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Detection of Cytochrome C Using a Selective and Sensitive Methylene Blue-Based Electrochemical Aptasensor

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Abstract: The present research aimed to detect the cytochrome C using methylene blue (MB)-anchored ultrasensitive electrochemical aptamer conjugated with Au nanoparticles (NPs), as a new electrochemical sandwich immunoassay. Aptamer-Au NPs could act as sandwich amplification element and MB accumulation reagent. According to the results, the interaction of MB and guanine occurred strongly on aptamer probe. After switching the aptamer structure by the cytochrome C, the MB- labeled aptamer probe was forced to detach from the sensing interface. Moreover, the cytochrome C concentrations (0.05-10.0 μM) with 5.0 nM detection limit reduced linearly the peak current of MB. In conclusion, an acceptable selectivity was observed for the constructed aptasensor in detecting the cytochrome C.
Keywords: Cytochrome C, Methylene blue, Aptamer, Aptasensor

1. Introduction

Cytochrome C (Cyt C) as mitochondrial redox protein contains a heme (Fe(III)), which accounts for oxidative phosphorylation [1] and carries electron in the mitochondrial intermembrane space between Cyt C reductase (complex III) and Cyt C oxidase (complex IV). The serum level of this protein is normally about 2 nM in human. Pathologically, different causes are involved in the translocation of Cyt C from mitochondria to cytosol and thus in the activation of caspases and subsequently cell apoptosis, highlighting the significance of testing its concentration to detect the possible consequences [2-6]. Accordingly, it is important to measure quantitatively the level of Cyt C in biological samples for preclinical diagnosis. There are two main methods to evaluate Cyt C electrochemistry, including the modification of electrode surface by adding a mediator (for example, the addition of 4,4′-dipyridyl disulfide on gold) and avoidance of contamination in the bare electrode surface using clean systems. Up to now, various surface modifiers and electrode materials are available in this regard [7, 8] for instance, the thin and ordered DNA films have been recently employed for Cyt C [9-11].

Among these, aptasensors have attracted further attentions nowadays [12]. Which are biosensors employing aptamers as a biorecognition element. The aptamers are a group of artificial nucleic acids binding to their targets with high affinity [13-22]. They were developed in 1990s and had different applications, such as the development of biosensors, evaluation of molecular interactions, and production of therapeutic agents [23-25]. These in vitro selected synthetic DNA or RNA oligonucleotides have the capability of linking to proteins, small molecules or even whole cells, detecting their target with high affinity and specificity, often adapting or even exceeding those of antibodies [26]. The detection trend, due to significant reversibility, is able to be returned and considered an important advantage against the immunosensors. There are multiple applications for the aptamers, including treatment purposes, molecular switches, affinity chromatography or biosensors. Many techniques have been introduced for detection-based aptamers, such as surface plasmon resonance, fluorescence, chemiluminescence, quartz crystal microbalance and electrochemistry; among these, the simple and sensitive approach is the electrochemical detection. The combination of high conductivity and stability characteristics of immobilized biomolecules has been used to develop the nano-scale electrochemical platform, an ordered layer of AuNPs into SPACEs [27-32].

The present study tried to investigate the interaction produced between the aptamer and its small ligand using MB^+ as an electrochemical indicator, which is a simple and regenerative electrochemical approach. The MB^+ from phenothiazine family is an organic dye as a redox indicator having potential within the range of -0.10 to -0.40 V (versus SCE) in pH

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values of between 4 and 11. This redox potential is close to numerous biomolecules, making it widely used a mediator for electron transfer. Recently reported applications for the MB^+ is to detect the DNA hybridization and to act as biosensor based on aptamer. The interaction established specifically between MB^+ and guanine is a basic reason for detecting the DNA hybridization, according to previous studies. In the current study, high sensitive and selective electrochemical biosensor have been introduced for Cyt C detection. Our sensor, like other normal MB^+-anchored aptasensores, reduces the current of MB^+ for detecting the interaction between the aptamer and small molecules.

2. Experimental

2.1 Materials and reagents

The oligonucleotide was purchased from Bio Basic Inc. (Canada), and the sequences of oligonucleotides were as follows: 5'-SH- CH_2-CH_2- CH_2-CH_2-CH_2-CH_2- CCG TGT CTG GGG CCG ACC GGCGCA TTG GGT ACG TTG TTG C -3'. Cytochrome C, Hydrogen tetra chloroaurate (III) ($HAuCl_4$), methylene blue and 6-mercaptohexan-1-ol (MCH) were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium hydroxide, sulfuric acid, phosphoric acid, hydrogen peroxide and ethanol were purchased from Merck (www.merck.com). All other chemicals were of reagent grade.

