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Adapting Selected Nucleic Acid Ligands (Aptamers) to Biosensors

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A flexible biosensor has been developed that utilizes immobilized nucleic acid aptamers to specifically detect free nonlabeled non-nucleic acid targets such as proteins. In a model system, an anti-thrombin DNA aptamer was fluorescently labeled and covalently attached to a glass support. Thrombin in solution was selectively detected by following changes in the evanescent-wave-induced fluorescence anisotropy of the immobilized aptamer. The new biosensor can detect as little as 0.7 amol of thrombin in a 140-pL interrogated volume, has a dynamic range of 3 orders of magnitude, has an inter-sensing-element measurement precision of better than 4% RSD over the range 0−200 nM, and requires less than 10 min for sample analysis. The aptamer-sensor format is generalizable and should allow sensitive, selective, and fast determination of a wide range of analytes.

Several bioanalytical instruments that use nucleic acid probes to bind to and detect specific sequences in RNA or DNA targets have been developed.[1−5] In particular, arrays of immobilized DNA have been synthesized,[6−13] and devices that can detect nucleic acid hybridization to addressable patches on the chips are now available for the rapid clinical diagnosis of genetic diseases.[11]

Nucleic acid aptamers that can bind to non-nucleic acid targets, such as small organic molecules or proteins, have been selected from random sequence pools.[14,15] Such aptamers can be adapted to diagnostic applications and have been shown to be useful for the detection of protein analytes. For example, radiolabeled aptamers were used for the detection of different protein kinase C isozymes.[16] Similarly, fluorescently labeled aptamers were shown to mimic the abilities of antibodies in ELISA-like applications[17] and in cell sorting.[18]

There are several potential benefits to using aptamers to sense non-nucleic acid targets. Aptamers bind targets with affinities and specificities comparable to those of monoclonal antibodies[19,20] and can be selected to bind a wide range of targets, including those that are toxic or not inherently immunogenic.[19,21] The in vitro selection process can be more precisely monitored than can organismal immunization, and the affinities and specificities of aptamers can thus be better tailored than can those of antibodies. Aptamers can be synthesized and stored until needed and are resistant to denaturation and degradation. Finally, aptamers can be more readily engineered than can antibodies for use as biosensors. For example, while it can prove difficult to incorporate a site-specific label into an antibody, it is trivial to do so with an aptamer.[16−18]

While aptamers have many features that make them attractive as recognition receptors in biosensors, there have been no attempts to develop devices that can harness the capabilities of immobilized aptamers. Here, we describe the first aptamer-based biosensor that can be used to detect free and nonlabeled non-nucleic acid targets.[22] An anti-thrombin aptamer was labeled with fluorescein isothiocyanate (FITC), covalently immobilized on a

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microscope cover slip, and protein binding was detected by monitoring the evanescent-wave-induced fluorescence anisotropy of the immobilized aptamer.

EXPERIMENTAL SECTION

Oligonucleotide Preparation. Oligonucleotides used in the study (the anti-thrombin aptamer, 5′ GGTTGGTGTGGTTGG, and a scrambled sequence, 5′-GGTGGTTGTTGTGGT) were synthesized on a DNA synthesizer (model ABI 391, Perkin-Elmer Applied Biosystems, Foster City, CA). Labels and linkers were added by including fluorescein phosphoramidite and a 3′-amino-modifier C7-controlled pore glass column during the synthesis. The synthesized oligonucleotides were electrophoretically purified on a 20% polyacrylamide gel (19:1 monomer:bis) containing 7 M urea and 1 × TBE (Tris−borate−EDTA buffer: 0.089 M boric acid, 0.089 M trizma base, and 0.01 M Na_2EDTA). Concentrations of the oligonucleotides were determined by optical absorption measurements at 260 nm.

Analyte Preparation. Thrombin from human plasma (Sigma Chemical Co., St. Louis, MO) was dissolved in a storage buffer (PBS containing 20% glycerol) to yield 2 and 25 μM solutions. These standards were divided into 50-μL aliquots and stored at −70 °C. Immediately before use, each aliquot was rapidly warmed to 20 °C and diluted with a PBS buffer solution to the required concentration. Elastase (porcine, Sigma) was dissolved in storage buffer, stored, and used in the same way as thrombin.

