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Parallel Analysis of Two Analytes in Solutions or on Surfaces by Using a Bifunctional Aptamer: Applications for Biosensing and Logic Gate Operations

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A bifunctional aptamer that includes two aptamer units for cocaine and adenosine 5′-monophosphate (AMP) is blocked by a nucleic acid to form a hybrid structure with two duplex regions. The blocked bifunctional aptamer assembly is used as a functional structure for the simultaneous sensing of cocaine or AMP. The blocked bifunctional aptamer is dissociated by either of the two analytes, and the readout of the separation of the sensing structure is accomplished by a colorimetric detection, by a released DNAzyme, or by electronic means that use Faradaic impedance spectroscopy or field-effect transistors. In one configuration, the blocked bifunctional aptamer structure is separated by the substrates cocaine or AMP, and the displaced blocker units act as a horseradish peroxidase-mimicking DNAzyme that permits the colorimetric detection of the analytes. In the second system, the blocked bifunctional aptamer hybrid is associated with a Au electrode. The displacement of the aptamer by any of the substrates alters the interfacial electron transfer resistance at the electrode surface, thus providing an electronic signal for the sensing process. In the third configuration, the blocked aptamer hybrid is linked to the gate of a field-effect transistor device. The separation of the complex by means of any of the analytes, cocaine, or AMP alters the gate potential, and this allows the electronic transduction of the sensing process by following the changes in the gate-to-source potentials. The different systems enable not only the simultaneous detection of the two analytes, but they provide a functional assembly that performs a logic gate "OR" operation.

Introduction

Aptamers are nucleic acids that specifically bind to low-molecular-weight substrates or to proteins. The aptamers are generated by the SELEX (systematic evolution of ligand by exponential enrichment) procedure, and are amplified to viable quantities by the polymerase chain reaction (PCR).[1] Substantial recent research efforts use aptamers as sensing units, which are called aptasensors.[2] Different electrochemical,[3] optical[4] or microgravimetric[5] aptasensors were designed in the recent years. For example, aptamers that were functionalized with redox labels were used to detect low-molecular-weight substrates or proteins, for instance, cocaine or thrombin, respectively.[3b,d] Similarly, nanoparticle-labeled proteins were employed as tracers to analyze the proteins by a competitive assay, through the dissolution of the nanoparticles and their electrochemical stripping.[6] Optical aptasensors were developed by controlling the fluorescence properties of the analyte-induced folded configuration of aptamers.[7] Also, the analyte-stimulated deaggregation of aptamer-modified Au nanoparticles was used for the colorimetric detection of low-molecular-weight substrates, for instance, cocaine or AMP.[8] The amplified microgravimetric detection of thrombin by its aptamer was accomplished by using aptamer-functionalized gold nanoparticles (Au NP) and their catalytic enlargement as "weight labels" that probe the formation of the aptamer–thrombin complex.[9] Two challenges exist, however, in the development of aptasensors: one challenge involves the label-less detection of the aptamer–substrate complexes. This has been recently demonstrated by the use of impedimetric methods and the use of field-effect transistors as a transduction means that follow the formation of aptamer–substrate complexes.[10] The second challenge involves the amplification of the substrate–aptamer detection event. Recently, a dynamic amplification procedure for the analysis of cocaine by its aptamer was reported.[11] The method is based on the formation of the cocaine–aptamer complex, and the subsequent cyclic replication–nicking process that accumulates a DNA product that activates a fluorescence signal.

DNAzymes are catalytic nucleic acids that mimic the functions of enzymes.[12] The possibility to hybridize nucleic acids, and simultaneously to use them as catalysts provides unique recognition and catalytic functions for amplified biosensing.[13]

The hemin–G-quadruplex assembly was reported to act as a DNAzyme that mimics the functions of horseradish peroxidase.[14] This DNAzyme was used as a label for the amplified detection of DNA.[15] Recently, "DNA-machines" for the amplified detection of DNA by the cyclic, autonomous synthesis of DNAzyme units were reported.[16] Similarly, a lead-ion-dependent DNAzyme was used for the optical detection of Pb^{2+} by

