Application Note

Wyatt Technology

Metacomplex Formation & Binding Affinity of Multivalent Binding Partners

Summary

Multivalent binding plays a crucial role in protein-protein interactions, including cell signaling pathways, immunological responses, and self-assembly of structural elements. However, standard analytical techniques for measuring macromolecule interactions require one binding partner to be immobilized on a surface, leading to errors when both binding partners are multivalent. Composition-gradient multi-angle light scattering (CG-MALS) is unique in its ability to quantify these interactions to yield affinity and stoichiometry of complexes forming in solution. We investigate metacomplex formation in the hetero-association of a multivalent antigen, streptavidin (SA), and a bivalent antibody (Ab) using CG-MALS.

Automated composition gradients were created using a Calypso® II connected to an inline UV/Vis concentration detector and DAWN® HELEOS® MALS detector. We used single-component concentration gradients to quantify any self-association and a dual-component "crossover" composition gradient to assess hetero-association. The data indicated that SA and Ab associate into complexes with higher molecular weight than a simple 1:1 or 2:1 stoichiometry. Since neither protein was found to self-associate, the higher-order stoichiometries must have resulted from the multivalent nature of the two binding partners.

The Calypso software enabled us to evaluate two hetero-associating models: the infinite self-association (ISA) of $[(SA)(Ab)]_n$ base units into n:n complexes and a more refined model that adds n+1:n and n:n+1 to ISA. Under the latter model, $(SA)(Ab)_2$, $(SA)_2(Ab)$, and $(SA)_3(Ab)_2$ complexes contribute significantly to the total light scattering. This more complete model provides the best fit, yielding affinities of 23±4 nM per binding site.

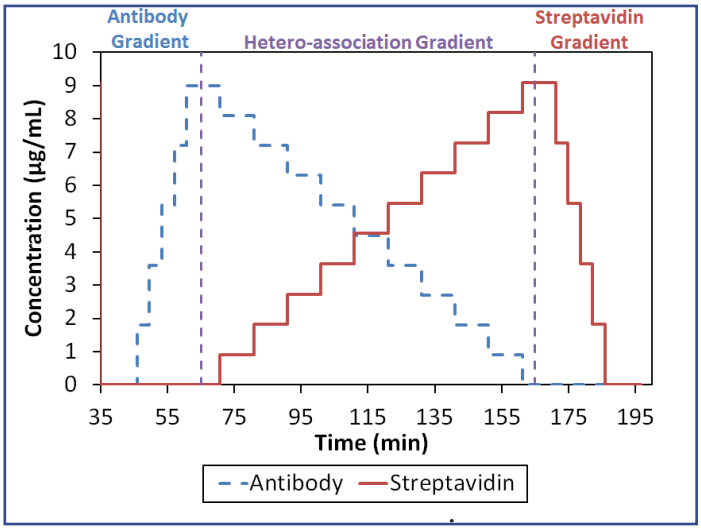

Figure 1. Composition-gradient method for quantifying the interaction between streptavidin and an anti-streptavidin antibody.

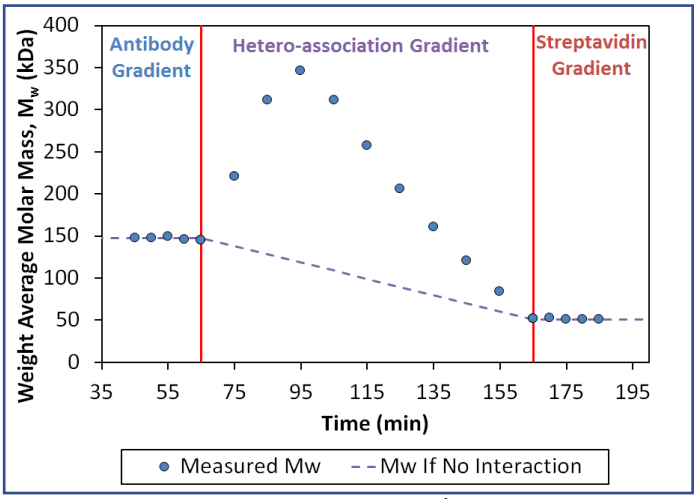

Figure 2. Weight-average molar mass (M_w) at equilibrium for each composition shown in the top figure. During the hetero-association gradient, the formation of SA-Ab complexes results in an increase in M_w.

