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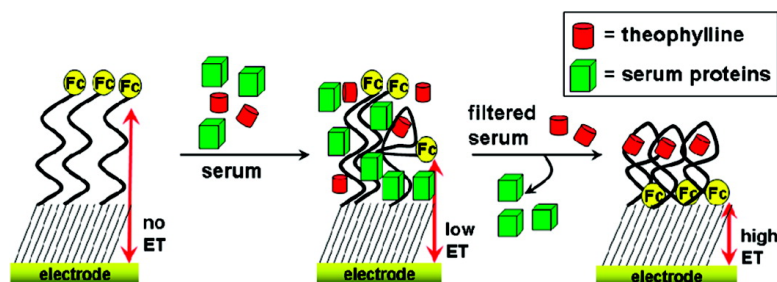
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Effect of Serum on an RNA Aptamer-Based Electrochemical Sensor for Theophylline

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Electrochemical performance of the ferrocene (Fc) redox-labeled RNA aptamer based sensor for theophylline (Th) is essentially inhibited in serum, but is restored in serum-free buffer solutions. This phenomenon is inconsistent with the data on methylene-blue-labeled aptamer beacon systems, which operational potential window is more negative compared to the Fc redox label. Electrochemical studies with a ferricyanide redox probe, having redox potential close to the Fc redox couple, and interfacial capacitance measurements unambiguously demonstrate that it is adsorption of serum proteins at positively charged electrode surface that slows down the kinetics of the electrode reactions in serum and interferes with the biosensor performance. In filtered serum solutions, in the absence of serum proteins, the Fc-labeled aptamer-based biosensor performed similarly to the pure buffer solutions, ad the signal for Th could be linearly calibrated versus Th concentration. These results on interfacial effects of serum are of particular importance for future research and development of the beacon-type biosensors for *in vivo* applications.

We have reported on the application of an RNA aptamer [1] in a selective and sensitive electrochemical biosensor for a label-free detection of theophylline,[2] a bronchodilator used for treatment of acute and chronic asthma conditions.[3,4] The biosensor exploited the molecular conformational change of the RNA aptamer upon interaction with theophylline. The aptamer was immobilized via a disulfide at the 5′-terminal at a gold electrode, and the amino-modified 3′-terminal was conjugated to a ferrocene (Fc) electrochemical reporter group. In the presence of theophylline the RNA aptamer changes conformation from an open and unfolded state to a folded structure bringing the Fc redox probe closer to the electrode surface. This leads to increased electron transfer (ET) efficiency between Fc and the electrode.[2] The read-out of the biosensor electrochemical response upon theophylline binding enabled selective and sensitive detection of theophylline in the presence of structurally related caffeine and theobromine. The specificity of the biosensor was advantageous compared to the existing gas/liquid chromatographic methods and commercial immunoassays,[3,4] and the stability of the biosensor exceeded that of the theophylline oxidizing enzyme assays.[5−9]

Unfortunately, the response of the aptamer-based theophylline sensor is essentially inhibited when measuring in dilute serum samples, and the signal could only be restored by placing the aptamer in a serum free buffer solution. It was a surprising result, especially, taking into account the report by Plaxco et al. on the efficient function of their electrochemical DNA aptamer-based biosensor for cocaine in serum.[10] We have speculated that there might be a potential-induced adsorption process at the electrode|serum solution interface that affected the electrochemical response of our biosensor. We have studied the gold electrode|serum solution interface by cyclic and differential pulse voltammetry (CV and DPV) and impedimetrically, under conditions similar and/or approaching those of the theophylline biosensor function, and this is the subject of the current Letter.

Serum is a complex biological fluid of a varying composition and with an essential content of albumins, globulins and antibodies. The adsorption of the serum proteins at the electrode|solution interface is expected to affect its electrochemical characteristics. We used potassium ferricyanide as a standard redox probe, which has the redox potential close to that of our biosensor label, the Fc redox couple. Protei adsorption is generally favored at hydroxyl-terminated alkanethiol SAMs.[5,6,8,11] Therefore, bare gold electrodes and gold electrodes, modified by 6-mercapto-1-hexanol similarly to the conditions used in the design of the theophylline aptamer-based biosensor (the Supporting Information),[2] were studied, to clearly show that the adsorption of serum proteins is a general phenomena: it proceeds not only on mercaptohexanol SAMs (which can be expected for proteins), but also at bare

