Talanta 92 (2012) 65–71

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A label-free aptasensor for the sensitive and specific detection of cocaine using supramolecular aptamer fragments/target complex by electrochemical impedance spectroscopy

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ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 21 January 2012
Accepted 29 January 2012
Available online 3 February 2012

Keywords:
Aptamer fragment
Cocaine
Supramolecular complex
Label-free
Electrochemical impedance spectroscopy

ABSTRACT

A simple and label-free aptasensor for sensitive and specific detection of cocaine was developed by measuring the change in electrochemical impedance spectra (EIS), based on the formation of a supramolecular aptamer fragments/substrate complex. An anticocaine aptamer was divided into two fragments, Cx and Cy. Three different sensing interfaces, called Au/Cx5S/MCE, Au/Cy3S/MCE and Au/Cy5S/MCE, were fabricated by immobilizing Cx or Cy on a gold electrode through modifying their 5′ or 3′ end with a thiolated group followed by the treatment with mercaptoethanol (MCE). The formation of the corresponding supramolecular aptamer fragments/cocaine complex was investigated via monitoring electrochemical impedance spectra in the presence of $[Fe(CN)_6]^{3-/4-}$. The interfacial electron transfer resistance (R_{et}) was found to depend strongly on the cocaine concentration. Since the supramolecular aptamer fragments/cocaine complex was formed on the electrode surface, the sensing interface strongly affected the sensitivity of the aptasensor. Au/Cx5S/MCE was shown to have good sensitivity within a cocaine detection range of 0.1–20 μM. Moreover, MCE was shown to improve the sensitivity of the aptasensor greatly. Even without the help of amplification or labeling, cocaine concentrations as low as 100 nM could be easily detected by the impedimetric aptasensor developed. The specificity and regeneration of the cocaine aptasensor were also investigated and satisfactory results were obtained. The developed aptasensor was successfully applied to detect the cocaine in biological fluids.

1. Introduction

Aptamers are artificial functional oligonucleic acids which are in vitro selected through SELEX (systematic evolution of ligands by exponential enrichment) from random-sequence nucleic acids libraries [1,2]. They can bind their respective targets with high affinity and specificity ranging from small inorganic or organic substances to even proteins or cells [3]. Aptamers are simply and reproducibly synthesized by automated synthesis, easily labeled, and chemically stable. They often experience significant conformational changes during the interaction with their targets. All of these advantages make aptamers ideal recognition probes for biosensors [4]. Various aptasensors (aptamer-based biosensor) have been developed based on different technologies, such as fluorescence [5–7], colorimetry [8–10], quartz crystal microbalance (QCM) [11,12], surface plasmon resonance (SPR) [13], and electrochemistry [14–20]. Among them, the electrochemical methods have attracted substantial attention in the development of aptasensors because of their high sensitivity, simple instrumentation, fast response, portability, and low production cost.

Most of the electrochemical-based aptasensors developed require labeling of the aptamers [14,15,21]. Since the labeling process would make the experiments relatively complex and might affect the bioaffinity between the aptamers and their targets, there has been interest in developing label-free and low cost aptasensors in recent years [22]. Some label-free aptasensors have been developed using electrochemical impedance spectroscopy (EIS) [23–27], square wave voltammetry [28], chronocoulometry [29] and other techniques. Among them, EIS, which is based on the change of the electron transfer resistance (R_{et}) using a redox couple, has received much attention due to its high sensitivity and label-free characteristics.

Three main strategies in the design of aptasensors based on EIS have been exploited, including charge switching [23,30–32],

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doi:10.1016/j.talanta.2012.01.049

target-induced strand displacement [33–35] and the formation of a supramolecular aptamer fragments/target complex [36]. In the charge switching strategy, a negatively charged target, such as thrombin, binds with a negatively charged aptamer immobilized on the surface of an electrode at neutral pH. The binding results in a change of R_{et}, by which thrombin could be detected as low as 2.0 nM [23]. But this strategy is not suitable for detecting small molecules since the binding of aptamers to small molecules cannot influence the apparent charge or interfacial changes on the electrode surface. Therefore, the target-induced strand displacement strategy has been developed, in which the aptasensor is fabricated by assembling the complementary DNA-aptamer duplex on the electrode. Upon binding to their target molecule, the aptamer or complementary DNA is displaced from the electrode, resulting in a significant change of R_{et}. This strategy has been successfully developed to detect proteins and small molecules [35]. The key requirement for using this strategy in the design of aptasensors is that the affinity constant between the aptamer and its target must be higher than that of the complementary DNA and aptamer. The third strategy is based on the formation of a supramolecular aptamer fragments/target complex. An aptamer can be fragmented into two fragments that can self-assemble into a supramolecular aptamer fragments/target complex in the presence of the target [36–40]. The formation of the complex increases the negative charge on the electrode, resulting in the increase of R_{et}. Sharon et al. developed an impedimetric aptasensor using anticocaine aptamer fragments that self-assembled in the presence of cocaine, to a supramolecular aptamer fragments/cocaine complex on the electrode surface. They also showed that gold nanoparticles could be used to amplify the response of the aptasensor leading to a device capable of detecting cocaine concentrations down to 10 μM [36]. This strategy is quite promising since it could be generalized for detecting different kinds of targets by cleaving an aptamer into two suitable fragments.