I. Introduction

Standard analytical techniques for probing macromolecular interactions, such as SPR and ELISA, require one binding partner to be immobilized on a surface in order to quantify the binding affinity. For simple 1:1 association and for some 1:*n* interactions, this physical tethering usually does not significantly impact the equilibrium dissociation constants (K_D) that are measured. When both binding partners are multivalent, however, immobilizing one ligand can lead to erroneous estimates of the binding affinity by orders of magnitude due to avidity effects at the surface, mass transport limitations, and incorrect assumptions about the stoichiometry of the interaction. In contrast, composition-gradient multi-angle light scattering (CG-MALS) measures interactions entirely in solution, allowing all possible binding stoichiometries to occur and providing simultaneous quantification of self- and hetero-interactions, metacomplex formation, and other multivalent interactions.[1] Here, we investigate the complex stoichiometries that result from the binding of an anti-streptavidin antibody (Ab) to the homotetramer streptavidin (SA) (Figure 3).

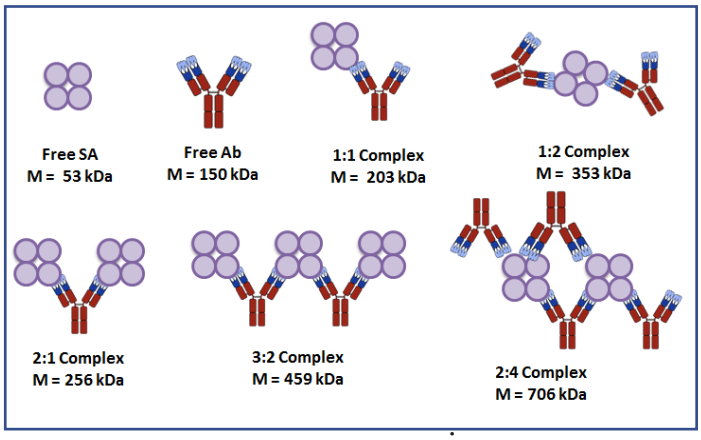

Figure 3. Some of the possible complexes formed between SA and Ab. The two identical binding sites of Ab can bind one or two SA molecules. SA's homotetrameric structure provides four possible binding sites and enables additional self-assembly of complexes in solution.

