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Aptamer-Based Potentiometric Measurements of Proteins Using Ion-Selective Microelectrodes

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We here report on the first example of an aptamer-based potentiometric sandwich assay of proteins. The measurements are based on CdS quantum dot labels of the secondary aptamer, which were determined with a novel solid-contact Cd^{2+}-selective polymer membrane electrode after dissolution with hydrogen peroxide. The electrode exhibited cadmium ion detection limits of 100 pM in 100 mL samples and of 1 nM in 200 μL microwells, using a calcium-selective electrode as a pseudoreference electrode. As a prototype example, thrombin was measured in 200 μL samples with a lower detection limit of 0.14 nM corresponding to 28 fmol of analyte. The results show great promise for the potentiometric determination of proteins at very low concentrations in microliter samples.

Aptamers are nucleic acid ligands that have been designed through an in vitro selection process called SELEX (systematic evolution of ligands by exponential enrichment).[1] Such aptamers hold great promise as affinity ligands for the biosensing of disease-related proteins and for developing protein-sensing arrays.[2−7] Owing to their relative ease of isolation and modification, good stability, and wide applicability, they appear to be excellent alternatives to antibodies.[8,9] The attractive biosensing properties of aptamer recognition elements have been illustrated in connection with a colorimetric method, but the lower detection limit was only in the micromolar range.[10,11] Another detection scheme has been based on changes in fluorescence properties upon binding the fluorophore-labeled aptamer to the target.[12−16] However, this fluorescence response is usually weak, and owing to the difficult design of signaling aptamers, the method is not easy to generalize. Lower detection limits in the 10 nM range have been obtained with piezoelectric analyzers.[17]

In recent years, different electrochemical strategies have been developed for monitoring the interaction between aptamer and target analytes. The electrochemical methods are, in general, superior to the optical ones because of rapid response, simple handling, and low cost.[18−20] Electrochemical aptamer biosensors are based, among others, on a binding-induced label-free detection,[21−25] on enzymes,[26,27] or on nanoparticle labels.[28] Excellent values in the femtomolar range have been achieved with impedance spectroscopy and amplification by chemical means to denature the protein captured by an aptamer on the electrode

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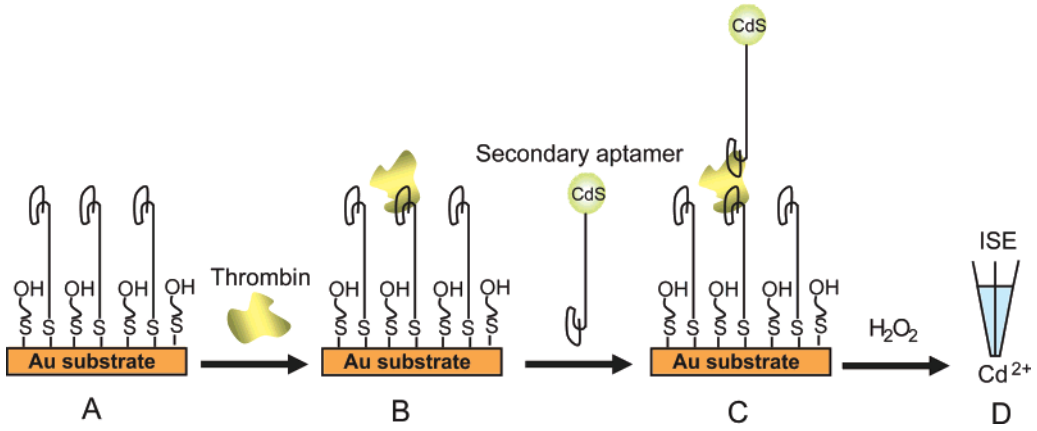
Scheme 1. Representation of the Analytical Protocol[a]



[a] (A) Formation of a mixed monolayer of thiolated aptamer on gold substrate; (B) thrombin addition and binding with aptamer; (C) secondary binding with CdS-labeled aptamer; (D) dissolution of CdS label followed by detection using a solid-contact Cd^{2+}-selective microelectrode.

