APPLICATION NOTE

AN3010: Analysis of hetero-oligomers with CG-MALS

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Summary

Molecules A and B may bind with different stoichiometries at different molar ratios of A and B. Adding to this complexity, A and B may also self-associate into homo-oligomers. Here we discuss a method that disentangles all these possibilities in a single experimental run using multi-angle light-scattering.

Composition-gradient multi-angle light scattering (CG-MALS) provides easily interpreted data for complicated macromolecular interactions. Here we show how a simple protocol provides information-rich data with a completion time of a few hours.

Introduction

Biomolecular interactions are commonly studied using binding assays. Quantitative binding assays hopefully provide the investigator with a *unique* value for binding affinity. Affinity is a key energetic parameter required to model how molecules A and B interact. In cases where A and B form a 1/1 complex, i.e. $A + B \rightleftharpoons AB$, multiple methods provide a reliable measurement for affinity. Microcalorimetry, surface plasmon resonance, radioligand binding assays and countless more methods all provide reliable values for affinities of a 1/1 complex.

But what if stoichiometry is unknown or merely *assumed*?

Naturally, any assumption needs to be confirmed. If molecules A and B combine with multiple stoichiometries, measuring *unique* binding parameters can be very difficult, if not impossible, using binding curves. With multiple binding sites, using a standard binding curve model to fit the data provides infinite solutions [1,2]. Thus, it is important to use the best tools possible to identify all the complexes that can form between A and B prior to determining which model provides reasonable values for the binding energies in a system.

Consider the possibility of heterotrimers for molecules of similar masses, i.e. the mass of ligand A is not too different than the mass of receptor B (Figure 1). There are two general binding models that can produce heterotrimers:

- In Scheme 1, B has two binding sites for A, and A has two binding sites for B.
- In Scheme 2, a dimer of A has a single binding site for B, and a dimer of B has a single binding site for A.

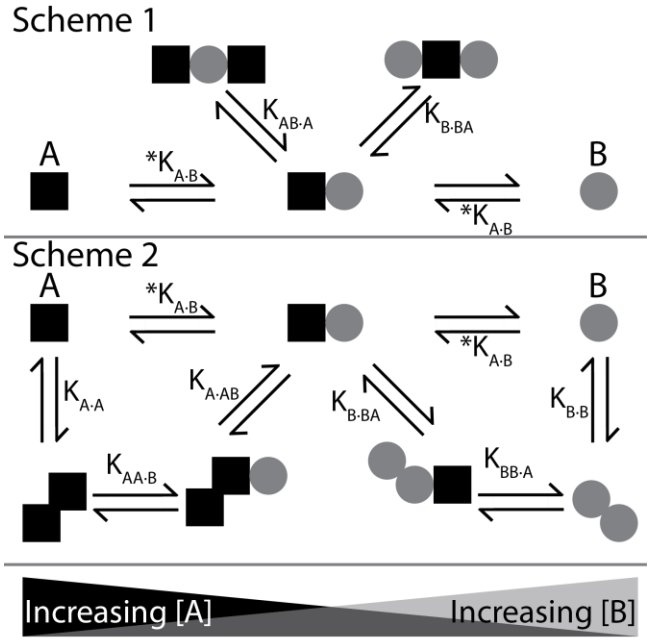


Figure 1. Schemes for trimer formation across a range of molar ratios.

$K_{A\text{-}A}$ refers to the association constant to form a dimer, $K_{AA\cdot B}$ is the association constant for a preformed dimer of A to bind molecule B, and so forth. $*K$'s are the same process, i.e. $*K_{A\cdot B}$ and $*K_{B\cdot A}$ are identical.

It would be useful to know which model best describes our system of interest. For example, if dimers of A do not form in solution, Scheme 2 seems unlikely. How can we determine which model is appropriate to use?

CG-MALS is fast and easy to interpret

An ideal method for assaying stoichiometry needs to provide data that are easily understood. In addition, macromolecules may break down in solution over time, so the method is ideally fast and controllable with a high turn-around time to collect the next data set.

Here we provide an example of how a CG-MALS system from Wyatt is capable of characterizing the formation of hetero-trimers.