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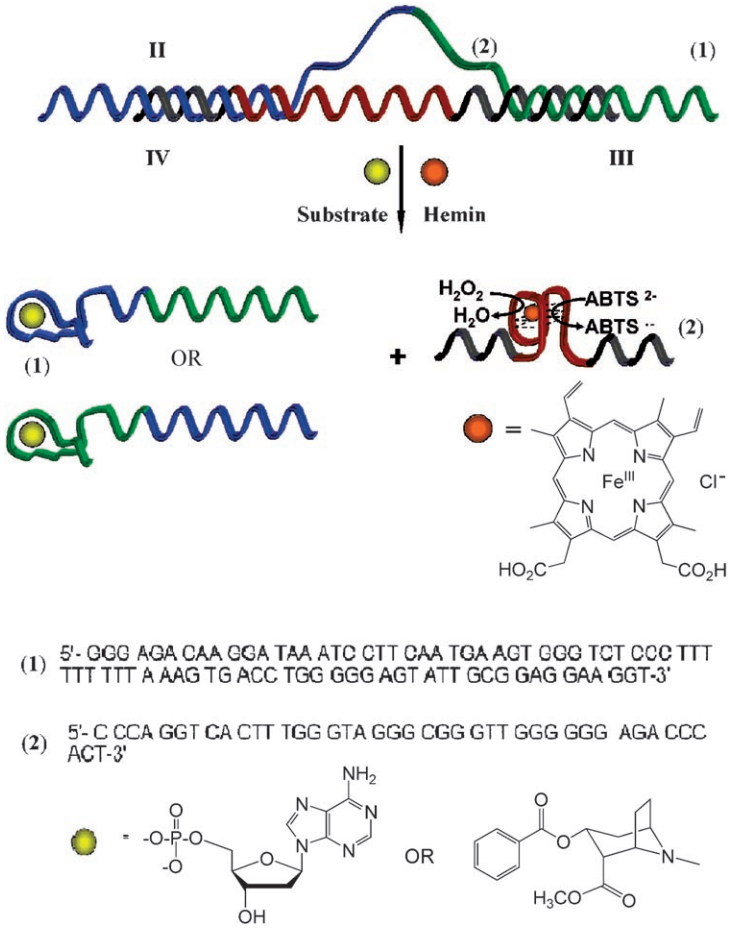
the DNAzyme-mediated cleavage of a Au nanoparticles aggregate.[17] Also, the release of a DNAzyme-tethered aptamer upon forming the aptamer–substrate (AMP) complex was used for the amplified colorimetric detection of the substrate.[18]

In the present study we report on the use of a blocked bifunctional nucleic acid that consists of the cocaine and AMP aptamers; this complex acts as an active assembly for the separate analysis of cocaine or AMP. The formation of the substrate–aptamer complexes releases the blocking nucleic acid, which subsequently acts either as a DNAzyme, or it enables the electronic transduction of the sensing processes by means of Faradaic impedance spectroscopy or by field-effect transistors. We also address the possibility of using the different optical or electronic transduction means as systems that perform logic gate operations. It should be noted that the present study, which demonstrates the utility of the bis-aptamer system to sense in parallel cocaine or AMP has no immediate practical application. Nonetheless, the system is aimed to introduce the concept of multiplexed analysis of other substrates by composite aptamers, and to highlight the different colorimetric and electrical methods that can be used to follow the sensing processes. Also, the demonstration of the cooperative function of the bis-aptamer system for logic gate operations adds a new dimension to the bis-aptamer system.



Scheme 1. The amplified analysis of cocaine or AMP by the substrate-induced separation of a blocked bifunctional aptamer–nucleic acid complex, and the formation of a horseradish-peroxidase-mimicking DNAzyme

Results and Discussion

Scheme 1 outlines the principle to use a blocked bifunctional aptamer unit for the amplified detection of cocaine or AMP by the formation of the horseradish-peroxidase-mimicking DNAzyme.

The nucleic acid **1** includes two units, I and II that consist of the aptamer units for cocaine and AMP, respectively. The nucleic acid **2** is designed to include the DNAzyme sequence and appropriate regions III and IV that hybridize with the complementary regions of **1**. The blocking unit **2** is designed by following two basic considerations: 1) the domains III and IV each include a hybridization sequence of 11 and 9 bases, respectively. These three domains act cooperatively in the stabilization of the two regions of the duplexes, and the resulting duplex structure reveals higher stability than the self-assembly of the DNAzyme to the G-quadruplex structure. 2) Each of the duplexes in regions I/III and II/IV is opened by the respective substrate, and the resulting substrate–aptamer complex exhibits a higher stability than the respective duplex. 3) The separation of each of the duplexes I/III or II/IV by cocaine or AMP results in an unstable single duplex that is spontaneously dissociated at room temperature. This results in the complete dissociation of **2,** and its self-assembly in the presence of hemin to the active DNAzyme structure.

Thus, upon the detection of either cocaine or AMP, the hybrid structure consisting of **1** and **2** is separated, and the resulting DNAzyme transduces the sensing events through the catalyzed H_2O_2-mediated oxidation of 2 mM 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid, $ABTS^{2-}$, to the colored product, $ABTS^{\bullet-}$. Because the separation of each of the aptamers is controlled by the concentration of the respective substrate, the activity of the released DNAzyme provides a quantitative measure for the concentration of the substrates. Figure 1 A shows the time-dependent evolution of the absorbance of $ABTS^{\bullet-}$ at $\lambda = 415$ nm, upon analyzing different concentrations of cocaine. Similarly, Figure 1 B depicts the time-dependent formation of the colored product, $ABTS^{\bullet-}$, upon analyzing different concentrations of AMP. Control experiments indicate that in the absence of cocaine or AMP, only a slow evolution of the colored product is generated (Figure 1 A and B, curves a). Similarly, hemin by itself catalyzes the H_2O_2-mediated oxidation of $ABTS^{2-}$ to a small extent, Figure 1 A and B, curves f. In a further control experiment, the **1**/**2** duplex was treated with the foreign nucleotide, for example, cytidine 5′-monophosphate (CMP), at a high concentration (1×10^{-3} M), and the response of the system was similar to that of **1**/**2** in the absence of any added analytes, Figure 1 B, curve e. Thus, we conclude that only cocaine or AMP dehybridize the supramolecular structure **1**/**2** to result in the formation of the DNA-