For the construction of impedimetric aptasensor based on the formation of a supramolecular aptamer fragments/target complex, the quality of the sensing interface plays a vital role on the sensitivity of the aptasensor since it influences the surface density of immobilized aptamer and their ligand capture efficiency. Therefore, in this work, the construction of efficient sensing interface was the focus. Cocaine was chosen as a model of target and anticocaine aptamer fragments (Cx and Cy) were taken as molecular recognition elements for the construction of impedimetric aptasensor based on aptamer fragments/target configuration. The sensing interface was fabricated by self-assembly of one fragment and mercaptoethanol (MCE) on a gold electrode surface through an Au—S bond. There exist three main factors which might influence the sensing interfaces to recognize the cocaine: (1) the adsorption efficiencies of aptamer fragments modified with a thiol group at its 5′ or 3′ end on Au electrode surface. (2) The negative charges of the binding aptamer fragment since the increase of R_{et} is caused by the increase of the negative charge on the electrode. (3) The efficiency of the formation of the aptamer fragments/target complex on the electrode surface. Three different sensing interfaces (Au/Cx5S/MCE, Au/Cy5S/MCE and Au/Cy3S/MCE) were designed according to the above three factors as shown in Scheme 1 and the responses of the different models to cocaine due to the formation of the aptamer fragments/cocaine complex were investigated via monitoring the change of R_{et} by EIS. The effect of MCE on the sensitivity of the aptasensor was also investigated. A 100 fold improvement in the detection limit for cocaine was obtained by optimizing the sensing interface compared with that developed by Sharon et al. based on the same configuration [36]. At the same time, the selectivity, regeneration and the application of the aptasensor in real samples were investigated in detail.

2. Experimental

2.1. Chemicals and materials

Oligonucleotides with the following sequences were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China):

5′HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TTCGTTCTTCAATGAAGTGGGACGACA3′, Cx5S
5′GGGAGTCAAGAACGAA3′, Cy
5′HO-$(CH_2)_6$-S-S-$(CH_2)_6$-GGGAGTCAAGAACGAA3′, Cy5S
5′GGGAGTCAAGAACGAA-$(CH_2)_6$-S-S-$(CH_2)_6$-OH3′, Cy3S
5′TTCGTTCTTCAATGAAGTGGGACGACA3′, Cx
5′CCAACCACACCAACC3′, random DNA

Cocaine hydrochloride and morphine were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, KCl, $MgCl_2$, and MCE were purchased from Sigma–Aldrich and used without further purification. The oligonucleotides were dissolved in phosphate buffer (0.10 M, pH 7.4) containing 5 mM KCl and 5 mM $MgCl_2$. A $[Fe(CN)_6]^{3-/4-}$ redox probe solution was prepared as follows: the $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1, each 5 mM) mixture was dissolved in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M KCl as the supporting electrolyte. The solutions were stored at 4 °C before use. All samples and buffer solutions were prepared using ultrapure water (18.2 MΩ cm) obtained from a Milli-Q water purification system.