Materials and Methods

We used two recombinant proteins with known molar masses. Analyte A is an 11 kDa peptide fragment of an SK2 protein ion channel (SKp), and analyte B is a 17 kDa full-length calmodulin (CaM). Both were characterized previously[3].

For the gradient mixer we used a Calypso® composition-gradient instrument. Since molecule A is slightly smaller than B, a modestly higher stock concentration of A is used. Analyte A, in Pump 1, was made at a stock concentration of 0.96 mg/mL. Analyte B, in Pump 2, was made at a stock concentration of 0.9 mg/mL. Pump 3 contained as diluent a saline solution typical for protein analyses.

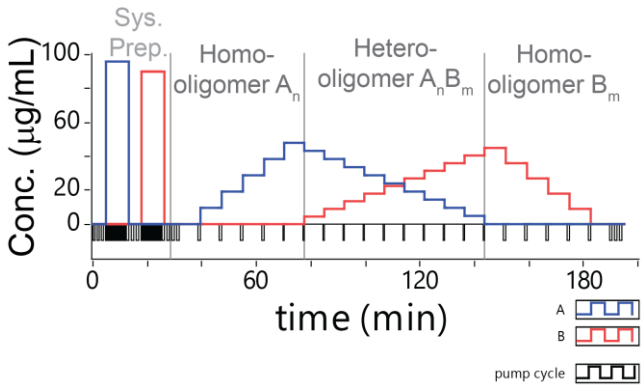
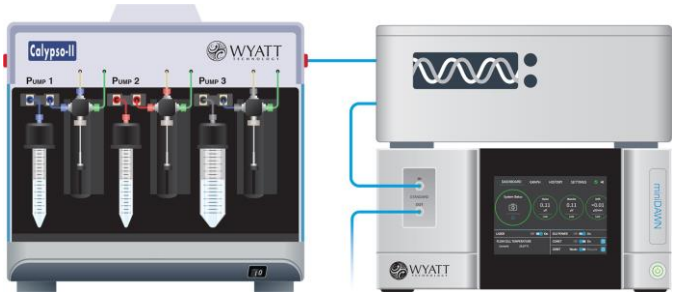


Figure 2. Modified experimental method using CALYPSO software. The system prep uses a higher concentration determined empirically in our case to saturate the inline filters with molecules.

To set up our binding method, we used CALYPSO™ software. CALYPSO has built-in methods that are easily modified to suit experimental paradigms. Figure 2 shows the method applied, which preps the system, then runs the experiment. The homo-oligomer steps at the beginning and end test for self-association of each molecule. The cross-over range tests for A-B hetero-oligomers at different molar ratios.

The Calypso injects the protein directly into a temperature-controlled DAWN® multi-angle light scattering detector. In all our experiments, we maintain a constant temperature of 25°C and include a delay time of 120 seconds after injection of each mixture to ensure protein binding reaches equilibrium. We also placed a Bio-Rad UV detector following the DAWN to monitor the total protein concentration.



Modeling the weight-average molar mass of analytes and analyte complexes in solution was accomplished using CALYPSO software. We selected the “Arbitrary Stoichiometry” option to build a model that best fit the data, resulting in the association constants K_{AB}, K_{AAB} and K_{ABB}.

Prior to fitting the hetero-complexes, we analyzed the concentration dependence of individual analytes (“homo-oligomer” regions) to determine whether self-association occurs and if our experimental concentrations match what we expected. Next, we fit the data in the cross-over range. The association constants for the 1/1, 1/2, and 2/1 complexes were the only variable parameters we used in these analyses. Though the software can account for the effects of virial coefficients and incompetent fractions, these were negligible and ignored. We obtained similar results regardless of whether the molar masses of the analytes were constrained, or varied to optimize the fit.

Results and Discussion

MALS measures the weight-average molar mass of macromolecules in solution. The weight-average molar mass of analyte A does not increase with increasing concentration (Figure 3). The same is true for analyte B. Hence, neither molecule forms a homodimer. The lack of dimer formation is a partial argument against Scheme 2.

