Label-free impedimetric aptasensor based on epoxy-graphite electrode for the recognition of cytochrome c

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Abstract

In this work, we present a label -free impedimetric aptasensor for the recognition of cytochrome c at a pM concentration level based on a epoxy-graphite composite electrode. The technique employed for the detection of the protein cytochrome c is electrochemical impedance spectroscopy (EIS).Detection occurs when the protein interacts with the immobilized aptamer on the aptasensor. The aptamer immobilization technique is based on its wet physical adsorption onto the electrode surface, which assures a simple, ready-to-use preparation of the biosensing platform. The work first optimizes concentration of immobilized aptamer, followed by blocking agent to avoid non specific interactions, and finally performs the label-free detection of cytochrome c. Amount of protein is quantified by the observed increase of the electron-transfer resistance, determined employing EIS and the $[Fe(CN)_6]^{3-,4-}$ redox marker. Results demostrate that the aptasensor has a good detection range for cytochrome c between 50 pM and 50 nM, as well as a high sensitivity with a low detection limit of 63.2 pM, well below levels of this protein in serum. Cross response of the developed aptamer biosensor versus potential interfering proteins also present in human serum has been fully characterized.

Keywords: cytochrome c, aptasensor, impedance, aptamer, label-free

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1.Introduction

Cytochrome c is primarily known as an electron-carrying mitochondrial protein. The transition of cytochrome c between the ferrous and ferric states within the cell makes it an efficient biological electron-transporter whereas it plays a vital role in cellular oxidations in both plants and animals. It is generally regarded as a universal catalyst of respiration, forming an essential electron-bridge between the respirable substrates and oxygen. This protein has been identified as an important mediator in apoptotic pathways.The release of mitochondrial cytochrome c into the cytoplasm stimulates apoptosis, therefore it is used as an indicator of the apoptotic process in the cell [1]. For this reason, it is important to evaluate its concentration. Normal non-pathological level of this protein in human serum is around 2 nM [2] .

In recent years, there has been great interest in the development of aptasensors [3]. Aptasensors are biosensors that use aptamers as the biorecognition element. Aptamers are artificial DNA or RNA oligonucleotides selected *in vitro* which have the ability to bind to proteins, small molecules or even whole cells, recognizing their target with high affinity and specificity, often matching or even exceeding those of antibodies [4]. Furthermore, because of a marked reversibility, the recognition process can be reverted and is stable in broad terms, an important advantage in front of immunosensors. Among described cases in the literature, there are two different configurations of aptamers: linear and molecular beacon. Aptamers with a linear configuration maintain, in certain physicochemical conditions, a typical 3D shape conformation with a specific binding site for the target molecule. Obviously recognition employs host-guest principles [5]. On the other hand, aptamers with a molecular beacon configuration initially form a loop that changes conformation following binding to the analyte of interest [6]. Aptamers can be used in a wide range of applications, such as therapeutics [7], molecular switches, drug development, affinity chromatography [8] or biosensors [9] .

In biosensor field, different transduction techniques such as optical [10] , atomic force microscope [11], surface plasmon resonance [12], electrochemical [13] and piezoelectric [14] aptasensors have been reported. In the last years, among the different electrochemical techniques reported, the use of Electrochemical Impedance Spectroscopy (EIS) has grown among these studies [15]. EIS is a characterization technique that is based on applying an AC potential to an electrochemical cell and measuring the current that crosses through the cell [16]. Thus, this tool is very sensitive to changes of the interfacial properties of modified electrodes upon biorecognition events taking place at electrode

surfaces [17, 18]. Other important features presented by EIS are that it does not require any special reagent for the analysis, it has the capacity for label-free detection and is a cost-efficient technique. For all these reasons, EIS is becoming a popular electrochemical technique for numerous applications such as immuno [18], genosensing [19], enzyme activities [20] or studies of corrosion [21] or surface phenomena [22].

In this communication, we report a label-free impedimetric aptasensor for the direct recognition of cytochrome c. The transducer employed consists of a graphite-epoxy composite (GEC) electrode, of general use in our laboratories which has already been extensively studied and applied to amperometric, enzymatic, immuno and genosensing [23-25] assays. The uneven porous surface of the GEC electrode allows the immobilization of aptamers on its surface by simple wet physical adsorption. This surface can be renewed after each experiment by simply polishing with different abrasive papers. This type of sensor has been already used for impedimetric detection of DNA hybridization [26] and very recently for the first aptasensor [3]. The transduction principle used is based on the change of electron transfer resistance in the presence of the $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ redox marker, which can be measured by EIS [16]. The proposed aptasensor showed appropriate response behaviour values to determine cytochrome c in the picomolar range.