surface (10 fM)[25] and, very recently, by electrogenerated chemiluminescence via target protein-induced strand displacement (1 fM).[29] Recently, the nanomaterial-based electrochemical detection of proteins has received considerable attention. The methods include the use of gold nanoparticles[30,31] or semiconductor nanocrystal tracers.[32,33] Usually, detection is made by anodic stripping voltammetry (ASV), which due to its intrinsic preconcentration step allows one to achieve ultralow detection limits.[34]

Potentiometry with ion-selective electrodes (ISEs) represents an attractive tool for trace metal analysis in confined samples. Since, with this method, the direct relationship between analyte activity and observed potential is independent of the sample volume, no deterioration of the signal or lower detection limit is expected upon reducing the volume. This is rather unique and establishes potentiometry as a preferred method when dealing with miniaturized analytical microsystems.[35,36]

Recent improvements in the detection limits of ISEs based on polymeric membranes containing selective receptors (ionophores) have yielded sensors for the direct measurement in the subnanomolar concentration range.[37] It is now possible to use miniaturized ISEs for detecting subfemtomole amounts of ions in microvolume samples.[35,36]

Recently, we have demonstrated that such potentiometric microsensors are very attractive for ultrasensitive immunoassays in connection with nanoparticle amplification labels.[38] By reducing the sample volume and using quantum dot tags, the lower detection limit has been improved to <10 ppb.[39] Here, for the first time, we demonstrate the use of a potentiometric microsensor for monitoring biomolecular interactions of an aptamer coupled to nanocrystal tags. As illustrated in Scheme 1, the target protein is captured by the thiolated aptamer anchored on the surface of a gold substrate. Then, a secondary aptamer with CdS nanoparticle labels is added, upon which CdS is dissolved with H_2O_2, yielding a dilute electrolyte background suitable for the potentiometric detection of the released Cd^{2+} with a polymer membrane Cd^{2+}-selective microelectrode.

In this work, we use an aptamer known to bind the blood-clotting protein thrombin as a model system.[40] Thrombin, the last enzyme protease involved in the coagulation cascade, converts fibrinogen to insoluble fibrin, which forms the fibrin gel either in physiological conditions or a pathological thrombus. The concentration of thrombin in blood can vary considerably. However, since a trace level of thrombin (high picomolar range) in blood has been found to be associated with coagulation abnormalities,[41] it is important to assess this protein with high sensitivity.

EXPERIMENTAL SECTION

Reagents. Thrombin from human plasma, TRIS·HCl, 6-mercapto-1-hexanol, tris(carboxethyl) phosphine (TCEP), potassium dihydrogenphosphate, and dipotassium hydrogen phosphate were purchased from Sigma (St. Louis, MO). The nucleic acid aptamers were obtained from Integrated DNA Technologies Inc. (Coralville, IA). The following oligonucleotide sequences were used: aptamer 1 (primary aptamer), 5′-HS−TTT TTT TTT TGG TTG GTG TGG TTG G-3′; aptamer 2 (secondary aptamer), 5′-HS−TTT TTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′.

Chemicals for the synthesis of CdS quantum dots, sodium bis(2-ethylhexyl) sulfosuccinate (AOT), $Cd(NO_3)_2$, Na_2S, cystamine, sodium 2-mercaptoethane sulfonate, and the solvents were obtained from Sigma. The ionophores, *N*,*N*,*N*′,*N*′-tetradodecyl-3,6-dioxaoctanedithioamide (ETH 5435), *N*,*N*-dicyclohexyl-*N*′,*N*′-

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dioctadecyl-3-oxapentanediamide (ETH 5234), the lipophilic cation exchanger, sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB), the lipophilic salt, tetradodecylammonium tetrakis(4-chlorophenyl)borate (ETH 500), 2-nitrophenyl octyl ether (*o*-NPOE), poly(vinyl chloride) (PVC), and tetrahydrofuran (THF) were purchased in Selectophore or puriss. p. a. grade from Fluka (Buchs, Switzerland). The solvent CH_2Cl_2 and H_2O_2 were obtained from Fisher (Pittsburgh, PA). Poly(3-octylthiophene) (POT) was synthesized as reported[42] and purified according to the patent application.[43] The methyl methacrylate−decyl methacrylate (MMA−DMA) copolymer matrix was obtained as described previously.[44] All stock and buffer solutions were prepared using doubly deionized water (18.2 MΩ cm).

