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LETTER

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Measurement of Aptamer–Protein Interactions with Back-Scattering Interferometry

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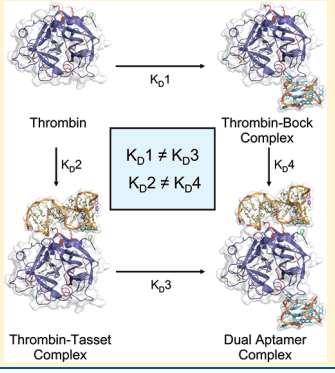
Supporting Information

ABSTRACT: We report the quantitative measurement of aptamer–protein interactions using backscattering interferometry (BSI) and show that BSI can determine when distinct binding regions are accessed. As a model system, we utilized two DNA aptamers (Tasset and Bock) that bind to distinct sites of a target protein (human α-thrombin). This is the first time BSI has been used to study a multivalent system in free solution wherein more than one ligand binds to a single target. We measured aptamer equilibrum dissociation constants (K_d) of 3.84 nM (Tasset–thrombin) and 5.96 nM (Bock–thrombin), in close agreement with the literature. Unexpectedly, we observed allosteric effects such that the binding of the first aptamer resulted in a significant change in the binding affinity of the second aptamer. For example, the K_d of Bock aptamer binding to preformed Tasset–thrombin complexes was 7-fold lower (indicating higher affinity) compared to binding to thrombin alone. Preliminary modeling efforts suggest evidence for allosteric linkage between the two exosites.



Multivalent binding interactions form the foundation of many important biological functions found in nature. For example, ethanol binds to the allosteric site of $GABA_A$ and glycine receptors[1] to up-regulate activity, while influenza viruses exploit multivalent protein-carbohydrate interactions to enter host cells.[2] Multivalent binding is also critical for *in vitro* molecular diagnostics, and the most widely used methods, such as the enzyme-linked immunosorbent assay (ELISA) and its variants,[3,4] are "sandwich" assays that target multiple epitopes to achieve specific detection of analytes.

The capability to accurately measure multivalent binding interactions is important for diverse biotechnological applications, and numerous analytical methods have been developed along these lines. For example, few mode fiber surface plasmon resonance (FMF-SPR)[5] and planar optical waveguides[6,7] are useful for the capture and detection of cancer biomarkers in a sandwich assay format, while lipid bilayer sensors[8] enable measurement of multivalent interactions between antibodies and membranes. Though powerful, the above methods require either chemical labeling of the target or surface immobilization of affinity reagents, which can alter the characteristics of molecular interactions.[9,10] Thus, there remains an urgent need for a label-free analytical measurement technique that can measure multivalent binding reactions in solution.

In this letter we demonstrate the capability to rapidly measure multivalent aptamer–target binding in a manner that could aid in the development of reagents for sandwich assay methods such as ELISA. This assay is performed with small quantities of analyte in free-solution, without any labeling or tethering, by simply forming aptamer–protein complexes and then measuring the binding affinity for a second aptamer using BSI. As a model, we have applied this technique to measure the binding of human α-thrombin by a pair of well-characterized aptamers (Bock[11] and Tasset[12]) that interact with distinct regions of the protein.[12,13] We found that the K_d values of each aptamer were in agreement with those found in the literature. Surprisingly, we also observed that the two aptamers exhibit allosteric effects, that is, the formation of a single aptamer–thrombin complex results in a significant change in the binding affinity of the second aptamer.

EXPERIMENTAL SECTION

As we have described previously,[14−18] BSI utilizes Fourier analysis of interference patterns arising from binding reactions in solution to obtain equilibrium dissociation constants (K_d)[14] with picomolar sensitivity and a dynamic range of over 6 orders of magnitude.[14,18] Briefly, the instrument is comprised of a helium–neon (HeNe) laser, a microfluidic chip, and a linear

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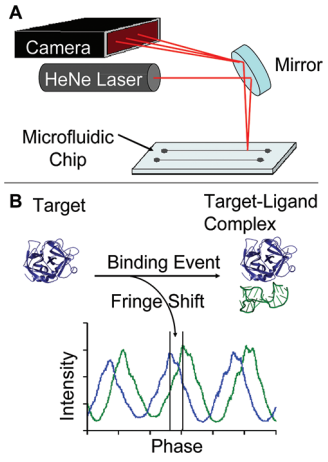

