



Live Webinar Q&A Sheet: Understanding Absolute Stoichiometry of Oligomeric Protein Complexes using SEC-MALS

The recorded webinar may be viewed from the [SEC-MALS](#) webinars page. These questions were submitted by live viewers. Additional information on DLS,FFF, 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 with any additional questions.

SEC-MALS technique

Q: What is the advantage of the RI detector compared to the UV detector for oligomer analysis? Could the analysis be done by using only a UV detector?

A: There are three primary reasons for using refractive index (RI) detection:

- It is sensitive (and versatile) for measuring the concentration of proteins or other biomolecules (sugars, small molecules etc.) that do not have a strong absorbance at 280 nm. Histones have a low aromatic residue composition and hence do not absorb well at 280 nm, that is why we use RI to measure concentration accurately, instead of the UV at 280 nm.
- The RI response, dn/dc , is nearly universal for pure proteins in aqueous buffer: 0.185 mL/g. Hence, with dRI we do not need to know which protein is present in each peak in order to measure concentration and calculate MW.
- RI detection was also important for analyzing heterocomplexes, since the extinction coefficients of the two constituents are usually different, whereas dn/dc is identical for almost all proteins to within 1-2%. Hence with RI the concentration of heterocomplexes can be measured in order to analyze MW by MALS, whereas this would not be possible with UV.

Q: How is dn/dc constant for all proteins?

A: dn/dc , or refractive index increment, is an intrinsic property of a solute-solvent pair. For proteins, it is the weight-average of the dn/dc values of the amino acids that make up the protein. Assuming an approximately random assortment of amino acids, by the law of averages, the dn/dc values of most proteins comes out to about the same value to within 1-2%: 0.185 mL/g (see Zhou, Browning and Schuck,



Biophys. J. 2011). Small proteins tend to have a somewhat wider spread due to averaging over a smaller number of amino acids, and certain proteins have evolved to have a larger dn/dc e.g. eye lens crystallins which biologically fulfill an optical function. But for the most part, the standard value will be an excellent approximation.

Q: Which SEC column is used for your analysis?

A: We use a GE Superdex S200 10/300 Increase column for our analysis.

Q: What factors did you consider to lower the % error with the MW obtained from SEC-MALS?

A: Equilibrating the column/MALS setup for 2-3 hours (or overnight) gives us the best signal-to-noise and low errors after processing the data. High concentrations of sample also reduce the % error.

Q: Did you have any issues with aggregation that impacted your SEC-MALS analyses? If so, how did you overcome them?

A: We did not have any issues with aggregation in our protein samples. If aggregation is an issue, the best way to overcome this is to test out different buffers, protein concentrations, and ratios. We routinely centrifuge our samples before analysis to remove particulates and it would be an issue if your sample spun out also; you can use the ASTRA software to quantify protein recovery.

Q: What volume of samples did you inject onto the column? Did you do this manually?

A: We loaded 100 μL of sample. It was injected using a GE Healthcare AKTA.

Q: What is the molecular weight of the largest complexes? Are you approaching the void volume of the Superdex column?

A: We did not approach the void volume of our Superdex column. Our largest complex was ~ 350 kDa (ScNAP1 with excess H3-H4) and the separation range of our column is 100-600 kDa.

SEC-MALS analysis

Q: At 300 mM NaCl both yeast and worm NAP1 proteins are shown to be dimeric but exhibit long tails in the SEC profile. Do those tails correspond to dissociation to monomers? If not, why do they appear?

A: This is likely due to oligomerization heterogeneity or degradation of the NAP-dimer. NAP1 is a constitutive dimer and cannot exist as a monomer (unless denatured). The MW of the tail region is significantly higher than the MW of a NAP-monomer which confirms that the tailing peak is not



monomeric NAP. The MW of the peak is selected across the peak to obtain the MW of the major species.

Another possible reason for the tails is the dimers are sticking to the column, and in this case the MALS analysis would show the dimer MW along the tail.

Q: Why is the MW vs Retention Volume plot for the CeNAP tetramer-dimer mixture sloped in the early elution portion, and then flat?

A: SEC-MALS produces weight averages of the MW at each elution volume in the peak. The early part shows MW decreasing with elution volume, which means that that part is transitioning from (partially resolved) tetramers to dimers. Later the MW is uniform, indicating a homogenous dimer population across most of the peak.

Q: About the first (NAP) oligomers shown: why does the MALS plot show a continuous line that changes between the molecular weight of tetramer and dimer, instead of two lines at the same elution volume, corresponding to the tetramer and the dimer?

A: We cannot observe two lines of MW because SEC-MALS measures the weight-average MW of the species present at each elution volume, giving a single continuous line. MALS sees distinct populations only when species are separated by the column.

Q: Is it possible to identify, with SEC-MALS, a protein metal-bridged dimer from a regular dimer, of the same protein?

A: You will be able to tell a difference by SEC only if there is a significant conformational difference such as globular dimer versus elongated dimer. If the two dimers differ by only a single metal then it would likely fall within the MALS error.

