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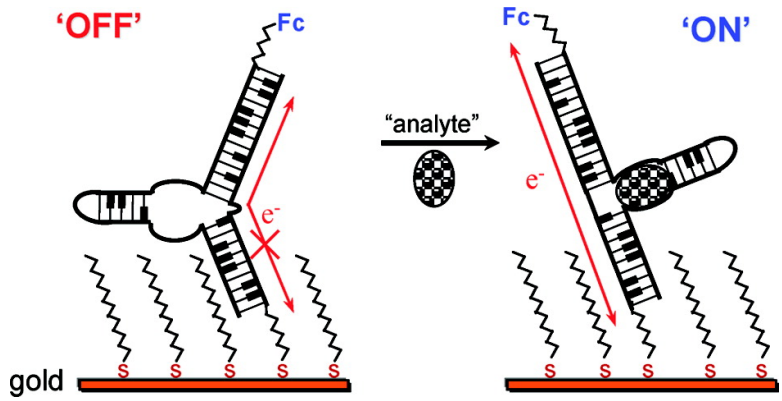

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Immobilized DNA Switches as Electronic Sensors for Picomolar Detection of Plasma Proteins

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Abstract: The sensing principle of a new class of DNA conformational switches (deoxyribosensors) is based on the incorporation of an aptamer as the receptor, whose altered conformation upon analyte binding switches on the conductivity of an adjacent helical conduction path, leading to an increase in the measured electrical signal through the sensor. We report herein the rational design and biochemical testing of candidate deoxyribosensors for the detection and quantitation of a plasma protein, thrombin, followed by surface immobilization of the optimized sensor and its electrochemical testing in both a near-physiological buffer solution and in diluted blood serum. The very high detection sensitivity (in the picomolar range) and specificity, as well as the adaptability of deoxyribosensors for the detection of diverse molecular analytes both small and macromolecular, make this novel sensing methodology an extremely promising one. Such synthetic and robust DNA-based electronic sensors should find broad application in the rapid, miniaturized, and automated on-chip detection of many biomedically relevant substances (such as metabolites, toxins, and disease and tumor markers) as well as of environmental contaminants.

Introduction

Contemporary research in the life sciences, including recent advances in genomics and proteomics, has led to the discovery of thousands of proteins with potential diagnostic and/or therapeutic significance. For example, some of these proteins are biological markers for organ activity, disease processes, or drug action. The ability to monitor slight differences in the amounts of these proteins as well as other biological macromolecules, in the smallest possible detection volumes, down to the level of single cells, is of utmost importance not only for proteomics research but also for biomedical diagnostics in general.[1] To date, antibody-based immunological assays (e.g., enzyme-linked immunosorbent assay, or ELISA) are the most commonly used diagnostic methods used for protein detection; typically, they are not as sensitive or specific as methods for detection of specific nucleic acid sequences, such as DNA microarrays, for instance.[2] In this paper, we describe a broadly adaptable electronic approach for the detection and quantitatation of concentrations as low as picomolar of a plasma protein (thrombin), based on a ligand-binding induced modulation of charge flow through DNA double helices. The design of such a deoxyribosensor typically incorporates a ligand-binding receptor (a DNA aptamer) functionally linked to a helical DNA charge conduction path.[3] Deoxyribosensors work by showing greater charge flow through them in the presence of bound ligand (analyte) than in its absence.[3,4]

Aptamers are molecular receptors made of single- and/or double-stranded oligonucleotides, capable of specifically binding a variety of molecules including many that normally do not interact with DNA or RNA.[5] As reviewed recently by Hermann and Patel,[6] aptamers are obtained by in vitro selection out of large pools of random-sequence oligonucleotides; they typically bind their specified ligands by induced-fit mechanisms. In 1992, Toole and co-workers[7] described their isolation of a single-stranded DNA aptamer to the protease thrombin of the blood coagulation cascade and reported binding affinities in the range of 25−200 nM. In a subsequent publication, Paborsky et al.[8] further demonstrated the aptamer-binding site on thrombin using a solid-phase plate binding assay as well as chemical modification of the protein. Due to the ease with which novel, made-to-order aptamers can be selected from large, random-sequence DNA and RNA libraries, and their generally impressive selectivity and affinity, they are widely regarded as ideal recognition elements for biosensor applications.[9] In line with this claim, antithrombin aptamers have been explored recently as sensing entities in conjunction with either optical or electrical readouts.[10–16] Two state-of-the-art designs, both proposed by

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Xiao et al., reported nanomolar detection of thrombin in blood serum; their electronic sensing was based on the large-scale, binding-induced, conformational switch of the antithrombin aptamer.[12,13] Radi et al.[14] recently described their design of electrochemical aptasensors for thrombin with a detection limit of 2 nM, which was based on the use of a fluorescent molecular beacon to modulate the effect of counterions on quadruplex formation. In the meantime, Baker et al.[17] also reported an aptamer-based electrochemical sensor for the rapid, label-free detection of cocaine in adulterated samples and biological fluids.