Figure 1. Experimental setup of the BSI system: (A) A mirror directs a helium−neon (HeNe) laser onto an isotropically etched semicircular microfluidic channel. The impinging beam reflects and refracts within the channel, generating a high-contrast fringe pattern that gets collected using a linear CCD array. (B) Binding between the target and ligand yields a quantifiable spatial shift in the fringe pattern.

array detector (Figure 1). The sample is injected into the microfluidic chip, which is configured to create a resonance cavity with a long effective path length, and the incident coherent light is converted into an interferometric fringe pattern that can be captured by a charge-coupled device (CCD) camera. We use Fourier analysis to determine phase change (in radians)[18] and quantify spatial shifts in the pattern resulting from changes in the refractive index (RI), which have been shown to correlate with ligand−receptor binding.[14−18] We performed all experiments in solution without immobilization or labeling of molecules and utilized end-point measurements as previously described by our group.[14] BSI assays are performed as difference measurements, with binding curves derived from the differences among the signals from the sample, blank (usually the ligand in the same matrix), and a control (also in the same matrix). This allows specific binding to be quantified even in the presence of a high background bulk RI. Results presented in this manuscript represent the difference in signal between the binding sample or the control sample signal and an aptamer-only blank. Subtracting the blank allows us to compensate for changes in bulk RI as the concentration is increased during the assay. The blank signal was similar in all experiments, about 0.04 radians.

The Bock aptamer (5′-GGTTGGTGTGGTTGG-3′), Tasset aptamer (5′-CAGTCCGTGGTAGGGCAGGTTGGGG TGACTTCGTGGAA-3′), and randomized 80-mer (5′- AGCAGC ACAGAGGTCAGATG-Random[40]-CCTATGCGTGCTACCG TGAA-3′) and 100-mer (5′-AGCAGCACAGAGGTCAGATG-Random[60]-CCTATGCGTGCTACCGTGAA-3′) negative control sequences were synthesized by Integrated DNA Technologies (Coralville, IA). For negative control aptamer samples, each individual strand contained a unique, randomized sequence. Human α-thrombin was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). All other chemicals were obtained through Sigma-Aldrich (St. Louis, MO). We used a 50 mM tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 7.5) containing 100 mM NaCl and 1 mM $MgCl_2$ for all dilutions. Each aptamer was denatured at 100 °C for 10 min, rapidly cooled in an ice bath for 10 min, and finally allowed to

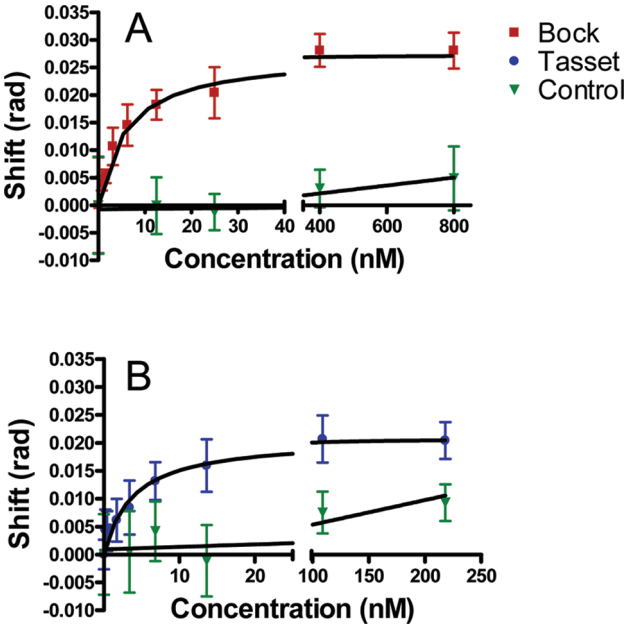

Figure 2. Measurement of dissociation binding constants of aptamers to thrombin using the single channel BSI setup: (A) Bock aptamer binding to thrombin shows a K_d value of 5.96 ± 0.57 nM and B_{MAX} = 0.027 radians; (B) Tasset aptamer binding to thrombin yielded a K_d value of 3.84 ± 0.68 nM and B_{MAX} = 0.021 radians. Error bars on all plots were derived from three independent experiments. Control data were generated using random 80-mer and 100-mer sequences for plots A and B, respectively.

reach room temperature. We incubated 2 nM thrombin at 10 °C overnight with aptamers prepared at a range of concentrations (0−800 nM) by serial dilution. After incubation, we brought the samples to room temperature over a 30 min period prior to BSI analysis.