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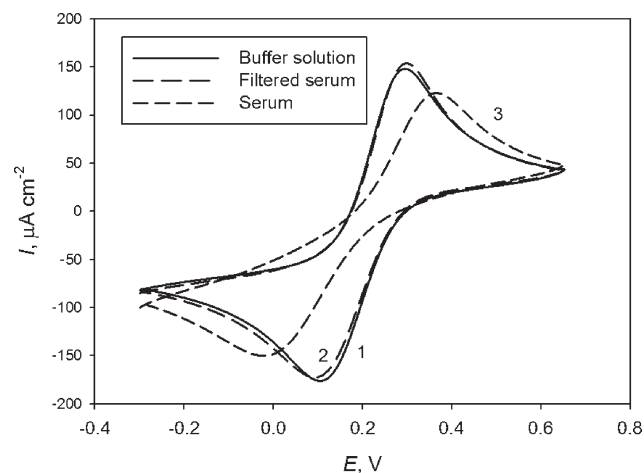
Figure 1. CVs of gold electrodes modified by a mercaptohexanol SAM in 5 mM K$_3$Fe(CN)$_6$ in the HEPES buffer solution (1), filtered serum (2) and nonfiltered serum (3) solutions. The scan rate is 50 mV s^{-1}.

gold, and in a quantitatively similar manner (i.e., that is not just the effect of the $-$OH head groups of SAM). The gold disk electrodes (CH Instruments, USA, diameter 0.2 cm) were mechanically polished to a mirror luster stepwise in 1 μm diamond- and in 0.1 μm alumna slurry on a microcloth, ultrasonicated in ethanol/water bath for 5 min, electrochemically polished by cycling in 1 M H$_2$SO$_4$ as described elsewhere,[11a] in 0.5 M H$_2$SO$_4$/10 mM KCl, and further kept in absolute ethanol for 30 min before use. Modification of electrodes with mercaptohexanol was performed overnight from the 5 mM solution in ethanol. The experiments were performed in a) an aqueous 50 mM HEPES, pH 7, containing 0.15 M NaCl and 5 mM MgCl$_2$ (HEPES buffer) b) in diluted serum (1:9 serum:HEPES buffer), and c) in diluted serum filtrated through a 3 kDa dialysis membrane, to extract the proteins present in serum. All potentials are cited versus an Ag/AgCl (3 M KCl) electrode.

As can be seen from Figure 1, the reversibility of the ferricyanide redox chemistry does not essentially differ when one changes from measuring in pure buffer to filtered serum (Table 1, also Figure S1 in the Supporting Information). However, in nonfiltered serum solutions the redox peak separation significantly increases, designating the lower ET rates (Table 1, Figure 1). The ET rate constant k_s for the ferricyanide redox process consistently decreases in serum compared to pure buffer and filtered serum solutions. This effect is much more pronounced on bare gold compared to mercaptohexanol-modified electrodes (Table 1, Figure S1 of the Supporting Information)

Impedance spectra of gold electrodes in serum demonstrated the same tendency, as compared to the spectra of the filtered serum and pure buffer solutions (Figure 2, Figure S2 of the Supporting Information): the charge-transfer resistance R_{ct} is correspondingly increased in serum. The values of the R_{ct} were estimated from the results of the fitting of the impedance spectra to the Randles circuit, with a Warburg impedance W included in series with the R_{ct} and a CPE in

place of the electric double-layer capacitance C_{edl} (Supporting Information)[16] and are presented in Table 1. The ET rate constants k_o extracted from the values of the R_{ct} are in good agreement with those obtained from the CVs except for the data obtained in serum. The lower values of k_o compared to those of k_s reflect the increased diffusion contribution in serum, which was not taken into account in calculations of k_s from CVs. (The same value for the ferricyanide diffusion coefficient has been used in all calculations.) Impedance spectroscopy inherently separates the charge transfer and diffusion contributions (R_{ct} and W) and thus gives more precise values for the ET rates. As follows from the obtained data, the electrochemical "permeability" of the electrode/solution interface diminishes in serum, first of all as a result of the presence of the bulky protein molecules, which are likely to adsorb onto the electrode and impede the ET process by blocking the electrode surface. When proteins (with a molecular weight of more than 3 kDa) are removed from serum, the electrochemistry of ferricyanide is more consistent with that in pure buffer solutions.