2. Experimental

2.1.Chemicals

Potassium dihydrogen phosphate, potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, sodium monophosphate and the target protein cytochrome c (Cytc), were purchased from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) 1.000 (PEG), sodium chloride and potassium chloride were purchased from Fluka (Buchs, Switzerland). All-solid-state electrodes (GECs) were prepared using 50 μm particle size graphite powder (Merck, Darmstadt, Germany) and Epotek H77 resin and its corresponding hardener (both from Epoxy Technology, Billerica, MA, USA). All reagents were analytical reagent grade. The DNA aptamer (AptCytc) used in this study had the following base sequence, extracted from the literature [2]:

5’AGTGTGAAATATCTAAACTAAATGTGGAGGGTGGGACGGGAAGAAGTTT ATTTTTCACACT-3’, was prepared by TIB-MOLBIOL (Berlin, Germany). Stock solutions of aptamer and cytochrome c were diluted with sterilized and deionised water, separated in fractions and stored at −20 °C until used.

All solutions were prepared using MilliQ water from MilliQ System (Millipore, Billerica, MA, USA). The buffer employed was PBS (187 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.76 mM KH_2PO_4, pH 7.0).

2.2. Apparatus

AC impedance measurements were performed with the ad of an Autolab PGStat 20 (Metrohm Autolab B.V, Utrecht, The Netherlands). FRA (Metrohm Autolab) software was used for data acquisition and control of the experiments. A three electrode configuration was used to perform the impedance measurements: a platinum-ring auxiliary electrode (Crison 52–67 1, Barcelona, Spain), an Ag/AgCl reference electrode and the constructed GEC as the working electrode. Temperature-controlled incubations were done using an Eppendorf Thermomixer 5436.

2.3. Preparation of Working Electrodes

The electrodes were prepared using a PVC tube body (6 mm i.d.) and a small copper disk soldered to the end of an electrical connector. The working surface is an epoxy-graphite conductive composite, formed by a mixture of graphite (20%) and epoxy resin (80%), deposited in the cavity of the plastic body [25]. The composite material was cured at 80 °C for 3 days. Before each use, the electrode surface was moistened with MilliQ water and then thoroughly smoothed with abrasive sandpaper and finally with alumina paper (polishing strips 301044-001, Orion) in order to obtain a reproducible electrochemical surface.

2.4. Procedure

The analytical procedure for biosensing consists of the immobilization of the aptamer onto the transducer surface using a wet physical adsorption procedure, the blocking to minimize non specific adsorption and the recognition of the protein by the aptamer. The scheme of the experimental procedure is represented in Figure 1, with the steps described in more detail below.

The first step consisted of aptamer immobilization onto the electrode surface.160 μL of aptamer solution in MilliQ water at the desired concentration was heated at 80–90 °C for 3 minutes to promote the loose conformation of the aptamer. Then, the solution was dipped in a bath of cold water and the electrode was immersed in it, where the adsorption took place for 15 minutes. This was followed by two washing steps using PBS buffer solution for 10 minutes, in order to remove unadsorbed aptamer.

After that, to minimize any possible nonspecific adsorption , the electrode was dipped in 160 μL of PEG 35 mM for 15 minutes. This was followed by two washing steps using PBS buffer solution for 10 minutes.

The last step is the recognition of cytochrome c by the immobilized aptamer. For this, the electrode was dipped in a solution with the desired concentration of cytochrome c. The incubation took place for 15 minutes. Then, the biosensor was washed twice with PBS buffer solution for 10 minutes.

2.5. Impedimetric measurements

Impedance experiments were carried out at an applied potential of 0.17V (vs. Ag/AgCl reference electrode), with a range of frequency of 50KHz-0.05Hz, an AC amplitude of 10 mV and a sampling rate of 10 points per decade above 66 Hz and 5 points per decade at the lower range. All measurements were performed in PBS buffer containing 0.01M $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture, used as a redox marker. In all cases impedance data were registered in the following order after each electrode successive modification: (1) bare electrode; (2) immobilization step; (3) Blocking step; (4) Biorecognition. The impedance spectra were plotted in the form of complex plane diagrams (Nyquist plots, imaginary, $-Z_i$, *vs.* real component, Z_r) and fitted to a theoretical curve corresponding to the equivalent circuit with Zview software (Scribner Associates Inc., USA). The equivalent circuit, shown in figure 2 , was formed by one resistor/ capacitor element in series with a resistance. The parameter R_1 corresponds to the resistance of the solution, R_2 is the charge transfer resistance (also called R_{ct}) between the solution and the electrode surface, whilst CPE is associated with the double-layer capacitance (attributable to the interface between the electrode surface and the solution). For all performed fittings, the chi-square goodness-of-fit test was thoroughly checked to verify calculations. In all cases, calculated values for each circuit remained in the range of 0.0003-0.15 much lower than the tabulated value for 50 degrees of freedom (67.505 at 95% confidence level). In this

