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# A Fiber-Optic Microarray Biosensor Using Aptamers as Receptors

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**A fiber-optic biosensor using an aptamer receptor has been developed for the measurement of thrombin. An antithrombin DNA aptamer was immobilized on the surface of silica microspheres, and these aptamer beads were distributed in microwells on the distal tip of an imaging fiber. A different oligonucleotide bead type prepared using the same method as the aptamer beads was also included in the microwells to measure the degree of nonspecific binding. The imaging fiber was coupled to a modified epifluorescence microscope system, and the distal end of the fiber was incubated with a fluorescein-labeled thrombin (F-thrombin) solution. Nonlabeled thrombin could be detected using a competitive binding assay with F-thrombin. The aptamer beads selectively bound to the target and could be reused without any sensitivity change. The fiber-optic microarray system has a detection limit of 1 nM for nonlabeled thrombin, and each test can be performed in ca. 15 min including the regeneration time.** © 2000 Academic Press

***Key Words:*** **aptamer; thrombin; microarray; biosensor; fiber optic.**

Aptamers are single-stranded oligonucleotides that can bind with high affinity to their respective target molecules such as nucleotides, drugs, proteins, and organic dyes. Aptamers are discovered by an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX)[2] (1, 2). These SELEX-derived aptamers generally have a high degree of similarity to antigen–antibody binding in both their specificity and their affinity. Aptamers have an advantage over antibodies because aptamers can be synthesized and reproduced in a short time frame. Since their original discovery, most aptamers have been explored for their potential use as drugs (3, 4). Only recently have the stability and convenience of DNA aptamers been exploited in diagnostic applications including biosensor (5) and flow-cytometry (6) assays. Even though RNA aptamers are not as stable as DNA aptamers, Drolet *et al.* (7) reported an ELISA-like assay using a modified nuclease-resistant RNA aptamer.

In recent years, there has been a great deal of interest in multiplexed, high-throughput assays and, as a result, many microscale chemical systems have been devised. Among them, DNA chip (microarray) technology has been growing most rapidly (8–10). These immobilized DNA arrays have proven useful for the rapid detection of mutations (11, 12), polymorphisms (13), and gene discovery and expression monitoring (14–16).

In this study, we employ an antithrombin aptamer as a receptor in a microarray format. Protein binding was successfully monitored in the microarray system. The antithrombin aptamer was covalently conjugated to the surface of microspheres, and these microspheres were distributed into the microwells of optical imaging fibers. A CCD (charge-coupled device) camera then measured aptamer–target binding after incubation with fluorescein-labeled thrombin. A more useful assay format was used to quantify nonlabeled thrombin through a competitive binding assay between nonlabeled and fluorescein-labeled thrombin. The sensor was reusable and did not show any sensitivity change during the experiment.

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[2] Abbreviations used: SELEX, systematic evolution of ligands by exponential enrichment; ELISA, enzyme-linked immunosorbent assay; CCD, charge-coupled device; PBS, phosphate-buffered saline; PEI, polyethyleneimine; SBB, sodium borate buffer; F-SE, fluorescein-succinimidyl ester; F-thrombin, fluorescein-labeled thrombin; BSA, bovine serum albumin.



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## EXPERIMENTAL SECTION

*Materials.* Silica microspheres (3.1 ± 0.06 μm diameter) were purchased from Bangs Laboratories, Inc. (Fishers, IN). Imaging optical fibers (500 μm diameter) were obtained from Galileo Electro-optics Corp. (Sturbridge, MA). Fluorescein protein labeling kit was obtained from Molecular Probes, Inc. (Eugene, OR). Oligonucleotides were synthesized by Tufts University Physiology Department (Boston, MA). An amino group with a spacer arm (C6) was attached to the 5′ end of the thrombin-binding aptamer (5′-$NH_2$-$C_6$-GGTTGGTGTGGTTGG-3′) and poly(T) (15-mer). Poly(A) (15-mer) was fluorescently labeled at the 5′ end using fluorescein phosphoramidite. All other chemicals including thrombin (human, 3000 NIH units/mg) were from Sigma Chemical Company (St. Louis, MO).

*Imaging instrumentation.* All fluorescence measurements were made using a horizontal modified epifluorescence microscope (Olympus, Lake Success, NY) and an intensified CCD (Princeton Instruments, Inc., Trenton, NJ) as described previously (17). A 75-W xenon arc lamp was used as a light source, and 20× microscope objective was used for collecting light. IPLab image processing software (Signal Analytics, Vienna, VA) was used for acquiring and analyzing data.