The versatility of deoxyribosensors, as reported in this paper as well as in prior reports,[3,4] is that they are able to incorporate and be functional with aptamers that show only modest ligand-induced conformational changes as well as those that show large changes, as is known to be the case with the thrombin aptamer.[6–8] Despite a lack of complete understanding of the mechanistic details of electron transfer through DNA, the molecular conductivity of double helices has been shown experimentally to depend on their conformational state.[18,19] We have recently demonstrated that such a dependence may be harnessed for the electronic sensing of external analytes.[3,4] This type of novel DNA-based biosensor (deoxyribosensor) incorporates an analyte receptor, typically an aptamer, whose altered conformation in the presence of bound analyte switches the conformation and, hence, the conductive path between two DNA double-helical stems. A wholly general design for such a deoxyribosensor, termed a coupled-ligand sensor (Figure 1a),[3] relies on the modulation of the current flow in a path directly adjacent to the receptor. For these sensors, irradiation of an appended anthraquinone (AQ) moiety results in efficient conduction in only the reporter stem in the absence of bound ligand. In contrast, the ligand- (analyte-) induced stacking of the reporter and detector stems results in enhanced conductivity of the detector stem, and this can be monitored biochemically.[3,4] To date, the successful design of deoxyribosensors has been demonstrated with trial analytes that are typically small organic molecules such as adenosine[3] and argininamide.[4] Nevertheless, the biochemical protocol for detection of enhanced charge flow in the detector stem is cumbersome, involving a base-catalyzed fragmentation of the deoxyribosensor DNA strands at sites of guanine oxidative lesions caused by the charge flow, followed by study of the resultant DNA fragmentation pattern by denaturing polyacrylamide gel electrophoresis (PAGE).[3,4]

Herein, we report on the feasibility of adapting the purely biochemical deoxyribosensor concept (Figure 1a) to a bona fide chip-based electronic device (Figure 1b), one capable of detecting the human serum protein thrombin in both an aqueous buffer and in blood serum, with ultrahigh sensitivity. Aside from the intrinsic popularity of thrombin as a model biomolecule for the evaluation of new analytical protocols, it is a biomarker of significant importance, given its role in coagulation and the need to monitor its levels for cardiovascular disease therapy and for surgical procedures.[20]

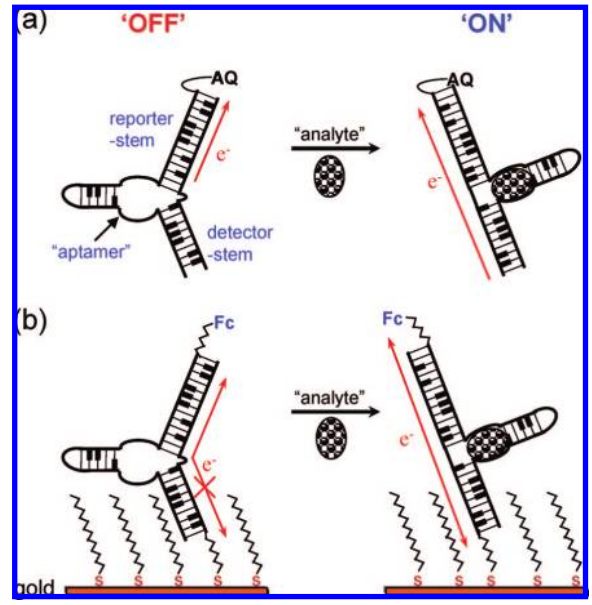

Figure 1. Design of DNA switches as electronic sensors (deoxyribosensors) for specific detection of molecular analytes, and illustration of the conduction path change upon analyte binding. (a) Biochemical mode of ligand/analyte detection. In the absence of ligand/analyte, the sensor adopts an open, unstacked conformation, which allows efficient charge transfer (indicated by arrows) only from the AQ (anthraquinone, tethered covalently to the end of the reporter stem) to the reporter stem. Analyte binding induces the adaptive folding and compaction of the aptamer, facilitating charge transfer from the detector stem. (b) Modification and immobilization of the deoxyribosensor constructs onto a gold chip for direct electronic detection of analyte. Ferrocene (Fc) is covalently attached to the end of the reporter stem for electrochemical monitoring of the binding event, while the end of the detector stem is modified with a $-(CH_2)_6SH$ functionality for immobilization on the electrode. The open area of the electrode is passivated with inert alkanethiols to reduce nonspecific adsorption and current leakage.

Results and Discussion

Deoxyribosensor Design and Testing by a Biochemical Protocol. A notable advantage of our protocol for deoxyribosensor design is that we are able to make use of pre-existing nucleic acid receptors (aptamers) for the binding of specified molecular analytes. The main design challenge, then, is the functional coupling of a DNA aptamer to a double-helical conduction path, such that conformational change wrought in the aptamer upon analyte binding results in a concomitant change in conductivity of the double-helical element. To construct a deoxyribosensor capable of detecting thrombin, a plasma protein, the DNA aptamer sequence in vitro selected by Bock et al.[7] (5′-GGTTG GTGTG GTTGG) was co-opted to construct a series of potential coupled-ligand sensors by combining it with double-helical reporter and detector stems used by Sankar and Sen[4] as the conduction path. The different sensor constructs varied in sequence at the three-way junction (3WJ) that serves to join the aptamer to the conduction path and are shown in Figure 2. The junctions in these constructs (3WJ-1−5) incorporate, variously, AA bulges at different locations, in addition to distinct elements of A-T and G-C base pairs (Figure 2). In practice, these putative deoxyribosensors were assembled by complementarity-determined hybridization of two distinct DNA strands: the first strand derivatized at its 5′-terminus with anthraquinone (AQ),[3,4] and the second strand radiolabeled at its 5′-hydroxyl end with a ^{32}P-phosphoester, incorporating the aptamer sequence.