Activation of Silica Surfaces. The surfaces of glass microscope cover slides were partially converted to diol silica by suspending the cleaned glass slides in a 10% aqueous solution of (glycidoxypropyl)trimethoxysilane, degassing for 10 min, and then allowing the reaction to proceed at 90 °C for 4 h with occasional shaking. During this time, the pH was maintained at 3.5 with 1 M HCl. The slides were then rinsed with distilled water, acetone, and ether and dried in vacuo overnight at 100 °C. The surface was then activated by suspending the slides in 0.5 M 1,1′-carbonyldiimidazole in dry acetonitrile and shaking the solution for 1 h at 20 °C. The slides were rinsed with acetonitrile and dried. The activated slides could be stored in air at 20 °C for several days.

Aptamer Coupling. The 3′-amine-modified ssDNA aptamer in 50 mM sodium phosphate (pH 8) buffer was applied as ∼30-μL microdroplets to the activated surfaces of the slides and incubated at 20 °C in air at ∼60% relative humidity. The droplets were allowed to dry,[23] and any unreacted aptamer was removed by rinsing with a PBS buffer solution. To block unreacted surface groups, a 0.1 M ethanolamine solution (brought to pH 9 with HCl) was applied, and the slides were incubated at 20 °C for 2 h. The slides were rinsed with a PBS buffer solution, dried in air, and stored at 4 °C. To obtain optimum background anisotropy, the concentration of the aptamer was varied from 20 to 200 μM.

Measurement of Fluorescence Anisotropy. All measurements were performed with an optical module of an SLM-AMINCO 4800C spectrofluorometer (SLM Instruments, Inc., Urbana, IL). An argon ion laser (model 171, Spectra-Physics, Mountain View, CA) operating at 488 nm was used as the excitation source. A 488 ± 5-nm band-pass filter was placed in the excitation path to eliminate plasma discharge lines. The vertical polarization of the laser beam was scrambled for calibration purposes by passing it through a coiled 2-m-long multimode optical fiber (SPC800N, Fiberguide Industries, Stirling, NJ). Light from the fiber output was collimated with a microscope objective, passed through an excitation Glan-Thompson polarizer, and directed onto a trapezoidal prism (BK-7 glass) which was arranged in the sample compartment of the optical module of the spectrofluorometer. The aptamer-coated glass slide was optically coupled to the prism and positioned on top of a 10-μL built-in-house flow microcell, as shown in Figure 1. The angle of incidence of the laser beam at the glass slide−solution interface was 63.5° from the normal (the critical angle is 61.0°). The laser beam was focused to a spot of 0.6 mm × 2 mm at the interface plane. The radiant power incident on the glass slide was limited to 30 μW. Fluorescence emission was collected from the top of the prism with a lens ($f = 50$ mm), passed through a set of optical emission filters (a long-pass 495-nm filter and a 530 ± 30-nm band-pass filter and an emission Glan-Thompson polarizer, and imaged onto the photocathode of a photomultiplier tube (PMT, Hamamatsu R928). The PMT output was read by a low-noise current preamplifier (model SR570, Stanford Research Systems, Inc., Sunnyvale, CA). Data were collected with a Macintosh computer and analyzed by means of commercial software packages. Fluorescence intensities were determined for selected positions of the excitation and emission polarizers. For solution studies, the biosensor assembly was replaced with a holder for a conventional fluorometric cuvette. Fluorescence measurements were performed with a single-channel 90° emission collection geometry. The fluorescence anisotropy r was calculated from measurements of the emission intensity I as[24] $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$, where G is the instrumental correction factor, $G = I_{hv}/I_{hh}$, and the subscripts v and h refer to the vertical and horizontal orientation of the polarizer, respectively. The first and second subscripts refer to the orientation of the excitation and the emission polarizers, respectively. All measurements, unless stated otherwise, were performed with a detection time constant of 1 s and a sampling rate of 2 Hz. All measurements were performed at 20 °C.