2.2 Apparatus

The electrochemical measurements were performed with an Autolab potentiostat/galvanostat (PGSTAT 302 N, Eco Chemie, the Netherlands). The experimental conditions were controlled with the General Purpose Electrochemical System (GPES) software. The screen-printed electrode (Drop Sens, DRP-110, Spain) consists of three main parts which are a graphite counter electrode, a silver pseudo-reference electrode, and a graphite working electrode. A Metrohm 710 pH meter was used for pH measurements.

2.3 The Procedure of preparing a modified screen-printed electrode

First, 6 mM of $HAuCl_4$ was used at a potential of -0.4 V to immerse the bare screen-printed electrode for 400 seconds. Then, the electrode was exposed to 5 µL of 10 µM aptamer solution and placed overturned for self-assembly within 30 minutes in a humid chamber. Next, 10 mM of PBS (pH=7.4) buffer was added, and the modified screen-printed electrode was placed in 1 mM of 1-HT solution for an hour for blocking the nonspecific sites and orienting the aptamer strands in a straight direction. Following each step, 10 mM of PBS solution (pH=7.4) was used to rinse the electrode successively. After that, 10 µl of Cyt C was covered on the electrode surface at different concentrations of PBS buffer for 30 min, followed by electrode immersion in aptamer-Au NPs solution for 30 min. Finally, the modified electrode was placed in 40 µM of MB solution for 30 min, and subsequently, differential pulse voltammetry (DPV) was used to measure the oxidation peak current of the accumulated MB. Figure 1 illustrates the preparation procedure of SPE-Au Np-Ap-MCH-C-MB to detect Cyt C.

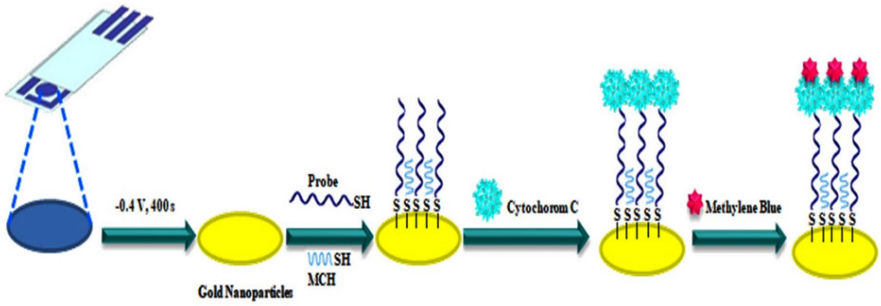

Figure 1. Schematic display of the different steps involved in the preparation of SPE-Au Np-Ap-MCH-C-MB for the determination of Cyt C

3. Results

3.1 The principle of detecting electrochemical aptasensor

The Au NPs initially immobilized on the screen-printed electrode surface, as a sensing surface, bind with aptamer in

the aptamer-Au NPs conjugates, resulting in adsorption of aptamer-Au NPs complex on the electrode surface containing Cyt C and enabling the MB accumulation on the electrode surface through binding with G bases found in aptamer-Au NPs conjugates. The result is the amplification of the electrochemical signal of MB. The MB through Cyt C could bind with G bases found in aptamer; the probe capturing decreased the MB background signal on the electrode. The level of MB adsorption on the electrode surface can be elevated significantly via aptamer-Au NPs conjugates due to the presence of high amount of G bases, enhancing the sensitivity of the electrochemical biosensor. A potential of detecting other Cyt C by MB occurs with the sandwich method using aptamer.

3.2 The synthesis and characterization of sensing interface

The CV was used to characterize the modified electrode and to validate the production of the sensing interface. The interfacial electron-transfer kinetics of modified electrode after each modification step were determined using $[Fe(CN)_6]^{4-/3-}$ anions as a redox probe. Figure 2 shows the CV of Fe (CN)6 $^{3-/4-}$ at the bare SPE (curve a), SPE-Au Np (curve b), SPE-Au Np-Ap (curve c), SPE-Au Np-Ap-MCH-C (curve d), respectively. A very large current of $[Fe(CN)6]^{4-/3-}$ could be seen for the modification of SPE with Au Np lead to a considerable increment in peak current of Au Np /SPE. The presences of Au Np evidently improve both conductivity and stability. The response of CV was reduced after electrode modification by Ap because of the poor electron-transfer capability of the modified electrode. The immobilization of Cyt C clearly resulted in reduced CV response due to the electrode surface mostly covered by Cyt C through blocking the electron-transfer efficiency of $[Fe(CN)_6]^{4-/3-}$ at solid/liquid interface.

