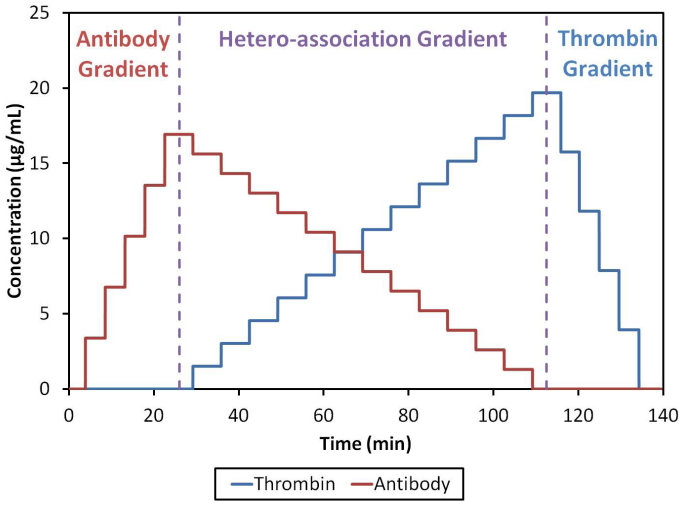
Measuring the Interaction Between Thrombin-α and an Anti-Thrombin Antibody

Summary

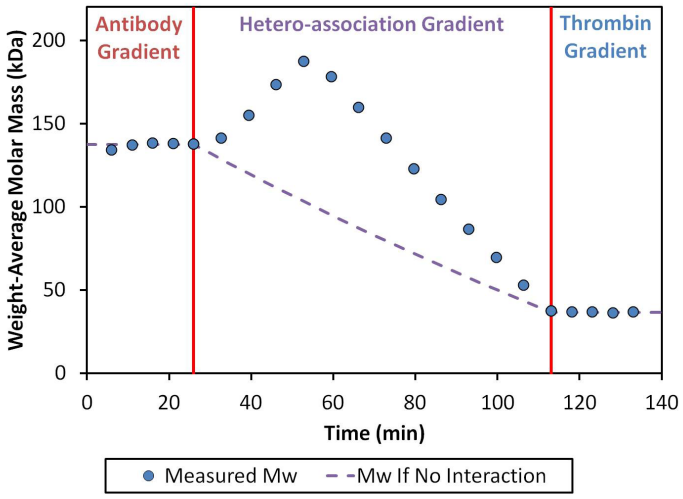
Antibody-antigen binding, hormone-receptor interactions, and many other common biomolecular interactions occur at stoichiometries other than 1:1. The Wyatt Calypso system utilizes composition-gradient multi-angle light scattering (CG-MALS) to determine both the affinity and binding stoichiometry for macromolecules in solution with no need for immobilization or tagging, which may influence the interaction.[1] In this study, we measured the interaction between an anti-thrombin antibody (Ab) and human thrombin-α (Thr).

To determine the interaction between Thr and Ab, composition gradients were created using a Calypso II and delivered to an online UV/Vis concentration detector and DAWN HELEOS. The automated Calypso method consisted of single component concentration gradients to quantify any self association and a dual component "crossover" composition gradient to assess the heteroassociation behavior. Heterocomplex formation was evident in the light scattering profile which peaked as the solution composition approached the expected 1:2 Ab:Thr stoichiometry.

The light scattering data from each plateau was best fit in the Calypso software by an association model which describes two equivalent thrombin binding sites per antibody molecule. The equilibrium dissociation constant determined by CG-MALS, K_D = 8 nM, agreed well with the manufacturer's data of K_D = 15 nM, as measured by ELISA. Furthermore, both thrombin and the antibody exhibited no propensity for self-association, a finding that could not be evaluated by conventional ELISA.



Composition-gradient method for quantifying the interaction between Thr and Ab.



Weight-average molar mass at equilibrium for each composition shown in the top figure. During the hetero-association gradient, the formation of Ab-Thr complexes results in an increase in Mw which reaches a maximum at 2:1 molar ratio, indicating an overall 2:1 stoichiometry.