Studies with Free and Immobilized Aptamers. In studies with free oligonucleotides, the protein concentration was varied by adding different volumes of standard solutions of thrombin or elastase. Dissociation constants were calculated as described elsewhere.[25] In studies with immobilized oligonucleotides, solutions were introduced into the flow microcell by means of a peristaltic pump (25 μL/min flow rate). Each aptamer-coated glass slide was washed with a PBS buffer solution for 2 h prior to measurement. Thrombin or elastase solutions were pumped into the flow cell, and the fluorescence anisotropy was monitored as a function of time. Thrombin was stripped from the aptamer-coated glass slide by sequentially applying a PBS buffer solution, a guanidinium hydrochloride solution, and a PBS buffer solution. Photostability was assessed by recording the change in the total

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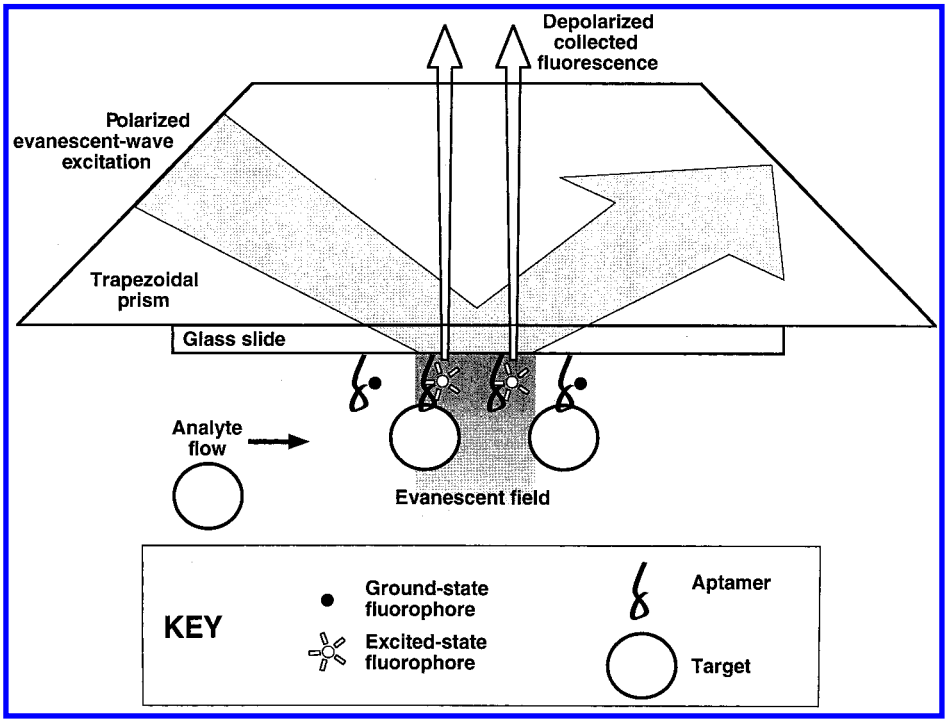

Figure 1. Detection scheme for the aptamer biosensor. The FITC label incorporated into an aptamer is excited by the evanescent field resulting from the total internal reflection of polarized 488-nm excitation light at the boundary between the aptamer-coated glass slide and an aqueous solution. The two polarization components of the depolarized fluorescence emission are monitored in rapid (2−4 s) sequence to indicate changes in the fluorescence anisotropy.

(polarization-independent) fluorescent signal with a 10-s time constant and a 0.1-Hz sampling rate over a 10-h time period.

RESULTS AND DISCUSSION

Design Strategy. In many biosensor schemes, the analyte is fluorescently labeled and its interactions with an immobilized receptor, such as an antibody, are detected.[26] In contrast, a nucleic acid aptamer can be fluorescently labeled without loss of function, so its interaction with free nonlabeled analytes can be detected. In our strategy, the fluorescently labeled aptamer is tethered to a glass slide via a flexible linker, and protein binding is detected, as shown in Figure 1. The aptamers have a characteristic rotational diffusion rate which is reflected in a characteristic fluorescence anisotropy.[24] Interactions between the aptamer and a cognate target result in an increase in the size of the fluorescent complex and a concomitant change in the rotational diffusion rate and the measured fluorescence anisotropy.