2.2. The pretreatment of the electrode

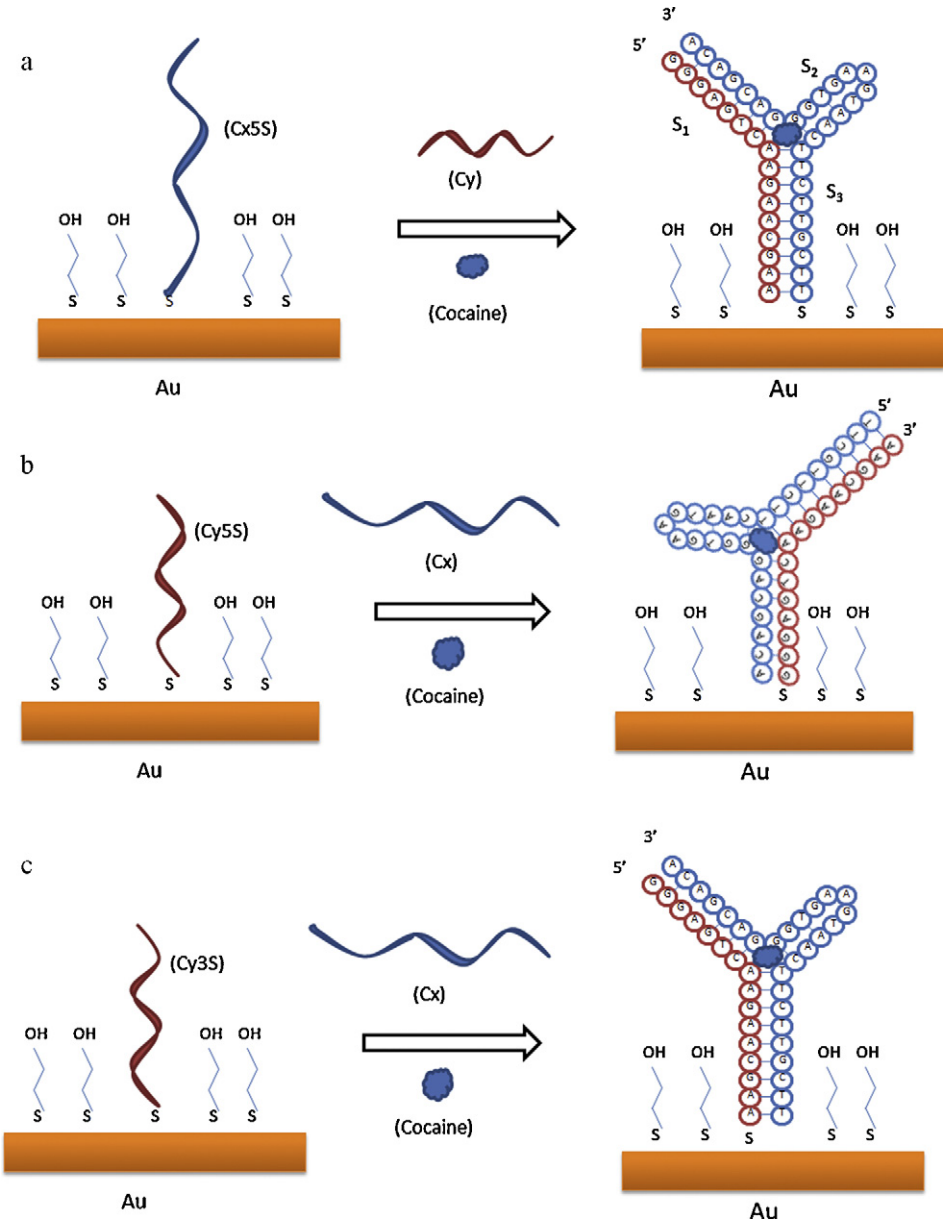
Gold electrodes with 1-mm diameter were purchased from Aida Instrument Inc. (Tianjin, China) and cleaned prior to use. The gold electrodes were first polished with 0.05 μm alumina slurry using a P1 gold polisher (Laizhou Weiyi Experiment Machine Manufacturing Co., Ltd, Shangdo, China) and washed with ultrapure water three times. Then the electrodes were cleaned in piranha solution (v/v, 3:1 H_2SO_4/H_2O_2; WARNING: piranha solution reacts violently with organic solvents) for 20 min, washed thoroughly with ultrapure water, and dried under nitrogen. Afterward, the electrodes were electrochemically cleaned in 0.5 M H_2SO_4 by potential cycling between −0.2 V and 1.6 V until a reproducible cyclic voltammogram was obtained, followed by rinsing them with pure water and drying under nitrogen.

2.3. Fabrication of the modified electrodes

For the formation of the self-assembled monolayers (Au/Cx5S/MCE), 10 μL solution of 1 μM Cx5S (in 0.10 M pH 7.4 phosphate buffer containing 5 mM KCl, 5 mM $MgCl_2$ and 20 μM TCEP,) was placed on a freshly cleaned gold electrode. The electrode was fitted with a vitreous cap to protect the solution from evaporation. The assembly was kept standing overnight at room temperature and then rinsed with phosphate buffer several times. After drying with nitrogen, the Au/Cx5S interface was covered with 5 μL of MCE (100 μM in pH 7.4 phosphate buffer) and kept at room temperature for 30 min, followed by rinsing with pure water and phosphate buffer. The modification of the gold surface with Cx5S/MCE was finished by drying the electrodes with nitrogen. The Au/Cy5S/MCE and Au/Cy3S/MCE were obtained using similar method.

2.4. Apparatus and electrochemical measurements

Electrochemical impedance spectroscopy (EIS) was performed using an Autolab PGSTAT302N (Eco Chemie, The Netherlands,



Scheme 1. The fabrication of an impedimetric aptasensor based on the formation of supramolecular aptamer–target complex.

controlled by GPES4 and FRA software). A conventional three-electrode system was used, with a gold electrode as the working electrode, an Ag/AgCl reference electrode, and a platinum wire as the counter electrode. The cell was housed in a Faraday cage to reduce electrical noise. Impedance spectra were measured at room temperature in a 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe solution, using a sinusoidal AC potential perturbation of 5 mV, in the frequency range from 100 kHz to 1 Hz. The applied potential was 0.22 V vs Ag/AgCl (formal potential of the redox probe $[Fe(CN)_6]^{3-/4-}$ in the buffer solution).

As shown in Scheme 1a, to detect cocaine, 5 μL solution containing 10 μM Cy and different concentrations of cocaine was placed on the Au/Cx5S/MCE sensing interface for 30 min at room temperature. Before measurement, the sensing interface was rinsed with pure water and phosphate buffer several times and dried with nitrogen. Similarly, the sensing abilities of the Au/Cy5S/MCE and the Au/Cy3S/MCE were investigated. In the control experiments, the same volume of solution containing 10 μM Cy and 10 mM morphine, or 10 μM random DNA sequences with 100 μM cocaine was placed on the Au/Cx5S/MCE surface for 30 min. The aptasensor was regenerated by soaking the electrode in water/methanol (40:60) (1 h, 60 °C); and the reusability of the electrode was investigated.