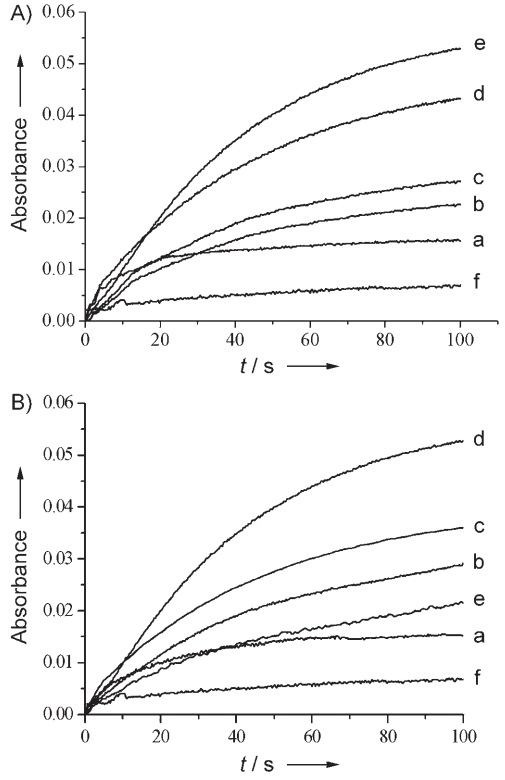


Figure 1. A) Absorbance changes that were observed upon the treatment of the bifunctional aptamer–nucleic acid complex (**1/2**) with different concentrations of cocaine: a) 0 M, b) 5×10^{-6} M, c) 1×10^{-5} M, d) 1×10^{-4} M, e) 1×10^{-3} M. Curve f corresponds to the absorbance changes that resulted in the buffer solution in the presence of hemin with no added (**1/2**). B) Absorbance changes that were observed upon the treatment of the bifunctional aptamer–nucleic acid complex, (**1/2**), with different concentrations of AMP: a) 0 M, b) 1×10^{-5} M, c) 1×10^{-4} M, d) 1×10^{-3} M. Curve e shows the absorbance changes upon treatment of the (**1/2**) complex with cytidine 5′-monophosphate (1×10^{-3} M). Curve f shows the absorbance changes in the presence of hemin only in the buffer solution, in the absence of the (**1/2**) complex. All experiments were performed in a 25 mM Tris buffer solution that included 100 mM NaCl, (pH 8.2), by using 2 mM $ABTS^{2-}$, 5×10^{-7} M hemin , 2 mM H_2O_2, and where appropriate, the nucleotides **1** (3×10^{-7} M) and **2** (3×10^{-7} M).

zyme that catalyzes the oxidation of $ABTS^{2-}$ by H_2O_2. The sensitivity limits for analyzing cocaine or AMP by the system correspond to 5×10^{-6} and 1×10^{-5} M, respectively. The detection limits for analyzing AMP and cocaine are comparable with the other optical aptasensor.[8,19]

Because the concentration of the blocking DNAzyme unit, **2**, in the system is known, we performed an activity assay in which the time-dependent oxidation of $ABTS^{2-}$ was monitored at different concentrations of the DNAzymes, and an appropriate calibration curve was derived (see the Supporting Information).

By using this calibration curve, we conclude that at cocaine and AMP concentrations that correspond to 1×10^{-3} M, approximately 100% of the duplex structure **1/2** was separated. The dissociation of the complex that was generated between the bis-aptamer **1** and the blocker **2** by means of the two substrates, cocaine or AMP, can be utilized not only for the sensing of the two substrates, but also to demonstrate the utility of the system to perform a logic gate operation. In fact, recently, several groups have used aptamers and their substrates as components that activate logic gate operations.[20]

Accordingly, the cocaine and AMP can be considered to be the inputs that activate an OR gate, and the dissociation of the blocking DNAzyme activates the colorimetric readout signal. Figure 2a shows that in the absence of the inputs (0,0 state)

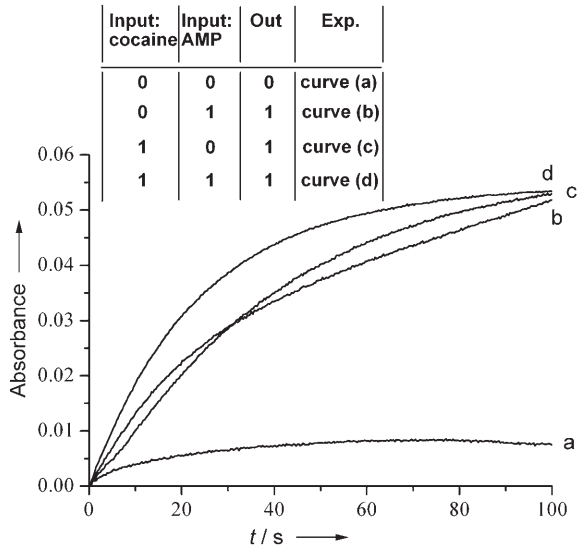


Figure 2. The optical readout of the "OR" logic gate operation of the bifunctional aptamer–nucleic acid complex by using cocaine or AMP (1×10^{-3} M each) as inputs: (a) The optical changes in the absence of any input. (b) and (c) The optical changes upon addition of cocaine or AMP as inputs, respectively. (d) Absorbance changes that were observed upon adding the two inputs to the (**1/2**) complex. The experimental conditions are detailed in the legend of Figure 1. Inset: Truth-table of the "OR" logic gate system.