Furthermore, the capacitance of the electrodes decreased in serum solutions, especially within the 0$-$0.5 V potential range (Figure 3). This decrease in capacitance, which may be roughly correlated with the properties of the formed electric double layer (i.e., its thickness and dielectric permittivity),[13] is reversible and is likely to be connected with the adsorption of serum proteins at the electrode/solution interface. This decrease was actually absent in the filtered serum solutions (Figure 3, curve 2 vs 3). The observed adsorption of the serum proteins exactly covers the potential window of our theophylline biosensor, and theoretically, it would not affect the response of any sensor exploiting redox probes with potentials more cathodic than 0 V versus Ag/AgCl (the start potential for the intensive "anodic" adsorption process). We suggest that this was the case for Plaxco's aptamer biosensor for cocaine using methylene blue as the redox label,[10] and our later research with the methylene blue-labeled theophylline aptamer is consistent with this suggestion.[17]

Finally, we investigated the previously developed electrochemical RNA aptamer-based biosensor for theophylline[2] under filtered serum conditions. The design of the biosensor, namely modification of the gold electrodes with the RNA aptamer (the sequence 5′-GGC GAU ACC AGC CGA AAG GCC CUU GGC AGC GUC-3′,[18] having the 5′-end alkanethiol and 3′-end amine modifications) and further aptamer "on-surface" labeled with a Fc redox probe, was performed similarly to the method in our previous work.[2] More specifically, electrochemically polished and ethanol-treated gold electrodes were incubated for 24 h in a solution of the RNA aptamer and further on-surface coupled to the Fc redox label through amine$-N$-hydroxysuccinimide ester chemistry (Supporting Information).[2] After modifications, the RNA-aptamer-modified electrodes, carefully rinsed with a buffer solution and treated with a 0.01% solution of mercaptohexanol for 1 h, were washed with a buffer solution and used in the electrochemical experiments. RNase-free water has been used in all experiments with RNA (Supporting Information).

Our biosensor showed a significantly inhibited electrochemical signal from the Fc redox probe in serum solutions

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Table 1. Kinetic Parameters for the Electrochemical Transformation of 5 mM K$_3$Fe(CN)$_6$ on Au Electrodes in the Buffer, Filtered Serum and Nonfiltered Serum Solutions, Derived from the Data in Figures 1, 2, S1, and S2

	Au/SAM, pure buffer	Au/SAM, filtered serum	Au/SAM, serum	bare Au, pure buffer	bare Au, filtered serum	bare Au, serum
ΔE, mV	177 ± 3	192 ± 2	242 ± 5	79 ± 2	92 ± 3	221 ± 4
k_s, cm s^{-1} [a]	0.14×10^{-3}	0.11×10^{-3}	0.056×10^{-3}	1.16×10^{-3}	0.525×10^{-3}	0.067×10^{-3}
R_{ct}, kΩ	3.44	5.15	50.70	0.73	1.10	54.50
k_o, cm s^{-1} [b]	0.157×10^{-3}	0.12×10^{-3}	0.012×10^{-3}	1.00×10^{-3}	0.58×10^{-3}	0.012×10^{-3}

[a] ET rate constant k_s calculated from the peak separations ΔE in CVs using Nicholson's approach.[12] The diffusion coefficient for ferricyanide was 0.76×10^{-5} cm^2 s^{-1}.[13] [b] ET rate constant k_o calculated from the values of the exchange current i_o derived from charge-transfer resistance R_{ct}[13] and related to the individual electrode surface area determined from the peaks of surface oxide reduction in 1 M H$_2$SO$_4$.[14,15]
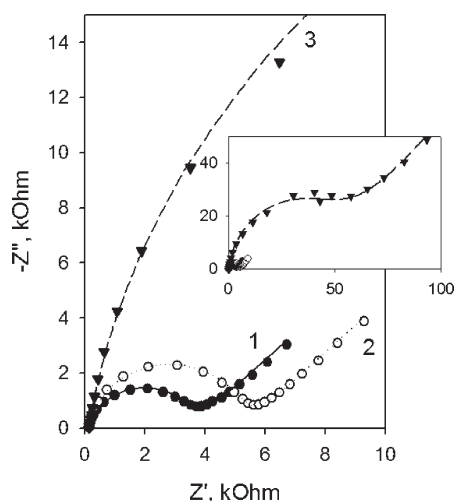

Figure 2. Impedance spectra for gold electrodes modified with mercaptohexanol in 5 mM K$_3$Fe(CN)$_6$ in the HEPES buffer (1), filtered serum (2), and nonfiltered serum (3) solutions. The measurements were performed at open-circuit potential within the 100 kHz– 0.1 Hz frequency range; the amplitude (rms) was 10 mV. Lines correspond to the theoretical fitting.