3. Results and discussion

3.1. Fabrication and characterization of Au/Cx5S/MCE

As can be seen in Scheme 1a, the aptamer of cocaine was split into two fragments, Cx5S and Cy. Cx5S was chemically modified with a thiol group at its 5′ end, in order to form an Au—S bond with the gold electrode. After that, MCE was used to displace any non-specifically adsorbed Cx5S and to increase the order of the Cx5S monolayer by occupying any remaining binding sites on the gold surface. The formation of the Au/Cx5S/MCE monolayer was monitored using EIS (Fig. 1) in a buffer solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ in a frequency range from 100 kHz to 1 Hz. A modified Randles equivalent circuit was used to fit the impedance spectra and to determine electrical parameters for each step. As

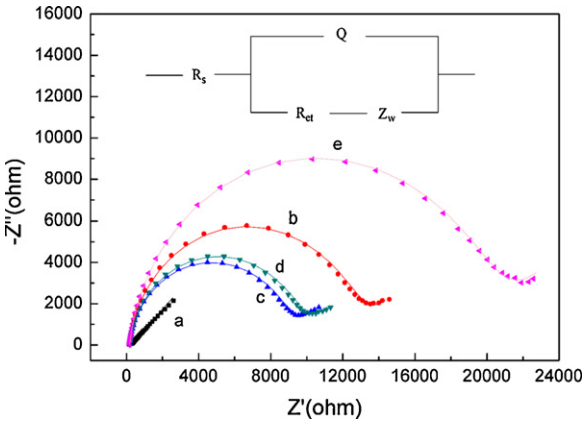


Fig. 1. EIS of (a) the bare Au electrode, (b) the Au/Cx5S electrode, (c) the Au/Cx5S/MCE electrode, (d) the Au/Cx5S/MCE electrode incubated with 10 μM Cy and (e) the Au/Cx5S/MCE electrode incubated with 100 μM cocaine and 10 μM Cy. The lines represent the fits with the equivalent circuit shown in the inset.

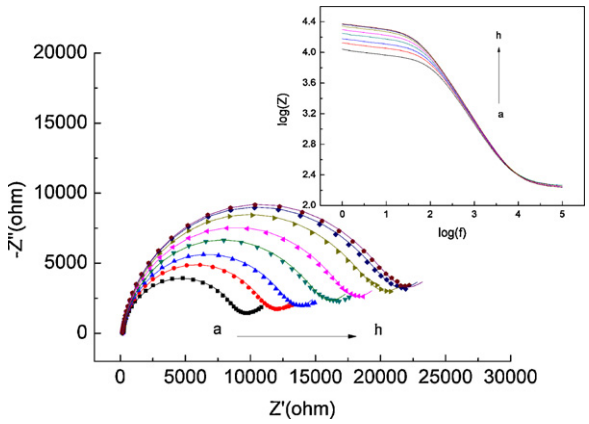


Fig. 2. EIS of the Au/Cx5S/MCE electrode after incubation with Cy and different concentrations of cocaine: (a) 0 μM, (b) 0.1 μM, (c) 0.5 μM, (d) 1 μM, (e) 5 μM, (f) 10 μM, (g) 50 μM, and (h) 100 μM. The lines represent the fits with the equivalent circuit shown in the inset in Fig. 1. The inset shows Bode plots of the same impedance spectra.

shown in Fig. 1 (inset), the circuit included the electrolyte resistance between working and reference electrodes (R_s), the Warburg impedance (Z_w), a constant phase element (Q) representing the double layer capacitance for an unmodified electrode or the capacitance of the self-assembled monolayer for the modified electrodes and the electron-transfer resistance (R_{et}). The results of an equivalent circuit fit of the impedance spectra presented in Fig. 1 are shown in Table 1. The bare gold electrode showed a small electron transfer resistance indicating a fast electron transfer process. The diffusion limited behavior at low frequencies is described by a Warburg impedance (Fig. 1, curve a). The self-assembly of Cx5S onto the Au electrode effectively retarded the interfacial electron-transfer kinetics of $[Fe(CN)_6]^{3-/4-}$ anions due to the negatively charged aptamer repulsing $[Fe(CN)_6]^{3-/4-}$ from the sensor surface, which was reflected by the increase of R_{et} from 0.1 kΩ to 13 kΩ and the decrease in the capacitance (Table 1 and Fig. 1, curve b). The modification of Au/Cx5S with MCE resulted in a relatively small R_{et} (8.8 kΩ), which was consistent with the literature [23,35]. The thiol-modified aptamer was adsorbed on the Au electrode surface specifically through the formation of an Au—S bond and nonspecifically due to the relatively weak interaction between Au and nitrogen atoms of DNA bases. After treatment with MCE, the nonspecifically adsorbed aptamer was largely removed from the surface. The more orderly monolayer of aptamer mixed with MCE could result in the decrease of R_{et} of Au/Cx5S/MCE [23]. The above results indicated that Cx5S was immobilized effectively on the Au electrode. Then the Au/Cx5S/MCE electrode was incubated with 100 μM cocaine in the absence and presence of 10 μM Cy. An incubation time of 30 min was chosen, which was reported to be sufficient for the formation of a supramolecular aptamer complex [37]. The reaction of Au/Cx5S/MCE with 100 μM cocaine and 10 μM Cy (Fig. 1, curve e and Table 1) caused a large increase of R_{et} by 127%, indicating the self-assembly of the supramolecular Cx5S/cocaine/Cy complex on the surface since the formation of the supramolecular complex increased the negative charge on the electrode surface. However, in the absence of cocaine, the interaction between Cx5S and Cy was too weak to form a stable supramolecular complex, resulting only in a very small change of R_{et} (9.2 kΩ) in the impedance response of Au/Cx5S/MCE (Fig. 1, curve d) close to the noise level. The whole process was also monitored using cyclic voltammograms (CV) of $[Fe(CN)_6]^{3-/4-}$ to investigate the kinetic barrier of the interface (Fig. S1). The performance of the electron transfer kinetics measured by CV was consistent with that by EIS.