work, we focused on the variation of change in resistance transfer (R_{ct}). In order to compare the results obtained from the different electrodes used, and to obtain independent and reproducible results, relative and normalized signals were needed [16]. Thus, the Δ_{ratio} value was defined according to the following equations:

$$\Delta_{ratio} = \Delta_s / \Delta_p \quad (1)$$

$$\Delta_s = R_{ct\ (AptCytc\text{-}Thr)} - R_{ct\ (electrode\text{-}buffer)} \quad (2)$$

$$\Delta_p = R_{ct\ (AptCytc)} - R_{ct\ (electrode\text{-}buffer)} \quad (3)$$

where $R_{ct(AptCytc\text{-}Thr)}$ was the electron transfer resistance value measured after incubation with the cytochrome c protein, $R_{ct\ (AptCytc)}$ was the electron transfer resistance value measured after aptamer immobilization on the electrode and $R_{ct\ (electrode\text{-}buffer)}$ was the electron transfer resistance of the blank electrode and buffer.

3. Results

3.1. Optimization of aptamer and PEG concentrations

The first step of the experiment was the optimization of the experimental concentrations of aptamer immobilized on the electrode and poly(ethylene glycol) (PEG) on the electrode surface by constructing its relative response curves.

The calibration curve was carried out by increasing amount of concentration of AptCytc used in the from 1 to 2,5 µM. The different concentrations were evaluated by the changes in the Δ_p. As can be seen in the figure 3, the Δ_p increased with the AptCytc concentration until a saturation value. This is due to the physical adsorption of the aptamer onto the electrode surface, which followed a Langmuir isotherm; in it, the variation of R_{ct} increased to reach a saturation value, chosen as the optimal concentration. The value selected for the aptasensor corresponded to a concentration of aptamer of 1.75 µM, as the compromise between the two extremes.

The use of blocking agent (PEG) on the electrode surface was introduced to minimize the non specific adsorption of other species, that would alter the observed impedimetric signal. Apart, these other species can potentially interact or disturb the binding of the Cyt c on the AptCytc in the further steps and thus must be avoided. For the optimization of

the PEG concentration we proceeded as above. PEG concentrations were increased from 20 until 70 mM and the electrochemical characteristics of the electrode were determined. As it can be observed in figure 4, the Δ_p increased up to a certain saturation value. The value selected as the optimal PEG concentration was 35 mM, corresponding to the intersection of the two extreme behaviors.

3.2. Detection of Cytochrome c

The aptamer of cytochrome c forms a linear single-strand oligonucleotide, in a sequence that recognizes the protein by a specific folding. During this folding, weak interactions between the aptamer and protein of the host-guest type are created, leading to the complex AptCytc-Cytc. One example of the obtained response after each biosensing step is shown in Figure 5. As can be seen in this figure, Rct increased after any modification of the electrode surface. This is due to the increased difficulty of the redox reaction of $[Fe(CN)_6]^{3-/4-}$ to take place, due to the sensor surface alteration [27]. Two different factors may be taken into account to explain that: the electrostatic repulsion and the steric hindrance. The electrostatic repulsion is more significant in the first step of protocol; when the AptCytc is immobilized onto the electrode surface, a first layer is formed, where negatively charged phosphate groups of aptamer DNA skeleton are responsible of the electrical repulsion towards the negatively charged redox marker, thus inhibiting the interfacial transfer process and resulting in Rct increment. When the protein is added to form the aptamer-protein complex Rct still increases, essentially due to the steric hindrance brought by the protein large size. In this instance, electrostatic effects are not so significant given the net charge of the protein at this pH is essentially positive (pI value for cytochrome c, pI=10-10.5 [28]). Probably, this fact justifies the small observed change in Rct (a negative charge would be more manifest with the negative redox marker used). For a better detection ability, this bioassay would need a labeled format, for example with the use of a sandwich scheme and nanoparticle labeling.

3.3. Sensitivity of aptasensor

In order to characterize the sensitivity of the developed aptasensor, a complete calibration curve was built defined by using increasing concentrations of Cyt c. Figure 6 illustrates the increasing impedimetric signal with increasing Cyt c concentration. Normalized

relative signal Δ_{ratio} were plotted for the different Cyt c concentration assays, as shown on figure 7. As it can be observed, a logarithmic relationship was obtained, with a linear range from 50 pM to 50 nM for the protein. Moreover, a good linear relationship (r = 0.993) between the relative analytical signal (Δ_{ratio}) and the cytochrome c concentration in this range was obtained according to the equation: $\Delta_{ratio} = 1.130 + 5.236 \cdot 10^{8}$ [Cytc] (M). The detection limit was estimated as three times the standard deviation of the intercept obtained from the linear regression, which was 63.2 pM. The reproducibility of the method showed a relative standard deviation (RSD) of 6.8%, obtained from a series of 5 experiments carried out at a concentration of 500 pM Cytc, each one employing a new/renewed sensor surface. These are satisfactory results for the detection of cytochrome c in real samples, given this level is exactly the concentration in human serum.

