

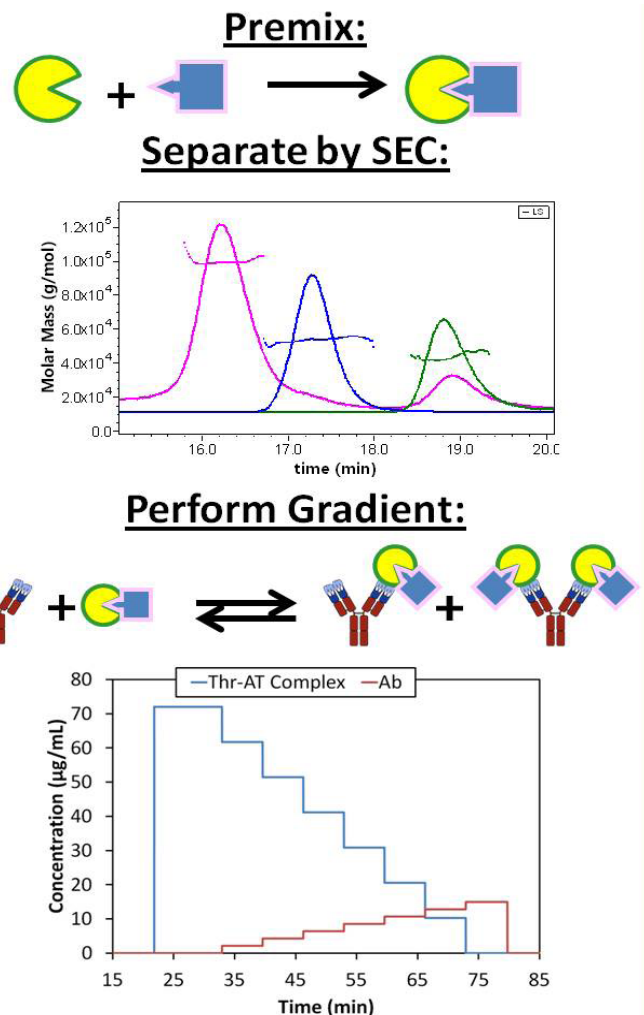
Does Antithrombin III Block the Action of a Monoclonal Anti-Thrombin Antibody?

Summary

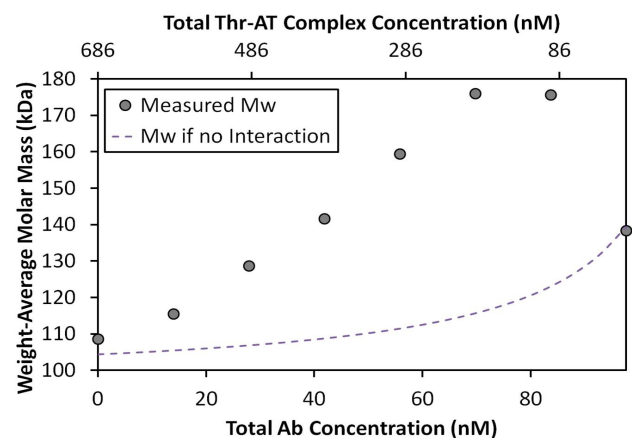
Multi-angle light scattering (MALS) is a powerful tool for quantifying multiple types of protein-protein interactions. In a previous application note, we described how composition gradient MALS (CG-MALS) quantified the equilibrium interaction between thrombin and an anti-thrombin antibody, revealing the expected 2:1 stoichiometry and equilibrium dissociation constant, $K_D = 8.8$ nM.¹ In another application note, we calculated the second order association rate constant for the covalent association of human thrombin- α to antithrombin III using time-dependent MALS (TD-MALS).² Here, we probe the interactions between three proteins: human thrombin- α (Thr), antithrombin III (AT), and an anti-thrombin monoclonal antibody to evaluate whether the antibody can recognize AT-inactivated Thr.