RESULTS AND DISCUSSION

BSI Measurement of Equilibrium Binding Constants of Aptamers. We measured the binding affinity of Bock and Tasset aptamers for thrombin in solution using an end-point assay.[14] As negative controls, we used random DNA sequences that were 80 and 100 nucleotides in length. For the BSI measurement, we drew 1 μL of the aptamer−thrombin sample into the microfluidic channel. We then stopped the flow and collected 30 s of fringe position data for each aptamer concentration, after which we eluted the sample and repeated the process for increasing aptamer concentrations. To minimize possible measurement errors arising from long-term drift of the instrument, we performed the entire assay sequentially in triplicate. To do so, we first ran the control and/or blank solutions in order of ascending concentration and then evaluated the binding pair for the same series of ligand concentrations. This data set constitutes an entire determination of K_d. This procedure was repeated three times to generate the binding curves displayed in Figure 2. Since the assay is performed in this manner, the error bars seen on the binding curves represent the assay reproducibility of the method and are thus a conservative estimate of binding affinity.

Using Graphpad Prism software and a single site binding fit, we determined K_d values for Bock and Tasset aptamers to be

Table 1. Comparison of Binding Affinities Obtained Using BSI

binding species	K_d (literature)	K_d (BSI)
Bock−thrombin	1.4 − 6.2 nM[19]	5.96 ± 0.57 nM
Bock−[Tasset complex]	not available	0.87 ± 0.18 nM
Tasset−thrombin	0.5 − 1.0 nM[14]	3.84 ± 0.68 nM
Tasset−[Bock complex]	not available	1.9 ± 0.2 nM
aptamer−self complex	not available	negligible binding

5.96 ± 0.57 nM and 3.84 ± 0.68 nM, respectively. These values compare reasonably well to literature values of 1.4−6.2 nM for Bock[12,19] and 0.5−1.0 nM for Tasset[12] (Table 1). It is well established that different methods can yield a range of K_d values, and the slight discrepancy between experimental and literature K_d values for the Tasset aptamer can most likely be attributed to the fact that literature values were generated using the fundamentally different nitrocellulose filter-binding technique.

Multivalent Interactions Affect Exosite Binding Characteristics. After establishing the affinity for each aptamer, we evaluated the use of BSI to study multivalent binding interactions and explore whether allosteric interactions exist in this system. To do so, we first formed the Tasset−thrombin complex and then measured the affinity of the Bock aptamer for the complex. We formed the Tasset−thrombin complex by incubating 500 nM Tasset aptamer with 2 nM thrombin at 10 °C overnight to saturate the binding site. Then, we performed an end-point assay with increasing concentrations of Bock aptamer to obtain the binding curve (Figure 3A). Finally, we measured the binding affinity of the Tasset aptamer to the Bock−thrombin complex in the same manner (Figure 3B).

To ensure that the BSI signals originated from aptamer−thrombin interactions and not aptamer−aptamer interactions, we performed a measurement in which aptamer−thrombin complexes were formed and then incubated with increasing concentrations of the same aptamer. We reasoned that if the majority of binding sites on thrombin are occupied during formation of the complex, the introduction of excess aptamers should only produce a refractive index (RI) change similar to that generated by the blank. The difference in BSI response for the aptamer complex−complementary aptamer and the blank, as measured by the slope, was approximately 1×10^{-5} radians for both aptamers (Figure 3). This level of signal is nearly 3 orders of magnitude lower than a typical binding signal and is therefore considered negligible, indicating that no appreciable binding took place after formation of the complex. As noted by others,[12,20,21] the Bock and Tasset aptamers do not bind to each other. In any case, we performed binding investigations as end-point assays, where first complex formation was allowed to progress over a relatively long incubation period (overnight) further ensuring excess unbound aptamer would self-associate or become unreactive to other aptamers.[20] Once the thrombin-single aptamer complex is formed, we introduce the second aptamer which is allowed to bind to the second thrombin exosite.