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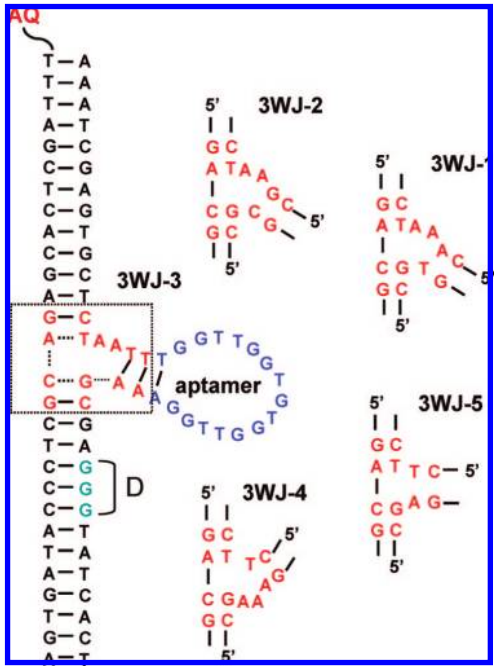

Figure 2. Rational design of candidate deoxyribosensors for thrombin detection: their sequences and secondary structures. Five different configurations of the junction base pairs (3WJ-1−5) were examined to select the one showing optimal performance. Guanine triplets (D) in the 5′-end ^{32}P-labeled strand were used to biochemically monitor charge transfer to the aptamer and/or detector stems from the reporter stem upon photoexcitation of the AQ.

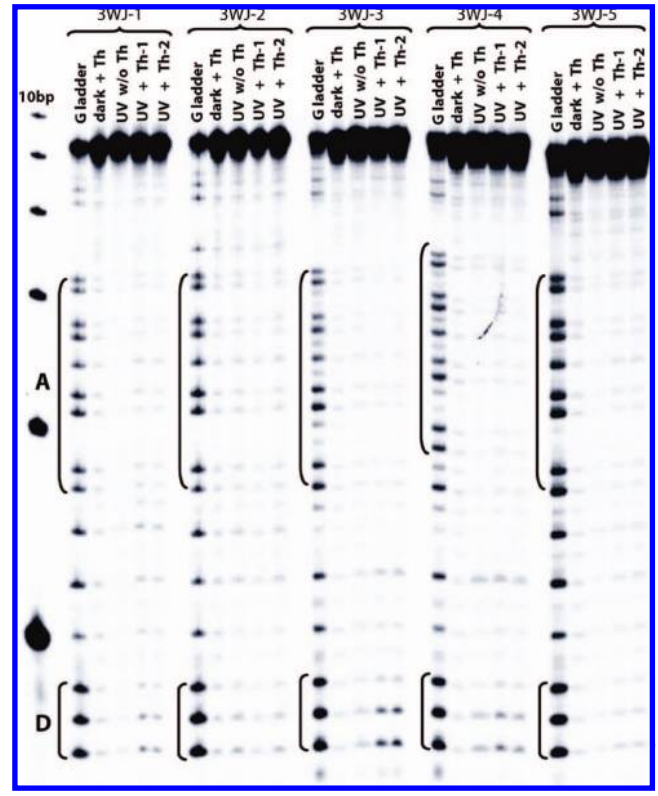

Figure 3. Biochemical testing of candidate deoxyribosensors for thrombin detection: phosphorimager traces of strand-cleavage data. A and D indicate, respectively, the positions of the guanine doublets in the aptamer and the triplet in the detector stem shown in Figure 2. The lanes for each construct correspond to no irradiation but added thrombin (dark + Th), irradiation but no added thrombin (UV w/o Th), and irradiation plus added thrombin (UV + Th-1, UV + Th-2). Reference G ladders are shown to the left for each of the constructs. The concentration of thrombin used in the experiments shown in the two Th lanes is 160 nM in each case.

The expectation in testing these five candidate sensor constructs was that the overall design of one or more constructs might encourage the stacking of the reporter and detector stems concomitant to the binding of thrombin to the aptamer loop. The design of these different three-way junction elements were based, in part, on empirical rules established by Welch et al.[21] for the construction of regular DNA three-way junctions (regular junctions consist of three fully base-paired helical stems of DNA, rather than two helical stems and an aptamer loop as shown in Figure 2). The presence of a two-nucleotide bulge within a regular junction typically leads to preferential stacking of two of the three component helices. However, the stacking rules strictly hold only where three regular helices (along with the unpaired two-nucleotide bulge) constitute the three-way junction. We envisioned that replacement of one of the three helical arms by a very short stem of two base pairs terminating in an aptamer loop might result in the disruption of stacking between the two stems, which stacking might be restored upon tightening of the aptamer loop and adjoining 2-bp stem by the binding of thrombin to the aptamer.

