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# Development of an Electrochemical Aptasensor for the Detection of Human Osteopontin

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**Abstract**

Electrochemical aptasensors, an emerging technology, enables the detection of protein biomarkers, which may be indicative of tumour activity. Osteopontin is a protein present in body fluids, being a possible biomarker since its overexpression has been related with breast cancer progression. An RNA aptamer, described in the literature, with affinity for human osteopontin, was synthetized, immobilized in a microelectrode gold surface and used for development electrochemical aptasensor for human OPN detection in standard solutions. Cyclic voltammetry results showed that this aptasensor allowed detecting human osteopontin with a detection limit of 8 nM, showing a satisfactory selectivity towards the target in the presence of others proteins, except for thrombin.
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## 1. Introduction

Biomarkers are produced by normal and tumour cells and can be indicative of tumour activity when detected in high amounts in the blood or other body fluids [1,2]. Osteopontin (OPN) is a possible protein biomarker because it is found in all body fluids and its overexpression has been related with breast cancer evolution and metastasis [3,4]. A simple and sensitive electrochemical RNA aptasensor with high affinity for human OPN (rhOPN) was developed to enable the detection of this non-invasive protein biomarker in real samples and ultimately to be used for cancer prognosis. This study presents preliminary results concerning aptasensor's performance for rhOPN detection in standard samples

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as well as selectivity towards others proteins (bovine serum albumin, lysozyme, recombinant bovine OPN and thrombin).

## 2. Materials and methods

### *2.1. Material*

Recombinant human OPN (rhOPN), recombinant bovine OPN (rbOPN) and thrombin (THR) were purchased from R&D Systems. Bovine serum albumin (BSA), lysozyme, diethypyrocarbonate (DEPC), 3,3-dithiodipropionic acid (DPA), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), ethanolamine (ETA), sulfuric acid 99.999% and streptavidin were obtained from Sigma-Aldrich. Potassium hexacyanoferrate (III) $[K_3Fe(CN)_6]$ and potassium hexacyanoferrate (II) $[K_4Fe(CN)_6]$·were obtained from Acros Organics and potassium dihydrogen phosphate ($KH_2PO_4$) from Merck. Sodium chloride (NaCl), potassium chloride (KCl) and sodium hydrogen phosphate ($Na_2HPO_4$) were acquired from Panreac.

All chemicals were of analytical grade and used as received. Deionized water (18.2 MΩ) purified by a milli-Q$^{TM}$ system (Millipore) was used throughout the experiment for aqueous solutions preparation.

Phosphate buffer saline (PBS) was prepared to contain 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$ and 1.47 mM $KH_2PO_4$, with adjusted pH=7.4. The redox probe was always freshly prepared in order to obtain a solution with concentration of 5 mM of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (1:1) and 10 mM of KCl in 100 mL of PBS, at pH 7.4.

Stock solutions of 200 mM of EDAC, 100 mM of NHS, as well as the stock solution of 1mg/ml septravidin in PBS (pH 7.4) were prepared and stored -20ºC before use. Stock solutions of 200 nM of DPA and 100 mM of ethanolamine were prepared and stored at 4ºC. Stock solutions of protein were prepared according to manufacturer specifications and stored at -20ºC. The protein working solutions were prepared and diluted to desired concentrations with phosphate buffer and stored at 4ºC before use.

### *2.2. RNA aptamer*

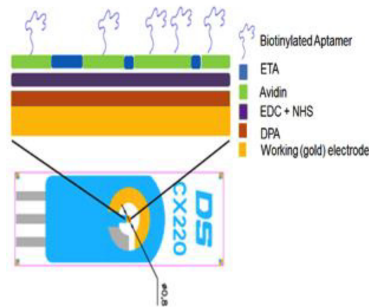
A RNA aptamer against rhOPN has been isolated by SELEX process as described by Mi *et al*. [5]. The sequence of the biotinylated RNA aptamer that was synthesized by Integrated DNA Technologies (Belgium) is as follows: 5'-Biotin- CGGCCACAGAAUGAAAAACCUCAUCGAUGUUGCAUAGUUG-3'.

Stock solutions of the synthetic oligonucleotides were prepared with deionized water containing 1% of DEPC (v/v) to remove the interference of RNase. The working RNA aptamer solution was prepared every day by dilution to the desired concentration (4 nM) using fresh PBS.

### *2.3. Immobilization of the DNA aptamer on a gold surface*

To construct an electrochemical-based aptamer biosensor, the biotinylated RNA aptamer was immobilized on a streptavidin-modified gold electrode (Fig. 1). The detailed procedure used was as follows: firstly, the working gold electrode surface was cleaned successively with three solutions (0.5 M $H_2SO_4$, 0.01 M KCl/0.1 M $H_2SO_4$ and 0.05 M $H_2SO_4$) under electric potential in the range of -0.3 to 1.5 V, and with a scan rate of 100 mV/s. Etching with $[Fe(CN)_6]^{-3/-4}$ solution was carried out to enhance the uniformity of the working gold surface. Next, the self-assembled monolayer (SAM) was spontaneously formed through an incubation of 200 mM of DPA for 30 min. After washing with deionized water, the working electrode was treated, during 1 hour, with same volumes of 100 mM of EDC and 1 mM of NHS to activate the carboxyl groups, so that they can bind with the amino terminal of streptavidin. Afterwards, the working electrode incubated overnight with streptavidin solution at 4ºC. The working electrode was then exposed to ethanolamine (100 mM, pH 8.5) for 20 min to block any remaining activated -COOH groups. Finally, the biotinylated RNA aptamer in PBS buffer (pH 7.4) was attached to the modified gold surface using the streptavidin–biotin interaction for 40 min. All reactions were carried out at room temperature.