3.2. Sensitivity of Au/Cx5S/MCE

The sensitivity of this impedance-based cocaine analysis protocol was investigated by incubating Au/Cx5S/MCE with solutions containing 10 μM Cy and different concentrations of cocaine for 30 min. The impedance increased significantly with increasing concentration of cocaine (Fig. 2), which was consistent with an increased coverage of the supramolecular complex on the electrode surface. As indicated in Table 1, the electron transfer resistance R_{et} showed the largest change upon exposure of the electrode to cocaine and Cy while changes in the capacitance and the Warburg impedance were comparatively small. Therefore, $\Delta R_{et} = R_{et} - R_{et}^0$ (R_{et}^0 was the blank R_{et} of Au/Cx5S/MCE) was used for evaluating the response to cocaine. As shown in Fig. 3 (curve a), there was a linear relationship between ΔR_{et} and the logarithm of the concentration of cocaine from 0.1 μM to 20 μM fitting the equation $\Delta R_{et} = 3.9 \times 10^3 \log(C_{cocaine}) + 29 \times 10^3$ ($R^2 = 0.98$). R_{et} reached a plateau at higher concentrations of cocaine (>50 μM), indicating that the amount of supramolecular Cx5S/cocaine/Cy complex formed on the electrode surface reached its saturation. An alternative to estimating R_{et} from impedance spectra would be to record the impedance at a single frequency. The Bode plots (inset in Fig. 2) show that the maximum impedance change can be observed at frequencies <30 Hz. Recording the impedance change at a single

Table 1
Results of an equivalent circuit fit for the impedance spectra shown in Fig. 1.

	R_s/Ω	$Q[Z_Q = -1/(i\omega C)^n]$		R_{et}/Ω	Z_W/Ω
		C/F	N		
	($\times 10^2$)	($\times 10^{-7}$)		($\times 10^2$)	($\times 10^{-4}$)
Au	1.4	14	0.84	1.3	3.9
Au/Cx5S	1.6	3.7	0.93	130	1.6
Au/Cx5S/MCE	1.7	3.8	0.92	88	1.7
Au/MCE/Cx5S/cocaine/Cy	1.7	2.4	0.92	2.0	0.99

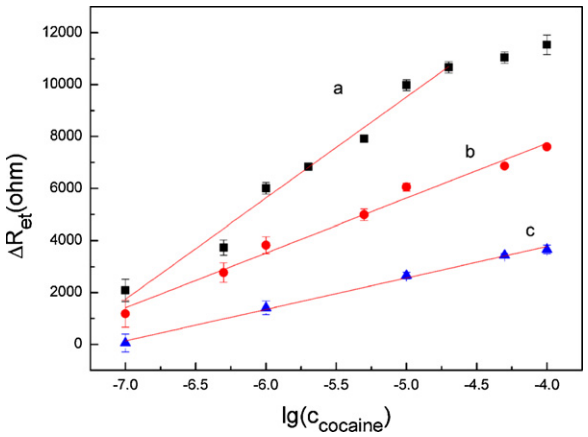


Fig. 3. Response of (a) the Au/Cx5S/MCE, (b) Au/Cy3S/MCE and (c) Au/Cx5S to different concentrations of cocaine. $\Delta R_{et} = R_{et} - R_{et}^0$: R_{et}^0 represents the blank R_{et} of the Au/Cx5S/MCE, Au/Cy3S/MCE or Au/Cx5S surfaces; R_{et} represented the R_{et} of the Au/Cx5S/MCE, Au/Cy3S/MCE or Au/Cx5S after incubation with different concentrations of cocaine. The error bars represent the average standard deviation of three measurements.

frequency <30 Hz would be a practical solution for a potential commercial device.

During the experiments, MCE was found to play an important role in the improvement of the sensitivity and the detection limit of this aptasensor. The response of the Au/Cx5S electrode to different concentrations of cocaine is shown in Fig. 3, curve c. As we can see, 100 nM cocaine did not cause an impedance increase of the Au/Cx5S electrode. ΔR_{et} was much smaller than that obtained with an Au/Cx5S/MCE electrode at the same concentration of cocaine. For example, ΔR_{et} was 1.4 kΩ at Au/Cx5S when the concentration of cocaine was 1 μM, while at Au/Cx5S/MCE, ΔR_{et} was as high as 6.0 kΩ, indicating the sensitivity was improved ca. 4-fold. The above results indicated that the conformation and density of aptamer on the surface greatly influenced its ability of recognizing its target. In Au/Cx5S, Cx5S did not orientate very orderly since some of the molecules inclined towards the Au surface or coordinated with Au through nitrogen atoms by nonspecific adsorption [30]. After treatment with MCE, the nonspecifically adsorbed aptamers were largely removed from the surface, and only one end of the aptamers was bound to the electrode surface with all the relevant bases freely available for reaction with cocaine. At the same time, the less densely packed monolayer of MCE left enough space for aptamer fragments to form the supramolecular complex on the electrode surface with cocaine. The above results indicated the quality of the sensing interface played an important role in the sensitivity of the aptasensor.