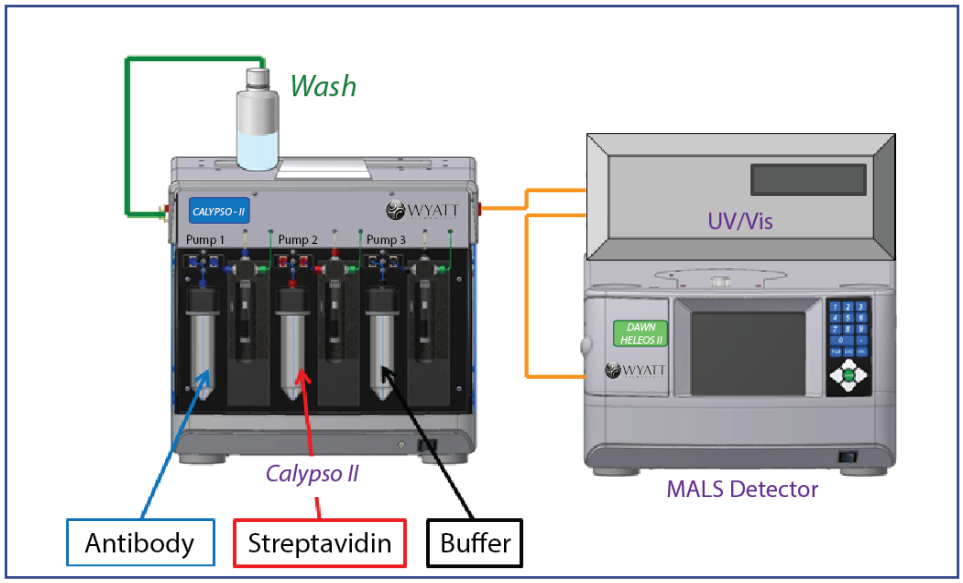

Figure 4. Calypso system hardware setup with inline UV/Vis concentration detector and DAWN HELEOS MALS detector.

II. Materials and Methods

Reagents and Instrumentation

The anti-streptavidin antibody was kindly provided by Dr. Shawn Cao at Amgen. For CG-MALS experiments streptavidin (Sigma-Aldrich) and antibody were diluted to a stock concentration of 10 µg/mL in a phosphate buffered saline (PBS; 25 mM NaH_2PO_4, 25 mM Na_2HPO_4, 50 mM NaCl, 200 ppm NaN_3 pH 6.7) for a total of ~100 µg each protein per experiment. Each solution was filtered to 0.02 µm using Anotop syringe filters (Whatman) and loaded on the Calypso II.

CG-MALS experiments were performed with a Calypso II composition-gradient system (Wyatt Technology) that prepared and delivered different compositions of protein and buffer to an inline UV/Vis detector (Waters) and Wyatt DAWN HELEOS MALS detector (Figure 4). Polycarbonate (Millipore) filter membranes with 0.1-µm pore size were installed in the Calypso for sample and buffer filtration.

Determination of Equilibrium Association Constant and Stoichiometry

An automated Calypso method was run, consisting of single-component concentration gradients to quantify any self-association and a dual-component "crossover" composition gradient to assess the hetero-association behavior. For each composition, 0.8 mL of protein solution at the appropriate concentration was injected into the UV/Vis and MALS detectors. The flow was then stopped to allow the solution to come to equilibrium within the MALS flow cell. For single protein gradients the flow was stopped for 120 s. The stop-flow time was increased to 500 s for the crossover gradient. A single experiment had an unattended run-time of ~3.5 h. Data collection and analysis of equilibrium association constants were performed using the Calypso software.

III. Results and Discussion

The increased light scattering signal in the hetero-association gradient region indicates that SA and Ab associate into complexes with a higher molecular weight than a simple 1:1 or 2:1 stoichiometric ratio (Figure 5). Since neither protein was found to self-associate under these conditions, the higher-order stoichiometries must have resulted from the multivalent nature of the two binding partners.

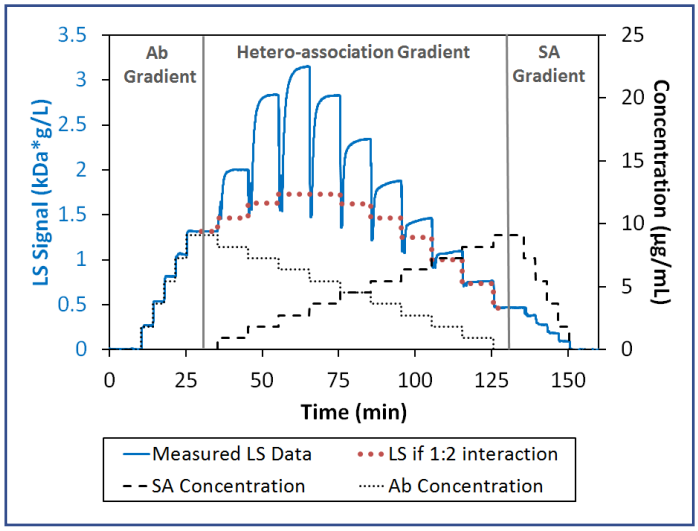

Figure 5. Light scattering and concentration data for the interaction between SA and Ab. The association is greater than can be explained by 1:2 interaction (bold-dotted red line).

