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Live Webinar Q&A Sheet:

Screening and Characterizing Biomolecular Interactions via Light Scattering for Biotherapeutic R&D: Part 2 – In-Depth Characterization by CG-MALS

The recorded webinar may be viewed here. These questions were submitted by live viewers. Additional information on CG-MALS and the Calypso II may be found on the Wyatt Library under Webinars, Application Notes, Featured Publications and Bibliography, as well as on the corresponding Product and Theory pages of our website.

Please contact info@wyatt.com with any additional questions.

Q: *What are the parameters to select Protein X and Protein Y concentration? What is the optimum concentration for MALS studies? Could it be possible to analyze the stoichiometry of two proteins, if they will interact in 3:1 ratio? What could be the best ratio for two different proteins? K_D is about 5μM.*

A: The appropriate concentration for a CG-MALS experiment, like any interaction analysis, depends on the actual affinity and stoichiometry as well as molar masses. When the proteins are not too disparate in terms of molar mass and the stoichiometry not too different from 1:1, the optimum concentrations of the stock solutions are 5-10x K_D for hetero-association and 2-3x K_D for self-association. Those solutions are then mixed and diluted at each step.

If the molar masses are disparate or the expected stoichiometric ratio greater than 2:1, we will typically use the simulation page in the CALYPSO software to design an optimal gradient which balances a few parameters.

It is straightforward to analyze a 3:1 interaction and discriminate from other stoichiometries, as shown in slide 15. If the molar masses of the two

proteins are similar, a good choice of stock solutions for K_D ~ 5 μM would be ~60 μM : 30 μM.

Q: *What is the tightest antigen/mAb interaction that has been measured by CG-MALS (assuming a 50 kDa antigen and 150 kDa mAb: 1 pM, 10 pM, 100 pM, 500 pM)?*

How low in protein concentration can CG-MALS typically work?

A: The lowest concentration and K_D depend on molar mass, since light scattering intensity is proportional to molar mass * (mass/volume) concentration. With sufficient care in filtering and preparing the buffers and samples, etc., you can measure antibodies below 1 μg/mL and so characterize K_D down to ~ 100 pM. For smaller proteins on the order of 10 kDa, the lower limit is ~ 1 nM, and for peptides on the order of 1 kDa, 10 nM.

So far the tightest antigen/mAb interaction we have measured was 300 pM. We should be able to go to 100 pM but have not yet seen an appropriate sample.

Of course, the limit of sensitivity depends on the detectors as well as sample preparation. Wyatt MALS detectors are exquisitely sensitive and usually limited by sample and solvent cleanliness rather than by the detector. UV detectors are usually not quite as sensitive as our MALS detectors but there are ways to overcome this.

Q: *Is it capable of measuring the dn/dc in real time? If not, how do you measure it in order to calculate the M_w?*

A: It is possible to combine, in a single gradient, analysis of dn/dc and interactions. However, dn/dc is usually known so this is not necessary. Most unmodified proteins in typical aqueous solvents have the same dn/dc to within 1-2%, and the same is true for most oligonucleotides. If, e.g., the protein is glycosylated with a known ratio of sugar to protein, dn/dc can be calculated readily.

If you have a complex buffer, e.g. high salt concentration, or an otherwise unknown dn/dc, it must be measured. That is accomplished by injecting solutions with accurately known concentrations into the Optilab T-rEX differential refractive index detector to measure the change in RI (dn) per unit change in concentration (dc). The Calypso can automate this process if provided a single stock solution with accurate concentration and the appropriate diluent.

The same gradient, analyzed differently, may be used to characterize self-association.

Q: *Can you measure CG-MALS under step gradient of salt concentration instead of step gradient of two proteins?*

A: Certainly you can mix three components any way you like and make the measurement, the question is what the analysis might tell you. If the analysis algorithms in the CALYPSO software do not suit your needs it is easy to export the processed MALS data to a text file for your own math software such as MatLab®.

One way we utilize the capability to work with three solutions is to measure self-association as a function of buffer modifier. This is done by setting up a series of protein concentration gradients. A fixed amount of buffer modifier, e.g. high-salt solution or acidic solution to change pH, is added to each concentration step, and the fraction of buffer modifier is varied at each new gradient. The entire series is automated so you can set up a large variety of conditions, run over several hours or overnight, walk away and then come back at the end to analyze the data. A method template to do this is provided with the software, as are templates for other types of gradients.

Q: *Are there opportunities for small molecule-protein binding dynamics with CG-MALS?*

Does the technique also work for small molecule-protein interactions; is there any examples of this?

If the size difference between binding partners is severe can this system effectively measure binding and if not what are the limitations?

A: Because CG-MALS measures changes in the solutions' weight-averaged molar mass, it will generally not be sensitive to a single small molecule binding to a protein, or a single protein binding to a liposome. The general comfort range for the analysis is a change of at least 10%, though with care this can be extended. A 10% change could mean forming a complex between a 10 kDa protein + a 100 kDa protein, or between 5 peptides of 2 kDa each + the same 100 kDa protein.