To identify the three-body interaction, we first pre-mixed thrombin and antithrombin and allowed the reaction to come to completion before separating the covalently bound complex from unreacted monomer by SEC. We then quantified the interaction between purified thrombin-antithrombin complex and the antibody by CG-MALS.

In this study, automated CG-MALS was used for the first time to assess the binding epitope of a monoclonal antibody. Binding of the antibody to the thrombin-antithrombin complex confirmed the antibody does not bind the thrombin active site; however, the antibody exhibits ~28x decrease in affinity for the thrombin-antithrombin complex compared with its affinity for free thrombin. We suspect that steric hindrance by antithrombin is the most likely cause for the reduced affinity.



Strategy for quantifying the binding between the covalent thrombin-antithrombin complex and an anti-thrombin antibody.



The measured Mw of the solution increases as the antibody binds the Thr-AT complex. The maximum molecular weight occurs at an overall molar ratio of 1 Ab: 2 Thr-AT complex, indicating a 1:2 stoichiometry.

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Introduction

Composition-gradient multi-angle light scattering (CG-MALS) and dynamic light scattering (CG-DLS) quantify the affinity and stoichiometry of equilibrium protein-protein interactions in solution as well as nonspecific interactions resulting from thermodynamic nonideality.³ In previous studies, we applied CG-MALS to characterize the reversible equilibrium between human thrombin- α and an anti-thrombin antibody¹ and the irreversible association of thrombin- α with antithrombin III.² In this application note, we further extend the technique to probe the recognition of the bound thrombin-antithrombin complex by the same antibody.

Materials and Methods

Reagents and Instrumentation

Human thrombin- α (Thr), human antithrombin III (AT), 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 Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4).

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 1). Inline filter membranes with 0.1- μ m pore size were installed in the Calypso for sample and buffer filtration.

Size exclusion chromatography (SEC) was performed using a column with 300 Å pore size (030S5 column, Wyatt), with injections performed by an HPLC pump and autosampler. The separation between the covalent Thr-AT complex and Thr and AT monomers was confirmed by SEC coupled with multi-angle light scattering (SEC-MALS) using a UV detector, DAWN HELEOS, and Optilab rEX.

Determination of Three-Component Binding

For initial determination of Ab binding to bound Thr-AT complex, CG-MALS experiments were performed such that Ab solution was exposed to an unfractionated mixture of Thr, AT, and Thr-AT complex, as follows. Thr and AT were diluted to stock concentrations of 24 μ g/mL and 80 μ g/mL, respectively, in PBS, and each solution was filtered to 0.02 μ m. Equal volumes of filtered stock solution were combined and allowed to incubate at room temperature for ~2 hr with gentle shaking. Ab was diluted to 22 μ g/mL in PBS and filtered to 0.02 μ m. A composition gradient was performed, consisting of six injections of premixed AT+Thr solution at constant concentration of 25 μ g/mL and Ab concentrations from 0 to 8 μ g/mL. The measured weight-average molar mass was used to determine qualitatively whether the antibody would recognize the bound Thr-AT complex.

To quantify the affinity of the Ab-complex binding, a second composition gradient was performed with multiple compositions of Ab and purified Thr-AT complex. Thr and AT were diluted to 2 mg/mL each in PBS, mixed at a 1:1 ratio, and allowed to incubate at room temperature 0.5-1 h. Pre-mixed aliquots of 0.1 mL were injected onto an SEC column, and fractions of bound Thr-AT complex were collected at the outlet of the UV detector. Multiple injections were performed and the fractions pooled and filtered to 0.1 μ m, with a final protein concentration ~70 μ g/mL. The purified Thr-AT complex was then used in further CG-MALS experiments. Ab was diluted to 15 μ g/mL in PBS and filtered to 0.02 μ m. The affinity and stoichiometry for the interaction between purified Thr-AT complex and Ab was quantified using a single crossover gradient with eight injections.