Figure 3. Potential dependence of the capacitance of the mercaptohexanol-modified gold electrode in the HEPES buffer, pH 7 (1), filtered serum (2), and nonfiltered serum (3) solutions. The frequency is 1 Hz.

compared to that in buffer solutions without any serum (Figure 4A,B); very poor calibration of the electrochemical signal versus theophylline concentrations was observed in serum at theophylline concentrations below 4 μM.[2] However, when the biosensor was transferred from serum to pure buffer solutions, the electrochemical signal from theophylline aptamer was restored (Figure 4A). In filtered serum solutions, the signal from the Fc label of the aptamer was well resolved; the biosensor readily responded to the addition of theophylline (Figure 4C). Though a ca. 90 mV cathodic shift of the Fc redox potential (relative to the Fc redox potential in pure aqueous buffer solutions) has been observed, the current response to theophylline actually approached that in the pure buffer. The system appeared to operate in filtered serum similarly to the operation in buffer solutions,[2] and the signal could be linearly calibrated versus theophylline concentration within the 0.2– 16 μM range.[2]

The interfacial properties of the aptamer-modified electrode were probed in the same way as had been done with bare gold and SAM-modified electrodes. In general, the electrochemistry of ferricyanide at the aptamer-modified electrodes followed the same pattern as observed with the bare gold and SAM-modified electrodes. Peak separation ΔE in CV and charge-transfer resistance R_{ct} estimated from the impedance spectra (Figures 5 and 6) increased when the electrodes were transferred from the pure buffer to serum solutions (Table 2). Therewith, certain peculiarities have been observed with the aptamer-modified electrodes. First, the reversibility of the ferricyanide transformation at the aptamer-modified
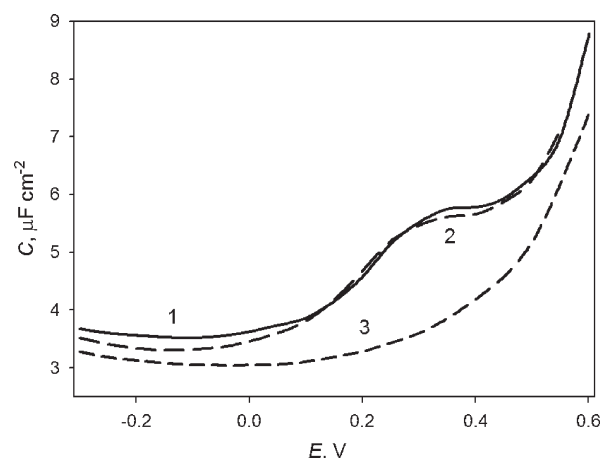
electrodes was much lower than that at the bare gold or SAM-modified electrodes (Figures 1, 5, and S1). This is consistent with a high surface density of the negatively charged RNA aptamer molecules, contributing to slow ferricyanide ET kinetics through both electrostatic repulsion of the negatively charged ferricyanide anion (similar to the observed decreased rates of the anion reduction at negatively charged electrode surfaces[19,20]) and the additional blocking effect of the bulky RNA molecules, decreasing the permeability of the biomolecular layer. Second, the kinetics of the ferricyanide redox reaction at the aptamer-modified electrodes depended on the aptamer conformational state (Table 2, Figures 6 and S3). For an unfolded aptamer in the absence of the theophylline ligand, the ET efficiency of the ferricyanide transformation, correlating with ΔE and R_{ct} values (Table 2, the characteristic k_o values derived from R_{ct} according to the reference in Table 1), was higher than that for a folded aptamer structure in the presence of a saturating concentration of theophylline. This phenomenon was observed in all studied solutions (in pure buffer, in filtered serum, and in serum solutions) and is likely to be connected with the increased density/decreased permeability of the folded, negatively charged aptamer layer, thus slowing down the ET reaction of ferricyanide (though the contribution from some "promotional" effect of theophylline also cannot be excluded).