The measurement of fluorescence anisotropy also has several advantages over techniques such as surface plasmon resonance (SPR) and total internal reflection fluorescence (TIRF) spectroscopy. The evanescent-wave-induced detection of fluorescence anisotropy effectively discriminates between different surface-bound targets and is insensitive to variations in the refractive index of the sample solution. In contrast, SPR and TIRF often suffer from these complications.[27,28]

Since the receptor rather than the analyte is the signal-transducing molecule, aptamer biosensors should not be limited to in vitro measurements, should be capable of monitoring real-time changes in analyte concentration, and should require fewer sample preparation steps. In addition, since fluorescent labels and linkers can be introduced at almost any position in an aptamer by using standard synthetic reagents and methods, it should prove possible to modify many different aptamers for use in biosensors.

Thrombin-Binding FITC-Labeled Aptamer. A 15-mer (5′-GGTTGGTGTGGTTGG) single-stranded DNA (ssDNA) aptamer that binds to the blood-clotting factor thrombin[29] was chosen as an example of the use of an aptamer for biosensing. The anti-thrombin aptamer folds into a chair-form quadruplex with the adjacent 5′ and 3′ ends in the corners of the quadruplex and two stacked G-quartets linked by TT and TGT loops.[30,31] The interaction with thrombin is thought to occur adjacent to the TT loops.[32] Based on these structural considerations, it seemed likely that derivatization of the 5′ or 3′ termini should minimally perturb thrombin-binding.

The fluorescence anisotropy of 5′-FITC-GGTTGGTGTGGTTGG was found to increase as a function of thrombin concentration, and a dissociation constant of 1.1 μM was calculated for the FITC-aptamer/thrombin complex (Figure 2A, curve 1). This value is roughly 10-fold higher than the published K_d for the unlabeled aptamer.[33] Given that the aptamer is quite small and fits snugly in the thrombin exosite, the apparent increase in K_d might be

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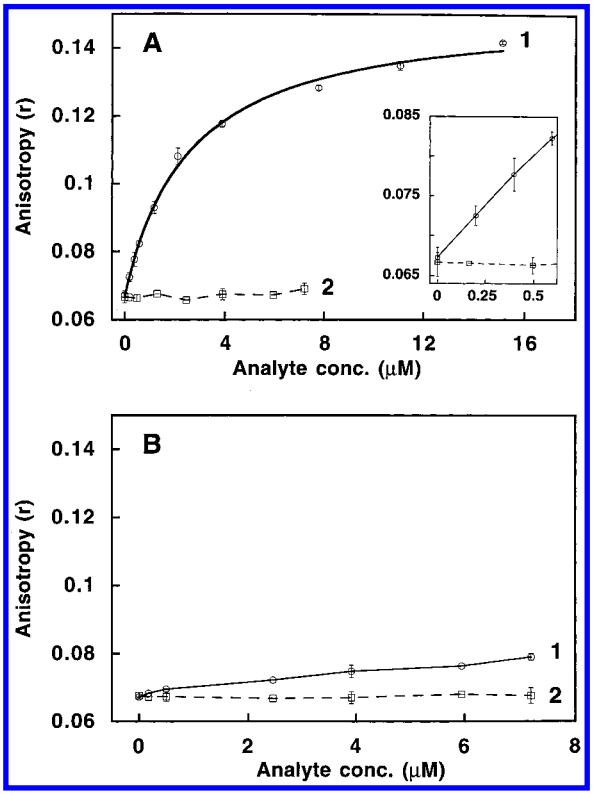

Figure 2. FITC-labeled anti-thrombin aptamers interact selectively with thrombin. Fluorescence anisotropy of free FITC-labeled anti-thrombin aptamer (A) and a scrambled sequence oligonucleotide (B) is shown as a function of thrombin (1) or elastase (2) concentration. The inset in (A) is an expanded view of the data for low thrombin and elastase concentrations. Every data point is the mean of 3−5 measurements. The error bars represent one standard deviation.

due to partial steric occlusion of binding by the FITC label or to an alteration of the solution conformation of the aptamer by the label. Alternatively, the oligonucleotides might have associated with one another and formed inter- rather than intramolecular quadruplexes; this behavior has previously been observed in G-rich quadruplexes.[34,35] Intermolecular quadruplex formation may have been facilitated by destabilization of the monomer structure by the fluorescein label.