The above designs were first evaluated biochemically by studying the eletrophoretic patterns and intensities of charge-flow generated guanine oxidative damage (determined by piperidine-catalyzed DNA cleavage) within them, notably at a specific trapping sequence[22] consisting of three consecutive guanines (marked as D in Figure 2) within the detector helix. Constructs 3WJ-1−5 were each irradiated at 366 nm to set up charge flow within them. Experiments were carried out in a test buffer [50 mM Tris, pH 7.4, 5 mM KCl, 50 mM NaCl, and 0.02 mM ethylenediaminetetraacetic acid (EDTA)], with or without added thrombin. Irradiated sensor samples (as well as unirradiated or dark controls) were then treated with 10% (v/v) piperidine at 90 °C[3,4] and lyophilized to dryness, and their fragmentation patterns were analyzed by denaturing PAGE. Figure 3 shows the data for each construct: the lane marked dark + Th indicates a dark control, where the deoxyribosensor was incubated in the dark in the presence of 160 nM thrombin; the lane marked UV w/o Th shows irradiated samples in the absence of protein; and the two lanes marked UV + Th show duplicate experiments on irradiated sensors in the presence of 160 nM thrombin. It can be seen that the different sensor constructs showed distinct oxidative cleavage patterns, especially at the detector guanine triplet (marked as D). It can be seen that with both constructs 3WJ-3 and 3WJ-4, notably higher band intensities were seen in the UV + Th lanes (UV irradiation plus added thrombin), relative to the dark + Th (no irradiation but added thrombin) and UV w/o Th (irradiation but no added thrombin) controls. Of these two constructs, 3WJ-3 provided a higher signal at the D sequence in response to the presence of thrombin, and this sensor construct was carried forward for further studies.

First, buffer solutions and temperature were systematically varied for the 3WJ-3 sensor, to determine the optimal conditions for its detection of thrombin. We found a superior response when

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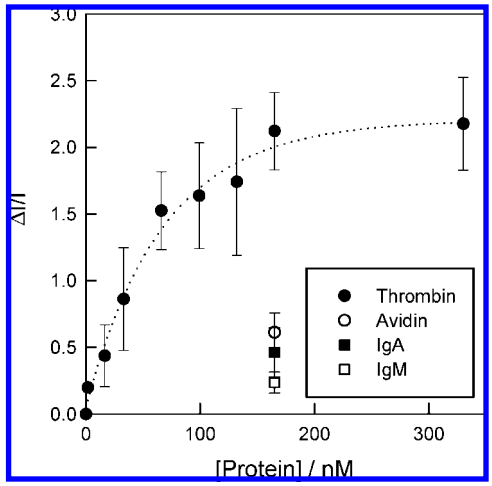

Figure 4. Relative biochemical signal (guanine damage at the detector stem G triplet) ($\Delta I/I$) of the deoxyribosensor as a function of protein concentration. Samples were photoirradiated for 60 min at 4 °C in 50 mM Tris, pH 7.4, 50 mM NaCl, and 0.02 mM EDTA, in the presence of different concentrations of thrombin. Following irradiation, the deoxyribosensor samples were piperidine-treated and analyzed with sequencing gels. The sensor signal was quantitated as a ratio of the change of DNA cleavage (ΔI) at the detector stem guanine triplet (corrected in each case, for unirradiated cleavage) in the presence of thrombin relative to that in the absence of thrombin (I).

sensor irradiation was carried out at 4 °C rather than at ambient (25 °C) or physiological (37 °C) temperatures. The addition of monocations (Na^+ and Li^+) ranging from 50 to 150 mM had no effect; increasing the Mg^{2+} concentration to >10 mM did not further enhance the sensor response either. The presence of bovine serum albumin (BSA) at 0.01% (w/w) in the buffer, however, improved the sensor performance by significantly reducing background DNA cleavage, presumably by discouraging nonspecific binding of thrombin to the sensor.

We then carried out the study of relative strand cleavage at the D sequence as a function of the concentration of added thrombin (under optimized solution and temperature conditions; see Experimental Section and Supporting Information). The sensor signal (increase in intensity seen in D, as determined by phosphorimagery, normalized to the background intensity, $\Delta I/I$) showed a monotonic increase initially and saturated when the thrombin concentration rose to ~160 nM (Figure 4). The dynamic range of the detection and the sensitivity demonstrated here are comparable with those previously determined with other optical protocols.[11,16] More importantly, control experiments with other proteins such as avidin, IgA, and IgG, all at fairly high concentrations (160 nM), produced insignificant sensor signals, which confirmed the specificity of the designed 3WJ-3 deoxyribosensor for thrombin. When tested with TTR, cytochrome *c*, and IgA, we observed slight decreases in the intensity at the D sequence (i.e., a negative response; see Supporting Information); this may relate to the nonspecific binding of these proteins to the detector stem (rather than to the aptamer), leading to protection of this sequence from charge-flow related damage and subsequent cleavage.