Preparation of Oligonucleotide Aptamer on Gold Surface. The immobilization of oligonucleotides was based on a previously reported protocol.[28] Thiolated aptamers were received with disulfide protecting groups.

Cleavage of Dithiol Protecting Group. The disulfide-protected nucleotides (100 μM, 10 μL) were diluted with autoclave water to 100 μL and treated with TCEP (1 mg) for 30 min, followed by purification using a MicroSpin G-25 column obtained from Amersham Biosciences (Buckinghamshire, U.K.).

Gold Substrates. The gold substrates were obtained from Denton Vacuum LLC (Moorestown, NJ), machine cut (Advotech Company Inc., Tempe, AZ) to identical pieces ($6 \times 3 \times 0.2$ mm^3), assuming a uniform thickness.

Preparation of Mixed Monolayers. Gold substrates were cleaned in Piranha solution and rinsed with water prior to use. (*Safety note*: the Piranha solution should be handled with extreme caution.) The oligonucleotide monolayer was generated by treating the gold substrates with a 1 μM thiolated oligonucleotide aptamer solution (100 μL) in a potassium phosphate buffer (1 M, pH 8.0) overnight, followed by removal of the solution. The surface of the gold substrates was then blocked by a 10 min treatment with 6-mercapto-1-hexanol (0.1 M, 100 μL), followed by washing with water.

Preparation of CdS Quantum Dot Nanocrystals. The quantum dot nanoparticles were prepared using a slightly modified procedure reported previously.[45] First, AOT (14.0 g) was dissolved in a mixture of *n*-hexane (200 mL) and water (4 mL). The resulting solution was separated into two subvolumes of 120 and 80 mL. A 0.48 mL aliquot of a 1 M $Cd(NO_3)_2$ solution was added to the 120 mL subvolume, whereas 0.32 mL of a 1 M Na_2S solution was added to the 80 mL subvolume. The two solutions were stirred for 1 h, then mixed and stirred for an additional hour under N_2. The quantum dots were capped by adding cystamine (0.34 mL, 0.32 M) and sodium 2-mercaptoethane sulfonate (0.66 mL, 0.32 M) and mixing under N_2 for 24 h. Evaporation of hexane in vacuo yielded the CdS quantum dot nanocrystals, which were washed with pyridine, hexane, and methanol.

Preparation of CdS Quantum Dot−Oligonucleotide Aptamer Conjugates. The CdS−oligonucleotide conjugate was prepared by using a modified protocol published earlier.[28,46] First, CdS quantum dot suspension (0.2 mg mL^{-1}, 500 μL) was exposed to 750 nM of the thiolated oligonucleotide secondary aptamer (aptamer 2). The mixture was stirred overnight at room temperature. The quantum dot−aptamer conjugate was collected by centrifugation at 10 000 rpm for 45 min, removal of supernatant, and resuspension in binding buffer (50 mM TRIS·HCl, 100 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$; pH 7.4)

Sandwich Aptamer−Protein Assay. The aptamer-modified gold substrates were incubated for 1 h with the desired amount of thrombin in binding buffer (100 μL) followed by washing with washing buffer (50 mM TRIS·HCl, 0.1% Tween 20; pH 7.4). Then, the gold substrates were incubated with quantum dot−oligonucleotide secondary aptamer for 1 h at room temperature. The supernatant was removed, the gold substrates were washed twice with washing buffer (each 100 μL), and transferred to new microwells, where they were washed four times again with the washing buffer (each 100 μL) and twice with water.

Dissolution and Detection. Hydrogen peroxide was used for the dissolution step since it was observed that it can efficiently oxidize the CdS quantum dots after carefully optimizing concentration and reaction time.[39] Preliminary experiments of dissolving CdS quantum dots with 0.01 M H_2O_2 and potentiometric detection of the released Cd^{2+} showed that it was fully oxidized after 15 min. In the final assay, dissolution of CdS was carried out by the addition of 0.01 M H_2O_2 in 10^{-4} M $CaCl_2$ (100 μL) for 1 h to ensure complete oxidation. The detection was performed in polystyrene microtiter plates (Corning Inc., NY). Prior to the measurements, each microwell was pretreated with 10% HNO_3 overnight and then washed at least five times with deionized water and left to dry.

