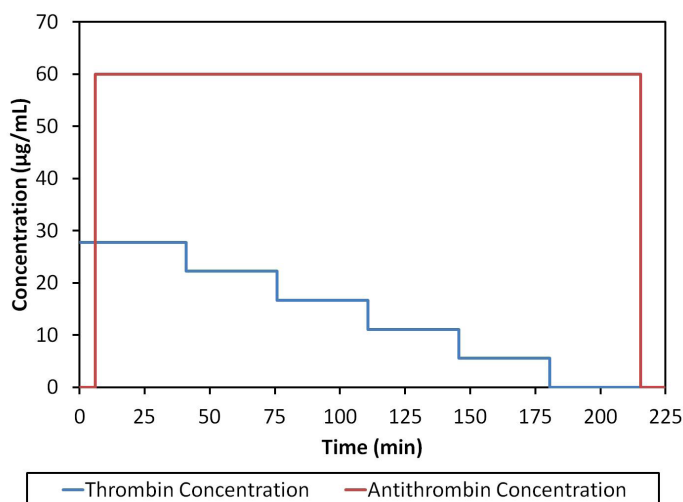


## Determining the Kinetics of Covalent Thrombin-Antithrombin Association

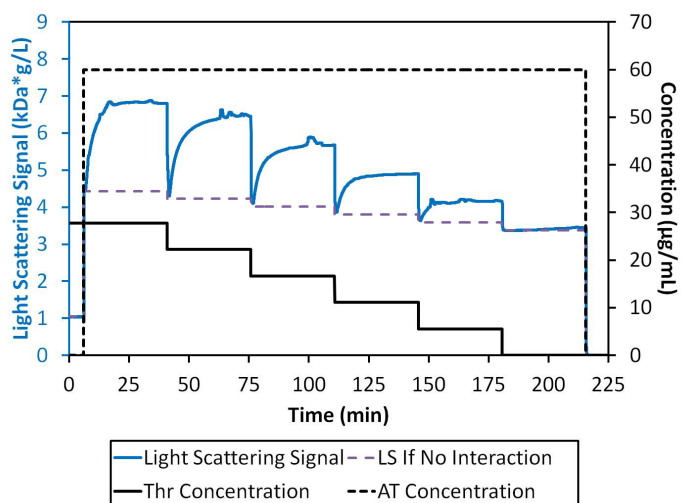
### Summary

Antithrombin III forms a covalent bond with the catalytic serine of several proteases involved in the coagulation cascade, and the rate of this reaction can increase up to 1000x in the presence of certain macromolecules. Here, we determine the second-order rate constant for the covalent association of thrombin- $\alpha$  (Thr) and antithrombin III (AT) by Composition Gradient Multi-Angle Light Scattering (CG-MALS).<sup>1</sup>

Composition gradients were created using a Calypso II and delivered to an online UV/Vis concentration detector and DAWN HELEOS. The method consisted of six injections at constant AT concentration of 60  $\mu\text{g/mL}$  and Thr concentrations from 0 to 30  $\mu\text{g/mL}$ . After each injection into the UV and MALS detectors, the flow was stopped for 2000 s to allow the reaction to come to completion. The light scattering data for all five measurements with nonzero thrombin concentrations were fit simultaneously to a model of 1:1 irreversible association between thrombin and antithrombin. Using this model, the second order rate constant was calculated as  $k = 6.09 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ , similar to results measured by other methods (e.g.,  $k = 5.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$  measured at 25°C by fluorescence<sup>2</sup>). The analysis simultaneously determined the fraction of thrombin capable of binding antithrombin. This calculated fraction, 77%, compared favorably with the manufacturer's reported specific activity of the antithrombin (0.79 mol Thr/mol AT).