The observed thrombin-dependent change in fluorescence anisotropy was specific to both the analyte and the receptor. Little or no change in fluorescence anisotropy was seen with increasing concentrations of elastase, another serine protease with an isoelectric point and molecular weight similar to those of thrombin (Figure 2A, curve 2). Conversely, a scrambled-sequence oligonucleotide based on the anti-thrombin aptamer had previously been shown to have no inhibitory effect on thrombin activity,[29] and a FITC-labeled scrambled-sequence oligonucleotide correspondingly showed no thrombin-dependent change in fluorescence anisotropy (Figure 2B).

Optimization of Aptamer Immobilization. Immobilization of aptamers restricts their rotation and raises the initial level of fluorescence anisotropy. This increase in initial anisotropy can limit the dynamic range of the anisotropy measurements. Thus, the greatest sensitivity will probably be achieved with immobilized receptors that have the lowest initial anisotropy.

(Glycidoxypropyl)trimethoxy silane (GOPS) has been used to couple oligonucleotides to glass surfaces and generate sensors for nucleic acid hybridization.[36] In nonaqueous solution, the trimethoxysilane group at one end of GOPS covalently links to the silicate oxygens of glass, leaving a six-atom linker with an epoxide group at the other end. The newly formed epoxide[37] should preferentially react with primary amino groups. An alkylamino group was therefore appended to the 3′ end of the fluorescently labeled anti-thrombin aptamer described above, and the modified aptamer was coupled to a GOPS-activated microscope slide. However, in our hands this coupling procedure resulted in unacceptably high levels of initial anisotropy, possibly because the highly reactive epoxide group could form covalent linkages to a variety of chemical moieties on the aptamer.

A modified coupling procedure proved more successful. In aqueous solution at reduced pH, the epoxide ring in GOPS was opened, forming a diol (Figure 3A). The diol was subsequently activated with 1,1′-carbonyldiimidazole (CDI). The reactivity of the resulting carbonylimidazole moiety (Figure 3B) was much more restricted than that of the epoxide[38] and should have preferentially coupled to the 3′-amine (Figure 3C). Following immobilization of the aptamer, unreacted surface sites on the slide were blocked with 100 mM ethanolamine. The aptamer concentration in the immobilization solution was also adjusted to minimize the initial fluorescence anisotropy level. The smallest initial anisotropy [0.22 ± 0.01 (mean $\pm$ SD; $n = 6$)] was achieved with a 50 μM solution of aptamer.

Sensitivity and Specificity of Aptamer Biosensor. The dynamic range of the biosensor was almost 3 orders of magnitude, from nanomolar to micromolar concentrations of thrombin (Figure 4). The detection limit (at a S/N = 3) calculated from the slope of the calibration curve in the lowest concentration range was 5 nM.

An important advantage of using an evanescent wave over direct sample illumination is a greatly reduced illuminated volume and a similarly reduced background.[39,40] For the excitation conditions used here, the penetration depth of the evanescent field was about 140 nm; with a laser spot size of 0.01 cm^2 at the interface plane, a volume of only about 140 pL of the 10-μL sample was irradiated. The actual volume for generation of the analytical signal was less than 10 pL, because the recognition sites of the immobilized aptamers were less than 10 nm from the glass-sample interface. This analysis implies that from 0.7 to 700 amol of thrombin can be detected.

Multiple (5−8) measurements with a single aptamer-coated glass slide produced ≤1% relative standard deviation (RSD) over a thrombin concentration range from 0 to 200 nM (Figure 4, inset).