WYATT TECHNOLOGY
6300 Hollister Avenue
Santa Barbara, CA 93117
Tel: +1 (805) 681-9009
Fax: +1 (805) 681-0123
Web: www.wyatt.com

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Introduction

Thrombin-α catalyzes multiple coagulation-reactions and interacts with proteins and other macromolecules at its active site and multiple exosites. Numerous techniques exist for probing these types of biomolecular interactions in vitro, including mass spectrometry, fluorescence anisotropy, and cell-based techniques, and so-called "label-free" techniques, such as SPR, analytical ultracentrifugation (AUC), and isothermal titration calorimetry (ITC). Composition-gradient multi-angle light scattering (CG-MALS) enables the characterization of macromolecular interactions in solution by quantifying the change in apparent weight-average molar mass (M_w) as a function of composition. Here, we apply CG-MALS to characterize the reversible equilibrium between human thrombin-α (Thr) and an anti-thrombin antibody (Ab).

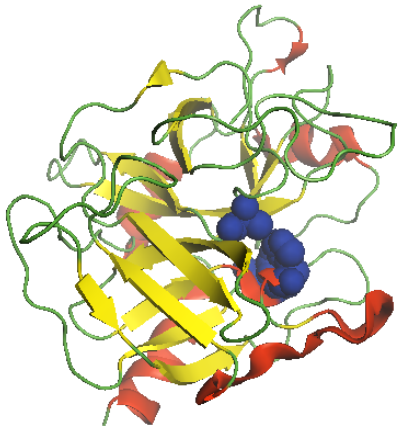

Figure 1: Thrombin-α with catalytic triad highlighted in blue (PDB ID 1TB6)

Materials and Methods

Reagents and Instrumentation

Human thrombin-α (Thr) and mouse monoclonal anti-human thrombin antibody (Ab) were purchased from Haematologic Technologies, Inc. All experiments were performed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) with or without 200 ppm sodium azide (NaN_3) as a preservative. Prior to analysis, all protein solutions were filtered to 0.02 µm using using Anotop syringe filters (Whatman).

CG-MALS experiments were performed with a Calypso II composition gradient system to prepare different compositions of protein and buffer and deliver to an online UV/Vis detector and HELEOS MALS detector (Figure 2). Anodisc (Whatman) or polycarbonate (Millipore) filter membranes with 0.1-µm pore size were installed in the Calypso for sample and buffer filtration.

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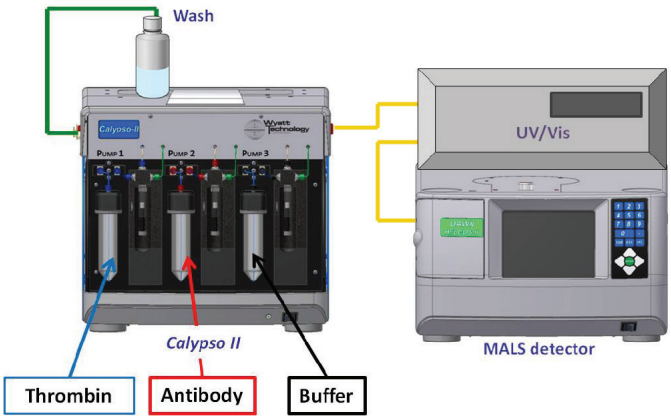

Figure 2: Calypso system hardware setup with inline UV/Vis concentration detector and DAWN HELEOS MALS detector.

Determination of Equilibrium Association Constants

Thr was diluted to a stock concentration of 20 µg/mL in PBS (for a total of ~200 µg Thr per experiment), and Ab was diluted to a stock concentration of 10 µg/mL or 20 µg/mL in PBS (for a total of ~100 or ~200 µg Ab per experiment). Each solution was filtered to 0.02 µm and loaded on the Calypso II. 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 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 60 or 180 s, and for the crossover gradient, the stop-flow time was increased to 120 or 300 s. A single experiment had an unattended run-time of ~2.5 h. Data collection and analysis of equilibrium association constants were performed using Calypso Software. Data from each replicate experiment was fit separately to yield an equilibrium association constant and stoichiometry.

Results and Discussion

Automated Calypso methods measured multiple protein compositions to probe the potential interactions present in a solution of thrombin (Thr) and a monoclonal anti-thrombin antibody (Ab). A single-species concentration gradient for each protein was used to determine whether Thr or Ab homo-oligomerized. This data combined with light scattering and concentration measurements at 8-12 ratios of Thr and Ab were then used to determine the affinity and stoichiometry of the Thr-Ab binding.