CG-MALS Method for measuring the time-dependent, irreversible interaction between thrombin and antithrombin



The slow, irreversible interaction between thrombin and antithrombin is measured for ~20 minutes after the flow to the detectors has stopped. For each composition of thrombin and antithrombin, the measured LS signal (blue) increases from the level indicated for no interaction (dashed purple line) to the final value at the completion of the reaction.

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### Introduction

Thrombin (Thr) is a serine protease that plays a key role in the coagulation cascade, and its activation is the terminating step for both the intrinsic and extrinsic pathways. The serpin antithrombin III (AT) inhibits thrombin by covalently binding the enzyme's catalytic serine and trapping it in this stable intermediate state.<sup>3</sup> This reaction is known to be slow with a second order rate constant  $k \sim 10^3 \text{ M}^{-1}\text{s}^{-1}$ , which increases  $>1000\times$  in the presence of physiological concentrations of heparin.<sup>4</sup> Although previous studies with stopped-flow light scattering have measured the kinetics of large assemblies such as fibril formation<sup>5,6</sup> and protein binding to lipid vesicles,<sup>6</sup> this study of the Thr-AT interaction represents the first quantification of the second-order association constant for a 1:1 protein-protein interaction by time-dependent CG-MALS.

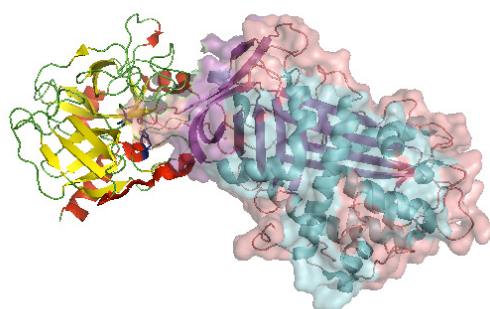


Figure 1: Structure of antithrombin (surface, right) bound to the active site of thrombin (ribbons, left). PDB ID 1TB6.

### Materials and Methods

#### Experimental

Human thrombin- $\alpha$  (Thr) and human antithrombin III (AT) from Haematologic Technologies, Inc., were prepared at stock concentrations of 60  $\mu\text{g/mL}$  and 120  $\mu\text{g/mL}$ , respectively, in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.76 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4) and filtered to 0.02  $\mu\text{m}$  using Anotop syringe filters (Whatman). The total amount of protein required was  $\sim 250 \mu\text{g}$  Thr and  $\sim 600 \mu\text{g}$  AT. 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). Filter membranes with 0.1- $\mu\text{m}$  pore size were installed in the Calypso for sample and buffer filtration. A composition gradient was performed, consisting of six injections at constant AT concentration of 60  $\mu\text{g/mL}$  and varying Thr concentrations from 0 to  $\sim 30 \mu\text{g/mL}$ . After each injection into the UV and MALS detectors, the flow was stopped for 2000 s to allow for the reaction to come to completion. The Calypso software was used to run the method, acquire MALS and UV signals, and calculate the apparent weight-averaged molar mass as a function of time. The data were exported and fit to an appropriate time-dependent association model.

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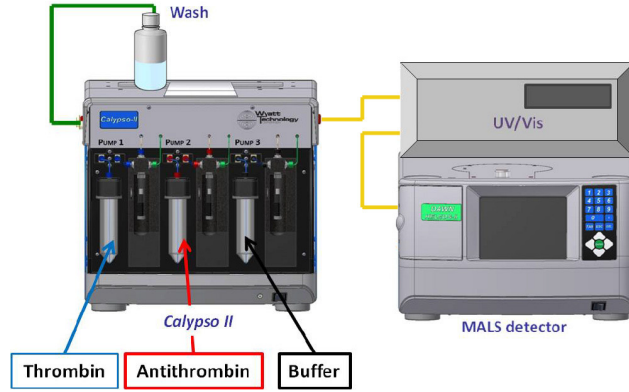


Figure 2: CG-MALS instrument setup for measuring the irreversible interaction between thrombin and antithrombin