Chip-Based Sensor Design and Electrochemical Thrombin Assay. Following the above biochemical demonstration of thrombin detection by a coupled-ligand deoxyribosensor, 3WJ-3, we constructed a version of the sensor that is appropriate for chip-based thrombin detection, via a direct electrochemical readout. Figure 5a (left) shows such a sensor, whose sequence was adapted from 3WJ-3 (notably, the reporter/AQ stem was

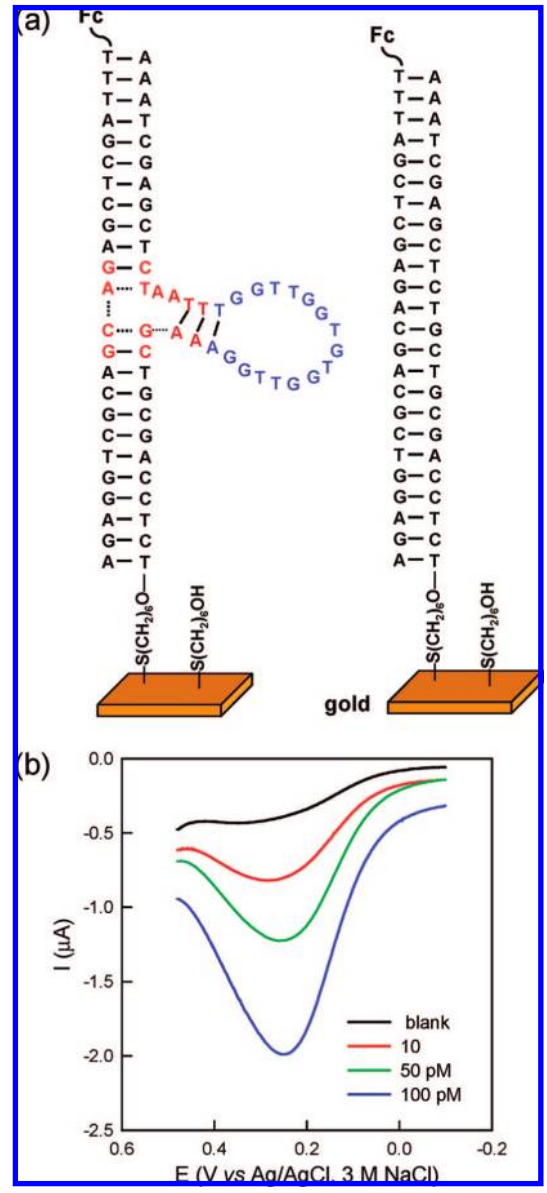

Figure 5. Design of the chip-based deoxyribosensor for thrombin and electrochemical test. (a) The secondary structure of the sensor is shown on the left, while a purely Watson−Crick base-paired double helix shown on the right (used as a control). On the basis of biochemical tests, the electron transfer direction is expected to be from the gold chip to the redox indicator Fc (ferrocene); Fc needs to be preoxidized to form Fc^+ to accept an electron. Upon immobilization of DNA constructs, the gold surfaces were passivated with 6-mercaptohexanol (MCH) to prevent nonspecific adsorption and to reduce leakage currents. (b) Square wave voltammetry (SQW) response of deoxyribosensor-modified electrodes in the presence of different concentrations of thrombin. The experiments were carried out in 50 mM Tris, pH 7.4, and 50 mM NaCl. The electrodes were incubated in 20 μL of various thrombin concentrations in 50 mM Tris, pH 7.4, and 50 mM NaCl at 4 °C for 10 min and then scanned in protein-free buffer from +0.48 to −0.1 V with precondition at +0.48 V for 5 s.

shortened and the guanine triplet in the detector stem was eliminated to facilitate electron transfer from electrode to redox center). Instead of an electron acceptor, such as the anthraquinone (AQ) used in the biochemical experiments, ferrocene (Fc) was covalently tethered to the terminus of the reporter stem as a redox indicator. As an electrochemically reversible, one-electron redox molecule, Fc was preferentially selected in comparison to AQ that has a multistep, protonation-

coupled redox process.[23] Furthermore, the distal end of the construct was now modified with a thiol group, enabling the formation of robust DNA monolayers on a gold electrode via sulfur−gold linkages.[24,25] We expected that, upon the binding of thrombin to the aptamer element of the deoxyribosensor, the induced switch in the conduction pathway could be measured directly with conventional electrochemical methods.

Before the performance of the thrombin deoxyribosensor itself was tested, the electrochemical behavior of a control Fc-terminated DNA double helix (duplex) immobilized on a gold chip (Figure 5a, right) was investigated. For the purpose of comparison, the sequence of this double helix was identical to that of the conduction path within the deoxyribosensor, but it lacked the aptamer loop and stem. Cyclic voltammetry (CV) and square wave voltammetry (SQW) were both carried out as parts of the initial investigation. Both experiments showed that the reduction current was more pronounced than the oxidation current (see Supporting Information). For CV, the reduction current at first scan was larger than that at later scans; furthermore, the redox reaction was more pronounced at lower scan rate (0.1 V/s) than at a higher scan rate (5 V/s). These results imply that the electron transfer in the immobilized duplex may prefer one direction [from the gold chip to the ferricinium ion (Fc^+), the oxidized state] over the other (from the Fc, i.e., reduced state, to the gold chip).