Under these conditions, no self-interactions were observed for Thr or Ab. The weight-average molar mass at each step in the self-association gradient for Ab was measured as 139±3 kDa, comparable to the monomer molecular weight measured by size exclusion chromatography coupled with MALS (SEC-MALS). Likewise, $M_{w,app}$ for Thr was measured as 36.8±0.3 kDa at all steps in the Thr self-association gradient, in good agreement with the formula molecular weight of 36.7 kDa. Heterocomplex formation was evident in the crossover gradient light scattering profile. In the absence of a Thr-Ab interaction, the light scattering signal would consist of a linear "staircase" of values (dashed purple plot, Figure 3). In fact, we observed an increase in scattering intensity indicating complex formation, and this intensity reaches a maximum at the step where the Ab concentration becomes limiting (solid blue plot, Figure 3). Binding kinetics, though clearly visible in the data, were not utilized to evaluate equilibrium constants.

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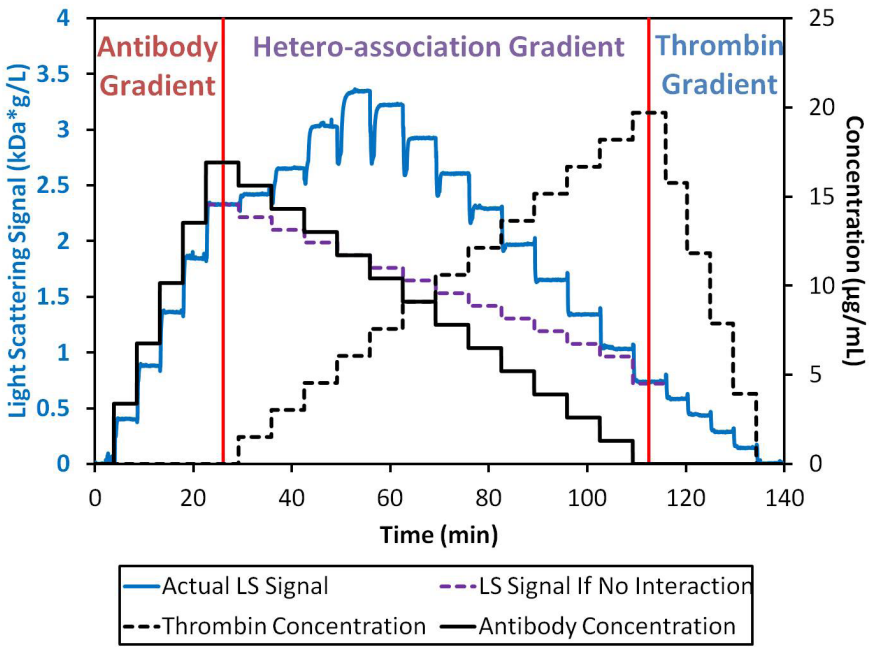

Figure 3: Typical light scattering signal and nominal concentrations describing the Ab-Thr interaction. During the hetero-association gradient, the measured LS signal (blue) was in excess of the calculated LS signal (purple dashed) for a mixture of two non-interacting proteins with M_w = 37 and 140 kDa each. The maximum light scattering signal occurs at the 2:1 molar ratio, indicating an overall 2:1 stoichiometry.

The light scattering intensity at equilibrium (plateau portion of each injection) and concentration data for each of two experiments were analyzed to determine binding affinity and stoichiometry for the interaction between Thr and Ab. For both experimental trials, the LS signal peaked at a Thr:Ab ratio of 2:1, indicating that the interaction was best described by an association model of two equivalent thrombin-binding sites per antibody molecule. The equilibrium association constant for the 1:2 interaction was calculated as $K_{A,12}$=(1.31±0.25) x 10^{16} M^{-2},which corresponds to a single-site binding affinity K_D = 8.8±0.8 nM. This affinity determined by CG-MALS agrees well with the manufacturer's data of K_D = 15 nM, as measured by ELISA. Furthermore, the CG-MALS data confirm the 1:2 stoichiometry that is not evident by ELISA or other surface-based techniques. There was no need to account for an incompetent fraction of thrombin or antibody monomers that was incapable of binding.