### Mathematical Models

The slow irreversible association of thrombin and antithrombin is an attractive model for analysis by time-dependent CG-MALS. For the elementary reaction  $AT + Thr \rightarrow C$ , the time-dependent accumulation of complex can be described by a second-order rate constant  $k$ :

$$\frac{d[C]}{dt} = k[AT][Thr] \quad (1)$$

where  $[AT]$ ,  $[Thr]$ , and  $[C]$  are the molar concentrations of antithrombin, thrombin, and the covalent Thr-AT complex, respectively. In the case where some fraction of each protein is incompetent for binding (e.g., misfolded, inactivated, etc.), Equation 1 is re-written as follows:

$$\frac{d[C]}{dt} = k[AT]_{comp}[Thr]_{comp} \quad (2)$$

where  $[AT]_{comp}$  and  $[Thr]_{comp}$  are the concentrations of antithrombin and thrombin, respectively, that are competent for binding. For each injection, the total concentrations of AT and Thr are fixed and related to the concentration of complex by stoichiometry:

$$[AT]_{total} = [AT]_{comp} + [AT]_{incomp} + [C] \quad (3)$$

$$[Thr]_{total} = [Thr]_{comp} + [Thr]_{incomp} + [C] \quad (4)$$

We define competent fraction of each monomer  $i$  as follows:

$$x_i^{comp} = \frac{[i]_{comp}}{[i]_{total}} \quad (5)$$

Substituting these relationships into Equation 2 and solving for the case where the thrombin concentration is limiting ( $[AT]_{total} > [T]_{total}$ ) yields the following relationship for the concentration of complex:

$$[C] = \frac{x_{Thr}^{comp}[Thr]_{total} \left( 1 - e^{k(x_{Thr}^{comp}[Thr]_{total} - x_{Thr}^{comp}[AT]_{total})t} \right)}{1 - \frac{x_{Thr}^{comp}[Thr]_{total}}{x_{AT}^{comp}[AT]_{total}} e^{k(x_{Thr}^{comp}[Thr]_{total} - x_{AT}^{comp}[AT]_{total})t}} \quad (6)$$

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The measured light scattering intensity for a dilute solution is then related to the concentration of each species Equation 7:

$$\frac{R}{K^*} = (M_{Thr})^2([Thr]_{comp} + [Thr]_{incomp}) + (M_{AT})^2([AT]_{comp} + [AT]_{incomp}) + (M_{Thr} + M_{AT})^2[C] \quad (7)$$

where  $R/K^*$  is the scaled Rayleigh ratio and  $M_i$  is the molecular weight of each species. The measured light scattering signal as a function of time and varying  $[AT]_{total}:[Thr]_{total}$  ratios can be used to determine the second order rate constant  $k$ .

