

## Electrochemical Biosensors

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**Label-Free Electronic Detection of Thrombin in Blood Serum by Using an Aptamer-Based Sensor\*\***

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Aptamers are DNA or RNA sequences selected *in vitro* for their ability to bind specific molecular targets.<sup>[1–3]</sup> Due to the ease with which novel aptamers can be fashioned and their generally impressive selectivity and affinity, they are widely regarded as ideal recognition elements for biosensor applica-

tions.<sup>[4,5]</sup> Consistent with this claim, aptamers have been employed in a variety of sensing technologies,<sup>[6–11]</sup> including a very promising optical approach termed “aptamer beacons”. Aptamer beacons employ a large-scale, binding-induced conformational change in order to modulate the emission of a covalently bound fluorophore.<sup>[12]</sup> To date, aptamer beacons have been reported for such diverse targets as the small molecule cocaine<sup>[13]</sup> and the proteins Tat,<sup>[14]</sup> Taq DNA polymerase,<sup>[15]</sup> platelet-derived growth factor,<sup>[16]</sup> and thrombin.<sup>[6]</sup>

Aptamer beacons are rapid, label-free, and exceptionally selective.<sup>[12,17]</sup> As beacons are an optical approach, however, they suffer from several potential drawbacks relative to electronic-sensing strategies. These include a requirement for generally bulky, expensive, and power-intensive light sources, detectors, and monochromators, a susceptibility to photobleaching, and potential false signals arising from contaminating fluorophores or quenchers.<sup>[18]</sup> In contrast, the impressive miniaturization of modern microelectronics and the relative stability and environmental insensitivity of electroactive labels suggest that electronic sensors might avoid many of these pitfalls.<sup>[19]</sup> Previously described electronic aptamer-based sensors, however, require either the addition of exogenous reagents or are susceptible to interference from contaminants. For example, while impressive sensitivity and detection speed have been demonstrated by using aptamer sensors based on the quartz crystal microbalance<sup>[10]</sup> or on surface plasmon resonance,<sup>[20]</sup> both approaches are prone to false positives arising from nonspecific binding. Similarly, while the electrochemical detection of an aptamer–thrombin interaction has been reported, the approach requires complex, multistep preprocessing and the addition of an exogenous redox label.<sup>[11]</sup>

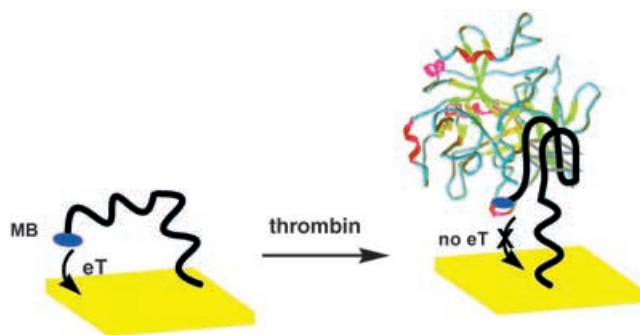
Here we describe a relatively general solution to the problem of electronic aptamer-based (E-AB) sensors. The sensor that we have developed, which is directed against the blood-clotting enzyme thrombin, is sensitive, selective, and reusable, and it does not require modification of the analyte. Instead, signal generation occurs upon a large, binding-induced conformational change in a redox-modified, electrode-attached aptamer upon target binding.

The E-AB sensor is analogous to the E-DNA sensor, a previously described, reagentless, electronic DNA sensing technology.<sup>[21]</sup> The E-AB sensor is constructed by covalently attaching a methylene blue (MB) labeled, thrombin-binding DNA aptamer (oligomer **1**) to a gold electrode by well-established self-assembling monolayer chemistry.<sup>[21]</sup> In the absence of target, the immobilized 32-base aptamer is thought to remain relatively unfolded,<sup>[6]</sup> thereby allowing the attached MB to collide with (or weakly bind to) the electrode and transfer an electron (Figure 1, left). Upon thrombin binding, electron transfer is inhibited, presumably due to a binding-induced conformational change in the aptamer (Figure 1, right)<sup>[6,22]</sup> that significantly alters the electron-tunneling distance and/or pathway (Figure 2, left). We assume that the immobilized aptamer (oligomer **1**) is in a conformational equilibrium between its unfolded state and the folded, binding-competent G-quartet conformation.<sup>[9]</sup> Since thrombin binds only the G-quartet conformation, the target protein