the DNAzyme is not dissociated, and thus no color of $ABTS^{\bullet-}$ is generated (output 0). In the presence of cocaine or AMP, input states (1,0) or (0,1), the DNAzyme is separated, and this leads to the formation of the $ABTS^{\bullet-}$ colored product, Figure 2, curves b and c, respectively (output 1). In the presence of the two inputs, state (1,1), the dissociated DNAzyme activates the oxidation of $ABTS^{2-}$, and the resulting colored product acts as a readout signal (output 1). The truth-table of the resulting OR gate is given in Figure 2, inset.

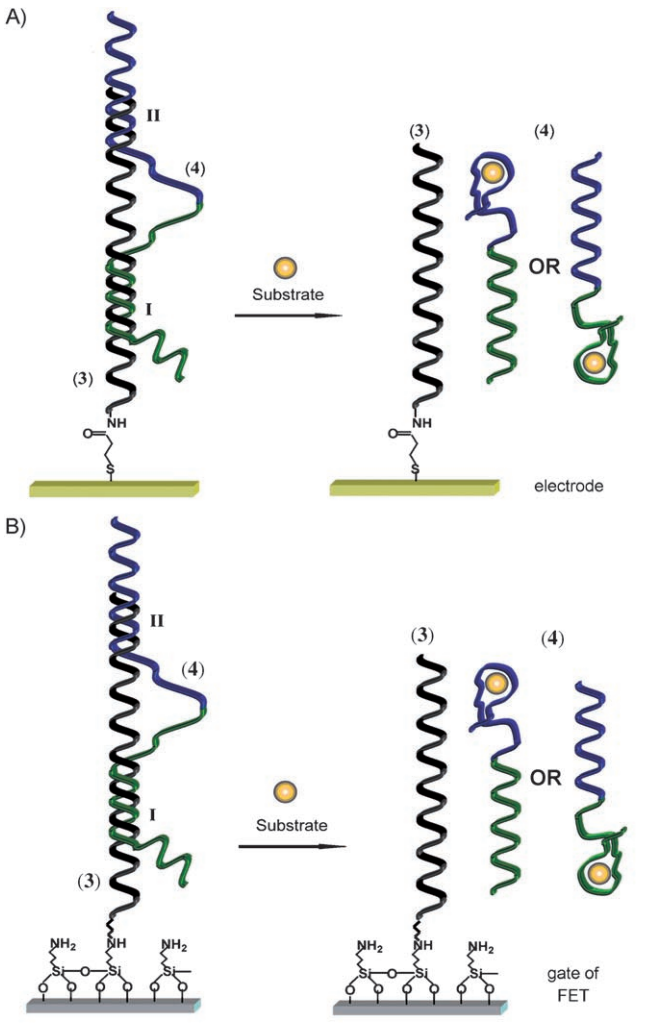
Impedance spectroscopy is a powerful method to probe chemically modified surfaces, and particularly, to characterize biomolecule-functionalized electrodes, and biorecognition events that occur on surfaces.[21] The formation of antigen–antibody complexes[22] or nucleic acid hybrids[23] was followed by Faradaic impedance spectroscopy by using a solution-solubilized redox label, for example, $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, as electrochemical indicator for the biorecognition events. The insulating effect that was introduced upon the formation of the antigen–antibody complex on the surface, or the electrostatic repulsion of the negatively charged redox indicator by the DNA hybrid, resulted in an increase in the interfacial electron transfer resistances between the electrode surface and the electrochemical probe in solution upon formation of the biomolecule recogni-

tion complex on the electrode. Recently, an impedimetric aptasensor for the detection of AMP was reported.[10] A nucleic-acid-blocked AMP-specific aptamer was immobilized on a Au electrode, and the separation of the blocked duplex by the formation of the AMP–aptamer complex was detected by Faradaic impedance spectroscopy. Scheme 2A depicts the method to apply the bifunctional cocaine–AMP aptamer to separately analyze cocaine or AMP. The amine-functionalized nucleic acid, **3** was covalently linked to the active ester monolayer-functionalized electrode. The nucleic acid **3** acts as a blocker unit and includes two complementary regions for the bifunctional aptamer **4**. The bifuncional aptamer **4** was then hybridized with the blocker nucleic acid **3** and immobilized on a Au electrode. The surface coverage of the resulting duplex on the electrode surface was determined by Tarlov's method,[24] and it corresponded to 1×10^{-12} mol cm^{-2}. The modified electrode was then treated with cocaine or AMP to stimulate the separation of the blocked aptamer unit. It is anticipated that either of the analytes can release the respective aptamer–substrate complexes, and consequently reduce the negative charge that is associated with the electrode. As a result, the dissociation of the blocked-aptamer structure that is associated with the electrode would decrease the interfacial electron transfer resistance in the presence of the negatively charged redox probe, $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, as compared to the primary duplex structure **3**/**4**, which is associated with the electrode. Figure 3A depicts the Faradaic impedance spectra (in the form of Nyquist plots) upon the treatment of the **3**/**4**-modified electrode with different concentrations of cocaine. As the concentration of cocaine increases, the interfacial electron transfer decreases because the negative charge that is associated with the electrode is diminished.