The observed relative variations of the ET kinetics of the ferricyanide redox reaction on unfolded and totally folded aptamer layers thus can be considered to be an indirect indicator of theophylline binding. Though direct quantification (and

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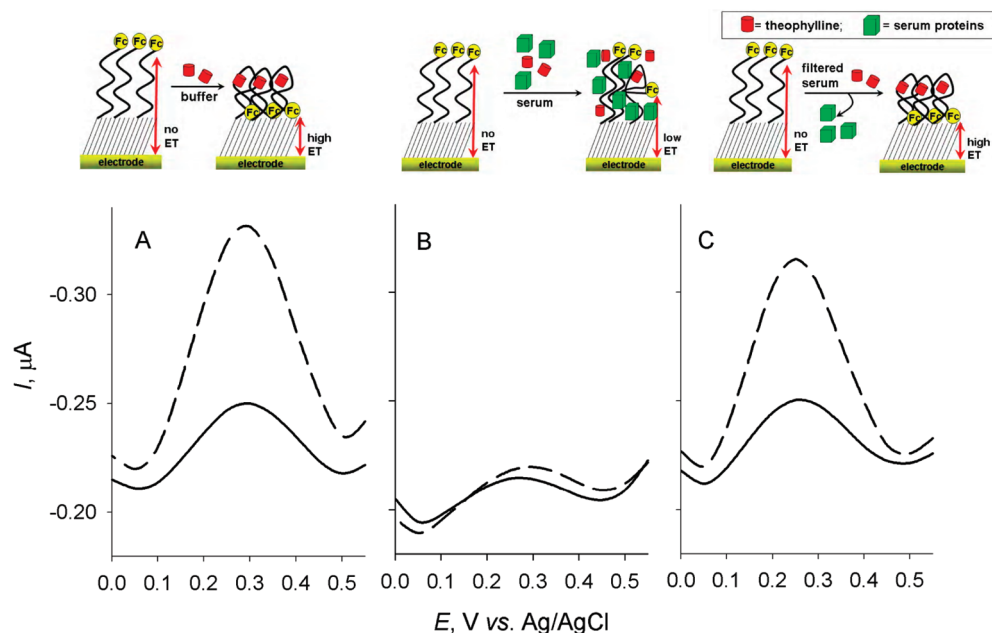(20) Ferapontova, E. E. *J. Electroanal. Chem.* **1999**, *476*, 37–45.

Figure 4. Representative DPVs recorded for the electrochemical RNA aptamer-based biosensor before (solid line) and after reaction with 40 μM theophylline (saturated conditions,[2] dashed line) in the HEPES buffer after measurements in buffer (A), in serum (B), and in filtered serum (C) solutions. The pulse amplitude is 50 mV, the sampling time is 50 ms, and the effective scan rate is 10 mV s^{-1}. The RNA surface coverage, calculated from the Fc signal in CVs, was 17.7 pmol cm^{-2}. (Insets) Schematic representation of the electrochemical RNA-aptamer-based biosensor for theophylline, operating in buffer, serum, and filtered serum solutions.
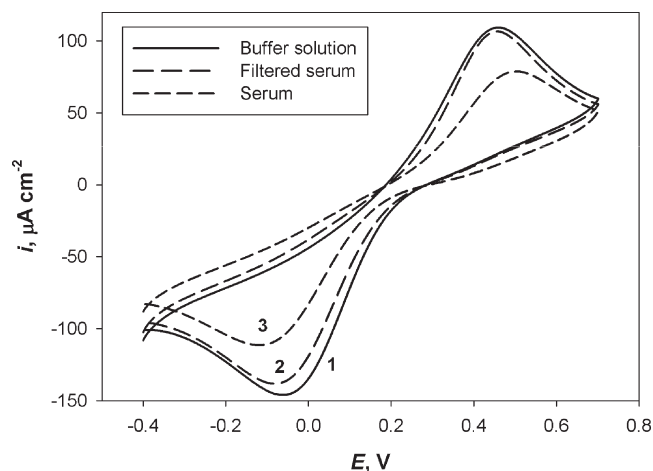

Figure 5. Representative CVs of the aptamer-modified electrode in 5 mM K$_3$Fe(CN)$_6$ and 1 mM theophylline in the HEPES buffer (1), filtered serum (2), and nonfiltered serum (3) solutions. The scan rate is 50 mV s^{-1}.