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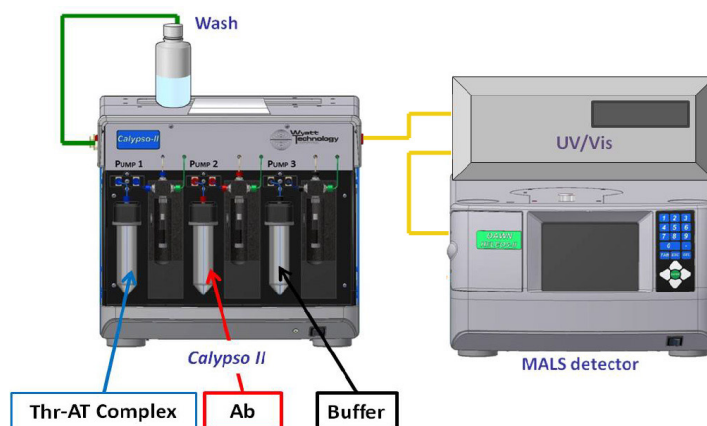


Figure 1: Hardware setup for investigating binding of Ab to Thr-AT complex. In one experiment, the Thr-AT mixture included free monomer Thr and AT. In the final experiment to quantify binding, the solution in Pump 1 contained only purified Thr-AT covalent complex.

Results and Discussion

Interaction of Ab with unpurified Thr-AT complex

Once Thr was bound irreversibly to AT, the ability of Ab to retain Thr-binding activity was measured with CG-MALS. In a preliminary experiment, AT and Thr were pre-mixed and the reaction allowed to come to completion before loading the unfractionated solution on the Calypso and performing a crossover gradient with this complex solution and Ab. Although binding was evident (Figure 2, left), the weight-average molar mass measured by light scattering was significantly less than expected for an interaction between Ab and Thr-AT complex with 2:1 stoichiometry and $K_D \sim 9$ nM per binding site, the affinity previously measured for Ab binding pure Thr (Figure 2, right, green triangles).

The decreased Ab-binding could be explained by two possible mechanisms: 1) Ab bound the Thr-AT complex with decreased affinity compared to free Thr; 2) Ab did not bind Thr-AT complex, and the observed increase in M_w resulted from Ab binding free Thr in solution. Previous experiments had shown that $\sim 23\%$ of the Thr was incompetent to binding AT;² Figure 2 (right) compares the measured M_w with that calculated for Ab binding this free Thr with affinity $K_D = 9$ nM and not binding the Thr-AT complex (blue X). Under these conditions, it was impossible to tell from which mechanism the measured M_w resulted.