In a "crossover" hetero-association gradient, the composition with the maximum light scattering signal (weight average molar mass, M_w) occurs at the overall stoichiometric ratio of the interaction. This ratio combined with the magnitude of Mw yields the absolute stoichiometry of the interaction. Figure 6 compares the measured Mw to three simple SA:Ab stoichiometries. For each simulation, the affinity per binding site was considered constant, K_D = 0.2 nM. Although the measured data reach a maximum near the 1:1 molar ratio, the maximum measured M_w (~350 kDa) is significantly larger than the maximum molecular weight for the 1:1 model (~200 kDa). A model considering two Ab bound per SA molecule approaches the correct maximum Mw (~330 kDa; Figure 4, solid purple line). However, this maximum occurs at the wrong composition compared to the measured data, and this simulated model significantly underestimates the measured M_w for all compositions with excess SA.

III. Results and Discussion (Continued)

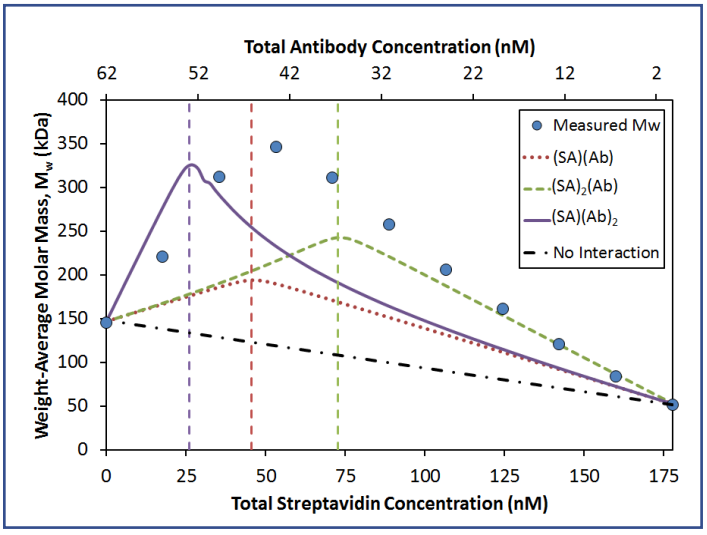

Figure 6. Measured M_w compared to simulated M_w for the indicated stoichiometries with K_D = 0.2 nM at each binding site. The position of the peak in the measured M_w indicates that the largest complexes form with stoichiometry n:n (dotted red line) compared to 2*n*:*n* (dashed green line) or *n*:2*n* (solid purple line). Figure 3 shows these complexes and their molecular weights.

Taken together, these data indicate the presence of higher-order complexes with an overall stoichiometric ratio of 1:1, i.e., $(SA)_2(Ab)_2$, $(SA)_3(Ab)_3$, etc. This result seems surprising as SA's four possible binding sites compared to the two on Ab suggest that complexes can occur with higher SA:Ab ratios, as shown in Figure 1. The complex that saturates the most binding sites would have an overall stoichiometric ratio of 2(SA):1(Ab). However, the crystal structure of streptavidin shows a dimer of dimers with two-fold symmetry, rather than a tetramer with four-fold symmetry,[2] which could explain this propensity to form 1:1 metacomplexes rather than 2:1 metacomplexes. Interestingly, a similar 2:1 stoichiometric ratio for macromolecular interactions has also been observed with DNA aptamers raised against streptavidin.[3,4,5]

To characterize the SA-Ab interaction, two different models are considered: (1) infinite self-association of [(SA)(Ab)] base units into n:n complexes and (2) a more refined analysis that includes n+1:n and n:n+1 complexes in addition to the n:n complexes.