It has been shown experimentally that SQW is more sensitive than CV; therefore, SQW along a reduction sweep was used to further characterize the deoxyribosensors. Figure 5b shows the SQW data measured with gold electrodes modified with the sensor constructs shown in Figure 5a (left). The sensor signal (now measured directly as an electrical current) increased significantly upon incubation of the sensor chip with thrombin at picomolar concentrations in the standard buffer solution (50 mM Tris, pH 7.4, and 50 mM NaCl). Figure 6 shows the dependence of the sensor signal on thrombin concentrations: the sensor signal was distinct at a concentration as low as 5 pM and increased with increasing thrombin concentrations (up to 200 pM). The linear range of response is up to 100 pM, and in comparison, the responses measured in the presence of other proteins such as BSA (67 kDa, pI = 4.8), avidin (68 kDa, pI = 10.5), IgA (350 kDa, pI = 3.5−5.5), and IgG (150 kDa, pI = 5.5−9) are negligible. Three of the above proteins are abundant in the serum. Even with concentrations as high as 80 pM, no evidence for binding of these noncognate proteins to the sensors were detected, relative to the clear signal seen in the presence of thrombin.

The results shown in Figure 6 are encouraging, not only because they demonstrate a successful translation of the coupled-ligand deoxyribosensor biochemical concept to a chip-based device with direct electronic readout, but also because of the unprecedented improvement in the sensitivity of detection. Upon moving from the solution-based biochemical tests to chip-based electrochemical detection, sensitivity was enhanced from the nanomolar range (which is at the same level as reported by others)[11−16] to the picomolar range. This may be partially due to a reduction in the background signal, which is always present at a fairly high level in the DNA fragmentation and gel

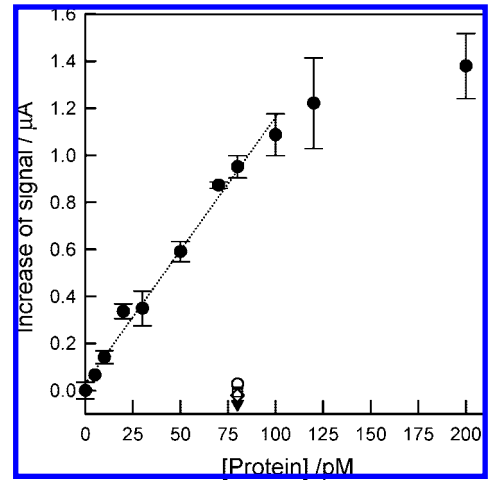

Figure 6. Performance of the electrochemical deoxyribosensor for detection of thrombin (●). The sensor response, that is, the increase in the reduction current upon incubation with thrombin at various concentrations, shows that the linear dependence is in the range of 0−100 pM. The sensor response in interferences of 80 pM BSA (○), avidin (□), IgA (◇), or IgG (▼) is also shown in this plot. Experimental conditions were the same as in Figure 5b.

electrophoresis experiments. The major factor in the sensitivity improvement, however, may be the use of a limited number of readily accessible sensor constructs on the electrode, given that the surface density of the DNA constructs on the gold chip is only 2.6 ± 0.3 pmol/cm^2 (determined by CV measurements in the presence of 5.0 μM $[Ru(NH_3)_6]^{3+}$ redox cations).[25,26] The electrode surface area is small, at 0.126 cm^2, and it is clear that even picomolar concentrations of thrombin in the solution are sufficient to activate the immobilized deoxyribosensor population. The dissociation constant, K_d, for the thrombin−aptamer interaction has been shown to be as low as 2.7 nM,[7,15] which is consistent with the data presented herein.

To date, reports of electrochemical probing of conductivity changes in DNA constructs are relatively few.[27−29] Kelley et al.[27] tested for lateral charge propagation through double-helical DNA films and found that the electrochemical signals decreased linearly with increasing percentages of mutated duplexes (i.e., containing C·A mismatches). Wong and Gooding[29] have recently shown that a long-range charge-transfer approach can be used to probe cisplatin-induced DNA perturbations, where differences in the charge flow could be attributed to changes in conformation of the DNA duplexes. Herein, we have provided evidence that structural changes to a DNA aptamer element located adjacent to a disrupted conduction path (two poorly stacking DNA double helices) helps to re-establish the stacking and hence repair the conduction path, a phenomenon that had so far been inferred only indirectly, from biochemical experiments.[3,4] Interestingly, the structural changes induced in this particular aptamer upon thrombin binding have also been used in the methodology of Xiao et al.,[12] which depends on altered distances between the redox center and the electrode surface upon ligand binding. However, the sensitivity observed in that paper was

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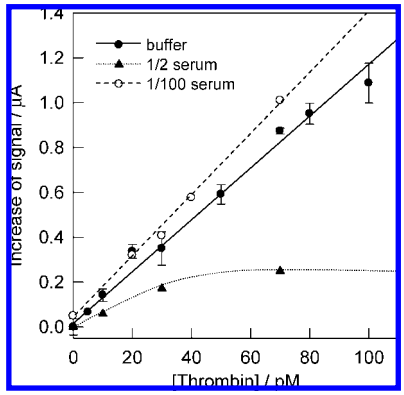

Figure 7. Investigation of the effect of serum on the chip-based thrombin deoxyribosensor signal. Serum was used after dilution with 50 mM Tris, pH 7.4, and 50 mM NaCl, either 2-fold or 100-fold. The thrombin solution was prepared either in serum-free 50 mM Tris, pH 7.4, and 50 mM NaCl alone or in the same buffer containing diluted serum as indicated. Other experimental conditions were the same as in Figure 6.