3.4.Selectivity of the aptasensor

Cytochrome c is present in blood serum, which can be described as a complex sample matrix containing hormones, lipids, blood cells and other proteins. To study the selectivity of the system, we evaluated the response of proteins present in serum such as fibrinogen, immunoglobulin G and albumin. All proteins showed a certain degree of interference for cytochrome c. For this reason, detailed calibration curves were built around the serum level concentration of each protein.

To evaluate the sensitivity of the aptasensor we compared the calibration plots for the different interfering proteins. Table 1 summarizes the parameters of the calibration curve of each protein in comparison with that of cytochrome c, including the respective slopes and detection limits. The aptasensor showed the highest sensitivity for its target molecule, Cyt c, with its slope being three orders of magnitude greater than the slope for fibrinogen and four orders of magnitude more than that of IgG. Therefore, it was demonstrated that the aptasensor exhibited a much higher sensitivity to Cyt c than that to regarding potential interfering proteins, which displayed this effect greatly due to the high level of concentration in which they were assayed.

Apart of previous data, a second treatment was used to characterize this interference. For this purpose, EC_{50} values for each protein and %Cross Response (%CR) were calculated

for all interfering proteins, and summarized on Table 2. The lowest EC_{50} value obtained corresponded to cytochrome c, the target protein with a value of $6.68 \cdot 10^{-10}$ M, and the larger, $3.63 \cdot 10^{-5}$ M, to IgG. The largest % CR value corresponded to Fbr, 0.05%, and the lowest to Alb, all with a very low relative value. Therefore, it was demonstrated that the aptasensor showed a much higher sensitivity and selectivity to Cytc, regarding potential interfering proteins, which displayed this effect due to the high level of concentration in which they are present in serum, and not in a secondary recognition by aptamer.

4. Conclusions

This comunication reported a simple label-free impedimetric aptasensor for the recognition of cytochrome c at picomolar concentration level based on GEC electrodes. EIS technique has demonstrated itself as being a very sensitive technique for monitoring the biosensing event and confirmation of steps performed for building the aptasensor. The uneven surface of GEC allowed Aptcytc to be absorbed by a simple wet adsorption procedure. The simple preparation and the possibility of renewal of the surface by polishing with abrasive paper makes it suitable for repeated use. From the results, it can be concluded that the aptasensor showed a low detection limit, 63.2pM, good range of concentration for cytochrome c detection, from 50nM to 50pM, and high sensitivity, $5.236 \cdot 10^{8} M^{-1}$. The interference produced by serum proteins, such as fibrinogen, immunoglobulin G and albumin, displayed some limitations in the operation of the aptasensor, although usable given the concentration excess at which they manifest.

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Table 1. . Summary of calibration results for cytochrome c and other major proteins presents in serum.

Protein	Regression Line	Sensitivity (M^{-1})	Detection limit	Typical conc. in serum
Cyt c	$\Delta_{ratio} = 1.130 + 5.236 \cdot 10^{8}$ [Cyt c]	$5.236 \cdot 10^{8}$	63.2 pM	2nM
Albumin	$\Delta_{ratio} = 0.672 + 8.377 \cdot 10^{3}$ [Alb]	$8.377 \cdot 10^{3}$	0.300mM	0.52–0.75 mM
Fbr	$\Delta_{ratio} = -0.909 + 5{,}3301 \cdot 10^{5}$ [Fbr]	$5{,}330 \cdot 10^{5}$	7.10 µM	6–12 µM
IgG	$\Delta_{ratio} = -0.6181 + 4.435 \cdot 10^{4}$ [IgG]	$4.435 \cdot 10^{4}$	0.143 µM	60–100 µM

Table 2. Summary of EC_{50} and %Cross-response values of each interferent protein.

Protein	EC_{50} (M)	% Cross response
Cytochrome c	$6.68 \cdot 10^{-10}$	100
Albumin	$6.06 \cdot 10^{-5}$	$1.01 \cdot 10^{-3}$
Immunoglobulin G	$3.63 \cdot 10^{-5}$	$1.84 \cdot 10^{-3}$
Fibrinogen	$1.37 \cdot 10^{-6}$	0.05

(%CR = (EC_{50} cytochrome c/EC_{50} interferent)·100).

