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Label-free electrochemical aptasensor for the detection of lysozyme

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ABSTRACT

This work reports the advantages of a label free electrochemical aptasensor for the detection of lysozyme. The biorecognition platform was obtained by the adsorption of the aptamer on the surface of a carbon paste electrode (CPE) previously blocked with mouse immunoglobulin under controlled-potential conditions. The recognition event was detected from the decrease in the guanine and adenine electro-oxidation signals produced as a consequence of the molecular interaction between the aptamer and lysozyme. The biosensing platform demonstrated to be highly selective even in the presence of large excess (9-fold) of bovine serum albumin, cytochrome C and myoglobin. The reproducibility for 10 repetitive determinations of 10.0 mg L^{-1} lysozyme solution was 5.1% and 6.8% for guanine and adenine electro-oxidation signals, respectively. The detection limits of the aptasensor were 36.0 nmol L^{-1} (if considering guanine signal) and 18.0 nmol L^{-1} (if taking adenine oxidation current). This new sensing approach represents an interesting and promising alternative for the electrochemical quantification of lysozyme.

1. Introduction

Since its discovery in 1990 [1–3], aptamers have demonstrated important advantages in the biosensing field, especially for the development of devices that allow the detection of proteins, toxins, peptides and neurotransmitters [4–9]. Aptamers are artificial single-stranded nucleic acids ligands, that present unique properties such as relatively easy production, multiple possibilities of modification, specific binding and high stability. These properties have made them very attractive recognition elements for a wide range of bioassays [4,5,10,11], and strong competitors of antibodies in the development of affinity biosensors.

The first aptamer-based biosensor has been proposed in 1998 by Ellington and co-workers [12] for the detection of thrombin and it has been based on the use of an aptamer labeled with a fluorescent marker. Aptamers modified with enzymes [13,14], fluorophores [15,16] and metallic nanoparticles [17,18] have been used as alternative to enhance the sensitivity of the determinations.

Different transduction modes have been reported for the development of aptasensors. Among them, Quartz Crystal Microbalance (QCM) has been proposed for the investigation of protein–nucleic acid and protein–aptamer interactions [19–21]. A comparative work using QCM and Surface Plasmon Resonance (SPR) for the transduction of the bioaffinity event has been also described [22]. Ostatná et al. [23] have proposed a sensitive and selective SPR-aptasensor for the quantification of thrombin [23]. A fiber-optic evanescent aptasensor for the detection of trinitrotoluene at the ppb level has been developed by Ehrentreich-Förster et al. [24]. Fourier Transform Infrared Attenuated Total Reflection (FTIR-ATR) has been used for the quantification of thrombin using an anti-thrombin DNA aptamer [25]. The advantages of using field effect transistor based on single-wall carbon nanotubes (SWCNT-FET) for the quantification of thrombin has been reported [26]. More recently, there has been an increasing interest for the development of electrochemical aptasensors for the detection of proteins [19,27–37] and small molecules as adenosine monophosphate, cocaine and potassium [38–41]. The electrochemical detection of thrombin and lysozyme using quantum dots has also been proposed [36]. Electrochemical impedance spectroscopy (EIS) has been used in several devices for the development of label-free aptasensors [28–31]. A highly sensitive thrombin quantification by employing EIS in combination with gold nanoparticles has been reported [42,43]. Numnuam et al. [44] have proposed the thrombin detection through the potentiometric quantification of Cd^{2+} using an anti-thrombin aptamer labeled with CdS quantum dots. Just few electrochemical aptasensors for the detection of lysozyme have been described [27,28,32].

In this work we propose an electrochemical aptasensor based on the use of carbon paste electrodes (CPE) modified with an anti-lysozyme aptamer for the direct detection of lysozyme without using electroactive markers. Lysozyme, also called muramidase or peptidoglycan *N*-acetylmuramoyl-hydrolase (EC 3.2.1.17), is an ubiquitous enzyme widely distributed in diverse organisms such as bacteria, bacteriophages, fungi, plants and animals. It catalyzes

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in vivo the hydrolysis of the β(1–4) glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine alternating sugar residues in the bacterial peptidoglycan and causes bacterial cell lysis. Its primary sequence contains 129 amino acid residues. The molecular weight is 14,351 Da and its isoelectric point is 11.0 [45,46]. This enzyme works as a natural inner body antibiotic because it possesses lytic activity against the polysaccharide wall of bacteria. Lysozyme also exhibits antiviral activity and could be a potential marker for rheumatoid arthritis [47–49].