There are certainly opportunities to look at small molecule-protein interactions, including dynamics, if the small molecule modulates a protein-protein interaction. A nice example is presented in this application note.

Q: *What the typical sample consumption for a CG-MALS interaction experiment? What total protein masses did this antibody-antigen experiment use?*

A: Depending on the complexity of the interaction, between 3-10 mL of stock solution where the stock concentration is as described above. That translates into a typical consumption of 100-200 μg for an antibody-antigen interaction with K_D on the order of tens of nM or less. If K_D is in the hundreds of nM, that would be more like 500-1000 μg, or 50-100 μg if the molar mass is ~ 10 kDa.

Q: *Is it possible to input your own model for fitting the data?*

A: The software provides some standard models, including monomer-dimer, isodesmic self-association, and 1:*n* hetero-association assuming equivalent binding sites. Beyond that, it allows you to build an enormous variety of complex models that combine multiple oligomers, multiple hetero-complexes, self-associating hetero-complexes, and certain kinds of constraints. It also provides correction for thermodynamic non-ideality and inactive fractions of stock solution.

If even those possibilities do not cover your needs, it is straightforward to export the processed data to a text file that can be imported into your favorite math software for fitting to models you build.

Q: *What do you mean by non-specific interaction? Do you have a range of Gibbs free energy values to define this?*

A: I think the distinction is more subtle than a range of free energy values, though there is some correlation. 'Specific interactions' means that there is a well-defined binding site on each binding partner, and once that site is occupied by the other partner, it is saturated and cannot interact with another molecule.

'Non-specific interactions' refers to the overall interactions due to a variety of charges, hydrophobic patches, dipoles, etc. spread across the surface of a macromolecule, as well as hard-core repulsion, van der Waals interactions and all the rest. The net interaction depends on intermolecular distance and orientation. In general these do not result in a complex with well-defined stoichiometry, and generate repulsion as well as attraction; depending on the concentration, the net behavior can even switch between repulsive and attractive.

The magnitude of typical non-specific interactions is equivalent to a K_D on the order of 1 mM, but they could be stronger e.g. between highly charged proteins in a low-ionic strength buffer.

Q: *Does the hydrodynamic radius of a protein depend on pH, provided that the salt concentration is constant across the pH range?*

A: To clarify, CG-MALS measures absolute molar masses (M_w), not hydrodynamic radii R_h (dynamic light scattering does that). R_h is of interest in CG-MALS at high concentration, in order to account for non-ideality.

With that said, there are two aspects to this question: apparent R_h and actual R_h. The actual R_h may certainly change with pH, e.g. if the protein unfolds. The apparent R_h – the value calculated in DLS by converting the measured diffusion constant – is in general dependent on intermolecular interactions and so may change with concentration and any change in buffer conditions including pH. You can determine the actual R_h under any given solvent by measuring apparent R_h as a function of concentration and extrapolating to zero concentration. Usually measurements at concentrations ~ 1 mg/mL or below will provide apparent R_h values that are quite close to actual values.

Q1: *How does the extinction coefficient of two proteins affect the interaction analysis?*

Can two proteins with similar extinction co-efficient be used in CG-MALS experiments?

A: It is crucial to know the stock solution concentrations. When appropriate, we use the known extinction coefficients and an in-line UV detector to measure those concentrations during the course of a CG-MALS experiment. Since a typical gradient includes an injection of 100% X and another of 100% Y, the stock solutions can be measured individually. Once those are known it is straightforward to calculate the ratios of intermediate mixtures based on the pump operation; this is all taken care of in the software. It does not matter if the extinction coefficients are similar.

There is another option in the software to determine the intermediate mixtures based on measuring the UV signals at each concentration and applying an extrapolation algorithm described in early CG-MALS paper by Attri & Minton (*Biophys. J.* 2005). For this algorithm to work, the total extinction of 100% X and 100% Y (i.e, extinction coefficient x concentration) must be sufficiently distinct from each other. This can usually be accomplished by selecting appropriate

concentrations if the extinction coefficients are known, but sometimes reality hits and though you intended different extinction values the samples end up with the same value…

Even if the extinction coefficients are not known or for some reason UV cannot be measured in-line, the CALYPSO software offers other options such as measuring with an Optilab RI detector or a fluorescence detector, or relying on user inputs of the actual stock solution concentrations without an in-line detector.

Q: *In practice, do polymerizing systems sometime create problems due to saturation (blow-out) of light-scattering detector signals? Can such effects make the analysis intractable, and if so, is there a rule-of-thumb guideline regarding what level of polymerization is tractable?*

A: If the signal saturates, the analysis will certainly be impacted. However, it takes a huge amount of polymerization to reach those conditions in a modern MALS detector provided by Wyatt which has a very large dynamic range and the option to turn down the laser power. For example, if your concentration is 1 mg/mL you could probably polymerize up to 50 MDa before saturating.