Figures

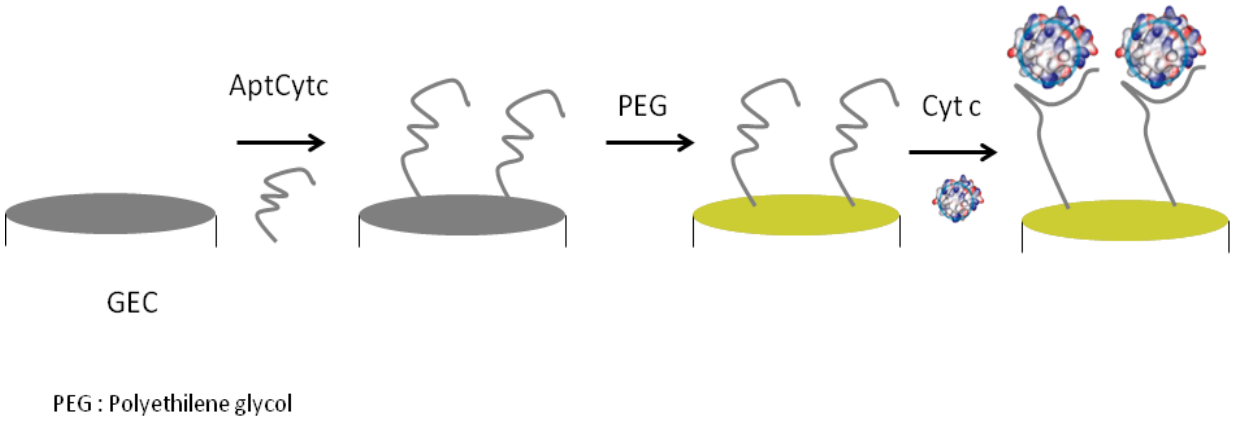

Figure 1. Scheme of experimental procedure.

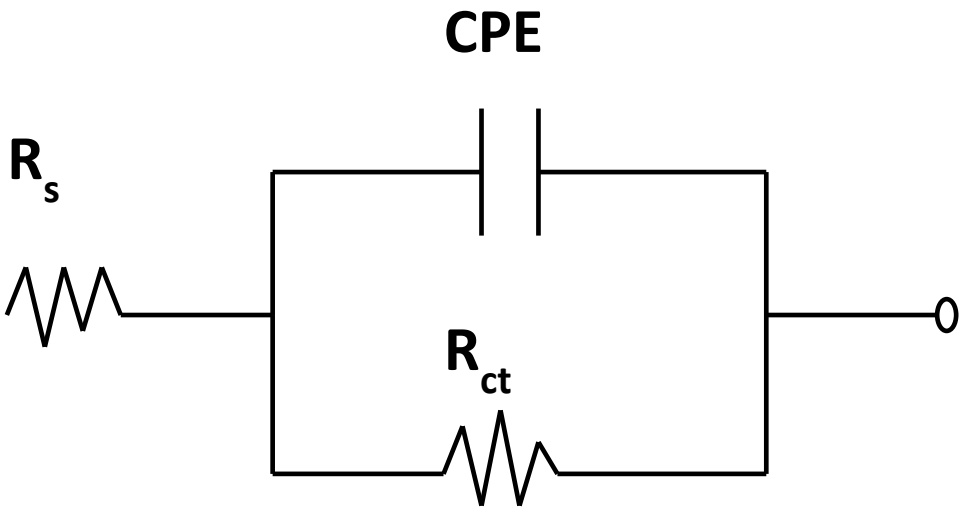

Figure 2. Equivalent circuit used for the data fitting. R_1 is the resistance of the solution, R_2 is the electron-transfer resistance and CPE, the capacitive contribution, in this case as a constant phase element.