Due to its relatively small size lysozyme can be taken as a model analyte for the development of aptamer-based platforms for the detection of proteins [46]. In this work, we propose the use of a lysozyme aptasensor as an innovative strategy for the quantification of lysozyme based on the changes in guanine and adenine oxidation signals once the protein was bound to the aptamer immobilized at a graphite paste electrode (CPE) previously blocked with mouse immunoglobulin G. The influence of the aptasensor preparation conditions on the analytical performance of the resulting electrode is also discussed.

2. Experimental

2.1. Materials

2.1.1. Oligonucleotides sequences

The synthetic sequence of the DNA-anti-lysozyme aptamer designed by Ellington et al. [50] (5′-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG-3′), and the scrambled control sequence (5′-AAG ATG CTG AAA TAG TGC GCA CTT AAG TCT CAA TGA ATG CGT-3′) were purchased from Invitrogen Argentina S.A. The anti-lysozyme aptamer was treated before using by heating at 70 °C for 3 min and allowed to slowly cool down at room temperature. Such heating and cooling steps are essential for maintaining the structural capabilities of the aptamer for the target capture [50]. The purified scrambled DNA sequence was treated following the same protocol. Lysozyme from chicken egg white, purified mouse immunoglobulin G (IgG), bovine serum albumin (BSA), cytochrome C (cyt C) and myoglobin (myo) from equine heart were obtained from Sigma–Aldrich. Other chemicals were reagent grade and used without further purification.

Ultrapure water (ρ = 18.2 MΩ cm) from a Millipore-MilliQ system was used for preparing all the solutions.

2.2. Apparatus

The electrochemical measurements were performed with an EPSILON potentiostat (BAS). The three electrodes system consisted of a carbon paste (CPE), a platinum wire and an Ag/AgCl, 3.0 mol L^{-1} NaCl (BAS, Model RE-5B) as working, counter and reference electrodes, respectively. All potentials are referred to the latter. The electrodes were inserted into the cell (BAS, Model MF-1084) through holes in its Teflon cover.

2.3. Aptasensor preparation conditions and biosensing procedure

2.3.1. Preparation of the aptamer-based biorecognition platform

2.3.1.1. Pretreatment of the CPE surface. The CPE was first pretreated by applying +1.700 V for 1.0 min in a 0.020 mol L^{-1} acetate buffer, pH 5.00.

2.3.1.2. Blockage of the CPE surface. This blocking step avoids unspecific interactions and ensures the efficient recognition of the target protein. In this case, a layer of mouse immunoglobulin G (IgG) was immobilized before adsorbing the DNA-aptamer. This molecule is widely used in immunoassay methodologies as blocking molecule to prevent the non-specific adsorption of the target antigen (protein) [57,58].

After a careful rinsing with 0.100 mol L^{-1} phosphate buffer solution pH 7.30, the pretreated CPE was immersed in a 0.100 M saline phosphate buffer solution pH 7.30 containing 100.0 mg L^{-1} IgG for 10.0 min at open circuit potential.

2.3.1.3. Aptamer immobilization. After a rinsing step with acetate buffer, the aptamer was immobilized at the CPE blocked with IgG at +0.500 V for 2.0 min from a solution containing 100.0 mg L^{-1} of anti-lysozyme aptamer (in 0.020 mol L^{-1} acetate buffer, pH 5.00). Finally, the electrode was carefully rinsed with acetate buffer solution for 10.0 s.

2.3.2. Lysozyme–aptamer association step

The CPE blocked with IgG and modified with the aptamer was immersed in lysozyme solutions of different concentration (from 0.0 to 100.0 mg L^{-1}, prepared in a 0.100 mol L^{-1} phosphate buffer solution pH 7.30) at open circuit potential for 10.0 min.

2.3.3. Electrochemical transduction

The aptasensor containing the protein was rinsed with acetate buffer solution for 10.0 s and then it was placed in a 0.020 mol L^{-1} acetate buffer solution pH 5.00. The transduction was performed by Square Wave Voltammetry (SWV) between −0.200 V and 1.500 V with a frequency of 25 Hz, amplitude of 0.025 V and a step potential of 0.010 V. The same procedure was followed when the target protein was captured in the presence of interferents (bovine serum albumin, cytochrome C and myoglobin). All measurements were conducted at room temperature.