*DNA immobilization.* Oligonucleotides were immobilized on the surface of microspheres following the method Ferguson *et al.* (18) with some modifications. Ten microliters of nonporous silica beads were silanized for 2 h using a 10% solution of (3-aminopropyl)triethoxysilane in acetone. After rinsing with acetone and 0.02 M PBS buffer (pH 6.8), the amino-silanized beads were allowed to react with 200 μL of 5% glutaraldehyde in PBS for 1 h. The beads were washed with PBS and then allowed to react with 200 μL of 5% polyethylenemine (PEI) for 1 h and then washed with PBS. In the meantime, 100 nmol of 5′-amino-terminal oligonucleotide was dissolved in 900 μL of freshly prepared 0.1 M sodium borate buffer (SBB) and activated with 100 M excess of cyanuric chloride in 100 μL of acetonitrile. The unreacted cyanuric chloride was removed by centrifugal filtration three times through an Amicon 3000 MW cutoff membrane (Millipore Corp., Bedford, MA). The final volume of the activated oligonucleotides was adjusted to 500 μL with SBB, and 100 μL of this solution was used for overnight reaction with the PEI-coated beads. The beads were then washed with 10 mM Tris–HCl, pH 8.3, 1 mM EDTA containing 0.1 M NaCl and 0.1% SDS and stored in buffer until used.

*Fluorescent labeling of thrombin.* Fluorescein-succinimidyl ester (F-SE) was used to fluorescently label thrombin following the manufacturer's instructions. Two-hundred microliters of thrombin solution (2 mg/mL in 0.1 M sodium bicarbonate buffer, pH 8.3) was allowed to react for 1 h with 25 M excess F-SE in 17 μL of dimethyl sulfoxide. Hydroxylamine (buffered to pH 8.5) was then added to stop the reaction and remove any weakly bound dye. The fluorescein-labeled thrombin (F-thrombin) was purified from unreacted dye by a supplied spin column. The degree of labeling was determined by using an absorbance method. The absorbance of F-thrombin was measured both at 280 nm ($OD_{280}$) and at 494 nm ($OD_{494}$ the wavelength for maximum absorbance of fluorescein). The conjugation degree of F in thrombin was calculated using the following equations

$$\text{F-thrombin concentration (mg/mL)} = (OD_{280} - 0.2OD_{494})/1.83 \quad [1]$$

$$\text{F per thrombin} = (OD_{494}/\varepsilon) \times [\text{MW of thrombin}/(\text{mg/mL of F-thrombin})], \quad [2]$$

where 0.2 is a correction factor, 1.83 is the absorbance of human thrombin at 1 mg/mL (19), and $\varepsilon$ is the molar extinction coefficient ($cm^{-1}$ $M^{-1}$) of F.

*Array preparation and fluorescence measurement.* To fabricate microwell arrays (3.1 μm diameter), imaging fibers were treated with a buffered hydrofluoric acid solution as previously described (20). After rinsing under running water, the fibers were then sonicated to remove any salts formed during the reaction. The oligonucleotide-attached beads were pipetted onto the microwells at the fiber's distal tip, and the proximal tip of the fiber was then coupled to the modified epifluorescence microscope. F-thrombin was diluted with PBS (pH 7.4) to the desired concentrations, and 10 μL of each concentration was brought to the fiber's distal end and incubated for 8 min. The distal tip was washed with PBS and an image was acquired in PBS with a 1-s acquisition time. Background signals were acquired in PBS prior to each test and subtracted from signals generated from the incubation with F-thrombin. To regenerate the beads, the fiber's distal tip was then incubated with 6 M guanidine hydrochloride solution for 5 to 10 min. Thrombin was stripped from the beads by this step, and the fiber was then rinsed with PBS. For a competitive assay format, F-thrombin was mixed with various concentrations of nonlabeled thrombin to give a final concentration of 200 nM F-thrombin. The rest of the steps were the same as before.

*Internal reference.* Poly(T)-attached beads were made by the same procedure as the aptamer beads. These two beads were mixed and distributed into the microwells. The signals resulting from nonspecific binding of thrombin to the poly(T) beads were subtracted from the signals measured from the aptamer beads. Fluorescence signals from 100 beads of each type were collected and averaged.