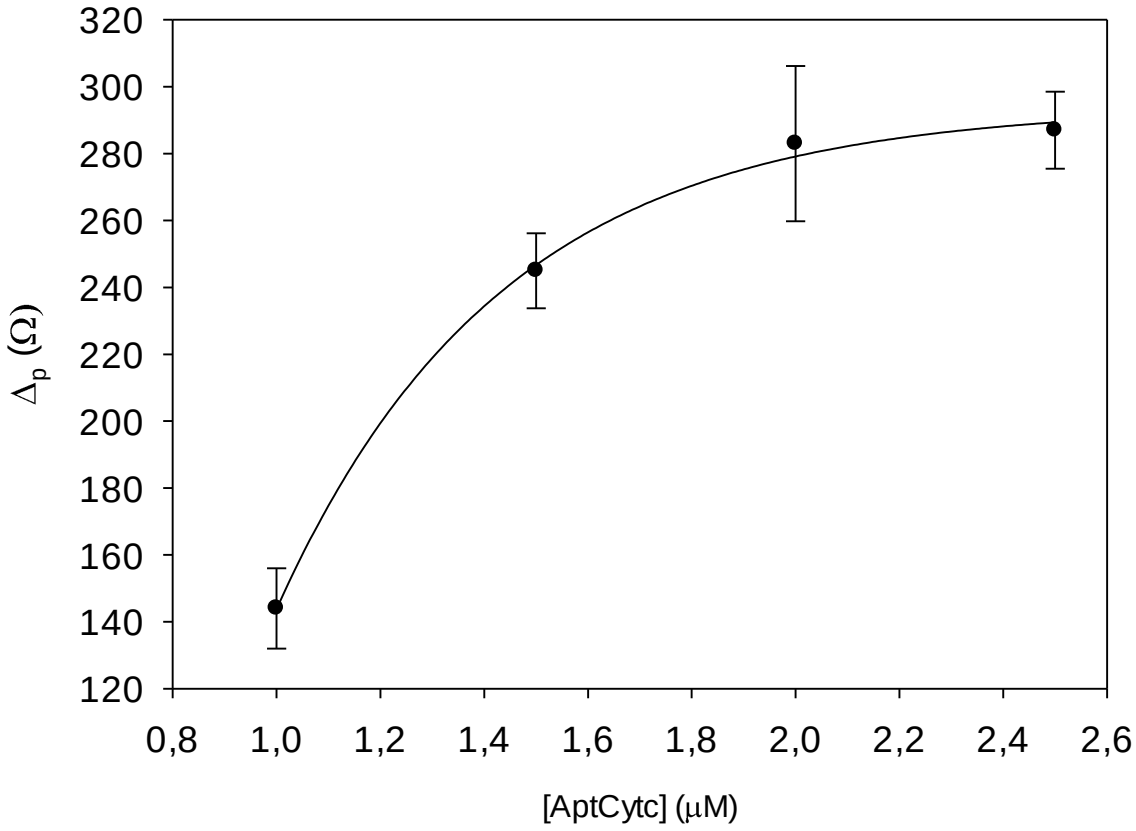

Figure 3. Optimization of the concentration of AptCytc. Uncertainty values corresponding to replicated experiments (n = 5).

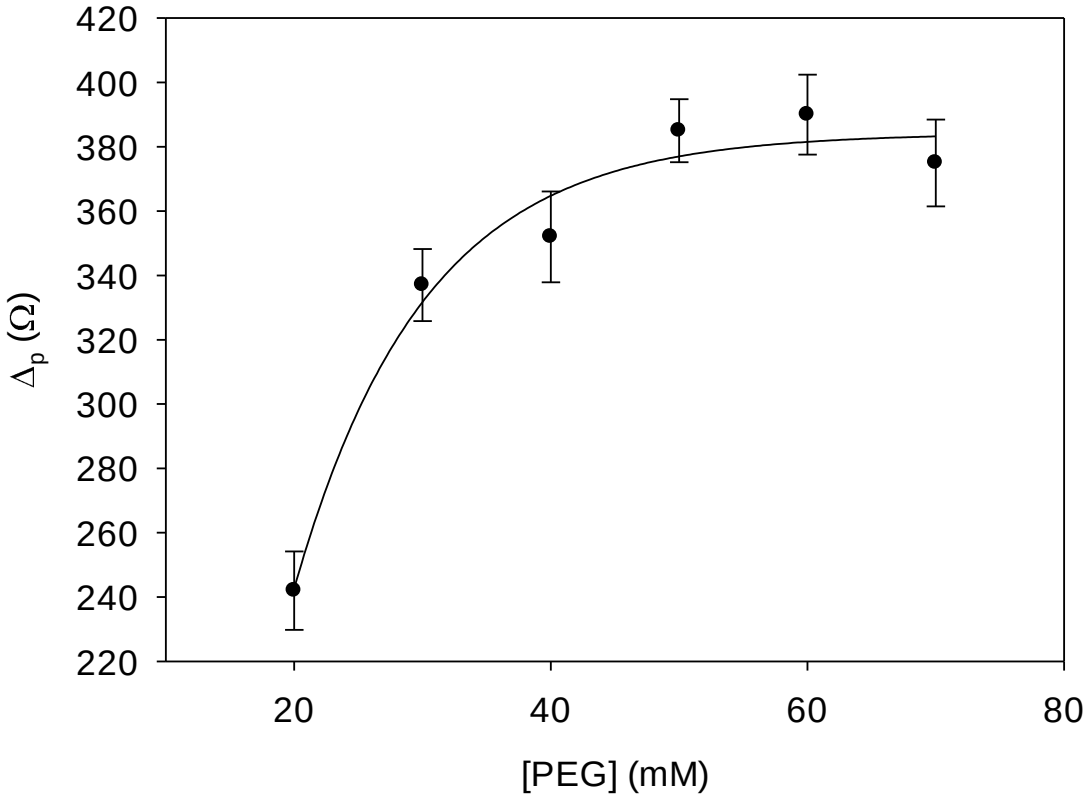

Figure 4. Optimization of the concentration of the blocking agent, PEG. Uncertainty values corresponding to replicated experiments (n = 5).