Fig. 1. Schematic illustration of a RNA aptamer immobilized onto an Au-SPE through streptavidin-biotin interaction.




### 2.4. Cyclic voltammetry analysis

Electrochemical analysis was performed at room temperature using a Potentiostat-Galvanostat device (PG580, Uniscan Instruments). The screen-printed electrodes chip (DropSens, S.L., Spain) used had a gold working electrode with a diameter of 0.8 mm, as well as a silver reference electrode and gold counter electrode.

Standard solutions of rhOPN in PBS buffer (between 25 nM and 1601.6 nM) were dropped on the working electrode and incubated for 1 hour. After washing the electrode with distilled water, 60µL of $[Fe(CN)_6]^{-3/-4}$ solution (redox probe) was dropped on the electrodes chip until all three electrodes were immersed. Then, cyclic voltammetry was performed and a electrochemical signal was generated due to the aptamer–target molecule interaction in the presence of $[Fe(CN)_6]^{-3/-4}$, under potential range of -0.5 to 0.6 V and with a scan rate of 50 mV/s.

Sensitivity of the RNA aptamer to other proteins was also assessed by means of the electrochemical signal generated from the interaction between the aptamer and a solution of each protein (BSA, LYS, rbOPN or THR; 200 nM ), under similar experimental conditions. Data treatment

The current decrease (relative current change, %) was calculated using the difference between the current values of the voltammogram oxidation peak recorded after analysing the protein solution and that recorded after aptamer immobilization divided by this last one, using the equation: $\Delta I = (I_0 - I_1)/I_0 \times 100$; where $\Delta I$ is relative current change; $I_0$ and $I_1$ represents the current (in mA) before and after the sample treatment, respectively.

## 3. Results

The electrochemical signal generated, during the cyclic voltammetry assays, for the aptamer–rhOPN interaction, recorded at different rhOPN concentrations, was monitored in the presence of $[Fe(CN)_6]^{-3/-4}$ as a redox probe. The cyclic voltammograms show a current decrease as a consequence of the increase of rhOPN concentration, thus indicating a signal-off sensing mechanism. Fig. 2 illustrates the performance of the aptasensor (relative current change, %) at different rhOPN concentrations. Preliminary results suggest a linear relationship at low concentration levels of rhOPN (<100 nM) with detection and quantification limits of 7.9 and 23.9 nM, respectively. Furthermore, and contrary to what was reported [5], the aptasensor was found to be not specific for rhOPN (Fig.3) since the current changes observed in the experiments conducted with other proteins (BSA, LYS, rbOPN and TRH) couldn't be neglected as compared to the target, especially for THR.

## 4. Conclusions

This preliminary work described suggests that the proposed electrochemical aptasensor is a simple and sensitive tool with satisfactory performance for the detection of rhOPN although not totally specific for this target protein. So, further studies are needed, namely in selecting new aptamers against rhOPN, in order to improve its detection performance and selectivity.

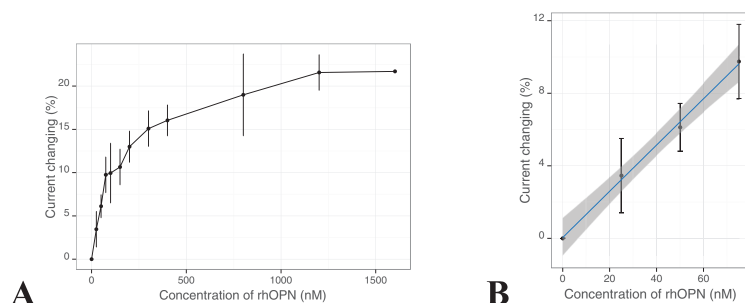



Fig. 2. Electrochemical aptasensor sensitivity analysis of hOPN using an aptamer-immobilized Au-SPE. The relative current change increase is proportional to the increase of rhOPN concentration in the: A) dynamic interval ranging from 0 nM to 1601.6 nM; B) interval ranging from 0 nM to 100 nM. Error bars indicate the relative standard deviation of three independent experiments.

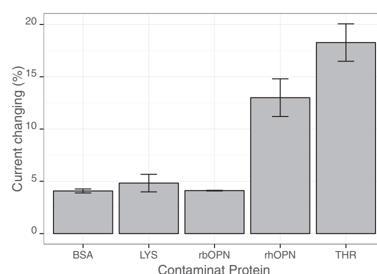



Fig. 3. Electrochemical aptasensor specificity response to different proteins at the same concentration (200 nM): BSA – bovine serum albumin, LYS – lysozyme, rbOPN – bovine osteopontin, rhOPN – human osteopontin and THR – thrombin. Error bars indicate the relative standard deviation of three independent experiments.

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