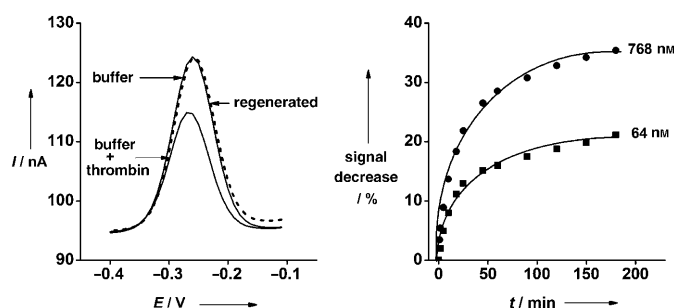
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**Figure 1.** A schematic of the E-AB sensor. In the unbound state (left), the aptamer is thought to be highly dynamic,<sup>[6]</sup> allowing rapid collisions (or complex formation) between the MB redox tag and the electrode. This, in turn, presumably allows the efficient electron transfer (eT) that is observed in the absence of target. Upon target binding (right), electron transfer is inhibited, presumably because the aptamer forms a stable, rigid structure.

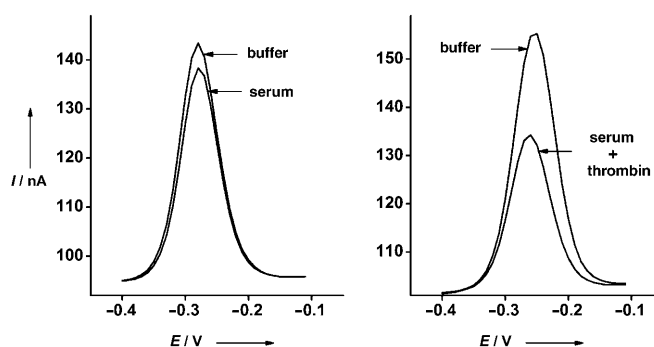


**Figure 2.** Left: The E-AB sensor responds robustly to 64 nM thrombin in buffered saline and can be regenerated by an eight-minute, room-temperature wash with 6 M guanidine hydrochloride. Even after two cycles of use and regeneration, we can recover 93 % of the original sensor signal (dashed line). Right: Time dependence of the sensor response after a 3 h incubation ( $t = 0$  min).

drives the equilibrium towards this state, thereby producing the observed signal.

As the modified aptamer is covalently attached to the sensing electrode, the E-AB sensor is readily regenerated (see dashed line in Figure 2, left). By contrast, several methods previously employed for the regeneration of antibody-based sensors (0.2 M NaOH; 0.25 % sodium dodecyl sulfate at pH 10; 6 M urea; 0.2 M HCl; 0.2 M glycine, 30 % methanol at pH 2; and 10 % isopropyl alcohol in phosphate buffer at pH 7) produced little, if any, regeneration (data not shown). When compared to other, commonly employed sensing strategies, such as ELISAs,<sup>[23]</sup> the E-AB sensor is reasonably rapid; while a 3 h incubation is required to achieve signal saturation, the majority of the signal change occurs within minutes (Figure 2, right). The use of the Laviron equation<sup>[24]</sup> to calculate the interfacial electron-transfer rate between MB and the electrode gives rate constants of  $88 \text{ s}^{-1}$  and  $58 \text{ s}^{-1}$  before and after the reaction with thrombin, respectively (data not shown). The similarity in these rates suggests that unbound aptamers produce a similar electron-transfer rate irrespective of whether neighboring aptamers are bound to thrombin and that the electron-transfer rate becomes undetectably slow upon binding.

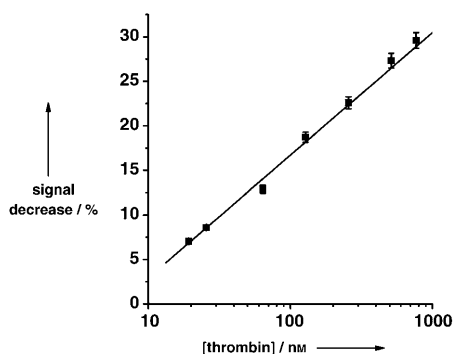
As signaling is based on a specific, binding-induced conformational change<sup>[25]</sup>—as opposed to a less specific physical change, such as adsorption—the E-AB sensor should be relatively insensitive to nonspecific binding. In order to test this, we have shown that 64 nM thrombin taken up in blood serum (diluted 50 % with buffered saline)—a realistically complex and contaminant-ridden clinical material—produces a large (35 %) reduction in peak current (Figure 3, right). By contrast, blood serum lacking exogenously added thrombin produces only a small ( $\approx 7$  %) reduction in peak current (Figure 3, left).