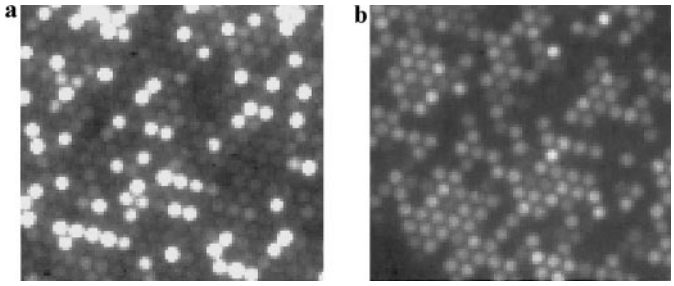


**FIG. 1.** Fluorescence images after incubation with 1 $\mu$M F-poly(A) (a) and 1 $\mu$M F-thrombin (b). White circles indicate beads and intensities of fluorescence. Poly(T)-attached beads were distinguishable after hybridization with their target. Nonspecific binding of F-thrombin to poly(T) beads was subtracted from total binding to antithrombin aptamer beads.

## RESULTS AND DISCUSSION

*Aptamer conjugation.* The antithrombin aptamer was prepared with an amino group at the 5′ end and this group was used to crosslink the aptamer to PEI-coated beads. PEI was used to immobilize a high concentration of aptamer on the surface of beads (18). PEI also provides a spacer so that the aptamer is more accessible to thrombin. In addition, the aptamer extends farther away from the surface as it has a six-atom spacer at the 5′ end. The 15-mer antithrombin aptamer folds into a chair-like structure with two G-tetrad stacks connected by two TT loops and a single TGT loop in solution (21–23). This tertiary structure has been reported to play an important role in binding to the anion-binding exosite on the serine protease thrombin, a key blood-coagulation enzyme. With our immobilization protocol, it seemed likely that there was enough space for the aptamer to fold into the two G-quartet stack structures and to interact with thrombin.

*Nonspecific binding of thrombin to the beads.* Initially, we used hydrophobic polymer microspheres (87% methylstyrene/13% divinylbenzene/R-$NH_2$); however, a significant amount of thrombin nonspecifically adsorbed to these beads. To reduce this nonspecific binding, hydrophilic silica microspheres with silanol (Si-OH) surface groups were used resulting in much less nonspecific protein binding (24). These beads still showed some nonspecific adsorption with thrombin. Thrombin has been reported to be an essentially neutral protein (p*I* 7.0, 7.3, 7.6) (19) and to readily adhere to both apolar and negatively charged surfaces (25, 26). Both beads showed less nonspecific binding after PEI derivatization because the amines impart a net positive charge to the surface. However, nonspecific binding could not be avoided completely and resulted in unreproducible results. We also tried to modify the beads with bovine serum albumin (BSA) before the binding experiments to reduce the nonspecific binding. Although BSA adsorption lowered the nonspecific binding significantly for the first few tests, during the binding experiment, the guanidine hydrochloride solution removed both BSA and thrombin and made the analysis more unpredictable. It seemed likely that the surface bound BSA also interacted nonspecifically with thrombin in solution (27). To circumvent this nonspecific binding problem, we employed poly(T)-conjugated beads, made by the same method as the aptamer beads, and used these beads as an internal reference.

An optical imaging fiber was etched so that its distal tip contained an array of microwells (20). Both sets of beads were mixed and then added to the microwells. The binding of thrombin to the poly(T) beads was considered as total nonspecific binding and subtracted from the fluorescence signals obtained from the aptamer beads. As shown in Fig. 1, binding of the fluorescein-labeled oligonucleotide target (Poly(A)) was more specific than that of the fluorescein-labeled protein target (thrombin). The poly(T) beads showed fluorescence after incubation with F-thrombin, but the aptamer beads did not show significant fluorescence after incubation with F-poly(A). As a result, poly(T) beads can be distinguished from the thrombin aptamer beads and used to measure the degree of nonspecific binding. In addition, preincubation with a high-concentration poly(A) solution enables the poly(T) beads to be identified. All other beads in the array were assumed to correspond to thrombin aptamer beads.