6.4 nM, and the dynamic range was from 6.4 to 768 nM. We believe that differences in design between our deoxyribosensors and the sensor constructs of Xiao et al.[12,13] are responsible for the observed differences in sensitivity and dynamic range of detection. There is no doubt that the exceptionally sensitive performance we observe is linked to the rational optimization of the overall design of the deoxyribosensor for thrombin. We have shown previously, on the basis of biochemical experiments, that high-performance deoxyribosensors can be constructed with a diversity of aptamers for other molecular analytes (e.g., adenosine[3] or argininamide[4]). To what extent the picomolar detection of thrombin by the deoxyribosensor described here is dependent on the specific structure and conformational change of its constituent antithrombin aptamer certainly deserves further investigation.

While the experiments reported above were carried out in the close-to-physiological standard buffer, we also examined the performance of the thrombin deoxyribosensor in the presence of blood serum. Given the very high sensitivity of this deoxyribosensor (detection in the picomolar range of analyte), practical application of such electronic sensors would typically be made only where blood serum (or other body fluids) have been diluted by thousands of times. In fact, the physiological concentrations of thrombin in resting and activated blood range from low nanomolar to low micromolar.[20] When we incubated and made current measurements with picomolar concentrations of thrombin in diluted serum (dilutions made with the standard buffer), we found no significant influence of serum on the sensor signal at serum dilutions of 100-fold (○ in Figure 7) and higher. The slightly higher current measurements made in these measurements may even reflect levels of endogenous thrombin originally in the serum.[12] In one experiment, picomolar concentrations of thrombin were first incubated with the immobilized deoxyribosensor in the standard buffer, following which the buffer was removed and replaced with undiluted serum, prior to the measurement of current. This set of data (▲ in Figure 7) shows that we can still get quantitative response from the sample, although the undiluted serum does influence the sensor signal to some extent, probably owing to the very high overall concentration of proteins in undiluted serum. However, this last result clearly demonstrates that the effect of nonspecific proteins and/or electrolytes in the original body fluid on this new sensor application may actually be very small.

Conclusions

We have described the direct electrical detection of a biomedical analyte, thrombin, using a unique design of a DNA switch, namely, a deoxyribosensor. We have designed a number of such sensors to demonstrate that charge transfer through a double-helical DNA conduction path can be modulated by conformational changes within it, resulting from the adaptive binding of a specified ligand/analyte to a DNA aptamer functionally appended to the conduction path. Such a measurable change in charge transfer concomitant to ligand binding results from both large and relatively subtle conformational changes in the aptamer upon ligand binding.[3,4] The change in charge transfer through the deoxyribosensor can be measured biochemically in solution, as well as electrochemically on a chip. Specifically, a deoxyribosensor originally designed for a solution-based sensing of a plasma protein, thrombin, can be translated to a chip-based electronic device, which detects and quantitates concentrations of thrombin as low as picomolar in both a defined buffer solution as well as in diluted blood serum. Owing to the generic and modular nature of the design of deoxyribosensors (coupling a DNA conduction path and an aptamer loop) and the established surface chemistry used to construct the biochip that utilizes the deoxyribosensor, we envision that this methodology will be widely applicable to the development of rapid, DNA-based electronic sensors for any number of small and macromolecular analytes of biomedical and/or environmental importance.

Experimental Section

Preparation of DNA Constructs and Biochemical Tests. 5′-Amino-C6-oligonucleotides and other DNA oligomers were purchased from Core DNA Services Inc. (Calgary, AB) and size-purified by denaturing (50% urea, w/v) polyacrylamide gel electrophoresis (PAGE) before use. DNA quantification was carried out in a Cary dual-beam spectrophotometer, by use of the absorbance at 260 nm estimated for single-stranded DNA. 5′-Amino-C6-oligonucleotides were coupled to anthraquinone (AQ) via a protocol published previously.[3] The covalent attachment was accomplished by reacting the *N*-hydroxysuccinimide ester of anthraquinone-2-carboxylic acid with the oligomer. The resulting AQ-oligonucleotides were purified on an Agilent HPLC system, with an Agilent Zorbax ODS RP-18 5-μm column, and eluted with a gradient formed between 0.1 M triethylammonium acetate (TEAA, pH = 7.0)/CH_3CN (20:1, buffer A) and 100% CH_3CN (buffer B) at 1.0 mL/min. To lower the levels of background cleavage from piperidine treatment, DNA strands to be ^{32}P-labeled were pretreated with 10% (v/v) piperidine at 90 °C for 30 min, followed by lyophilization, prior to the labeling reaction according to standard T4 kinasing protocols and PAGE purification.

The coupled-ligand deoxyribosensor constructs shown in Figure 2 were formed by annealing equimolar mixtures (0.125 μM each) of constituent strands in the binding buffer (50 mM Tris, pH 7.4, 50 mM NaCl, and 0.02 mM EDTA). The mixtures were heated to 90 °C for 2 min, cooled slowly to room temperature, made up to the desired concentrations of $MgCl_2$ and BSA, and then kept on ice. Finally, ice-cold diluted thrombin was added. The original thrombin stock (96 μM, stored at −80 °C) was freshly diluted either in storage buffer (50 mM sodium citrate, pH 6.2, 0.1% PEG 8000, and 50 mM NaCl) or in diluted serum solution before use. The diluted serum solution was prepared with standard buffer (50 mM Tris, pH 7.4, and 50 mM NaCl). All solutions were incubated in a water bath at 37 °C for 5 min before UV irradiation.