Ion-Selective Electrode Membranes. The Cd ISE membrane was prepared by dissolving 60 mg of the following components in CH_2Cl_2 (0.8 mL): ETH 5435 (1.27 wt %, 15 mmol kg^{-1}), NaTFPB (0.46 wt %, 5 mmol kg^{-1}), ETH 500 (1.15 wt %, 10 mmol kg^{-1}), and copolymer MMA−DMA (97.12 wt %). The membrane solution was deaerated by purging it with N_2 before coating the microelectrodes. The membrane for the Ca ISE used as a reference electrode was prepared by dissolving 140 mg of the following components in THF (1 mL): ETH 5234 (0.87 wt %, 10.9 mmol kg^{-1}), NaTFPB (0.47 wt %, 5.12 mmol kg^{-1}), PVC (32.2 wt %), and *o*-NPOE (66.3 wt %). The solution was left to evaporate for 1 h, after which it was filled into a 100 μL pipet tip and left to dry for at least 24 h. Then, the membrane was conditioned in 10^{-3} M $CaCl_2$ for 1 day.

Microelectrodes. The solid-contact Cd^{2+}-selective microelectrode was prepared by using a 2 cm long Au wire (200 μm diameter) as solid substrate soldered to a Ag wire for electric contact. Before use, the Au wires were thoroughly cleaned with 10% sulfuric acid and rinsed with water, then acetone, and left in $CHCl_3$ for 5 min. The solution of POT (25 mM with respect to the monomer in $CHCl_3$) was applied along the length of Au wire at least three times or until the color of the wire became black. After the Au wires were fully covered with POT they were left to dry. The wires were then inserted into a polypropylene tip so that they were level with the end of the micropipette tip. Finally, the membrane cocktail was applied to the top of the wire covered

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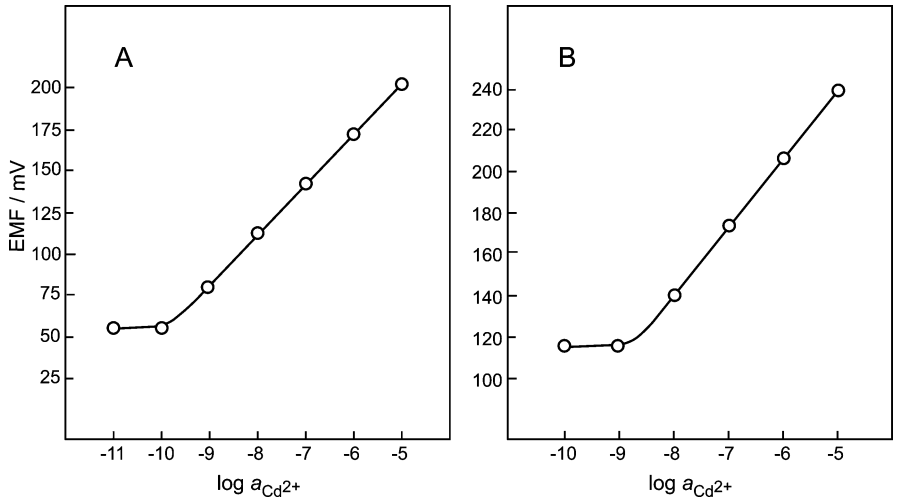

Figure 1. Calibration curves of a solid-contact Cd^{2+}-selective electrode in (A) 100 mL and (B) 200 μL samples with 10^{-4} M $CaCl_2$ as background using ELISA microplates.

with POT for three times at 15 min intervals and allowed to dry for 2−3 h until full evaporation of CH_2Cl_2. The microelectrodes were conditioned first in 10^{-3} M $CdCl_2$ and subsequently in 10^{-9} M $CdCl_2$ with 10^{-4} M $CaCl_2$ (1 day each). Measurements were performed in ELISA microwells containing 180 μL of 10^{-4} M $CaCl_2$ and adding 20 μL of sample, using a Ca ISE as reference and a small magnetic stirring bar. All stock solutions used for measurements were freshly prepared daily.