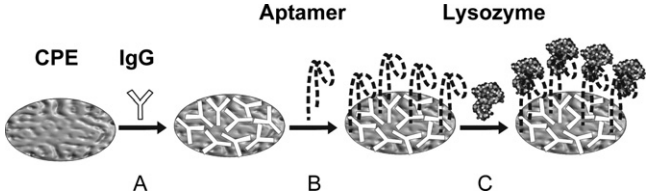
Scheme 1 shows the different steps during the preparation/operation of the aptasensor.

3. Results and discussion

3.1. Electrochemical behavior of carbon paste electrodes modified with the anti-lysozyme aptamer

Fig. 1 shows the electrochemical response of pretreated CPE modified with the anti-lysozyme–aptamer by immobilization for 2.0 min at 0.500 V from a 0.020 mol L^{-1} acetate buffer solution pH 5.00 containing 100.0 mg L^{-1} aptamer. Two well-defined oxidation processes are observed at +1.030 V and +1.330 V, attributed to the oxidation of guanine (a) and adenine (b), respectively [52–55].

The dependence of the peak currents for guanine and adenine oxidation on the accumulation time of the anti-lysozyme aptamer at 0.500 V was evaluated (not shown). For 10.0 mg L^{-1} aptamer solution the current increased slightly from 30.0 s to 5.0 min adsorption suggesting a fast adsorption of the aptamer under these conditions [56]. When performing the same study using a 100.0 mg L^{-1} DNA-aptamer solution, almost no changes were observed in the guanine and adenine oxidation currents for the different accumulation times. Consequently, in order to make sure



Scheme 1. Preparation steps of an anti-lysozyme aptasensor: (A) carbon paste electrode (CPE)-blocking step, (B) aptamer immobilization, and (C) lysozyme binding event.

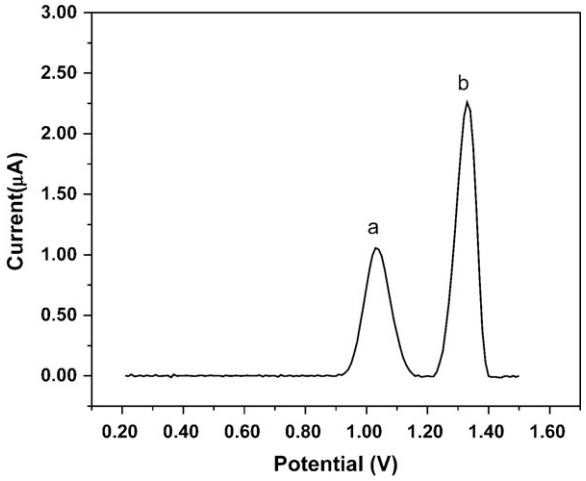


Fig. 1. Electrochemical response of the anti-lysozyme aptamer immobilized on CPE. CPE pretreatment: +1.700 V for 1.0 min in 0.020 mol L^{-1} acetate buffer, pH 5.00; accumulation conditions: +0.500 V for 2.0 min in the 100.0 mg L^{-1} aptamer solution prepared in 0.020 mol L^{-1} acetate buffer, pH 5.00; transduction step: square wave voltammograms were recorded between −0.200 V and +1.500 V, frequency: 25 Hz, amplitude: 0.025 V and step potential: 0.010 V. Supporting electrolyte: 0.020 mol L^{-1} acetate buffer solution, pH 5.00.

that the surface was completely covered and to avoid non-specific interactions, an immobilization time of 2.0 min from a 100.0 mg L^{-1} aptamer solution was selected for further experiments.

3.2. Lysozyme aptasensor

3.2.1. Analytical performance of the lysozyme aptasensor

The guanine and adenine oxidation signals obtained at CPE blocked with IgG and modified with the aptamer were compared with those obtained at the unblocked CPE (Section 3.1). The presence of the blocking agent produced a decrease of 23.0% and 35.0% in the guanine and adenine oxidation signals, respectively. This decrease is attributable to a lower accessibility for electro-oxidation of the electroactive residues of the aptamer. In the case of the blocked surface, a new small peak appears at around 0.80 V as a result of the oxidation of electroactive amino acids of IgG.