Upon incubation of a deoxyribosensor construct with the protein to be tested, the sample was placed under a UVP Black-Ray UVL-56 lamp (365 nm) for 60 min at a distance of 4 cm from the lamp at 4 °C. Temperature was maintained by having the sample tubes placed in a water bath set to the desired temperature. Following irradiation, samples were ethanol-precipitated and the DNA pellets were dissolved in 10% piperidine prior to heating for 30 min at 90 °C, as described previously in detail.[3,4] The samples were then lyophilized, dissolved in a denaturing gel loading buffer, heated to 100 °C, cooled, and loaded on a 12% polyacrylamide sequencing gel. Imaging and quantification of the gels were carried out on a Typhoon 9410 phosphorimager with ImageQuant 5.2 software; in this way, densitometry traces of entire lanes in a gel can be obtained. The density of a G8 peak was taken as a percentage of the total signal within a given lane. The relative signal of the sensor construct as a function of the thrombin concentration, as plotted in Figure 4, was calculated as the increase of percentage cleavage of the band in the presence of thrombin (ΔI) divided by the net percentage of the corresponding band in absence of thrombin (I).

DNA Immobilization and Electrochemical Tests. The synthetic DNA oligomer (strand **1**) containing the antithrombin aptamer (47-mer), HO-$(CH_2)_6$-SS-$(CH_2)_6$-O-5′-TCT CCA GCG TCG AAA **GGT TGG TGT GGT TGG** TTT AAT CTC GAG CTA AA-3′; the amino derivative of the shorter strand (24 mer), H_2N-$(CH_2)_6$-O-5′TTT AGC TCG AGA CGA CGC TGG AGA-3′ (strand **2**); and its complementary strand, HO-$(CH_2)_6$-SS-$(CH_2)_6$-TCT CCA GCG TCG TCT CGA GCT AAA (strand **3**) were also purchased from the Core DNA Services Inc. (Calgary, AB). The 5′-thiol modifier was acquired from Glen Research (Sterling, VA). The Fc modification of strand **2** was carried out in the same manner as the AQ modification, following an established protocol.[30]

The synthesized DNA strands were purified by reverse-phase HPLC on a Gemini 5-μm C18/110 Å column (Phenomenex, Torrance, CA), and eluted with a gradient of 0.1 M triethylammonium acetate (TEAA, pH 7.0)/CH_3CN (20:1, buffer A) and 100% CH_3CN (buffer B) at 1.0 mL/min. In order to reduce the disulfide bonds and generate thiol-terminated single-stranded DNA (HS-ssDNA; strands **1** and **3**), the HPLC-purified samples (0.5−2.0 nmol) were treated with 40 μL of 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Aldrich−Sigma) in 100 mM Tris buffer at pH 7.5 at room temperature for 4 h, followed by desalting in a MicroSpin G-25 column (Amersham Biosciences, England). The columns were equilibrated with deoxygenated water prior to use.

For the DNA constructs used to modify gold electrodes (Figure 5), 5.0 μM of freshly prepared HS-ssDNA (strand **1** for the sensor or strand **3** for the control) was hybridized with the 5.5 μM Fc-modified strand (strand **2**) in deoxygenated standard buffer (50 mM Tris, pH 7. Four and 50 mM NaCl) at 80 °C for 2 min, followed by slow cooling to room temperature.

Gold substrates (100 nm Au/5 nm Cr/glass) were purchased from Evaporated Metal Films Inc. (Ithaca, NY). Prior to modification, they were cleaned with freshly prepared piranha solution [3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2 (*WARNING: piranha solution reacts violently with organic solvents*)] at 90 °C for 5 min and then rinsed thoroughly with water. The freshly cleaned gold was modified by spreading a droplet of 10 μL of 5 μM DNA constructs in standard buffer on its surface. The gold chips coated with DNA were stored in a box at 100% relative humidity at room temperature, for 12−48 h. After modification, they were rinsed with standard buffer incubated in 1 mM 6-mercapto-1-hexanol (MCH, Aldrich) in the same buffer for 1 h (except where otherwise indicated) and thoroughly rinsed again with the same buffer.

Electrochemical experiments were carried out by use of a 1-mL three-electrode cell with a gold chip, modified with the DNA constructs, as the working electrode (with a geometric area of 0.126 cm^2). A platinum wire counterelectrode and a Ag | AgCl | 3 M NaCl reference electrode were used. Cyclic voltammetry (CV) and square wave voltammetry (SQW) measurements were performed at ambient temperature (21−23 °C) in standard buffer before and after incubation in different amount of thrombin (in the same buffer) by use of a μAutolab II potentiostat/galvanostat (Eco Chemie B.V., Utrecht, Netherlands). The reaction of chips with thrombin was performed in 20 μL of thrombin in standard buffer or in diluted serum solutions (prepared as described above), at different concentrations, at 4 °C for 10 min, and then the chips were washed with standard buffer three times.

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Supporting Information Available: Additional experimental data for biochemical and electrochemical testing of the candidate dexoyribosensors. This material is available free of charge via the Internet at http://pubs.acs.org.

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