3.3. Comparison of the sensitivity to cocaine at the different sensing interfaces

Since the formation of the supramolecular aptamer fragments/cocaine complex happened on the electrode surface, the sensing interface was expected to have a significant influence on the sensitivity of the constructed aptasensor. To obtain the optimum sensitivity, two other aptasensors called Au/Cy5S/MCE and Au/Cy3S/MCE (Scheme 1b and c) were constructed based on the following factors which might influence the sensitivity of the constructed impedimetric aptasensors: (1) the adsorption efficiency of an aptamer fragment on a gold electrode surface; (2) the negative charges of the binding aptamer fragment; (3) the efficiency of the formation of the Cx/cocaine/Cy complex on the electrode surface.

Fig. 4A shows the impedance spectra of Au/Cy5S/MCE in the presence of Cx and variable concentrations of cocaine. An

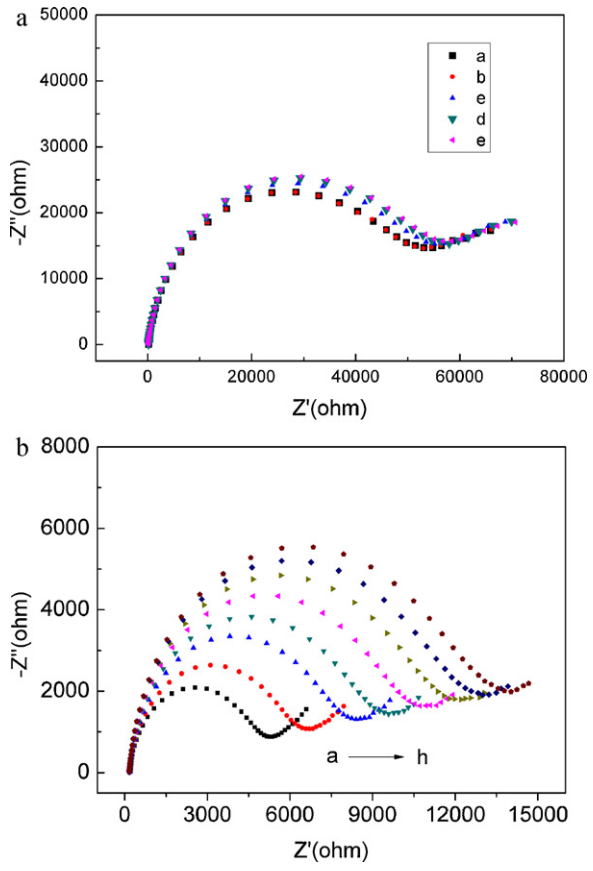


Fig. 4. (A) EIS of the Au/Cy5S/MCE after incubation with 10 μM Cx and different concentrations of cocaine: (a) 0 μM, (b) 0.1 μM, (c) 0.5 μM, (d) 5 μM and (e) 50 μM. (B) EIS of the Au/Cy3S/MCE after incubation with 10 μM Cx and different concentrations of cocaine: (a) 0 μM, (b) 0.1 μM, (c) 0.5 μM, (d) 1 μM, (e) 5 μM, (f) 10 μM, (g) 50 μM and (h) 100 μM.

impedance increase was observed at 500 nM cocaine. Then ΔR_{et} reached the plateau at 1 μM, indicating that the effective amount of Cy5S to react with Cx and cocaine was limited. Stojanovic demonstrated that the proposed cocaine binding pocket is located in the lipophilic cavity of a three-way junction [6], as shown in Scheme 1. The stems need enough length and complementarity to stabilize the three-way junction structure for the accumulation of negative charges and hydrophobic surfaces. There are three stems in the complex. The S3 stem consists of ideally matched bases, and the hydrogen bonds between the double helix structures of S3 stem are much more stable. But the S1 and S2 stems include noncanonical base pairs, which are likely formed only in the presence of cocaine. The immobilization of the aptamer fragment on a gold electrode might interfere with the interaction between cocaine and aptamer to some extent. Compared to the immobilization of S3 (Cx5S) on the surface, the immobilization of S1 (Cy5S) might perturb the formation of aptamer fragments/cocaine complex severely, which caused Au/Cy5S/MCE to have the poor recognition of cocaine. In addition, the loop of the supramolecular Cy5S/cocaine/Cx complex was closer to the gold electrode surface than that of the Cx5S/cocaine/Cy supramolecular complex, as shown in Scheme 1a and b. The larger steric hindrance made the formation of a supramolecular Cy5S/cocaine/Cx complex on the electrode surface more difficult.