1. First Pass Analysis: Infinite Self-Association of 1:1 Stoichiometries $[(SA)(Ab)]_n$

In the infinite self-association (ISA) model, SA and Ab bind with some affinity to form a 1:1 base unit [(SA)(Ab)]. This base unit then self-associates indefinitely, with each base unit adding to the growing chain with constant affinity

According to this model, Ab binds SA with affinity K_D = 22 nM, and the [(SA)(Ab)] base units self-assemble with K_D = 50 nM. An additional term accounting for two antibodies binding a single streptavidin molecule $(SA)(Ab)_2$ was required to fully capture the change in light scattering as a function of composition. Under these conditions, there is no appreciable concentration of $[(SA)(Ab)]_n$ complexes with *n*>3 (Figure 7).

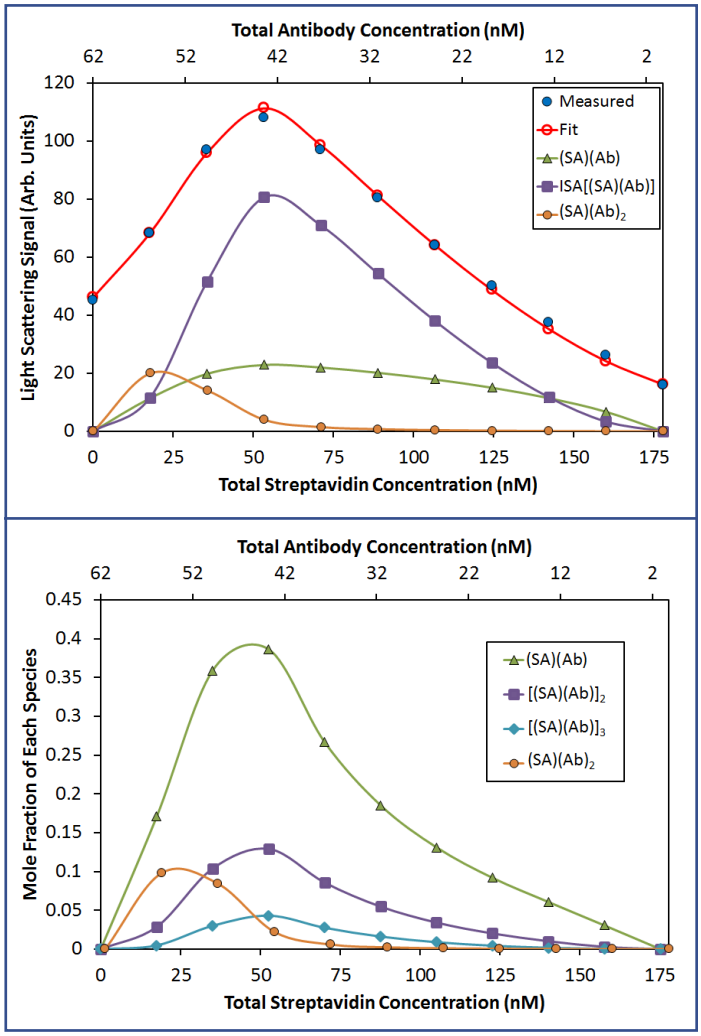

Figure 7. Best fit of CG-MALS hetero-association data using the ISA model. Top: The best fit (red unfilled circles) for the measured light scattering data (blue filled circles) includes contributions from (SA)(Ab), $(SA)(Ab)_2$, and ISA species. The purple squares give the overall contribution to the model by all species with stoichiometry $[(SA)(Ab)]_n$ with *n*>1. Bottom: For each composition, the molar distribution of species in solution can be calculated. In both graphs, SA and Ab monomer contributions are left off for clarity.