**Figure 3.** The E-AB sensor can detect thrombin even in a complex, contaminant-ridden sample such as blood serum. The sensor signals arising from thrombin-free buffered saline, from 50 % fetal calf serum, and from 50 % serum doped with 64 nM thrombin are shown.

The origin of the small signal drop observed in the absence of added thrombin is unclear. Possible sources include degradation of the aptamer or nonspecific interactions mimicking, to a limited extent, the binding-induced sequestration of the MB. However, neither an MB-tagged stem loop (oligomer **2**) nor a second, MB-tagged control oligonucleotide (oligomer **3**; of identical sequence composition and 81 % sequence identity to oligomer **1**, but known not to bind thrombin<sup>[1]</sup>) exhibit any measurable signal drop when incubated in serum (data not shown), a result suggesting that these mechanisms are not significant. It is difficult to rule out the possibility that the signal change arises due to the presence of thrombin which, as a blood-clotting enzyme, may be present in the undoped serum; while resting (non-clotting) serum thrombin levels are very low, active concentrations soar to several hundred nanomolar during clotting.<sup>[26]</sup> Given that the blood serum employed is fetal calf serum (a safe proxy for human blood) that was harvested from calves in utero, some activation of the blood-clotting cascade may have occurred, thereby producing detectable levels of thrombin.

The sensitivity and dynamic range of the E-AB sensor span the physiological concentrations of thrombin in resting and activated blood, which range from low nanomolar to low micromolar, respectively.<sup>[8]</sup> Except at the very lowest thrombin concentration that we have investigated (6.4 nM), peak currents are linear with the logarithm of thrombin concentration (Figure 4). This linear relationship holds up to the highest thrombin concentrations that we have investigated (768 nM). Relative signal changes, however, are dependant on



**Figure 4.** The dynamic range of the E-AB sensor covers physiologically relevant concentrations, which range from a few nanomolar (resting blood) to several hundred nanomolar (when the clotting cascade is activated). The error bars represent the standard deviation of four measurements conducted with a single electrode at each thrombin concentration. Multiple electrodes were used to collect this data set.

surface coverage; at lower surface coverage than that employed here, we observe smaller absolute signals but higher relative signal changes (data not shown). We presume this to be a consequence of reduced steric blocking of unbound aptamers by thrombin binding.

In conclusion, we have demonstrated a novel, label-free electronic method for the specific and quantitative detection of the blood-clotting factor thrombin. The E-AB sensor, which is based on a binding-induced conformational change in a highly selective, high-affinity thrombin-binding aptamer, detects the blood protein at nanomolar concentrations without any more cumbersome preprocessing than sample dilution with buffered saline. Moreover, because signal generation does not rely on simple physical adsorption, the sensor is quite insensitive to nonspecific binding and thus readily detects physiological thrombin levels, even in complex, contaminant-ridden samples such as blood serum. Lastly, the E-AB sensor is reasonably rapid and, given its label-free, fully covalent nature, readily regenerated.

The E-AB sensor platform is limited to targets for which DNA aptamers are available that undergo a large-scale, binding-induced conformational change. Such aptamers, however, may be relatively common. For example, as described above, a number of groups have reported optical aptamer-beacon sensors that utilize a ligand-induced folding event to segregate a fluorophore–quencher pair.<sup>[12]</sup> Additionally, Ellington and co-workers have reported an in vitro selection technique for the generation of aptamer-beacon-like molecules against arbitrary targets.<sup>[6b]</sup> The present study demonstrates a presumably general means by which the binding of such aptamers to their targets can be monitored electronically, thus opening a new direction for the development of rapid, reusable, and sensitive detection of multiple analytes in complex, contaminant-ridden clinical samples.