*Sensitivity of the system.* This system has a dynamic range of nanomolar to low micromolar concentrations of F-thrombin with an apparent $K_d$ of ca. 300 nM (Fig. 2), which is higher than the range of 50–100 nM reported previously (5, 21). The antithrombin aptamer sequence used in this study was originally

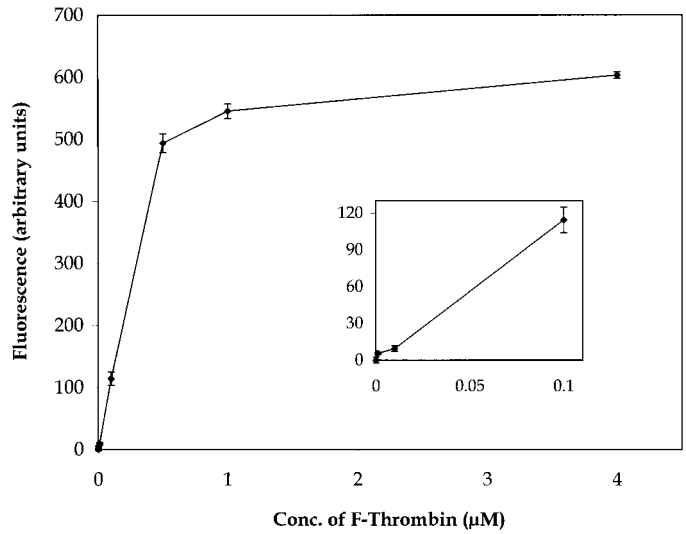


**FIG. 2.** Standard curve with F-thrombin. The inset is the response change over lower concentrations of F-thrombin. Every data point was measured in triplicate. Error bars represent ±1 SD.

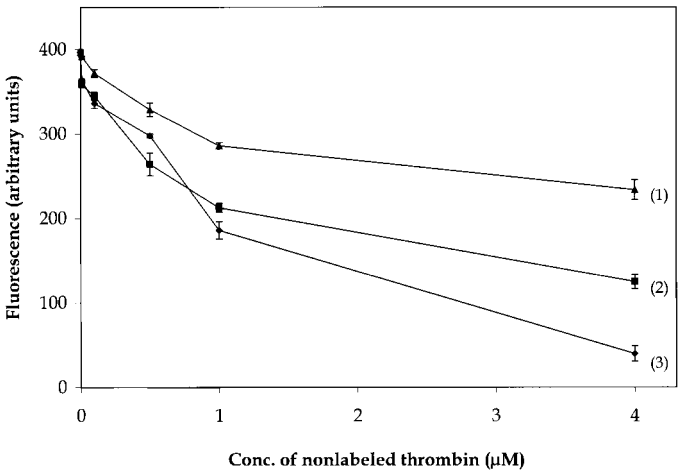


**FIG. 3.** Competitive binding of thrombin with F-thrombin. Fluorescence changes from competitive binding of 100 nM (1), 200 nM (2), and 500 nM (3) F-thrombin with nonlabeled thrombin. Every data point was measured in triplicate. Error bars represent ±1 SD. The largest response change is shown with 500 nM F-thrombin, even though higher sensitivity is achieved with 200 nM F-thrombin in the lower range of nonlabeled thrombin.

derived from binding to nonlabeled thrombin, and the binding to F-thrombin could be different. The fluorescein labels on thrombin may cause steric hindrance and thereby affect binding and/or conformational changes in thrombin. In this study, one thrombin molecule was labeled with ca. four fluorescein dye molecules. In addition, aptamer immobilization may also result in the slightly higher $K_d$. A detection limit of 10 nM was estimated from three standard deviations of background fluorescence signals.

A more practical assay would not employ labeled thrombin. To detect nonlabeled thrombin, we devised a competitive assay format. We mixed a fixed concentration of F-thrombin with various concentrations of unlabeled thrombin. As the amount of unlabeled thrombin increased in the mixture, the binding of F-thrombin to the aptamer beads decreased (Fig. 3). In order to measure reasonable response changes, the concentrations of F-thrombin used were chosen from the dynamic range of the standard curve as a function of the F-thrombin concentrations (Fig. 2). Three different concentrations of F-thrombin (100, 200, and 500 nM) were tested in the competitive format. It was determined that 200 nM F-thrombin showed the most sensitive response changes with nonlabeled thrombin. Even though the total fluorescence change was larger with 500 nM F-thrombin, this amount of F-thrombin was so high that unlabeled thrombin could not compete effectively with F-thrombin for the binding sites at lower concentrations. With 200 nM F-thrombin, a detection limit of 1 nM thrombin was achieved with a 10-$\mu$L sample volume. A concentration of 10 nM thrombin could be detected by competition with 100 and 500 nM F-thrombin. Physiologically relevant concentrations of thrombin in plasma are 5–500 NIH units/mL (28, 29). If we assume that the lyophilized thrombin used in this study retains its activity (3000 NIH units/mg) in solution, low nanomolar to low micromolar concentrations of thrombin solution fall into the physiological concentration range. Considering that the competitive assay format has a dynamic range in these concentrations (Figs. 3 and 4), this system should be readily adapted for the application of real plasma samples. Confirmatory validation studies comparing this approach to established methods are still required.