The calculated monomer molecular weights and K_D for each experimental replicate were used to determine the concentration and LS contribution for each species in solution during the crossover gradient (Figure 4B). As expected, at high Ab:Thr ratios, the (1 Ab):(1 Thr) species was the most abundant complex formed, and its concentration peaked at $[Thr]_{total}$ =$[Ab]_{total}$. As the thrombin concentration increased and antibody concentration decreased, the (1 Ab):(2 Thr) complex dominated with a peak at $[Thr]_{total}$=2$[Ab]_{total}$.

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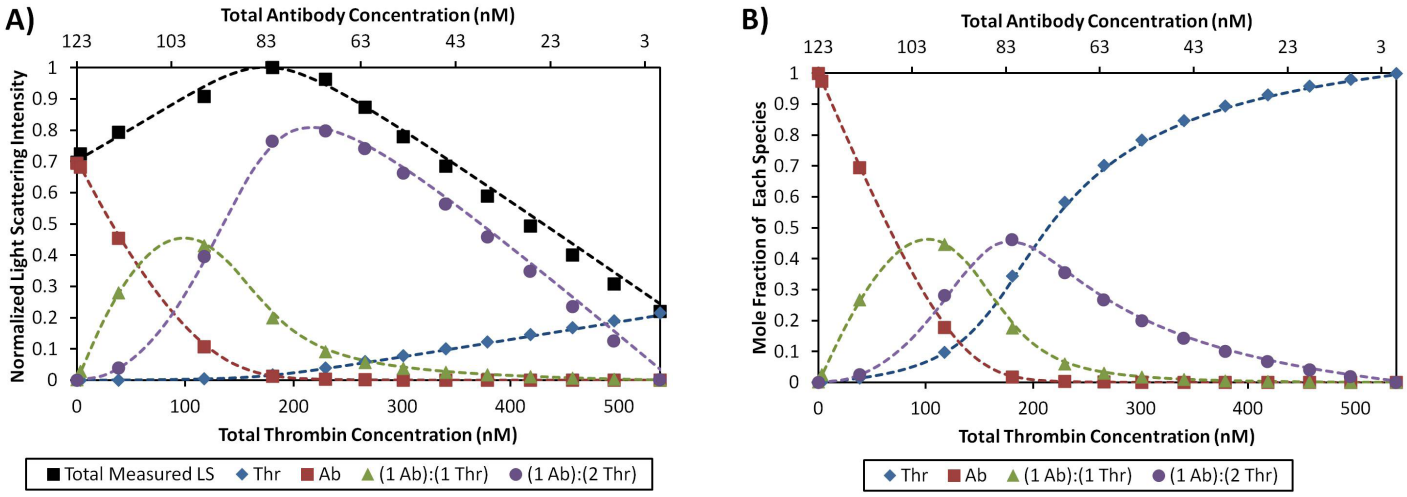

Figure 4: Typical best fit analysis for crossover gradient of Thr and Ab. A) Normalized total light scattering intensity (black) and contribution calculated from each species as a function of composition. B) Distribution of species at equilibrium as a function of composition. Dashed lines represent values calculated from a model of two equivalent binding sites per Ab with K_D = 8.8 nM, M_{Thr} = 36.8 kDa, and M_{Ab} = 139 kDa.

Conclusion

The simultaneous quantification of multiple species present in solution makes CG-MALS unique among biophysical characterization techniques and enables unambiguous quantification of macromolecular interactions. Here, CG-MALS quantifies the affinity and stoichiometry of monovalent and multivalent interactions existing at equilibrium without a secondary reporter or immobilization of either binding. Thus, probing macromolecular interactions by light scattering provides a robust, complementary biophysical technique for understanding complex protein-interaction *networks*.

References

(1) Some, D. and Kenrick, S. (2012). Characterization of Protein-Protein Interactions via Static and Dynamic Light Scattering. In *Protein Interactions*, Cai, J. and Wang, R.E. (Ed.), InTech, DOI: 10.5772/37240. (http://www.intechopen.com/books/protein-interactions/characterization-of-protein-protein-interactions-via-static-and-dynamic-light-scattering)

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