Fig. 4B shows the response of Au/Cy3S/MCE to different concentrations of cocaine in the presence of Cx. The impedance of Au/Cy3S/MCE was much smaller than that of Au/Cy5S/MCE (Fig. 4a), indicating that the amount of Cy immobilized on the electrode surface through the 3′ end was less than that of Cy through 5′

end. It has been reported that DNA modified with a thiol group at its 5′ end or 3′ end had different adsorption efficiencies on gold surface [41,42] and that at the 5′ end showed better adsorption. Therefore, the result was consistent with the literature. As shown in Fig. 4B, 100 nM cocaine could cause an impedance increase of Au/Cy3S/MCE, which was as good as that of Au/Cx5S/MCE. The similar detection limits might be due to the fact that the supramolecular Cx5S/cocaine/Cy and Cy3S/cocaine/Cx complexes had the same conformation on the electrode surface as shown in Scheme 1. R_{et} of the Au/Cy3S/MCE electrode increased significantly with increasing concentration of cocaine (Fig. 3, curve b). A linear relationship between ΔR_{et} and the logarithm of the concentration of cocaine from 0.1 μM to 100 μM was obtained, where the regression equation was $\Delta R_{et} = 2.1 \times 10^3 \log(C_{cocaine}) + 16 \times 10^3$ ($R^2 = 0.98$). The increase of R_{et} is caused by the increase of the number of negative charges on the electrode. Since Cx has more bases than Cy, which means more negative charges on the electrode surface and larger change of R_{et} when the same amount of Cx/cocaine/Cy complex is formed, binding Cx to Au/Cy3S/MCE was expected to result in larger sensitivities than binding Cy to Au/Cx5S/MCE. However, the sensitivity of Au/Cy3S/MCE electrodes was lower than that of Au/Cx5S/MCE electrodes. The reason might be that the surface concentration of Cy3S on Au/Cy3S/MCE was lower and accordingly the amount available for reaction was lower, which caused the smaller change of R_{et} at the same concentration of cocaine and Cx. Using a previously reported method [43] involving electrochemical quantification of DNA immobilized on gold electrode, the surface coverage of Cx5S and Cy3S on the gold electrode was estimated to be 1.0×10^{13} and 7.2×10^{12} molecules/cm^2, which confirmed our assumption. Therefore, the adsorption efficiencies of aptamer on the Au electrode surface had a more profound effect on the sensitivity of the above developed aptasensor than the negative charges of the binding aptamer fragment.

The performances of the Au/Cx5S, Au/Cx5S/MCE, Au/Cy5S/MCE and Au/Cy3S/MCE aptasensors for cocaine detection are compared in Table 2. Au/Cy5S/MCE showed the worst recognition to cocaine and Au/Cx5S/MCE had the highest sensitivity, indicating different factors had different effects on the performance of the aptasensors. Among the three factors mentioned above, the efficiency of the formation of the Cx/cocaine/Cy complex on the electrode surface had the biggest effect on the sensitivity of the aptasensor constructed. As shown in Table 2, the detection limit obtained at Au/Cx5S/MCE improved the detection limit for cocaine 100 fold compared with that using the same configuration [36], indicating the importance to obtain an efficient sensing interface. At the same time, without the help of amplification or labeling, the impedimetric aptasensor developed could easily detect cocaine concentrations as low as 100 nM, which made this aptasensor attractive and advantageous. Since Au/Cx5S/MCE showed a better performance than Au/Cx5S, Au/Cy3S/MCE and Au/Cy5S/MCE, all subsequent experiments were performed using Au/Cx5S/MCE electrodes.

3.4. Specificity of the aptasensor

To verify the specificity of our aptasensor, two control experiments were performed to confirm that the R_{et} change observed was a result of the aptamer fragments/cocaine binding (Fig. 5). The first one was to replace cocaine with morphine. After 30 min of immersion in the solution containing 10 mM morphine and 10 μM Cy, Au/Cx5S/MCE showed no significant change of R_{et} (Fig. 5, curve b). However, 100 nM cocaine caused an obvious R_{et} increase of 2 kΩ (Fig. 5, curve d), which clearly indicated that the aptasensor was highly specific to cocaine. To further verify that the supramolecular complex was formed by the reaction of Cx5S, Cy and cocaine, a random oligonucleotide sequence (random DNA) was used to replace Cy for another control experiment. Au/Cx5S/MCE was incubated with 100 μM cocaine and 10 μM random DNA for 30 min. No significant change in the impedance spectrum could be observed although the concentration of cocaine was increased significantly (100 μM) (Fig. 5, curve c), which was not surprising because the changed sequence had no affinity to cocaine, and the addition of cocaine could not induce the formation of a supramolecular complex. All the results confirmed the high specificity of this aptasensor.

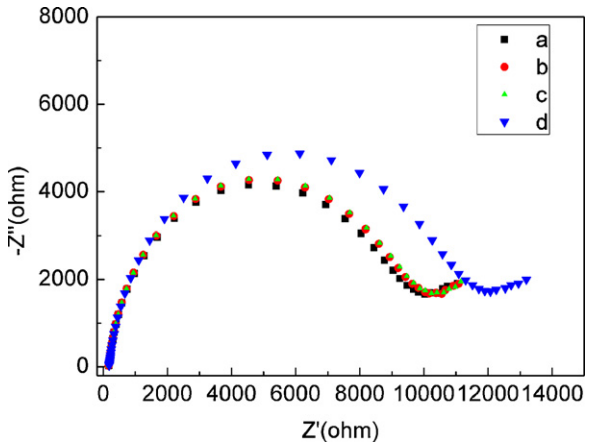


Fig. 5. EIS of the Au/Cx5S/MCE before (a) and after incubation with (b) 10 mM morphine and 10 μM Cy, (c) 100 μM cocaine and 10 μM random DNA and (d) 100 nM cocaine and 10 μM Cy.