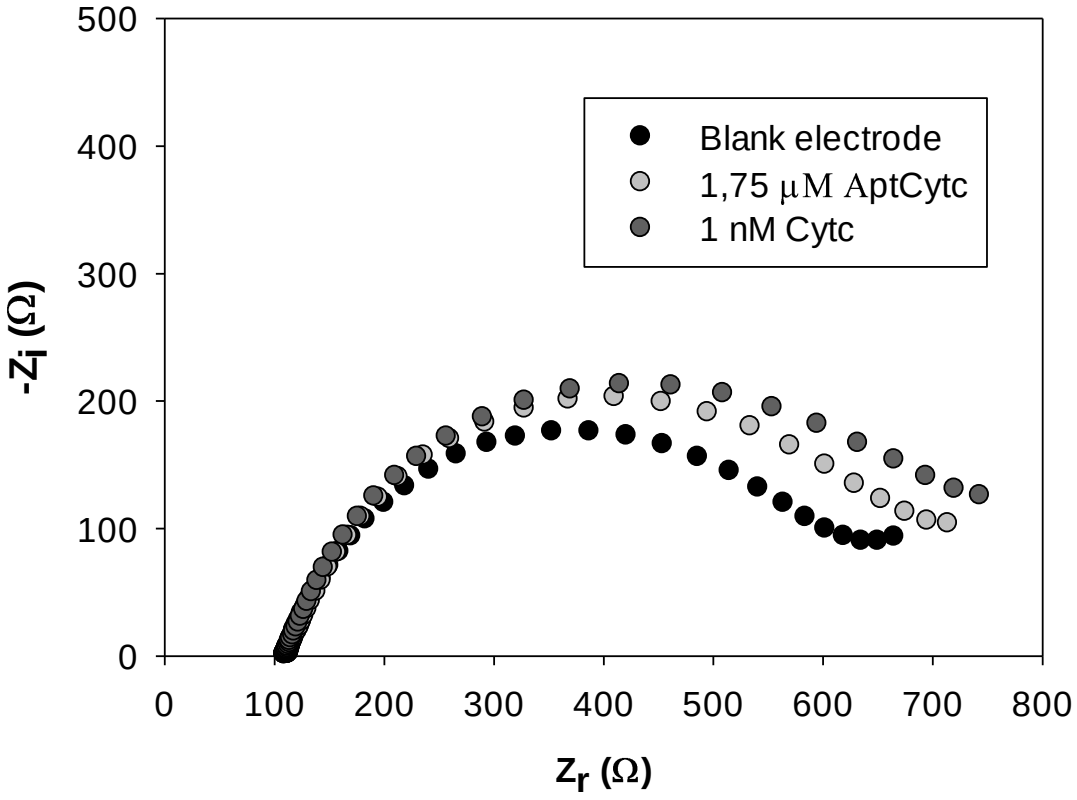

Figure 5. Nyquist Diagram obtained in the preparation and operation of the cytochrome c aptasensor.