Similarly, Figure 3B shows the Faradaic impedance spectra upon analyzing different concentrations of AMP by the functionalized electrode. As the concentration of AMP increases, the interfacial electron transfer resistances decrease.

For example, the electrode that was employed for the analysis of cocaine revealed an interfacial electron transfer resistance of 450 Ω prior to the reaction with cocaine. The resistance value decreased to $R_{et}=220$ Ω after treatment with a 1×10^{-5} M cocaine solution ($\Delta R_{et}=230$ Ω). Furthermore, it can be seen that the change in the electron transfer resistance of the electrode upon analyzing 1×10^{-5} M cocaine is substantially higher than the resistance changes that are observed upon analyzing AMP at a higher concentration (1×10^{-4} M, $\Delta R_{et}=60$ Ω).

These results are consistent with the fact that the dissociation constant of cocaine to its aptamer ($K_d<5$ μM)[19] is substantially lower than the association of AMP to its aptamer.[25] Thus, a higher concentration of the AMP is needed to separate the blocked aptamer, and the extent of separated aptamer is lower, which gives rise to the lower ΔR_{et} values. The sensitivity limits for the detection of cocaine and AMP by Faradaic impedance spectroscopy correspond to 5×10^{-6} M and 1×10^{-5} M, respectively, a value that is comparable to other configurations of AMP or cocaine aptasensors.[10,3d] The advantage of the impedimetric method is the fact that the system does not reveal any background interference.



Scheme 2. The analysis of cocaine or AMP by the substrate-induced separation of a blocked bifunctional aptamer–nucleic acid complex: A) Analysis of the substrates by using a monolayer-functionalized electrode and Faradaic impedance spectroscopy as a transduction means. B) The label-less detection of the substrates by the immobilization of the bifunctional aptamer–nucleic acid complex on the gate of a field-effect transistor device.

The cocaine- or AMP-stimulated separation of the complex that is formed between the bifunctional aptamer **4** and the "blocker" nucleic acid **3** on the electrode surface, and the transduction of the process by the Faradaic impedance spectra can be used to develop an "OR" logic gate with electronic readout, Figure 4. While the blocked aptamer complex in the absence of the cocaine/AMP inputs, state (0,0), reveals a high interfacial electron transfer resistance, output "0" (Figure 4,

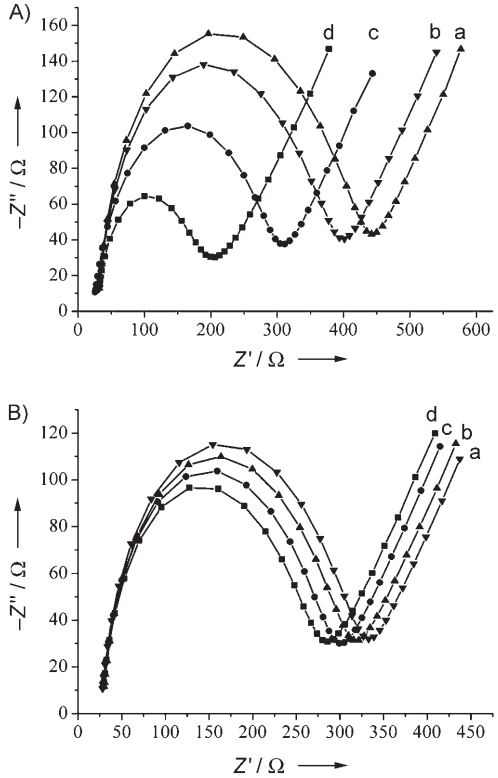


Figure 3. Faradaic impedance spectra (Nyquist plots) that correspond to the treatment of the bifunctional aptamer–nucleic acid complex **3**/**4** with: A) Different concentrations of cocaine: a) 0 M, b) 5×10^{-6} M, c) 5×10^{-5} M, d) 1×10^{-5} M. B) Different concentrations of AMP: a) 0 M, b) 5×10^{-5} M, c) 1×10^{-5} M, d) 1×10^{-4} M. All experiments were performed in a 25 mM Tris buffer solution that included 100 mM NaCl (pH 8.2). The solution also included 10 mM $Fe(CN)_6^{3-/4-}$. Measurements were performed in the frequency range of 100 MHz to 10 kHz, at $E=0.17$ V vs. SCE and by using the 5 mV alternating voltage.