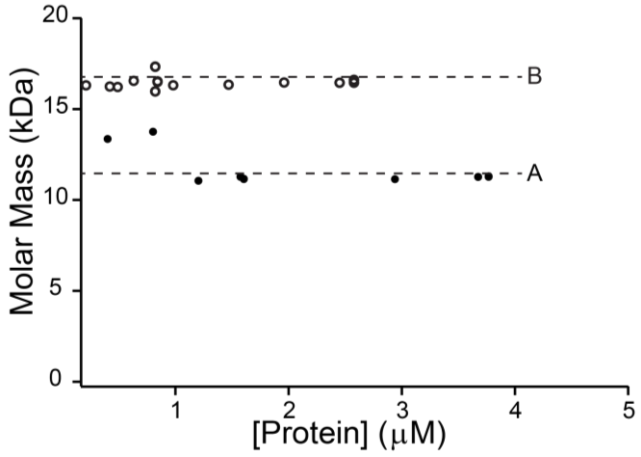


Figure 3. The molar masses of molecules A and B were calculated using data collected in the homo-oligomer regions. Protein concentrations were verified by UV absorbance using previously determined extinction coefficients of the proteins[3]. Analyte A, filled circle, has an average measured molar mass of 12±2 kDa. Analyte B, open circle, has an average measured molar mass of 16±1 kDa. Each symbol shows the calculated mass from 15-17 MALS detectors. The dashed lines correspond to theoretical molar masses determined from protein sequence using ExPasy ProtParam tool https://web.expasy.org/protparam/ .

The cross-over phase is used to measure the molar masses of complexes that form between A and B at different ratios. Figure 4 shows a characteristic 'M' shape for this binding reaction. At very low concentrations of B, the weight-average molar mass is dominated by that of monomer A in the solution, i.e., it is close to 11 kDa. Likewise, at high concentrations of B relative to A, monomer B dominates the measured weight-average molar mass.

At ratios where complexes can form, two peaks and a valley are observed. The interpretation is straight forward: analyte A forms 2/1, 1/1 and 1/2 complexes with analyte B. The larger peak is formed by the 2/1 complex, A-B-A while the smaller peak is formed by the 1/2 complex, B-A-B. Although B-A-B is larger than A-B-A, in this experiment A-B-A forms with higher affinity (log K_{AAB} = 17 M^{-2}) so a larger mole fraction of A-B-A forms in solution than of B-A-B. Thus, the weight-average B-A-B molar mass appears as the smaller peak because it forms with weaker affinity (log K_{ABB} = 16 M^{-2}).

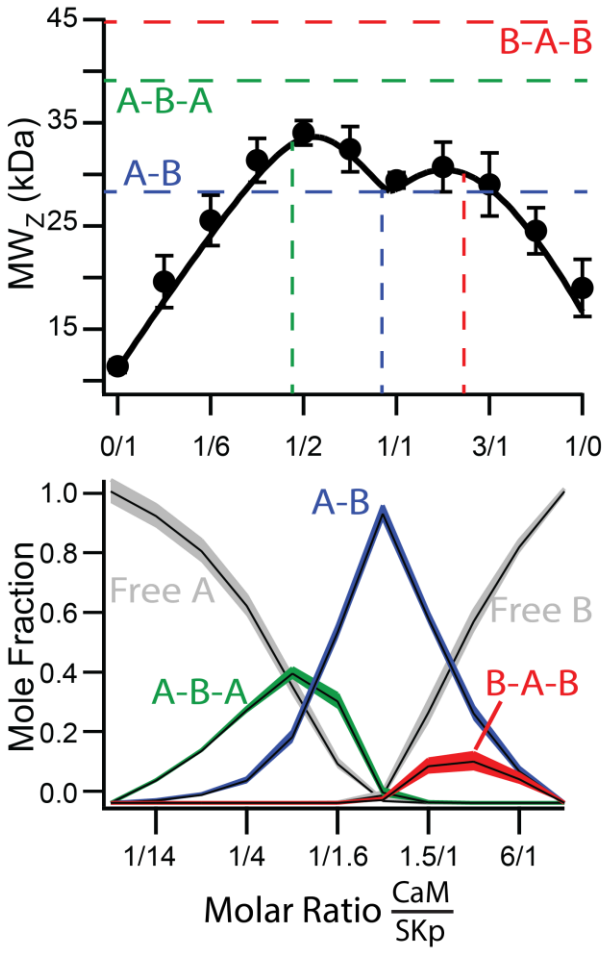


Figure 4. Top graph shows the weight-average molar masses (closed circles) calculated from data collected in the heterooligomer region of Figure 2. Error bars were established by performing five repeat trials. The solid line shows a modeled fit for the mole fraction of species in solution, shown in the bottom panel, that would be needed to produce the observed molar masses. All calculations were performed in CALYPSO™ software.