3.5. Regeneration of the aptasensor

Besides high sensitivity and specificity, the possibility to regenerate the sensing interface is another important aspect for the development of the aptasensor. The results showed that the sensing interface after detection could be regenerated completely after treating with water/methanol (40:60) for 1 h at 60 °C; and the regenerated interface could be used to detect cocaine again. The result of the second detection of cocaine was almost the same as the first one (Fig. 6) showing that there was no effect on the next detection after the former measurement. Therefore, the regeneration of the aptasensor was deemed to be feasible.

3.6. Application of the aptasensor in human serum

The applicability of the aptasensor developed to the detection of cocaine in real samples was investigated. Fresh human plasma was provided by hospital attached to Peking University.

Table 2
Comparison of the performance of Au/Cx5S/MCE, Au/Cy5S/MCE, Au/Cy3S/MCE and Au/Cx5S aptasensors for cocaine detection.

Interface	Regression equation for calibration curve	Linear range (μM)	Detection limit (μM)
Au/Cx5S/MCE	$\Delta R_{et} = 3.9 \times 10^3 \log(C_{cocaine}) + 29 \times 10^3$	0.1–20	0.1
Au/Cy3S/MCE	$\Delta R_{et} = 2.1 \times 10^3 \log(C_{cocaine}) + 16 \times 10^3$	0.1–100	0.1
Au/Cy5S/MCE	–	–	0.5
Au/Cx5S	$\Delta R_{et} = 1.2 \times 10^3 \log(C_{cocaine}) + 8.6 \times 10^3$	1–100	1

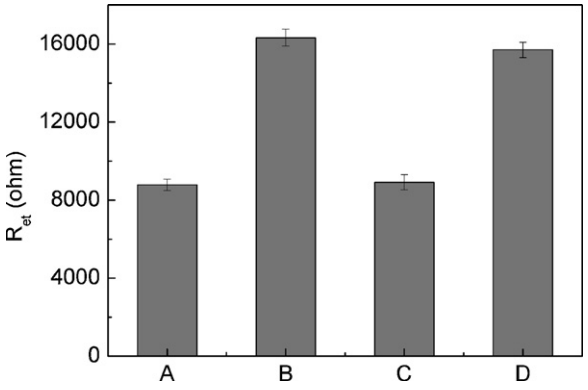


Fig. 6. Regeneration of the Au/Cx5S/MCE after detecting cocaine: (A) unused sensor; (B) after detecting 5 μM cocaine; (C) regeneration of the interface; (D) detection of 5 μM cocaine for the second time.

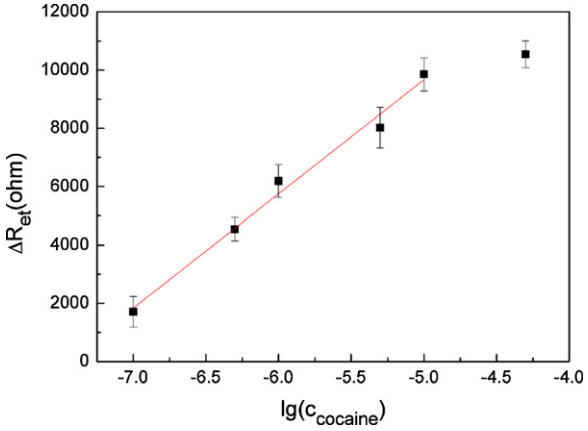


Fig. 7. EIS responses of the Au/Cx5S/MCE to different concentrations of cocaine (0.1–50 μM) in 25% human serum; $n = 3$.

As shown in Fig. 7, the EIS responses to the different concentrations of cocaine in 25% human serum were similar to those measured in buffer. R_{et} increased with increasing concentration of cocaine in the range of 0.1–10 μM and the regression equation was $\Delta R_{et} = 3.9 \times 10^3 \log(C_{cocaine}) + 29 \times 10^3$ ($R^2 = 0.98$), which was almost the same as that obtained in blank buffer.

4. Conclusions

In summary, a label-free impedimetric aptasensor for the detection of cocaine was developed based on the formation of a supramolecular aptamer fragments/substrate complex. We designed three different sensor architectures and found that Au/Cx5S/MCE performed best due to the efficient immobilization of aptamer fragments and the favorable conformation of the supramolecular complex on the electrode surface. The sensor with its optimized sensing surface showed high sensitivity, and could easily detect cocaine as low as 100 nM without the help of amplification or labeling. The aptasensor developed showed good specificity for cocaine and could be regenerated efficiently. The sensor performance in biological fluids was shown to be comparable to that measured in buffer solutions. The formation of a supramolecular aptamer fragments/substrate complex in this study provides a promising model for the impedimetric detection of small molecules.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 20805002, 30890142, 20975007 and 90713013) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, MOE, China.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.01.049.

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