitoring on a second time scale, or when the full distribution is not necessary, it is possible to monitor  $M_w$  by unfractionated MALS analysis. Simply run the sample continuously through a MALS and RI detector to calculate the weight-averaged molecular weight  $M_w$ .

## DLS

Q: *What concentration range can be used in DLS using Dynapro II?*

A: The concentration range is a function of size or molecular weight.

The lower limit of concentration may be calculated directly from the sensitivity specification, 0.15 mg/mL lysozyme. Just divide 0.125 mg/mL by the ratio of the molar mass of your molecule to that of lysozyme (14,400 Da). For example, a mAb with molar mass of 144,000 would have a lower limit of sensitivity on the DynaPro Plate reader II of 0.0125 mg/mL.

The upper limit of concentration is somewhat trickier to assess a priori, but with a maximum attenuation upwards of  $10^5$ , an unaggregated or lightly aggregated mAb could be measured up to hundreds of mg/mL. Analysis of such high concentrations, though, is not well established due to theoretical limitations. For particles the upper limit of accurate analysis is heavily dependent on size. Please contact Wyatt for more specific answers.

Q: *While freeze thaw study of monoclonal antibody, in DLS analysis, radius changes from 5.8 to 6.7 after 25 cycles of Freeze thaw. What will you conclude from this change in radius - is this significant? How can one verify that a conformational change or change in  $R_h$  is significant?*

A: This is generally considered a significant change, because the typical standard deviation of mAb  $R_h$  in repeat measurements should be on the order of 0.1-0.2 nm. The best way to confirm this is to make multiple measurements under identical conditions and verify that your actual standard deviation is well below the change you see.

Another parameter you should be looking at is the %Pd. If your mAb has undergone partial aggregation then you will see an increase in %Pd as well as a shift in the average  $R_h$ .

Q: *In DLS, if we see two peaks, can we determine the molecular weight of those two peaks separately?*

A: First let's recognize that DLS does not determine molecular weight, it determines the hydrodynamic radius, a measure of size. You can *estimate* molecular weight by assuming a model such as globular or random coil, which converts size to molecular weight. Given that assumption, you can apply it to both peaks, apply different models to different peaks, or make any other reasonable assumption to estimate molecular weight based on size—but DLS will not give you absolute molecular weight, regardless of how many peaks you see.

Q: *Why can the DynaPro give 110nm size instead 90nm to ps std (90nm) at 25c in 0.89 viscosity?*

A: One possibility is an error in the viscosity. Another is partial aggregation.

## SEC-MALS

Q: *Is the method used for protein-conjugate analysis different from that used for standard molar mass determination?*

A: Yes. Standard molar mass determination requires a single concentration detector, usually either RI or UV. Analysis of protein conjugates or copolymers requires two concentration detectors, usually RI and UV. One way to think about this is, in standard molar mass determination, the  $dn/dc$  of the molecule is known, whereas in the conjugate analysis, first we determine  $dn/dc$  by means of the two concentration detectors, and then proceed to calculate the molar mass.

Q: *Can SEC-MALS be used to determine molecular weight of 12 nm protein (expected molecular weight 480,000 Da)?*

A: Certainly, SEC-MALS is the go-to method for characterizing such molecules without making any assumptions about conformation.

Q: *What is the lowest concentration of target proteins that can be detected for aggregation?*

A: It all depends on molar mass of the aggregate as well as the technique used, see the response to a previous question. In general MALS is about 100x more sensitive than DLS, though SEC does tend to dilute by ~5x-10x which means SEC-MALS or FFF-MALS is only 10x-20x more sensitive than batch DLS relative to the stock concentration.

The sensitivity of a DAWN HELEOS is the highest, 0.2  $\mu\text{g/mL}$  BSA. Considering an aggregate consisting of 1000 IgG molecules, the sensitivity would be about 90  $\text{pg/mL}$ .

Q: *Does SEC-MALS or DLS take into consideration the actual shape of the macromolecule? In other words does it assume the molecule to be globular?*

A: Molar mass determination by SEC-MALS makes no assumptions regarding shape. DLS only determines size and therefore at best can estimate molar mass by assuming a shape, e.g. globular or random coil.

## CG-MALS

Q: *What are the viscosity and concentration limits for analysis of self-association measured using CG-MALS?*

A: Concentration per se is not a real limit for the analysis, it is viscosity. The Calypso II can mix samples with viscosities up to 5-6 cP. Higher concentrations are both too viscous to push through the tubing, and also tend to foul the flow cell. If higher viscosities must be analyzed then it is possible to pre-mix the requisite concentrations manually and load them onto the 10  $\mu$ L MALS cuvette, using the CALYPSO software to acquire and analyze the data.

Q: *Can we set the temperature in CG-MALS?*

A: If you have a temperature-controlled DAWN, the sample temperature may be controlled in the flow cell so that the light scattering measurement is made at the desired temperature. Prior to reaching the flow cell the temperature is generally not controlled.

If you need to keep the proteins cold before injection into the detectors, the conical tubes holding the stock solutions in the Calypso II may be placed in ice baths.

Q: *Can we do the self-association and cross-interaction analysis on batch-mode SLS measurements without using a Calypso? Can we simply install the software to do the analysis?*

A: Yes, it is possible to purchase the CALYPSO software separately from the Calypso II composition gradient system, and run self-association and cross-interaction analyses with manual sample preparation. A technical note on the Wyatt Support Center at [www.wyatt.com/Support](http://www.wyatt.com/Support) explains how.

Q: *What is the minimum protein concentration you need for MALS? I have a protein whose monomer is only about 6kd and it tend to dimerize or tetramerize when the protein concentration is increased, can we use one of your instrument to monitor this concentration dependent process?*

A: In order to monitor this kind of self-association, you would use a CG-MALS system consisting of a Calypso II, a DAWN HELEOS II and a UV detector. We have used this system to monitor self-association of a 3 kDa peptide with a minimum concentration of just a few  $\mu$ g/mL (diluted down from 10x solution by the Calypso), as well as insulin (6 kDa). A minimum concentration of 30  $\mu$ g/mL of a 6kDa protein could be considered fairly routine and has been carried out regularly by Calypso users in this field.