Another key feature is the dip in the 'M'. This feature can only be modeled if the 1/1 complex forms with very strong affinity. In fact, we measured affinity $K_{d,AB}$ of 100 pM (log $K_{AB} \geq 10$ M^{-1}) or less to form A-B. This further supports Scheme 1 for the binding mode of our proteins (Figure 5).

Scheme 1 indicates that either analyte has two binding sites for the other analyte. Since the affinity for forming the heterotrimer is weaker than forming the 1/1 complex, it suggests that either of the analytes binding to the 1/1 complex is not binding as strongly as the first one.

Because of possible cooperativity, we cannot yet rule out Scheme 2. More experiments are needed, but the current results help to guide future work on the more likely scenarios. More work is also needed to identify both B-binding sites on A and both A-bonding sites on B.

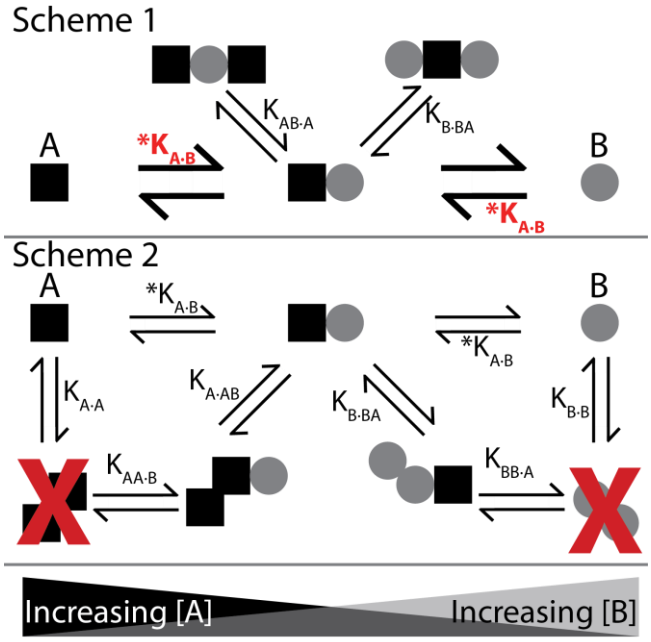


Figure 5. If cooperativity is ignored, Scheme 1 seems most likely to describe binding of A and B. Three association constants are sufficient to describe binding interactions (again noting that *K's are the same).

Conclusions

CG-MALS provides data on stoichiometry that is easier to interpret than most other binding techniques. The output is a light-scattering signal that is used directly to calculate weight-average molar mass of macromolecules in solution. Provided that the concentration of each analyte is well determined, the measurement of weight-average molar mass is a robust and interpretable result.

Modeling the mole fractions of each complex helps to quantify the amount of each complex in solution via a fitted apparent association constant. With complicated models, rigorously determined association constants are the end goal, yet more work is needed to fully understand binding models.

Acknowledgements

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References

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2. Middendorf, T. R. & Aldrich, R. W. Structural identifiability of equilibrium ligand-binding parameters. *J Gen Physiol* **149**, 105 (2017).
3. Halling, D. B., Kenrick, S. A., Riggs, A. F. & Aldrich, R. W. Calcium-dependent stoichiometries of the $KCa_{2.2}$ (SK) intracellular domain/calmodulin complex in solution. *J Gen Physiol* **143**, 231 (2014).

To learn more about CG-MALS and Calypso, schedule an application review or request a demo, please contact Wyatt Technology via https://www.wyatt.com/products/calypso-product-info-request.html or e-mail info@wyatt.com.