For selectivity measurements, the Cd ISE membranes were conditioned in 10^{-3} M $CaCl_2$ for 2 days in order to avoid primary ions leaching from the membrane.[47] For each ion, a calibration curve was recorded and the selectivity coefficients were calculated using the separate solutions method.[48] For measurements in 100 mL samples with a customary reference electrode, the electromotive force (emf) values were corrected for liquid-junction potentials according to the Henderson equation. Activity coefficients were calculated by the Debye−Hückel approximation.

Electromotive Force Measurements. Potentiometric measurements were performed in stirred solutions at room temperature (22 °C) with a PCI MIO16XE data acquisition board (National Instruments, Austin, TX) connected to a four-channel high Z interface (WPI, Sarasota, FL).

RESULTS AND DISCUSSION

Solid-contact ISEs with nanomolar detection limits can now be routinely prepared for different ions.[49] Such electrodes are easily miniaturized to operate in samples of very small volume.[35,36] In this work, a novel solid-contact Cd^{2+}-selective microelectrode has been developed, which is based on the copolymer matrix MMA−DMA and the ionophore ETH 5435.[50]

The novel Cd ISE was first characterized in large samples of 100 mL. As shown in Figure 1A, with a background of 10^{-4} M $CaCl_2$, it displays a very good lower detection limit of 100 pM. In microwell plates of 200 μL sample volume, the detection limit is less good by 1 order of magnitude (Figure 1B). Yet, it is still in the nanomolar range (1 nM) with the same background. Although we do not expect deterioration of the detection limit upon reducing the sample volume, the changes observed here are likely attributed to impurities due to the unfavorable surface-to-volume ratio of the small sample. The reproducibility of the solid-contact microelectrodes in 200 μL samples was also evaluated by recording three different calibration curves over the concentration range of 10^{-10} to 10^{-5} M. After each measurement, the electrode was washed for 5 min to eliminate possible memory effects. The standard deviation of the emf for each concentration was <1 mV. Another important performance parameter is the long-term stability of the ISE. During continuous experiments, it was observed that the electrodes are capable of measuring more than 45 samples with good response times and a standard deviation of <1.5 mV. After recalibration, they can be used for more analyses. After 1 month, the Cd ISEs showed a loss of detection limit by half an order of magnitude.

The membranes exhibit good selectivities for the relevant interfering ions, Ca^{2+}, Na^+, and H^+ with the corresponding logarithmic selectivity coefficients, log $K^{Pot}_{Cd,J}$, of −7.04, −3.88, and −4.59, respectively. Note that the selectivity over sodium ions is inferior relative to a recently reported liquid inner contact cadmium ISE based on the same ionophore.[39] This might be explained by intermixing of POT and the membrane during casting, resulting in some selectivity deterioration. Owing to its high level of discrimination, Ca^{2+} was selected as the background electrolyte together with a Ca^{2+}-selective electrode as the reference electrode. For this purpose, the selectivity of the Ca ISE for Cd^{2+}, log $K^{Pot}_{Ca,Cd} = -3.15$, is sufficiently high.

For aptamer-based protein detection, the CdS quantum dot labels were oxidized with H_2O_2 since HNO_3, the standard oxidizing agent for ASV,[28] would deteriorate the lower detection limit due to proton interference. The influence of H_2O_2 on the response of the solid-contact microelectrodes was examined by taking calibration curves in the range of 10^{-3} to 10^{-1} M Cd^{2+} at different background concentrations of H_2O_2 (10^{-3} to 10^{-1} M). Although an increase in its concentration accelerates the dissolution,[39] no influence on the potentiometric response was observed at $\leq 10^{-2}$ M H_2O_2. A concentration of 10^{-2} M H_2O_2 was selected for further