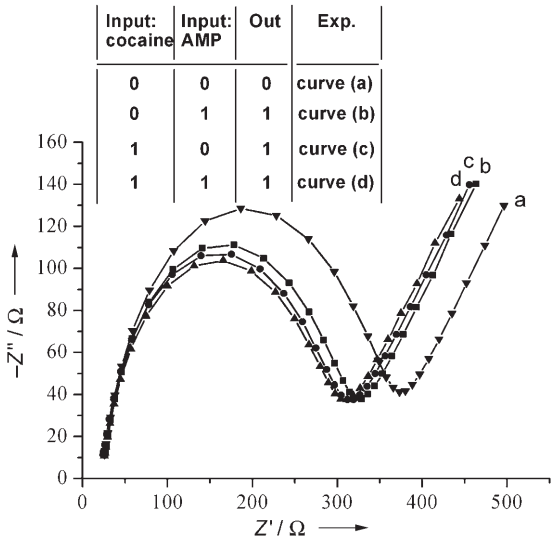


Figure 4. The Faradaic impedance readout of the "OR" logic gate operation of the bifunctional aptamer–nucleic acid complex by using cocaine and AMP (6×10^{-4} M) as inputs: a) Faradaic impedance spectrum of the **3**/**4** complex in the absence of inputs. b) and c) Impedance spectra in the presence of cocaine or AMP, respectively. d) Impedance spectrum in the presence of the two inputs. Experimental conditions as detailed in the legend of Figure 3.

curve a), the cocaine or AMP-induced separation of the aptamer states (1,0) or (0,1), respectively, leads to low electron transfer resistances, respectively (see Figure 4, curves b and c, respectively). Similarly, the addition of both inputs, state (1,1) yields a low interfacial electron transfer resistance (output "1", Figure 4, curve d). The respective truth-table of the resulting "OR" gate is given in Figure 4, inset.

Field-effect transistors are receiving growing interest as electronic devices that transduce biorecognition events.[26] The gate potential of the field-effect transistor can be changed by the charge that is associated with the gate. Recently, the separation of a blocked aptamer complex on the transistor gate by the aptamer substrate was used for the label-less detection of aptamer–substrate complexes.[10] Scheme 2B outlines the method to assemble the field-effect transistor for monitoring the dual analysis of cocaine and AMP. The oxide gate surface of the transistor device was modified with an aminopropylsiloxane layer, and the amine-functionalized nucleic acid **3** was covalently linked to the surface by the glutaric dialdehyde crosslinker. The modified gate was then hybridized with the nucleic acid **4**. The association of cocaine or AMP with the respective aptamer domains is then expected to release **4**, and the change in the charge on the gate gives rise to the response of the transistor device. Figure 5A and B shows the time-dependent changes in the gate-to-source potential, ΔV_{gs}, upon interaction of the device with 1×10^{-3} M cocaine and AMP, respectively. The gate potential changes for 7 min, and then levels off to the saturation value.

Accordingly, a time-interval of 15 min was used to analyze the two substrates, cocaine or AMP. Figure 6A shows the changes in the gate-to-source potential upon analyzing different concentrations of AMP. The ΔV_{gs} value increases as the concentration of AMP is elevated, and it reaches a saturation value at an AMP concentration of 1×10^{-4} M. At this concentration, most of the blocked aptamer units that are associated with the gate are separated to the aptamer–AMP complex. Similarly, Figure 6B depicts the change in the gate-to-source potential upon treatment of the **3**/**4**-functionalized device with cocaine. Similarly, the ΔV_{gs} values increase as the concentration of cocaine is elevated; the transistor response levels off to a saturation value at a cocaine concentration of 1×10^{-4} M. Control experiments revealed, as before, that the duplex **3**/**4** was not separated by the foreign nucleotide (CMP), because only minute changes in the device response ($\Delta V_{gs}=5$ mV) were detected with CMP.

The field-effect transistor could also be used to transduce the "OR" logic gate functions of the system. Figure 7 shows the readout signals of the device (in the form of a bar presentation) upon its activation by the two substrates as inputs. The interaction of the ISFET device that is modified with the complex **3**/**4** with only cocaine (state 0,1) or AMP (state 1,0) leads to a signal response, and the signal is also generated by the two substrates (state 1,1) in accordance with an "OR" gate configuration.

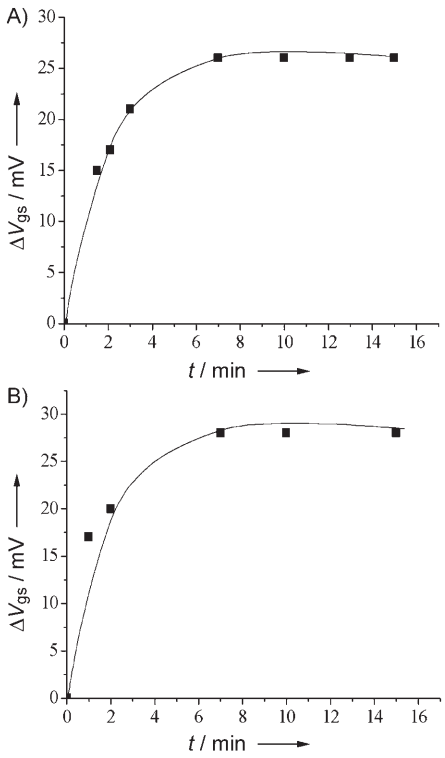


Figure 5. Time-dependent gate-to-source potential changes upon treatment of the **3**/**4**-functionalized field-effect transistor with: A) cocaine (1×10^{-3} M), B) AMP (1×10^{-3} M). Measurements were performed in a 25 mM Tris buffer solution that included 100 mM NaCl (pH 8.2). In all experiments $V_{SD}=1$ V and $I_{SD}=100$ μA.