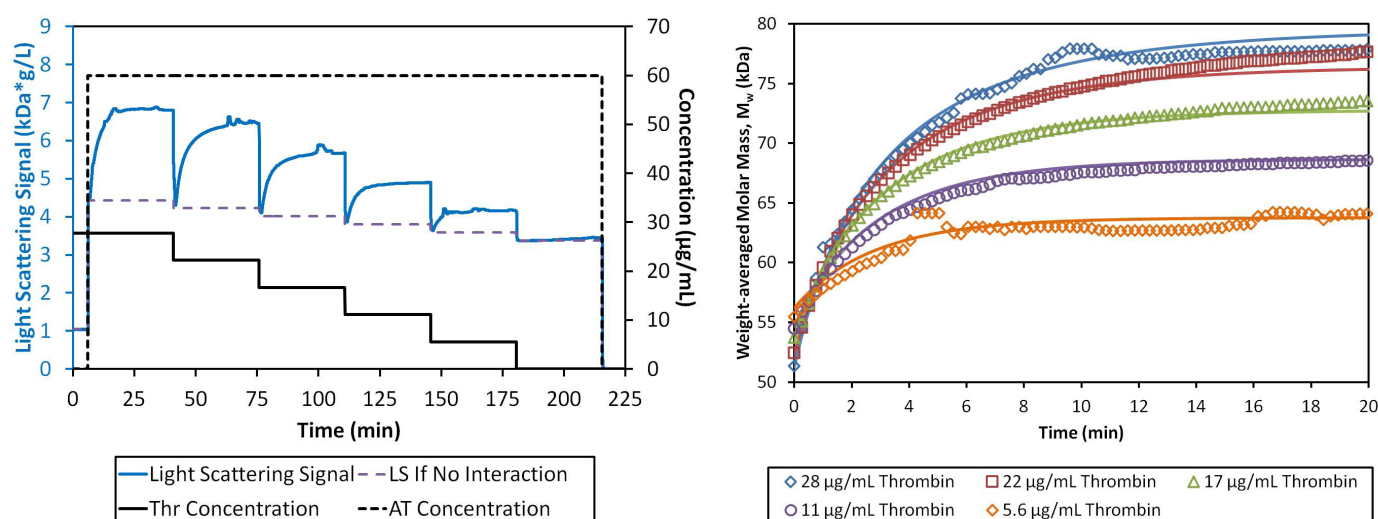


Figure 3: Left: Light scattering and concentration data for thrombin titration at constant antithrombin concentration. Right: The increase in  $M_w$  as a function of time was fit to the appropriate association model to calculate the second order rate constant for the inhibition of Thr by AT. Raw data (open symbols) and best fit curves are shown for varying Thr concentrations and constant AT concentration of 60 μg/mL.

## Results and Discussion

The slow association of Thr and AT is evident in the light scattering intensity for each injection of Thr and AT (Figure 3). For each combination of Thr and AT, the initial light scattering intensity corresponds to a system of non-interacting Thr and AT molecules with a weight-weight average molar mass,  $M_w$  between 50 and 55 kDa. The intensity increases over time as Thr-AT complex is formed, and a corresponding increase in  $M_w$  is calculated. The light scattering signal reaches a plateau as the reaction comes to completion in 10-20 minutes (Figure 3, left). The LS intensity at  $t = 0$  in Figure 3 (left) corresponds to the light scattered by pure Thr at ~30 g/mL; similarly the light scattered during the interval  $t = 180-215$  min represents the light scattered by pure AT at ~60 g/mL. The LS intensity from each pure species remains relatively invariant over time, indicating self-association is not occurring at these concentrations.



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In analyzing the light scattering data, it was determined that some fraction of Thr, AT, or both may be incompetent for binding. This was not unreasonable given the variable thrombin activity from lot-to-lot, as reported by the manufacturer, and stoichiometry other than 1:1 had been measured in previous studies.<sup>2</sup> Nonlinear least squares analysis was performed simultaneously for all five compositions with non-zero Thr concentration to solve for  $k$ ,  $x_{Thr}^{comp}$  and  $x_A^{comp}$  (Figure 3, right). The fit included LS data for the first 1000 seconds after flow into the MALS detector had stopped. The resulting second-order rate constant  $k = 6.09 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$  with  $x_{Thr}^{comp} = 0.7$  and  $x_A^{comp} = 1$  was consistent with previous measurements of thrombin inhibition by antithrombin III in the absence of heparin.<sup>2</sup> Moreover, the competent fraction of thrombin determined by the fit is consistent with the manufacturer's reported specific activity of the antithrombin (0.79 mol Thr/mol AT).

## Conclusion

Time-dependent CG-MALS enabled the observation of association kinetics in real time, without the use of fluorescent tags, surface immobilization, or other modifications that could influence the interaction. Moreover, the time dependent MALS data suggested that the extent of reaction was limited by a fraction of thrombin that was incompetent for binding with antithrombin. Thus, CG-MALS provided a complete picture of the association between thrombin and antithrombin in solution.

## References

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