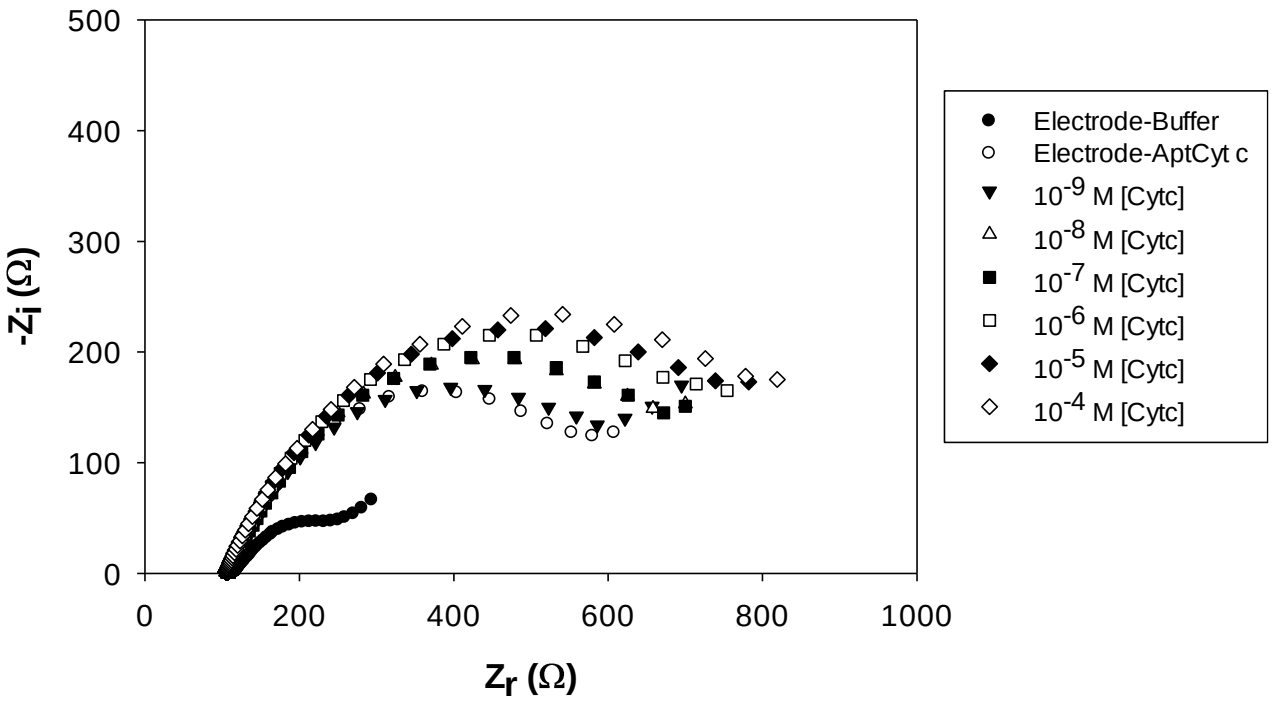

Figure 6. Nyquist diagrams for different concentrations of cytochrome c.

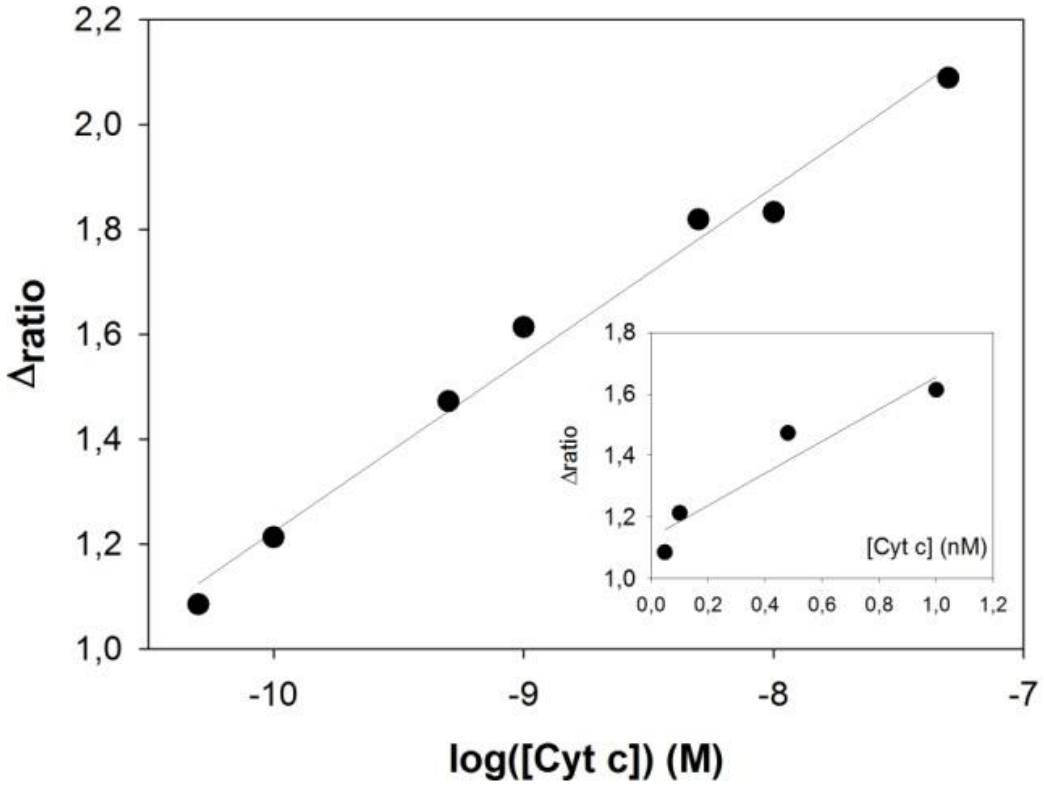

Figure 7. Calibration curve, relative signal *vs.* cytochrome c concentration.