Light Scattering for the Masses®

Because proteins are often multimeric in their native state, the biomarker targets of many pharmaceuticals present multiple binding sites. In the case of monoclonal antibody therapeutics, the pharmaceutical itself is multivalent, leading to multiple possible stoichiometries of the drug-target complex. Few techniques are capable of measuring the affinity and stoichiometry of these complex interactions in solution. In this application note we quantify the affinity and absolute stoichiometry of interactions between a DNA aptamer and a tetrameric target, streptavidin, using composition-gradient multi-angle light scattering (CG-MALS).

Experiments were performed with a Calypso II, HELEOS II, and UV concentration detector. The Calypso automated the generation of three composition gradients, as shown in the top figure. Individual concentration gradients in streptavidin and the aptamer were used to quantify the molecular weight of each monomer and identify any self-interactions. A “crossover” hetero-association gradient comprised of 15 compositions of streptavidin and aptamer was used to characterize equilibrium binding between the two macromolecules.

Light scattering and composition data were tested against different models of hetero-association stoichiometry using the Calypso software. At equilibrium, the aptamer binds streptavidin with affinity $K_D = 190$ nM at each binding site, in agreement with affinity measurements by other techniques. Furthermore, CG-MALS data indicated that the aptamer binds streptavidin with 2:1 aptamer:streptavidin stoichiometry, rather than the expected 4:1 stoichiometry, based on the tetrameric structure of streptavidin. This may indicate that streptavidin acts as a dimer of dimers and that the aptamer recognizes the dimer structure.

Summary

For the hetero-association gradient, the light scattering data indicate each SA molecule binds two aptamer molecules with equal affinity. No complexes with 1:3 or 1:4 stoichiometries are observed.
Introduction

With the success of monoclonal antibody therapeutics, research is expanding to a vast array of biological pharmaceuticals for the treatment and prevention of disease, including peptides, bispecific antibodies and siRNA, and drug delivery vehicles, such as virus-like particles and liposomes. Understanding and optimizing the efficacy of many biotherapeutics involves in vitro characterization of their structure and function and requires quantifying the binding to a target molecule. Although binding affinity can be measured reliably with many techniques under conditions of simple 1:1 association or with another assumed stoichiometry, few techniques are capable of simultaneously measuring the affinity and full stoichiometry of a complex macromolecular interaction.

Composition-gradient multi-angle light scattering (CG-MALS) is a rigorous biophysical technique for quantifying self- and hetero-interactions between macromolecules. The light scattering signal of various macromolecular compositions is measured and fit to various equilibrium association models in order to identify the complexes that are formed in solution and their respective equilibrium association constants, $K_A$. Because MALS provides an absolute measurement of the weight-average molar mass ($M_w$), CG-MALS is able to determine the absolute stoichiometry of an interaction, not just stoichiometric ratio. The measurements are made in solution, without tagging or immobilization that could interfere with the interaction. In this note we report applying CG-MALS to quantify the equilibrium stoichiometry and affinity for the interaction between streptavidin (SA) and a streptavidin-binding DNA aptamer (Apt) (Figure 1).

Materials and Methods

The streptavidin-binding aptamer$^1$ was kindly provided by Prof. H.T. Soh (University of California, Santa Barbara). Streptavidin (Sigma) and aptamer samples were diluted to ~20 µg/mL and ~50 µg/mL, respectively, in buffer (phosphate buffered saline, 1 mM MgCl$_2$, pH 7.4) and filtered to 0.02 µm. Composition gradients were automated by the Calypso II and delivered to downstream HELEOS multi-angle light scattering (MALS) detector and dual-wavelength UV detector. The Calypso method consisted of single component concentration gradients to quantify any self-association and a dual-component “crossover” composition gradient to assess the hetero-association behavior. Figure 2 shows the method used to assess the interaction and the expected light scattering signal during the hetero-association gradient for several SA:Apt stoichiometries of 1:n.

For each composition, 0.8 mL of solution was injected into the MALS and concentration detectors and the flow stopped for 60 s (single-component gradient) or 300 s (hetero-association gradient). Light scattering and composition data were fit to various association models using the Calypso software, in order to identify the best-fit model of specific hetero-association.

Possible Stoichiometries:

![Possible stoichiometries for DNA aptamer binding to tetravalent streptavidin.](image)

Figure 1: Possible stoichiometries for DNA aptamer binding to tetravalent streptavidin.
Results

The light scattering signal as a function of single-species concentration yielded molecular weight for each monomer. The calculated monomer molecular weight was 53 kDa and 10 kDa for SA and Apt, respectively, in good agreement with the sequence molecular weight. Neither molecular weight appeared to vary as a function of concentration, indicating neither SA nor Apt self-associates under these conditions (Figure 3, left).

When the two molecules were combined, excess light scattering was observed, as compared to the values expected for a mixture of non-interacting molecules. Complex formation leads to an increase in the weight-average molar mass of the solution of interacting species (Figure 4, right), reflected in the light scattering intensity. These data were analyzed to determine the affinity and stoichiometry for the SA:Apt interaction (Figure 4).
Figure 4: Light scattering and concentration data for interaction between SA and Apt. Expected light scattering signal in the absence of binding is shown for reference (dashed purple line).

The best fit of the CG-MALS hetero-association data indicated that each streptavidin molecule contained two equivalent aptamer-binding sites. Although a 1:4 stoichiometry was expected based on the structure of SA, the maximum light scattering signal occurred at the 1:2 molar ratio, indicating a 1:2 stoichiometry (Figure 5, left), confirmed by the weight-average molar mass of the solution. The excellent overall fit to the model indicated the presence of no additional species, e.g. 2:4 or 1:4. This unexpected result was recently confirmed by mass spectrometry.²

The best fit model also confirmed that the affinity at each aptamer-binding site was equivalent and independent. No cooperativity or steric hindrance was observed. The affinity at each binding site was quantified by its equilibrium dissociation constant, $K_D = 190 \text{ nM}$. The species composition of each plateau in the hetero-association gradient was calculated from the light scattering data based on this model (Figure 5, right). As the SA concentration decreased and the aptamer concentration increased, 1:1 and 1:2 SA:aptamer complexes formed in equilibrium with unbound monomers. The maximum concentration of $(SA)(Apt)$ complex occurred when the overall solution concentration of SA was equal to the overall concentration of aptamer. Similarly, the concentration of $(SA)(Apt)_2$ peaked at an overall molar ratio of 1:2, the same molar ratio at which the light scattering signal reached a maximum (Figure 5). Despite significant excess aptamer in solution, a best fit model cannot include any species with 1:3 or 1:4 stoichiometry.

Figure 5: Best fit indicates SA binds Apt with 1:2 stoichiometry. Left: The raw light scattering data are best fit with a model that includes free SA, free aptamer, 1:1 SA:aptamer complex, and 1:2 SA:aptamer complex; the “LS Fit” shown is the sum of the contributions from each of these species. Right: The calculated LS signal for each species is converted to concentration.
Conclusion
Composition-gradient multi-angle light scattering automated by the Calypso system provides rapid measurement of macromolecular interactions. Although a 1:4 SA:aptamer stoichiometry was expected, the data indicated 1:2 stoichiometry with two equivalent binding sites. This may indicate that streptavidin acts as a dimer of dimers and that the aptamer recognizes the dimer structure. In addition, CG-MALS confirmed that the aptamer binds with equal affinity at each site. This result could not be measured by surface-based techniques or conventional DNA binding-assays using fluorescence or radioactivity. Thus, CG-MALS is uniquely suited for measuring equilibrium affinity and absolute stoichiometry of macromolecular interactions in solution.

References