Fig. 2A shows SWV recordings for increasing concentrations of lysozyme from 0.0 to 30.0 mg L^{-1} at the aptamer-IgG-CPE. As the concentration of lysozyme increases, there is a decrease in the guanine and adenine oxidation signals as a consequence of the efficient capture of the protein by the aptasensor. The structural changes produced in the aptamer due to the interaction with lysozyme pockets would be responsible for the decrease in the oxidation signals of the electroactive residues. Aptamers preferentially interacts with pockets or clefts on proteins such as active and allosteric sites and the binding process involves the whole aptamer molecule and produces important structural changes in the aptamer and in the protein. For instance, the inhibition of the enzymatic action of the protein bound to the aptamer has been strongly demonstrated [51].

Fig. 2B displays calibration plots obtained from the electro-oxidation signal for guanine (I) and adenine (II). There is a linear relationship between lysozyme concentration and guanine (I) or adenine (II) oxidation signals up to 20.0 mg L^{-1} of lysozyme. The sensitivities for lysozyme are (-0.049 ± 0.003) μA mg^{-1} L and (-0.025 ± 0.002) μA mg^{-1} L obtained from adenine and guanine oxidation signals, respectively. The saturation was reached for lysozyme levels higher than 20.0 mg L^{-1} (Fig. 2B). The detection limits, taken as the ratio between 3.3 times the standard deviation of the blank solution and sensitivity of the calibration curve are 3.60×10^{-8} mol L^{-1} (0.52 mg L^{-1}) and 1.80×10^{-8} mol L^{-1}