III. Results and Discussion (Continued)

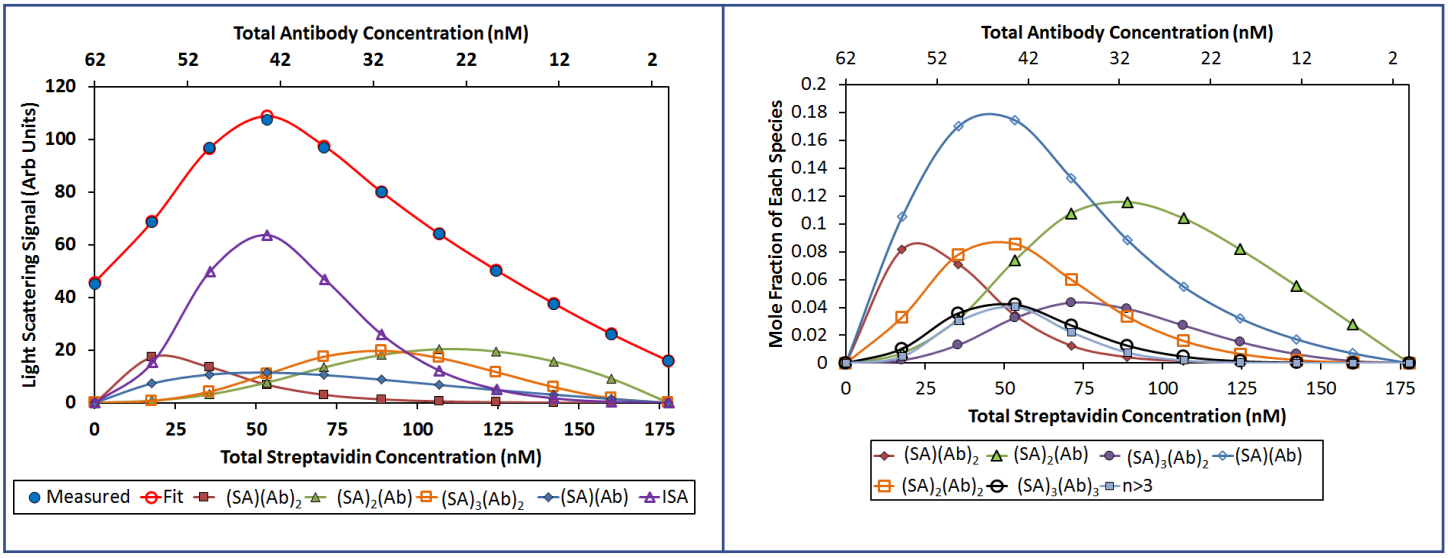

Figure 8. Best fit of CG-MALS hetero-association data assuming arbitrary $(SA)_i(Ab)_j$ stoichiometries. Left: The best fit (red unfilled circles) to the measured light scattering data (blue filled circles) is made up of a combination of the indicated stoichiometries. Right: Distribution of species across the hetero-association gradient. The $n>3$ includes all stoichiometries $[(SA)(Ab)]_n$ with $n>3$. In both graphs, SA and Ab monomer contributions are left off for clarity.

2. Refined Analysis: Inclusion of All $(SA)_i(Ab)_j$, for $i,j < 3$

Although providing a reasonable fit to the data, the ISA model implies that Ab binds SA with a lower affinity if that SA molecule is already bound to another Ab than if it is a free monomer. This lower affinity for complex formation could be explained by steric hindrance. However, one might expect these affinities to be the same since the same binding sites participate in both interactions. A more complete analysis includes the n+1:n and n:n+1 complexes that should form in addition to the n:n complexes. Under this analysis $(SA)(Ab)_2$, $(SA)_2(Ab)$, and $(SA)_3(Ab)_2$ contribute significantly to the total light scattering (Figure 8). Other stoichiometries, including $(SA)_2(Ab)_3$ and $(SA)_2(Ab)_4$, drop out of the fit and do not contribute to the overall light scattering signal. This suggests that it is more favorable to saturate the Ab binding sites with SA rather than the SA binding sites with Ab. This tendency could also reflect the nature of SA to act as a dimer of dimers.