Fitting these curves to a single site binding analysis, we measured the K_d of Tasset aptamer binding to the Bock−thrombin complex to be 1.9 ± 0.2 nM. Similarly, we measured the K_d of Bock aptamer binding to the Tasset−thrombin complex to be 0.87 ± 0.18 nM. These K_d values are considerably lower (indicating higher affinity) than for the individual aptamer−thrombin measurements, as summarized in Table 1.

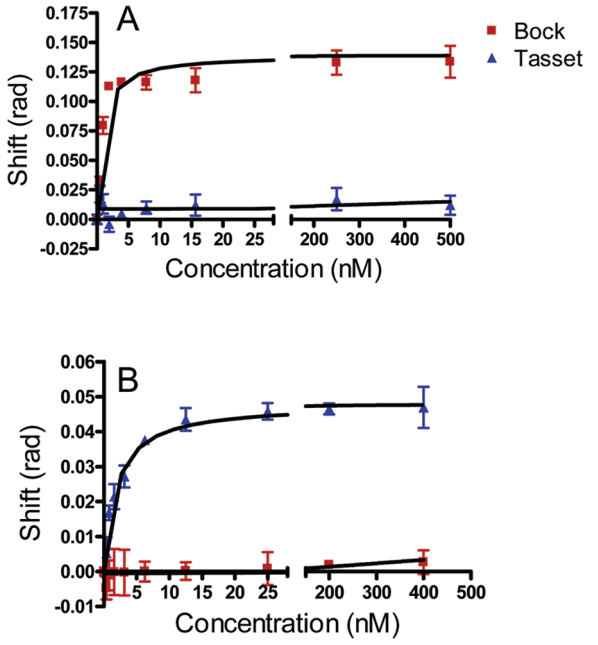

Figure 3. Measurement of aptamer binding to preformed aptamer−protein complexes: (A) Bock aptamer binding to Tasset−thrombin complexes yields a K_d value of 0.87 ± 0.18 nM and B_{MAX} = 0.139 radians. Tasset does not bind Tasset−thrombin complex; (B) Tasset aptamer binding to Bock−thrombin complexes yields a K_d value of 1.9 ± 0.2 nM and B_{MAX} = 0.048 radians. Bock does not bind the Bock−thrombin complex.

To explain these apparent allosteric interactions, we created a computational model using the AMBER 10 and RosettaDock programs. The model suggests that the binding sites of the two aptamers, which reside at opposite ends of the substrate-binding cleft, may be connected by a framework of rigid secondary-structural elements (Figure S-1 in the Supporting Information). While preliminary, these results are in reasonable agreement with similar studies that have found evidence for an allosteric linkage between exosites I and II using fluorescently labeled hirudin and a prothrombin fragment.[13]

CONCLUSIONS

In this work, we report the first use of BSI to measure the binding interactions among multiple DNA aptamers to a protein in solution. As a means of calibration, we first confirmed that the K_d values obtained with BSI for the Bock and Tasset aptamers against human α-thrombin are in reasonable agreement with those reported in the literature. Importantly, when we preformed the Tasset−thrombin complex, we observed a 7-fold increase in Bock aptamer affinity for this complex compared to binding to thrombin alone. Similarly, we observed a 2-fold increase in affinity for Tasset aptamer binding to the Bock−thrombin complex compared to thrombin alone. As negative controls, we verified that the Tasset and Bock aptamers have negligible binding to Tasset−thrombin and Bock−thrombin complexes, respectively. Although we have not conclusively identified the origin of this phenomenon, our preliminary modeling efforts suggest that a rigid secondary structure framework contributes to allosteric linkage between exosites 1 and 2, as has been observed with other species.[13]

In conclusion, we have demonstrated the capability to measure binding interactions of multiple aptamer ligands with a

single target in solution without labels using BSI. In order to increase the sensitivity and reproducibility of the measurements, we are currently optimizing the technique to maximize the signal difference arising from changes in conformation, waters of hydration, and/or charge density produced by the binding events. We believe such advances will benefit many areas of bioanalytical technology, especially with regard to assay design and optimization for molecular diagnostics.

■ ASSOCIATED CONTENT

Supporting Information. Description of computational modeling procedures and the corresponding figure of the docked system. This material is available free of charge via the Internet at http://pubs.acs.org.

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