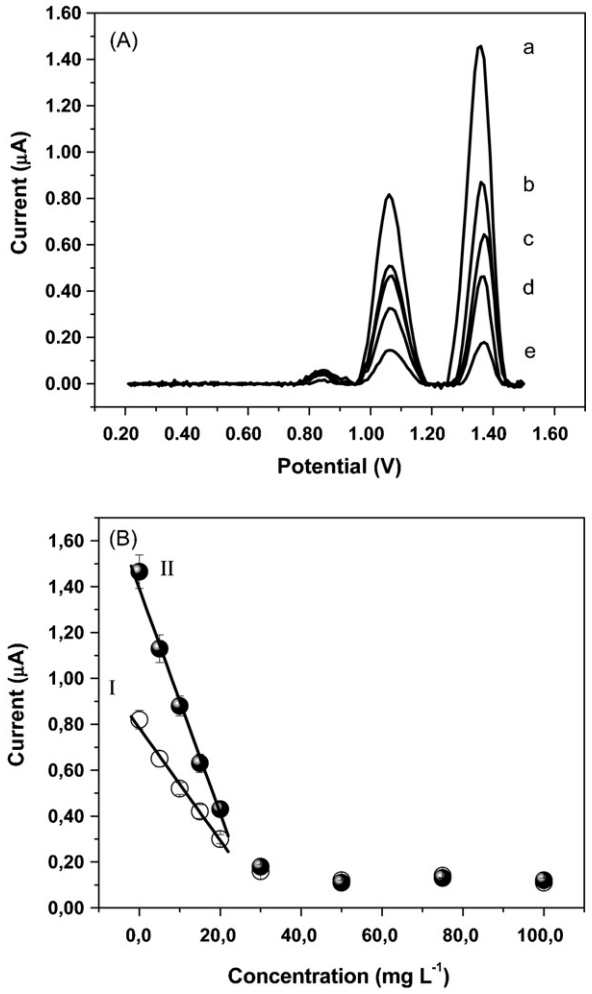


Fig. 2. (A) Square wave voltammograms for increasing concentration of lysozyme solutions: 0.0 mg L^{-1} (a), 10.0 mg L^{-1} (b), 15.0 mg L^{-1} (c) 20.0 mg L^{-1} (d) and 30.0 mg L^{-1} (e) obtained at the anti-lysozyme aptamer modified-Ig G blocked-CPE. CPE pretreatment: +1.700 V for 1.0 min in 0.020 mol L^{-1} acetate buffer, pH 5.00; blocking step: 10.0 min at open circuit potential in a 100.0 mg L^{-1} IgG in phosphate buffer solution 0.100 M pH 7.30; aptamer accumulation conditions: 2.0 min at +0.500 V in 0.020 mol L^{-1} acetate buffer, pH 5.00 containing 100.0 mg L^{-1} aptamer; capture step: in buffer phosphate solution 0.100 mol L^{-1} pH 7.30 containing lysozyme for 10.0 min at open circuit potential. Transduction step: square wave voltammograms: initial potential: −0.200 V, final potential: +1.500 V, frequency: 25 Hz, amplitude: 0.025 V and step potential: 0.010 V. Supporting electrolyte: 0.020 mol L^{-1} acetate buffer solution, pH 5.00. (B) Calibration plots for lysozyme. I and II Correspond to guanine and adenine oxidation signals, respectively.

(0.21 mg L^{-1}) depending if considering guanine or adenine oxidation signals, respectively. The quantification limits, taken as the ratio between 10 times the standard deviation of the blank solution and sensitivity of the calibration curve, are 1.10×10^{-7} mol L^{-1}, taken from guanine oxidation signal, and 0.60×10^{-7} mol L^{-1}, taken from the adenine oxidation response. The detection limits are one order of magnitude lower than that for the optical aptamer-based sensor array proposed by Ellington and co-workers [50] and comparable to those reported in the literature for electrochemical aptasensors (which goes from 0.2 μg mL^{-1} to 0.5 μg mL^{-1}) [27,28,32]. Therefore, the decrease in the oxidation signals for adenine and guanine observed when increasing lysozyme concentrations provides an excellent, easy and fast way to quantify lysozyme.

The platform containing the aptamer shows good reproducibility. Ten repetitive measurements obtained in the presence of

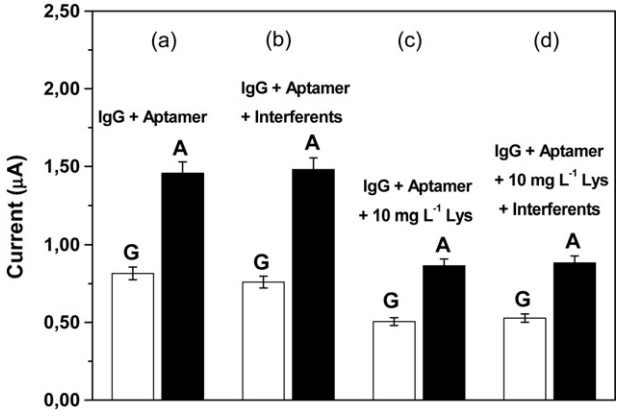


Fig. 3. Evaluation of the aptasensor selectivity. Electrochemical oxidation signals for anti-lysozyme aptamer modified-Ig G blocked-CPE prepared according to the conditions stated in Fig. 2 in different solutions: (a) in the absence of lysozyme and interferents, (b) in the presence of 30.0 mg L^{-1} BSA + 30.0 mg L^{-1} cytochrome C (cyt C) + 30.0 mg L^{-1} myoglobin (myo), (c) in the presence of 10.0 mg L^{-1} lysozyme, and (d) in the presence of 10.0 mg L^{-1} lysozyme + 30.0 mg L^{-1} BSA + 30.0 mg L^{-1} cyt C + 30.0 mg L^{-1} myo (d). Other conditions as in Fig. 2.

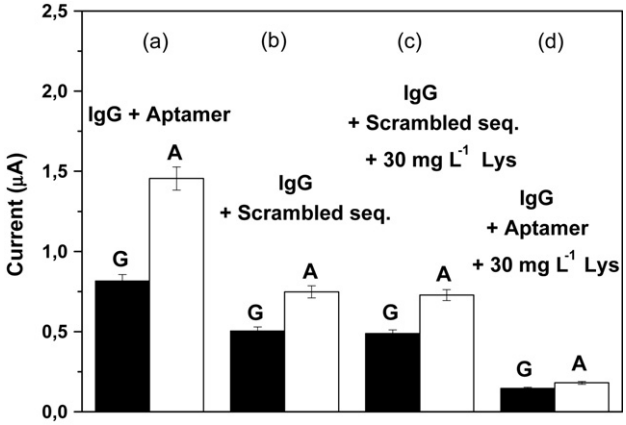


Fig. 4. Guanine and adenine oxidation signals obtained at an anti-lysozyme aptamer modified-Ig G blocked-CPE (a and d) and at a scrambled sequence modified-Ig G blocked-CPE (b and c) in the absense (a and b), and presence (c and d) of 30.0 mg L^{-1} lysozyme. Other conditions as in Fig. 2.