This analysis yields K_D values with a narrow range of 23±4 nM per binding site. The constant affinity per binding site shows that there is no negative cooperativity disfavoring metacomplex formation as predicted by the ISA model. This makes sense since both molecules are symmetric and no allostery is expected. Additionally, the residuals of this refined model are smaller and more random than those of the ISA model (Figure 9). The more systematic residuals of the ISA model, on the other hand, indicate an error in this description of the data.

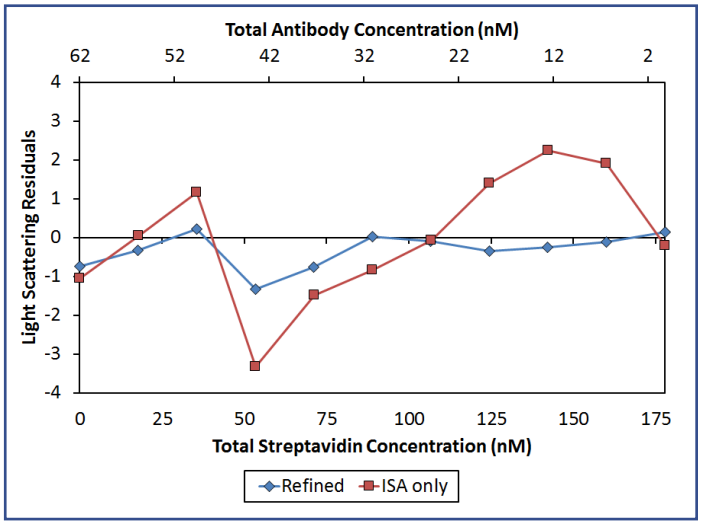

Figure 9. Residuals of the second, "Refined" model (blue diamonds) are smaller and more random than residuals of the first, "ISA only" model (red squares), indicating a better fit of the data.

Conclusion

Metacomplex formation by multivalent binding partners in solution is amenable to analysis by CG-MALS to obtain absolute stoichiometries and binding affinity. In this analysis, we found that homotetrameric SA molecules present two binding sites for each bivalent Ab, leading to the formation of multiple stoichiometries in solution.

By measuring the absolute molecular weight of the solution, CG-MALS determined the absolute stoichiometry of the complexes that were formed. We presented two different interpretations of the data: the infinite self-association (ISA) of $[(SA)(Ab)]_n$ base units into *n:n* complexes and a more refined model that added *n*+1:*n* and *n:n*+1 complexes to ISA. Under the latter model, $(SA)(Ab)_2$, $(SA)_2(Ab)$, and $(SA)_3(Ab)_2$ complexes contribute significantly to the total light scattering, providing strong evidence for their presence.

The symmetry of each binding partner is reflected in the near constant affinity per binding site, K_D = 23±4 nM, confirming that there is no cooperativity favoring the formation of higher-order complexes or appreciable inhibition to their formation under these conditions. The number of complexes, ratio of SA to Ab, and degree of self-assembly could not be predicted *a priori*. By this analysis, CG-MALS proves uniquely suited to investigating complex systems of macromolecules in solution.

References

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(2) Livnah, O., et al. Proc. Natl. Acad. Sci. (1993) 90, 5076.

(3) Ruigrok, V.J., et al. Chem. Biochem. (2012) 13, 829.

(4) "Binding Affinity and Stoichiometry of a Multivalent Protein-aptamer Association." Wyatt Technology Corp. Published 30 Nov 2012. (http://www.wyatt.com/files/literature/binding_affinity_stoichiometry_calypso.pdf)

(5) Some, D. Light-scattering-based analysis of biomolecular interactions. Biophys. Rev. March 2013. doi: 10.1007/s12551-013-0107-1/

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