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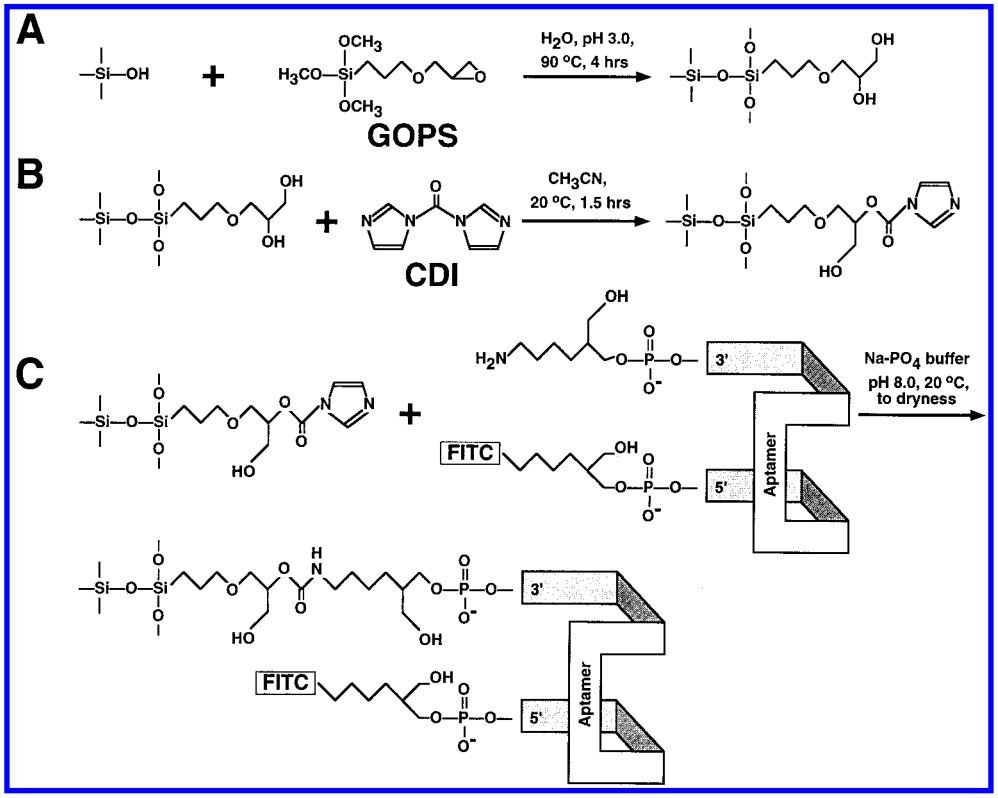

Figure 3. Fabrication of aptamer biosensor. (A) Attachment of (glycidoxypropyl)trimethoxy silane (GOPS) to glass surface at low pH results in the formation and subsequent cleavage of an epoxide. (B) The resultant diol is activated with 1,1′-carbonyldiimidazole (CDI) to form a carbonylimidazole. (C) A 3′-amino aptamer displaces the imidazole and forms a carbamate linkage to the tether.

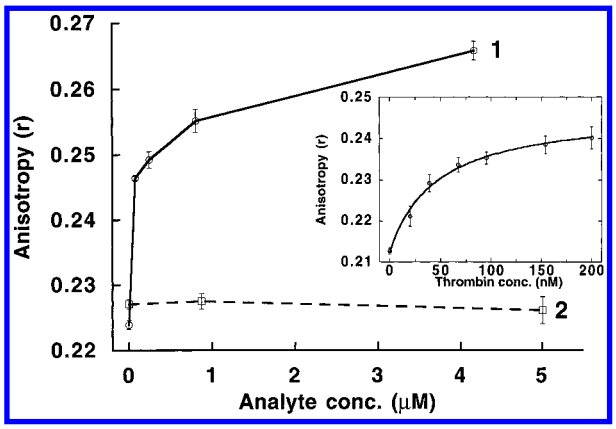

Figure 4. Immobilized aptamers interact with thrombin. Fluorescence anisotropy is measured as a function of the concentration of thrombin (1) or elastase (2). Every data point is the mean of 3−8 measurements. Error bars represent one standard deviation. The inset is the response of a second aptamer-coated glass slide over a 0−200 nM concentration range of thrombin.