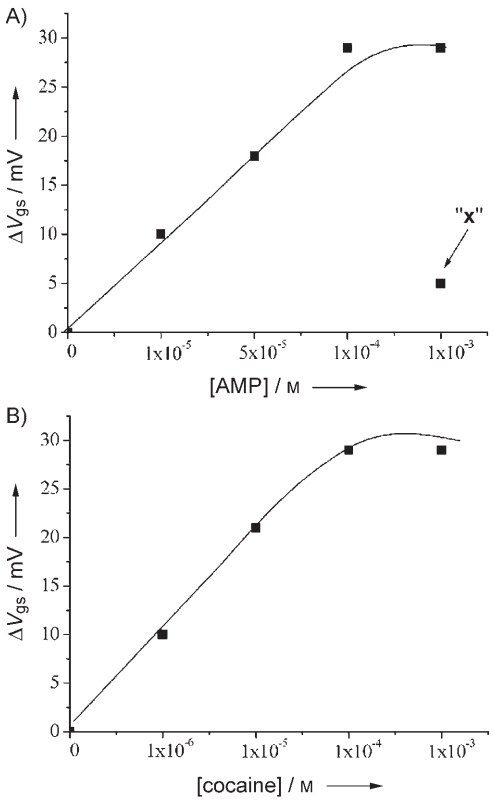


Figure 6. Gate-to-source potential changes upon the treatment of the **3**/**4**-functionalized field-effect transistor with different concentrations of: A) AMP B) cocaine. Experimental conditions were the same as detailed in the legend of Figure 5. Point "**X**" corresponds to the response of the **3**/**4**-functionalized field-effect transistor to cytidine 5′-monophosphate (1×10^{-3} M).

Conclusions

This study has introduced a bifunctional aptamer structure for the parallel analysis of two different molecular analytes, cocaine or AMP.

The different configurations for analyzing the aptamer–substrate complexes were presented, and these included the formation of a DNAzyme as a catalytic label, and the use of impedance spectroscopy and field-effect transistors. Besides the analytical significance of the study, which demonstrates the design of new aptasensors, the results suggest future possibilities to tailor bioelectronic logic gate systems. The availability of numerous aptamers and DNAzymes opens the way to construct nucleic acids that will act as other logic gates or as signal transduction cascades.

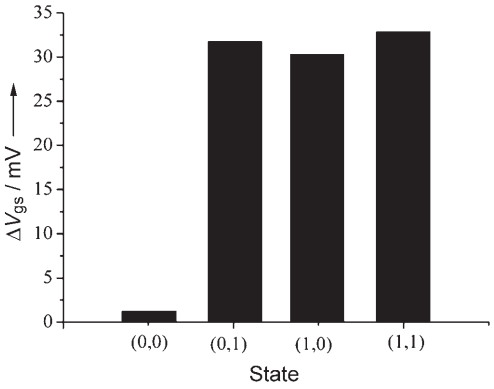


Figure 7. The use of the **3**/**4**-modified ISFET device as electronic readout system of the blocked aptamer hybrid as an "OR" logic gate. The readout signal is displayed in the form of a bar presentation of the changes in the gate-to-source potential upon treatment of the hybrid with cocaine (1×10^{-3} M) and/or AMP (1×10^{-3} M) as inputs. All other experimental conditions are given in the legend of Figure 5.

Experimental Section

Chemicals: Hemin was purchased from Porphyrin Products (Logan, Utah, USA) and it was used without further purification. A hemin stock solution was prepared in DMSO and stored in the dark at −20 °C. 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonate ($ABTS^{2-}$), 3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide ester), glutaric dialdehyde solution, (3-aminopropyl) triethoxysilane, tris(hydroxymethyl) aminomethane hydrochloride (Tris), 4-(2-hydroxyethyl)piperazine-1 ethanesulfonic acid sodium salt (HEPES), and adenosine 5′-monophosphate (AMP) were purchased from Sigma–Aldrich and were used as supplied. Oligonucleotides were custom-ordered from Sigma Genosys (Rehovot, Israel). Ultrapure water from NANOpure Diamond (Barnstead, Boston, USA) source was used in all of the experiments.

Colorimetric analysis: Absorbance measurements were performed by using a Shimadzu UV-2401PC UV/Vis spectrophotometer. Vari-

ous concentrations of AMP or cocaine were added to the system that included the hybridized oligonucleotides **1** and **2** (3×10^{-7} M) in a buffer solution that contained Tris (25 mM, pH 8.2), NaCl (0.1 M), and KCl (5 mM). After 1 h of the reaction hemin (7×10^{-7} M), $ABTS^{2-}$ (2 mM), and H_2O_2 (2 mM) were added to the reaction mixture (to yield a final volume of 200 μL). The rate of the biocatalyzed oxidation of $ABTS^{2-}$ was monitored at $\lambda = 415$ nm.