## Experimental Section

Labeled DNA oligonucleotides were synthesized by BioSource Int. (Foster City, CA) and purified by C18 HPLC and PAGE; their

structures were confirmed by mass spectrometry. The sequences of the three oligomers employed are:

**1:** 5'-HS-(CH<sub>2</sub>)<sub>6</sub>-TAAGTTCATCTCCCCGGTTGGTGTGGTTGGT-(CH<sub>2</sub>)<sub>2</sub>-MB-3'

**2:** 5'-HS-(CH<sub>2</sub>)<sub>6</sub>-GCGAGGTAAACGACGCCAGTCTCGC-(CH<sub>2</sub>)<sub>7</sub>-MB-3'

**3:** 5'-HS-(CH<sub>2</sub>)<sub>6</sub>-TAAGTTCATCTCCCCGGTTGGTGTGGTTGGT-(CH<sub>2</sub>)<sub>2</sub>-MB-3'

MB was conjugated to the 3' end of these probes through succinimide ester coupling (MB-*N*-hydroxysuccinimide was obtained from EMP Biotech, Germany).<sup>[27]</sup> Inspection of a model of the solution structure of the thrombin–aptamer complex suggests that bases 4–12 of the aptamer bind to the anion-binding exosite of thrombin and that the remainder of the aptamer is relatively solvent-exposed.<sup>[22,28]</sup> This, in turn, suggests that the MB label, which is covalently attached to the 3'-terminal base, should not interfere with thrombin binding.

The human  $\alpha$ -thrombin used in this study was purchased from Haematologic Technologies Inc. (Essex Junction, VT; specific activity: 3545 U mg<sup>-1</sup>) and diluted with sterile water as appropriate. Fetal calf serum was purchased from Sigma-Aldrich, Inc. (USA) and used without further processing.

The E-AB sensor was fabricated by using polycrystalline gold disk electrodes (1.6 mm diameter; BAS, West Lafayette, IN). The electrodes were prepared by polishing with diamond and alumina (BAS), sonication in water, and electrochemical cleaning (a series of oxidation and reduction cycling in 0.5 M NaOH, 0.5 M H<sub>2</sub>SO<sub>4</sub>, 0.01 M KCl/0.1 M H<sub>2</sub>SO<sub>4</sub>, and 0.05 M H<sub>2</sub>SO<sub>4</sub>) before being modified with the probe DNA. The clean gold surface was interacted with a 0.1  $\mu$ M solution of thrombin DNA aptamer (oligomer **1**) containing 1  $\mu$ M tris-(2-carboxyethyl) phosphine hydrochloride (TCEP, which is included to reduce disulfide bound oligomers) in 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4 for 16 h. The surface was then rinsed with deionized water and subsequently passivated with 6-mercaptohexanol (1 mM in 10 mM Tris buffer, pH 7.4) for 3 h. The electrodes were rinsed again with 100 mM Tris buffer at pH 7.4 before being measured by alternating-current voltammetry with a CHI 603 potentiostat (CH Instruments, Austin, TX) in a standard cell with a platinum counter electrode and an Ag/AgCl reference electrode. Control electrodes modified with oligomers **2** and **3** were prepared on the gold surface as described for the immobilization of the thrombin aptamer (oligomer **1**). The surface coverage of the DNA was  $(2.5 \pm 0.2) \times 10^{-12}$  mol cm<sup>-2</sup>.

Sensor measurements were conducted by monitoring the electrode in buffered saline (0.1 M Tris (pH 7.4) with 140 mM NaCl, 20 mM MgCl<sub>2</sub>, and 20 mM KCl). Neither increasing the final KCl concentration to 150 mM nor changing the pH value to 8.5 significantly affected the sensor response (data not shown). Thrombin detection was carried out either in buffered saline, or in fetal calf serum diluted with buffered saline. (The serum samples were diluted to 50% with 0.2 M Tris (pH 7.4). Saline (50  $\mu$ L), 2.94 M in NaCl, 0.42 M in MgCl<sub>2</sub>, and 0.42 M in KCl, was then added to this mixture (1 mL).) For all thrombin detection measurements, except the time-course study (Figure 2, right), electrodes were incubated in each sample for 3 h at room temperature. E-AB sensors were regenerated by soaking in 6 M guanidine hydrochloride (Pierce, Rockford, IL) for 8 min at room temperature, followed by rinsing with deionized water.

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