When measurements from different aptamer-coated glass slides were compared, the RSD was ≤4%. Variable surface coverage between manually produced sensing elements might account for this precision difference. Large-scale, automated fabrication of aptamer biosensors would likely yield much more uniform surface coverage and a correspondingly lower RSD.[41] Significantly, the immobilized FITC-labeled aptamer exhibited a high degree of selectivity for thrombin (Figure 4, curve 2), just as the free FITC-labeled aptamer had (Figure 2A, curve 2).

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Interestingly, the immobilized aptamers (Figure 4) were much more sensitive than the free aptamers (Figure 2) for detecting thrombin. The apparent K_d for the glass-bound complex was 47 nM, a value that is congruent with solution-phase studies (the K_d as determined by filter-binding analysis is ca. 100 nM,[33] while the IC_{50} for blood-clotting is ca. 70−80 nM[42]). It is possible that immobilization resulted in a more effective presentation of the aptamer conjugate. For example, immobilization would have favored the formation of intra- rather than intermolecular G quartets.

Kinetics of Aptamer/Thrombin Interaction. The new biosensor allows more rapid thrombin determinations than traditional techniques such as radioactive assay, ELISA, and clotting time measurements.[43] The sensor equilibrates within 4−8 min (Figure 5A). Moreover, the response is reversible. Thrombin can be stripped by rinsing the aptamer-coated glass slide with a PBS buffer solution followed by guanidinium hydrochloride. The sensor is then regenerated by reequilibration with PBS. The different signal changes for the two peaks in Figure 5A are due to the application of PBS and guanidinium at different times.

Photophysics and Photochemistry of Covalently Immobilized Aptamer. Evanescent wave fluorescence anisotropy is an extremely attractive detection method for use with aptamer biosensors. The total (polarization-independent) fluorescence of the aptamer label is almost completely unaffected by thrombin

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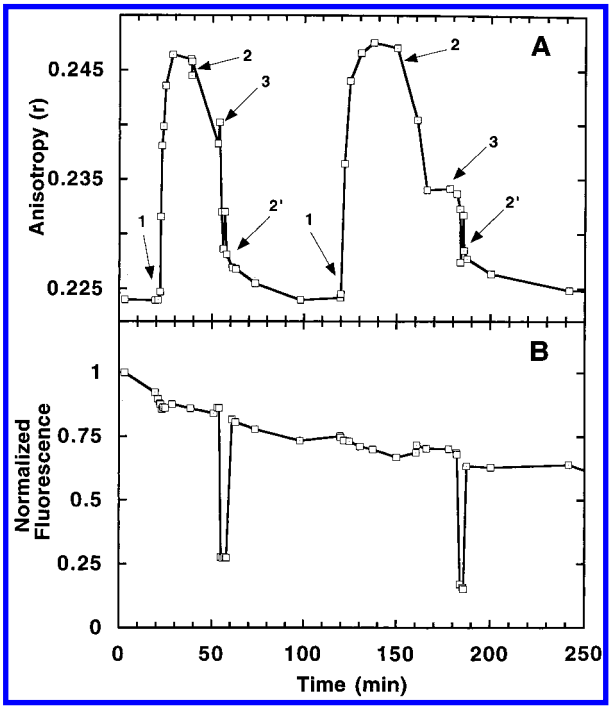

Figure 5. Reversible thrombin-binding kinetics of an ethanolamine-blocked aptamer-coated glass slide. (A) Fluorescence anisotropy signal. The following solutions were introduced into the flow microcell: (1) thrombin (420 nM injected at 20 min and 860 nM injected at 120 min), (2 and 2′) a PBS buffer solution, and (3) guanidinium hydrochloride. (B) Total background-subtracted fluorescence signal. The 35% drop in fluorescence intensity during the 4-h experiment is due to partial photobleaching of the FITC-labeled aptamer. The fluorescence dips at 55−60 and 180−185 min are due to quenching of the fluorescence of immobilized FITC by the guanidinium hydrochloride solution.

binding (Figure 5B). In addition, the photobleaching-induced signal drift apparent in Figure 5B is eliminated in Figure 5A because both fluorescence polarization components drop proportionally. Finally, since fluorescence anisotropy is a relative (ratioing) technique, it is inherently self-referencing and relatively immune to source fluctuations and electronic drift.