Figure 6. Impedance spectra of the aptamer-modified electrode in 5 mM K$_3$Fe(CN)$_6$ and 1 mM theophylline (saturated conditions) in the HEPES buffer (1), filtered serum (2), and nonfiltered serum (3) solutions. All other conditions are the same as in Figure 2. Lines correspond to the theoretical fitting. The RNA surface coverage, calculated from the Fc signal in CVs, was 31.7 pmol cm^{-2}.

calibration versus theophylline concentration) of the observed phenomena is not straightforward, having a complex dependence on the aptamer surface coverage and theophylline concentration, these kinds of changes in the properties of the aptamer layers deserve future detailed investigation.

To summarize, general variations of the ferricyanide ET reaction at the aptamer-modified electrodes with the transfer of electrodes from the pure HEPES buffer to serum solutions follow the same pattern as in the case of bare gold or SAM-modified electrodes and evidence the detrimental effect of the serum proteins on the ET kinetics (Tables 1 and 2). Electrode modifications strongly affect the rate of the ferricyanide ET reaction: compared to bare gold electrodes, lower k_o values and their less pronounced variations are observed at electrodes modified with mercaptohexanol SAMs or bulky, negatively charged aptamer molecules. For the
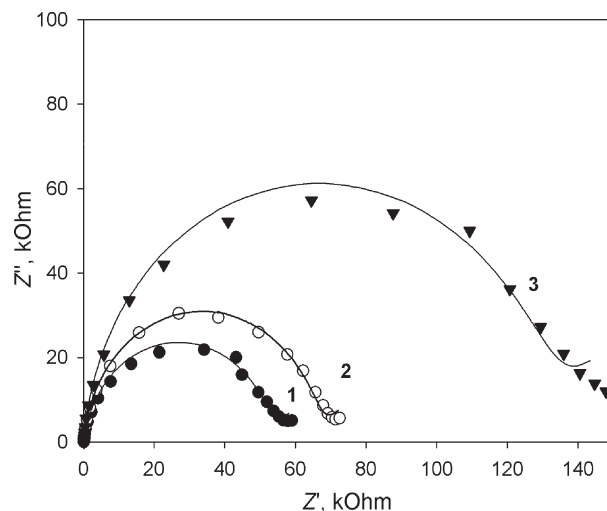
aptamer-modified electrodes, the interfacial capacitance approached that characteristic of the protein-blocked mercaptohexanol-modified surfaces, and further adsorption of serum proteins had a very small effect on the shape of the capacitance−potential curve but a significant effect on the ferricyanide permeation/interfacial ET properties (Table 2). Thus, the inhibition effect of serum on the aptamer biosensor function may be connected both with the effect of serum proteins on the folding properties of the aptamer molecules and with protein blocking of the surface. At present, it is difficult to separate these two interfering effects unambiguously.

In conclusion, the use of the nucleic acid beacon architectures and the Fc redox probes for the development of

Table 2. Kinetic Parameters for the Electrochemical Reaction of 5 mM K$_3$Fe(CN)$_6$ at the Aptamer-Modified Electrodes in the Buffer, Filtered Serum, and Nonfiltered Serum Solutions Derived from the Data in Figures 5 and 6

	unfolded aptamer, pure buffer	unfolded aptamer, filtered serum	unfolded aptamer, serum	with 1 mM theophylline, pure buffer,	with 1 mM theophylline, filtered serum	with 1 mM theophylline, serum
ΔE in CV, mV	447 ± 4	481 ± 3	540 ± 3	476 ± 3	498 ± 7	568 ± 8
R_{ct}, kΩ	46.1	58.9	88.4	52.4	65.8	129.2
k_o, cm s^{-1}	0.016×10^{-3}	0.012×10^{-3}	0.008×10^{-3}	0.014×10^{-3}	0.011×10^{-3}	0.0056×10^{-3}

biosensors capable of efficient operation in serum represents a certain challenge. Serum proteins affect the interfacial electrode/solution properties and the nucleic acid beacon switching characteristics, which results in poor biosensor response in serum; serum filtration improves the biosensor operational characteristics. Thus, for future in vivo applications of similar beacon architectures, the use of redox probes with more negative than Fc redox potentials[10,17] or the application of special on-electrode membranes should be considered.

Supporting Information Available: Details of the experimental procedures and CVs and EIS for bare gold electrodes and unfolded aptamer-modified electrodes. This material is available free of charge via the Internet at http://pubs.acs.org.

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