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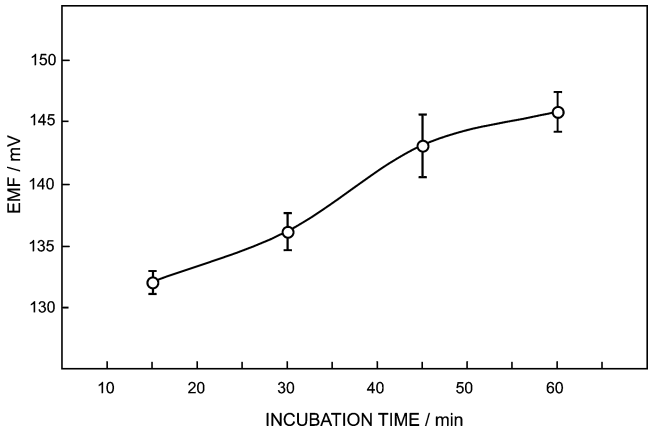

Figure 2. Response of different incubation times between immobilized 1000 nM primary aptamer and 100 ppb of thrombin in 15, 30, 45, and 60 min (error bars: SD, $N = 3$). Potentiometric measurements were performed in 200 μL samples with 10^{-4} M $CaCl_2$ as background electrolyte and a Ca ISE as reference electrode.

experiments since some signal drifts were observed with 10^{-1} M H_2O_2, which was probably due to the interaction of H_2O_2 with the conducting polymer.

Next, the possible interference by the other components of the assay was investigated. When performing the measurement according to Scheme 1 but without labeling the secondary aptamer, as expected, no change of the emf was observed upon addition of H_2O_2 after binding the secondary aptamer. On the other hand, when using the CdS quantum dot-labeled secondary aptamer, the control samples, i.e., zero target, lysozyme, and IgG, initially showed emf changes that were similar to those obtained with the target (data not shown). Hence, to reduce such nonspecific adsorption effects, the washing steps were improved including a transfer into new microwells (see the Experimental Section).

The effect of the incubation time of the aptamer−thrombin binding was examined over the range of 15−60 min (Figure 2) using a 100 ppb thrombin solution, which gives about half the maximum signal in this assay (see below). For this case, the emf increased with increasing binding time, but at times longer than 45 min, the increase was no longer significant. On the basis of these results, an incubation time of 60 min was chosen for all further experiments.

The concentration of the CdS-labeled secondary aptamer was varied between 250 and 1000 nM using 1000 nM primary aptamer and 100 ppb thrombin (Figure 3). The signal increased with increasing concentration, but this trend declined at >500 nM. Since the nonspecific absorption slightly increased at 1000 nM concentration, 750 nM was chosen for subsequent experiments.

The selectivity of the thrombin aptamer was tested with the assay parameters as selected above. As shown in Figure 4, 500 ppb of lysozyme or IgG showed emf responses that were not significantly higher than for the control, corresponding to about 0.25 pbb of cadmium ions. This residual cadmium ion level may partly originate from impurities in the water and reagents that were used. In contrast, the response to a 10 times smaller concentration of thrombin was found to be about 6 times higher in cadmium ion concentration (1.6 ppb, note the logarithmic response characteristics of the ISE).

The results of a typical series of measurements with thrombin concentrations from 5 to 1000 ppb are shown in Figure 5. The

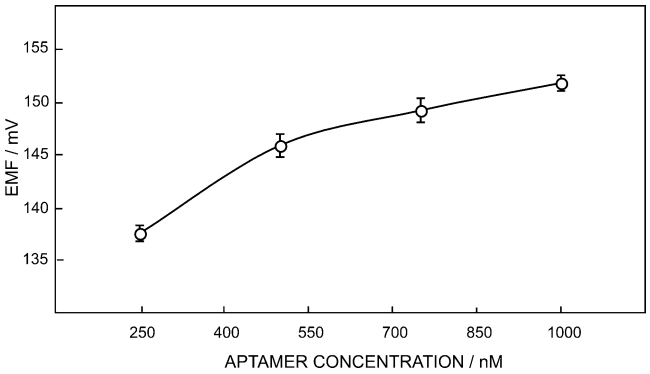

Figure 3. Response to 250, 500, 750, and 1000 nM secondary aptamer with 100 ppb of target thrombin previously bound to 1000 nM primary aptamer (error bars: SD, $N = 3$). Other conditions are as in Figure 2.