Chemical modification of the electrodes: Gold wire electrodes (0.5 mm diameter, ≈ 0.2 cm^2 geometrical area, roughness factor, $\approx$1.2–1.5) were used for the electrochemical measurements. The electrodes were cleaned by boiling them in a supersaturated hot KOH solution for 5 h, followed by treatment for 15 min with concentrated H_2SO_4. The electrodes were then rinsed with water, then soaked in concentrated HNO_3 for 10 min and rinsed again with water. The Au surface was treated with a 1 mM solution of 3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide ester) in DMSO for 1 h. The resulting electrodes were washed with DMSO and interacted with a solution of **3** (5×10^{-7} M) in HEPES buffer (0.1 M) at pH 7.4 for 1 h. The oligonucleotide-functionalized Au surface was then treated with a 5×10^{-7} M solution of **4** in a buffer solution that was composed of Tris (25 mM, pH 8.2) and NaCl (0.3 M) for 2 h, to yield the double-stranded DNA assembly on the surface. The resulting electrodes were then treated with different concentrations of AMP or cocaine in a buffer solution that contained Tris (25 mM, pH 8.2) and NaCl (0.1 M).

Electrochemical measurements: Faradaic impedance spectroscopy experiments were performed by using an electrochemical analyzer (PGSTAT12 Differential Electrometer, Autolab). The measurements were carried out in a conventional three-electrode electrochemical cell that consisted of the modified Au electrode as a working electrode, a glassy carbon auxiliary electrode, and a saturated calomel electrode (SCE) that was connected to the working volume with a Luggin capillary. The Faradaic impedance measurements were performed in the presence of a $K_3[Fe(CN)_6]/K_4[Fe(CN)_6$ (1:1, 10 mM) mixture, in a buffer solution that contained Tris (25 mM, pH 8.2) and NaCl (0.1 M). The Faradaic impedance spectra were recorded by applying a potential equal to the redox probe formal potential, 0.17 V. Electron transfer resistances were calculated by extrapolating the Nyquist plots and by assuming that the electron transfer resistances corresponded to the diameter of the semicircle lying on the Z′ axis. Also, the Nyquist plots that are shown in Figure 3 were fitted with an equivalent circuit, which is shown in Figure 8. The results of the fitting were very similar to the values that were extracted by the extrapolation of the semicircle regions. For example, for Figure 3 curves a and d, the fitting yields values of $R_{et} =$ 360 Ω and 160 Ω and a ΔR_{et} value of 200 Ω.

Preparation of the ISFET devices. The Al_2O_3 ISFET devices (20×700 μm^2, IMT, Neuchâtel, Switzerland) were preliminary functionalized with (3-aminopropyl)triethoxysilane, 10% (*v/v*) in toluene by dropping the silane solution (0.2 μL) on the top of the gate. The chips were rinsed with toluene and a Tris buffer solution (25 mM, pH 8.2), and then dried in air at room temperature for 20 min. Subsequently, the functionalized gate was modified with 10% (*v/v*) glutaric dialdehyde in 10 mM phosphate buffer (pH 7.4). Then, the functionalized chip was interacted with a 5×10^{-7} M solution of the amine-functionalized nucleic acid **3**, in a buffer solution that included 25 mM Tris (pH 8.2) and NaCl (0.1 M). Further partial hybridization was carried out with a 5×10^{-7} M solution of the nucleic acid **4** in a buffer solution that contained 25 mM Tris (pH 8.2) and NaCl (0.3 M). The resulting devices were then treated with the different concentrations of AMP or cocaine in a buffer solution that contained Tris (25 mM, pH 8.2) and NaCl (0.1 M).

ISFET measurements: ISFET devices with an Al_2O_3 gate interface (20×700 μm^2, IMT, Neuchâtel, Switzerland) were used in all the experiments. An Ag/AgCl electrode was used as a reference. The chip that was functionalized with the hybridized oligonucleotides was immersed in the working cell, which was filled with a buffer solution that consisted of Tris (25 mM, pH 8.2) and NaCl (0.1 M). The output signal between the source of the ISFET and the reference electrode was recorded by using a semiconductor parameter analyzer (HP 4155B). The system configuration enabled the measurements of the source-gate voltage (V_{gs}), while the source-drain voltage (V_{sd}) and the source-drain current remained constant ($V_{sd} = 1$ V, $I_{sd} = 100$ μA). The difference between V_{gs} values for the ISFET that was modified with oligonucleotides before and after the reaction with the different concentrations of substrates was plotted against the substrate concentrations.

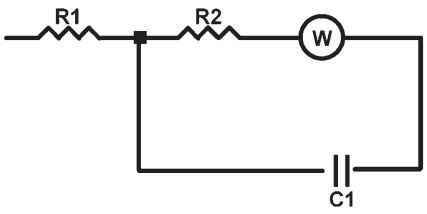


Figure 8. Equivalent circuit for fitting the impedance spectra shown in Figure 3.

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