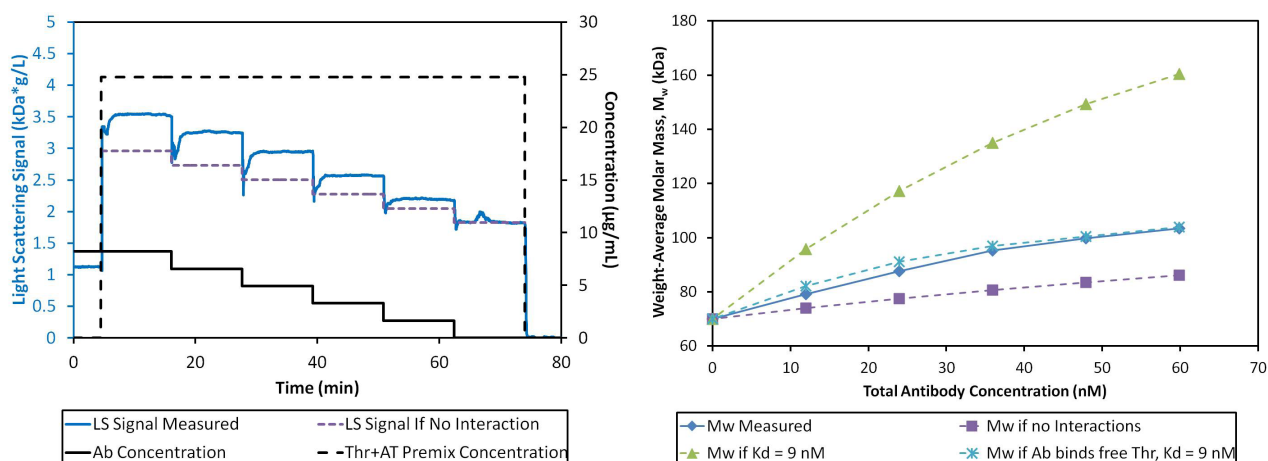


Figure 2: Left: LS and concentration data for composition gradient with constant concentration of unfractionated Thr, AT, and Thr-AT complex (25 µg/mL total protein) and varying concentrations of Ab. Right: Equilibrium M_w for experiment depicted at left indicated binding was occurring but could not differentiate between binding with decreased affinity and Ab binding free Thr in solution.

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Interaction of Ab with purified Thr-AT complex

In order to distinguish between the two possible mechanisms, bound Thr-AT complex was separated from unbound Thr and AT via SEC (Figure 3). The fraction of Thr that is incompetent for binding to AT is evident as a secondary peak in the SEC chromatogram for the pre-mixed solution (magenta, Figure 3). After collecting the appropriate peak, an aliquot was re-applied to the column to confirm that no dissociation had occurred and there was negligible contamination by free Thr.

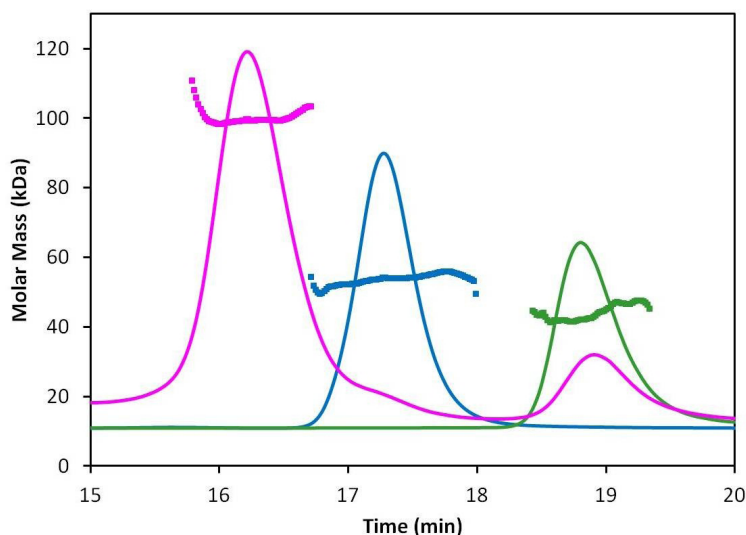


Figure 3: Overlay of size exclusion chromatograms for pure Thr (green), pure AT (blue), and a mixed solution of Thr and AT (magenta). Molecular weights measured by MALS correspond to expected values. Fractions of covalently bound Thr-AT complex were collected for CG-MALS analysis.

A CG-MALS experiment, consisting of a single crossover gradient with Ab and purified Thr-AT complex, revealed antibody binding with the expected 1:2 stoichiometry (Figure 4 and Figure 5). Since AT was covalently bound to the Thr active site, this possible binding site was inaccessible to the Ab, indicating the antibody recognizes an epitope on thrombin other than the active site. Previous binding assays also support this assertion. Quantifying the binding of the antibody to purified thrombin does not require accounting for an incompetent fraction of Thr, suggesting that the binding epitope is always accessible.¹ On the other hand, the binding of AT to Thr, which requires the active site to be accessible and/or properly folded, indicates that ~23% of Thr presented an active site that was incompetent for binding AT.² Taken together, these data confirm that the antibody binds thrombin away from the active site.