Q: Some of your chromatograms show significant aggregate present. Would you consider these to indicate higher-order oligomerization? What do you make of the aggregate populations and how they vary?

A: We don't see large aggregates in the void volume (8 mL elution), but we do see a small peak just upstream of the main peak. The signal is quite low so it accounts for a very small percent of the overall system. These could be disulfide bonded oligomers that are not native (there are exposed cystine residues, although we do have reducing agent present), or they could be a small population of higher order oligomers that may or may not be biologically relevant.



Q: What levels of higher-order complexes (tetramers and higher) could you measure (e.g. CeNAP1 tetramer, ScNAP1 N>4)?

A: At the concentration used for the study (20 μ M), we observed both proteins CeNAP1 and ScNAP1 were dimers at 300 mM NaCl and exhibits a dimer-tetramer equilibrium at 150 mM NaCl (mostly dimer, minor tetramer in dynamic equilibrium with each other). We could have measured higher oligomers if they were present. Detection is limited only by what enters and separates on the SEC column.

Q: Can MALS perform nanoparticle (100 nm to 200 nm) size determination?

*A: MALS can measure the size of nanoparticles. The range depends on the specific model: the **DAWN** can measure R_g within 10 - 500 nm, while the **miniDAWN** can measure R_g within 10 – 50 nm. So the DAWN would be needed. However, that size will probably not separate well on an SEC column and you would need **asymmetrical-flow field-flow fractionation (AF4-MALS)** with an **Eclipse** to perform the separation by size and subsequent downstream characterization.*

Q: You mentioned that you did not report data on small molecular weight species because of the error associated with the measurement. What is the upper and lower limit of the MALS detector?

A: Usually smaller proteins of under 20 kDa have low light scattering and at the concentration used for the study (20 μ M), the MWs had high % error (uncertainty), hence we did not report the MW value. We confirmed that the smaller species is histone H2A-H2B since it is the only other component that we add to our samples apart from NAP1 (2-protein complex). You can measure low-MW proteins like histones with better errors when you inject them at a higher concentration, so there is no “technical” limit aside from the instrument specification of 200 Da to 1 GDa/10 MDa for a DAWN/miniDAWN. It varies depending on the concentration of your samples.

Interaction studies

Q: How do you know your protein is a constituent dimer for all conditions or in the presence of other proteins?

A: The crystal structure of NAP with histones showed that histone-binding does not split the NAP dimer in typical buffer conditions. More extreme levels of pH, ionic strength or denaturant may cause NAP dimers to dissociate, but these are not representative of physiological conditions.



Q: How can the MW result of NAP by MALS be explained as tetramer even if the MALS signal is fairly far away from the tetramer line? Maybe a trimer?

A: NAP proteins are constitutive dimers and can only exist as dimers, tetramers etc. (NAP₂, NAP₄ etc). SEC-MALS analysis produces a weight-average MW at each elution volume across the peak, and since the line between dimer and tetramer but closer to the dimer MW, we conclude that the population consists of majority NAP dimer and minority NAP tetramer. The MW line is sloped across the peak, varying approximately with concentration, which means the species is heterogenous mix of mostly dimers and some tetramers which are in constant exchange (dynamic equilibrium).

Q: Are you concerned that column matrix interactions could confound your results? How can you confirm that these complexes exist in solution? Have you considered composition-gradient MALS (CG-MALS?)

A: The proteins have shown similar results with other in-solution experiments (AUC, EMSA etc.) that do not involve SEC. We have not tried CG-MALS but we can in the future.

Q: Can we determine stoichiometry of a protein complex with more than two components using SEC-MALS? If so, what would be a good experimental design?

A: Yes, we can. We keep two components constant and titrate the third. Of course, we need control runs of all the components alone and all of them in a binary-complex to verify the MWs, and then when we titrate the third component, we can dissect the observed MWs into possible stoichiometries of the ternary complex.

Q: Would you say the limitation of binding stoichiometry study is: if the two molecules are very close in MW, since MALS takes average MW, it would be hard to tell them apart from just MALS number, say, you won't know if it is AA or AB complex?

*A: If there is such a concern, you would run an experiment of A by itself at different concentrations and B by itself, to see if dimers form. If A and B have very similar molecular weights, *and* one or both form homodimers in equilibrium with monomers, then yes, it would be difficult to determine if AB heterodimers upon mixing A and B since all three forms (AA, AB and BB) may co-elute and the monomers also coelute. If homodimers do not form and the AB experiment indicates dimers, these can only be heterodimers. In the former case, CG-MALS or IEX-MALS may discriminate the formation of homo- or heterodimers.*



Q: *Can we investigate the protein-DNA interaction by SEC-MALS in a similar way?*

A: Yes, it is possible, though the analysis is a little more complex – you would need to use the *Protein Conjugate Analysis* method in ASTRA. Analysis of a protein-DNA conjugate is described in a white paper, [WP3001](#).