*Specificity of the system.* To test for specificity, BSA was mixed with the F-thrombin. As shown in Fig. 4, no effect on the interaction with the aptamer beads was observed at up to 1 $\mu$M BSA. Above this concentration, BSA caused a fluorescence change even though the F-thrombin concentration was held constant. BSA is nearly twice as large as thrombin and, above 1 $\mu$M, the concentration of BSA is more than 10 times higher than F-thrombin. These results suggest that HSA and other serum proteins may interfere as well.

*Stability and reproducibility of the system.* The aptamer beads did not show any degradation in activity during these 8-h-long experiments. The beads were stable for over 3 months storage. Fresh beads were used for each experiment and at the end of the experiment, aptamer beads were taken out of the image fiber by sonication. F-thrombin was stable for only ca. 2 weeks at 4°C in PBS. For nonlabeled thrombin solutions, lyophilized thrombin was dissolved in PBS, divided into aliquots, stored at −20°C, and used within a few days. The binding of thrombin to the aptamer beads was complete in 7 to 8 min, and the regeneration

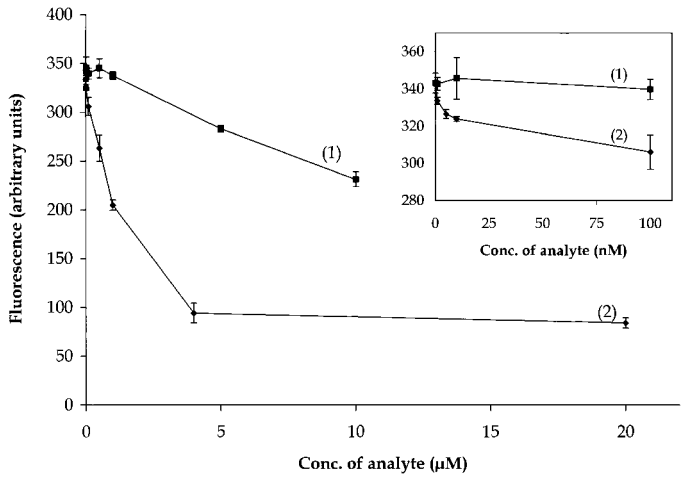


**FIG. 4.** Competitive assay with BSA (1) and nonlabeled thrombin (2). For both assays, F-thrombin was mixed with BSA or nonlabeled thrombin to give a final concentration of 200 nM. The fluorescence change over low analyte concentrations is shown in the inset. Every data point was measured in triplicate. Error bars represent ±1 SD.

of the aptamer beads was done in 5 to 6 min. Although fluorescence signals from the individual aptamer beads showed significant variability, the average signals of 100 beads provided more precise values with ca. 3% CV. The high density of the arrays enables such redundancy without sacrificing array diversity. Both false positive and false negative results can be avoided by using replicates of the aptamer beads.

## CONCLUSIONS

The fluorescence-based aptamer method reported here is complementary to conventional ELISA techniques. Both approaches require a combinatorial screening to obtain the binding agent (antibody or aptamer). While the approach described in this paper employs an optical microarray with specialized instrumentation, it should also be amenable to use in a microtiter plate format (7). The data presented here demonstrate that aptamer microarrays can be used for the analysis of target proteins. The system shows selectivity for its target and can be reused with good reproducibility. This study also suggests the possibility for multianalyte detection. We made four different oligonucleotide beads including aptamer beads. These beads were mixed and placed into the microwells of an imaging fiber, and each bead type could be distinguished from the others with over 90% accuracy after hybridization with their fluorescein-labeled target sequences (data not shown). Future development of a multianalyte detection microarray will depend largely on the selectivity and high affinity of aptamers for their targets and on nonspecific binding correction techniques.

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