10.0 mg L^{-1} lysozyme gave R.S.D of 5.0% and 6.8% for guanine and adenine redox signals, respectively.

To analyze the selectivity of the proposed protocol, the aptamer sensor was challenged with a large (9-fold) excess of common interferents such as bovine serum albumin (BSA), cytochrome C (cyt C) and myoglobin (myo). Fig. 3 shows the guanine and adenine electro-oxidation currents obtained at the aptasensor under different experimental conditions. The signal obtained in supporting electrolyte (a), was taken as 100.0%. The presence of 30.0 mg L^{-1} BSA, cyt C and myo (in the absence of lysozyme), (b) has a negligible influence on the response of the electrode, with interferences of 3.2% and 1.6% for guanine and adenine oxidation signals, respectively. When the aptasensor is challenged with 10.0 mg L^{-1} lysozyme (c) a clear decrease of the guanine and adenine oxidation signals is observed, as expected according to Fig. 2. If 10.0 mg L^{-1} lysozyme is determined in the presence of 30.0 mg L^{-1} BSA, cyt C and myo (d), the response remains almost constant. In fact, signals of (0.51 ± 0.02) μA and (0.53 ± 0.03) μA are attained for guanine oxidation signals; and (0.87 ± 0.04) μA and (0.88 ± 0.04) μA are obtained for the oxidation of adenine, in the presence and absence of interferents, respectively (c vs. d). These results are strong proof of the highly selective detection of lysozyme. No cross-reactions with interferent proteins were found even when they have very close structural similarities with lysozyme as in the case of cytochrome C (MW: 12 kDa and pI ~ 11) [59,60].

3.2.2. *Specificity of the anti-lysozyme aptamer sequence*

The specificity of the aptasensor was also evaluated from the aptamer sequence used for performing the biorecognition event by employing a DNA-scrambled sequence (disordered aptamer sequence) (Fig. 4). The oxidation signals for adenine and guanine are smaller than those obtained when using the right sequence (a). Even when the bases are the same, their particular array in the scrambled sequence leads to a structure with different exposure of the electroactive bases and makes more difficult the electron transfer with the electrode surface. A decrease of 3.2% and 2.7% for guanine and adenine oxidation signal, respectively, was obtained in the presence of 30.0 mg L^{-1} lysozyme at the electrode containing the scrambled sequence (c). This change in the signal is largely different from that found when using the right aptamer sequence where a reduction of 82.1% and 87.6% for guanine and adenine oxidation signals, respectively, is obtained (d). These results suggest that the unique structure of the anti-lysozyme aptamer sequence is absolutely required for the highly specific lysozyme binding.

4. Conclusions

This work proposes an elegant label-free electrochemical aptasensor for lysozyme detection based on the use of an anti-lysozyme aptamer modified CPE. The substantial drop in guanine and adenine electro-oxidation signals obtained after the recognition event takes place has allowed us to obtain a highly sensitive (detection at nM levels) and selective (no interference of BSA, cytochrome C and myoglobin) aptasensor for lysozyme quantification.

Compared to EIS, the most typical electrochemical transduction mode in aptasensors, the voltammetric transduction mode represents a faster detection and results-processing alternative. In addition, the use of CPE as electrode material offers multiples advantages such as easy surface renewal, fast conditioning and simple aptamer immobilization at variance with other electrode materials such as gold, glassy carbon or indium tin oxide, that present special requirements for surface preparation and the immobilization of the aptamer is time consuming. These features make the aptamer-IgG-CPE proposed here, an excellent biosensing platform for the development of a highly sensitive and selective lysozyme aptasensor with detection limits lower than those reached with optical aptasensors and comparable to those obtained by other reported electrochemical sensors.

This original and novel method of sensing aptamer–protein interactions at the transducer surface opens new promising routes for future applications in proteomics and diagnostics. The concept can be widely extended to the aptamer-based detection of a large range of proteins and small molecules taking advantages of several specific aptamer structures identified lately by SELEX.

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