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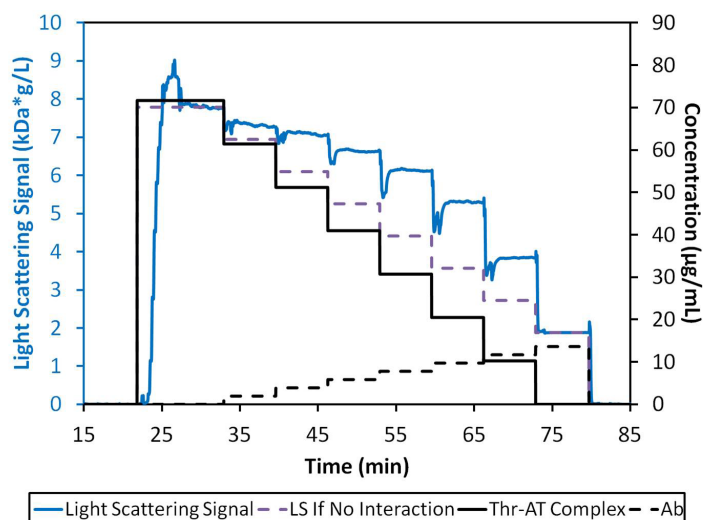


Figure 4: The measured light scattering data (blue) indicate an interaction between the antibody and purified thrombin-antithrombin complex.

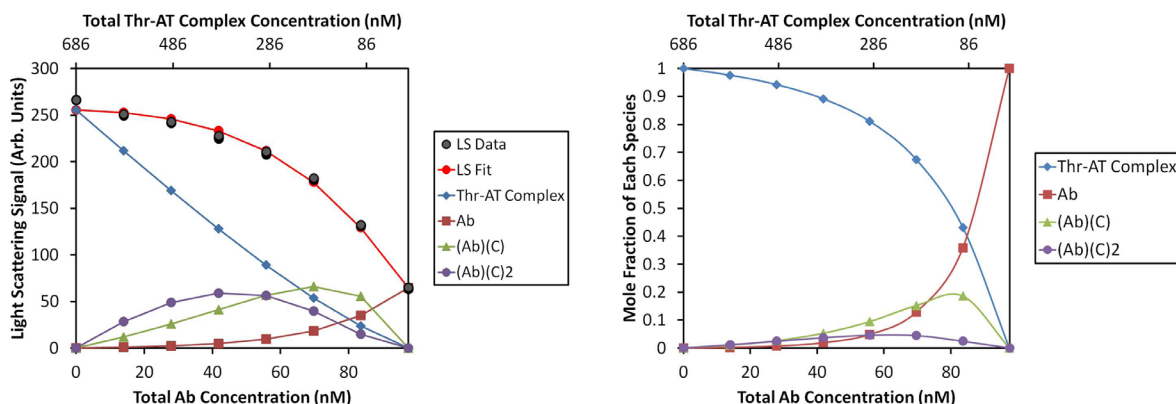


Figure 5: Left: Best fit of LS and concentration data for crossover gradient with purified Thr-AT complex and Ab indicates 1:2 (Thr-AT complex):(Ab) stoichiometry with decreased affinity compared to Thr:Ab alone. Right: Molar composition of species.

Although binding of the antibody to the Thr-AT complex is evident, the measured affinity of the antibody for the Thr-AT complex is $\sim 28\times$ decreased as compared to the affinity for pure Thr, with $K_D = 250$ nM. This may indicate that AT imparts some steric hindrance for antibody-binding. Alternatively, the binding of Thr to AT may lock the enzyme in an inactive conformation⁴ which has lower affinity for the antibody than the native or allosterically activated Thr.

Conclusion

The simultaneous quantification of multiple species present in solution makes MALS unique among biophysical characterization techniques. SEC-MALS confirmed the irreversible binding of AT and Thr and enabled unequivocal separation of the purified complex for further characterization. CG-MALS quantified the affinity and stoichiometry of the multivalent, equilibrium interaction between Ab and the Thr-AT complex, confirming the binding of the antibody to AT-inactivated thrombin but with decreased affinity. Thus, probing macromolecular interactions by light scattering provides a robust, complementary biophysical technique for understanding complex protein-interaction networks.

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