In practical terms, the sensor can be continuously operated for about 40 min before the fluorescence intensity drops to 1/e of its initial level (Figure 6). Since the total time required for analyte measurement is less than 10 min (Figure 5A), it is likely that individual aptamer-coated glass slides can be reused multiple times. In the best circumstances, a regime could be established for equilibration, baseline determination, sample introduction, measurement, and stripping that would require illumination of the sensing element for less than 1 min.

Understanding the photophysics and photochemistry of the immobilized aptamer should lead to future improvements in biosensor design. For example, the multiexponential photobleaching kinetics (see equation in Figure 6) of the immobilized aptamer label suggest that the fluorophore molecules undergo several different photobleaching processes, which might include dye-to-dye, dye-to-oxygen, and surface-induced mechanisms.[44−46]

CONCLUSIONS

Just as DNA arrays have been used to analyze genomic and expressed nucleic acid sequences,[6−8,10−12] aptamer arrays should

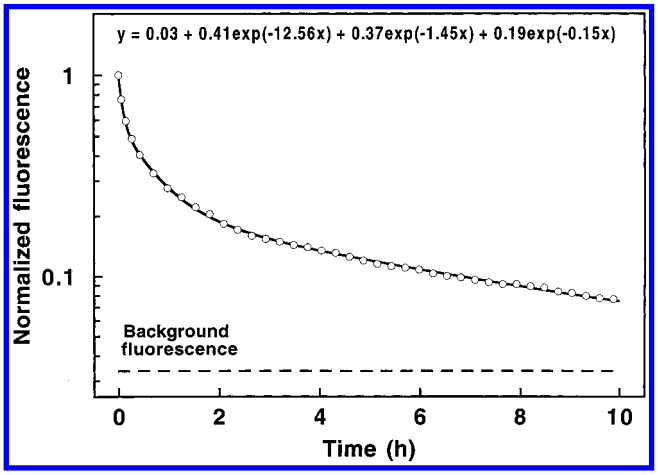

Figure 6. Photostability of the immobilized aptamer. Fluorescence was measured under continuous evanescent wave illumination at 488 nm: photobleaching data (open circles) and triple-exponential fit, $\chi^2 = 0.02$, (solid line).

be useful in the analysis of organismal "proteomes" or of levels of specific proteins, such as cytokines. There are several unique features in our implementation of aptamer biosensors; these features should yield biosensors with superior characteristics.

First, the receptor rather than the analyte is fluorescently labeled and immobilized. In this respect, the new method differs significantly from those previously reported,[17,18,47−51] because nonlabeled samples can be analyzed with minimal preparation steps and in real time.

Second, while previous diagnostic applications of aptamers have utilized avidin−biotin immobilization strategies,[51] the covalent immobilization strategies used here for aptamers resemble those developed for antibody immobilization[52] and should prove to be functionally superior. Avidin is a relatively large protein, and the relationship between the biotin−receptor conjugate and the surface is unknown and, hence, difficult to engineer.[53] In contrast, the length and chemical composition of the covalent linker we have used for immobilization can be varied at will.

Third, the use of fluorescence anisotropy as a detection method means that any target large enough to sizably affect the rotational momentum of an aptamer is amenable to detection.[24] Since the fluorescent tag can potentially be appended to many different positions on an aptamer, it should be possible to modify almost any anti-protein aptamer for use in a biosensor.

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In the future we will focus on the development of aptamer arrays for multianalyte detection. Numerous aptamers with unique selectivities and subnanomolar dissociation constants have already been identified, and additional binding sequences can be generated by established procedures. Different aptamers in an array could potentially be distinguished either by spatial separation, by the attachment of different fluorescent labels, or by some combination of these techniques.

For these and other applications, some straightforward advances are possible. For example, different background-correction techniques could be used to further improve selectivity. In addition, the surface density of the immobilized aptamer under continuous exposure to the analyte solutions could be investigated to increase the long-term stability of the biosensor.

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