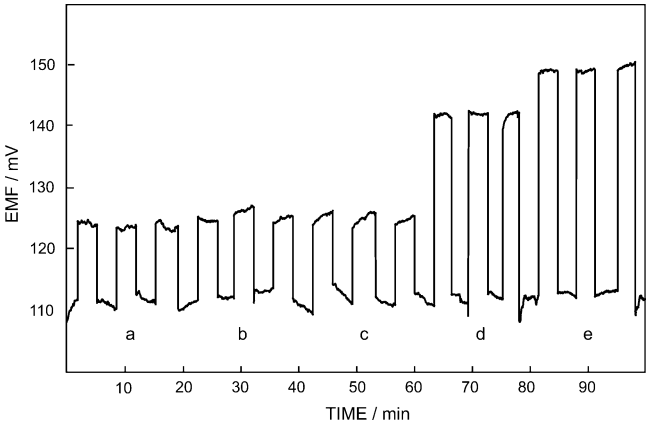

Figure 4. Potentiometric responses of the Cd^{2+}-selective electrode for (a) the control (zero target), (b) lysozyme, 500 ppb, and (c) 500 ppb IgG (as noncomplementary targets), (d) thrombin, 50 ppb, and (e) thrombin, 100 ppb (as complementary target) after aptamer−thrombin interaction. Other conditions are as in Figure 2.

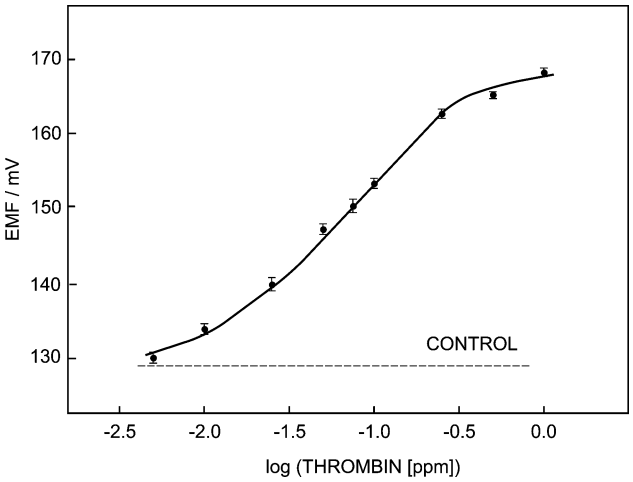

Figure 5. Potentiometric monitoring of thrombin concentration via CdS quantum dot label in 200 μL microwells with the aptamer−thrombin sandwich assay (error bars: SD, $N = 3$). Other conditions are as in Figure 2.

emf response of the Cd ISE versus log[thrombin] is close to linear and offers a sufficient concentration dependence suitable for thrombin measurements over a dynamic range of 10−250 ppb and with a lower detection limit of ca. 5 ppb. This corresponds to 28 fmol of thrombin in 200 μL samples or 0.14 nM and compares

well to results obtained with other reported sensors such as piezoelectric transducers (ca. 10 nM[17] and 1 nM[51]) or electrochemical sensors (of the order of 10 nM).[21,51,52] Significantly lower detection limits were obtained with impedance spectroscopy and amplification by chemical means to denature the protein captured by an aptamer on the electrode surface (10 fM)[25] and, very recently, by electrogenerated chemiluminescence via target protein-induced strand displacement (1 fM).[29]

CONCLUSIONS

For the first time, we demonstrate that ion-selective microelectrodes can be used for monitoring protein−aptamer interactions with semiconductor nanocrystal labels in an ELISA microplate format. It is important to emphasize that a low detection limit of 5 ppb or 28 fmol of thrombin was reached without a preconcentration step typically used in other electrochemical techniques. This was possible in conjunction with a reduction of the sample volume and the excellent lower detection limit of the Cd ISE used. It is expected that various biomolecular interactions can be monitored with similar assays based on different nanoparticle tracers and corresponding ISEs.

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