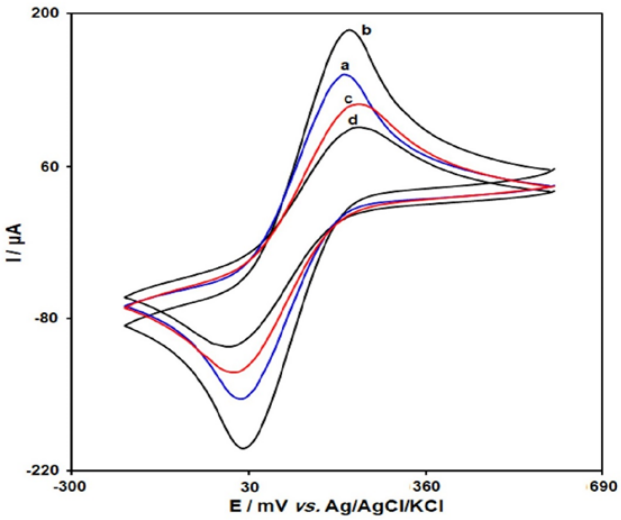

Figure 2. Cyclic voltammograms of different modification steps of the SPE in the presence of 5 mM $K_{3/4}Fe(CN)_6$ and 0.1 M KCl. a) bare SPE b) SPE-Au Np c) SPE-Au Np-Ap d) SPE-Au Np-Ap-MCH-C. Scan rate: 0.10 V s^{-1}

3.3 The optimization of experimental conditions

In this study, different parameters, including the various aptamer concentrations, time of interaction between aptamer and MB were analyzed to optimize the AP/SPE sensor efficacy. Figure 3 exhibits the effect of different aptamer concentrations (0.5 to 7.0 µM) on the Cyt C detection procedure in the presence of 0.1-M PBS (pH=7.0). As can be observed, 4.0 µM of aptamer concentration enhanced effectively the electrode response.

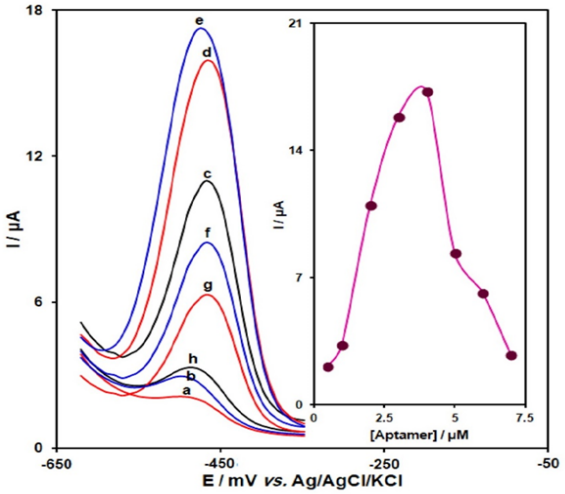

Figure 3. DPVs of SPE-Au Np-Ap-MCH-C-MB in 0.1 M PBS (pH 7.0) containing different concentrations of aptamer. a-h correspond to 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 µM of aptamer, respectively. Inset: plot of the peak current as a function of aptamer concentration in the range of 0.5-7.0 µM

Figure 4 displays the effect of times (7 to 180 min) required for aptamer interaction on the Cyt C detection procedure in the presence of 0.1-M PBS (pH=7.0); according to the results, 90 min of interaction time increases the electrode response at the maximum level.

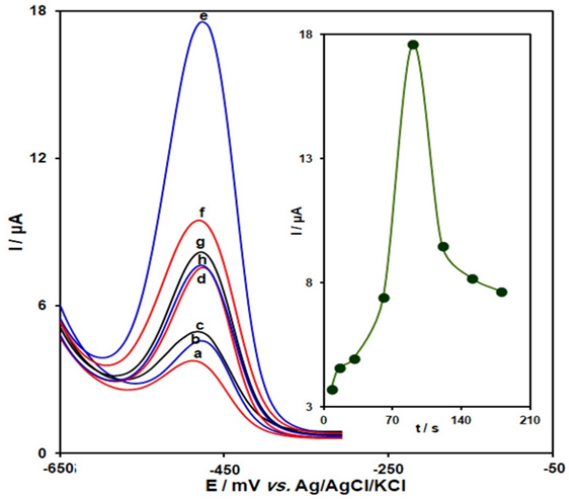

Figure 4. DPVs of SPE-Au Np-Ap-MCH-C-MB with a concentration of 4.0 µM of aptamar in 0.1 M PBS (pH 7.0) containing different times interaction of aptemer with Cyt C. a-h correspond to 7, 15, 30, 60, 90, 120, 150 and 180 min, respectively. Inset: plot of the peak current as a function of times Interaction in the range of 7-180 min

Figure 5 shows the effect of times (7 to 120 min) for MB interaction on the Cyt C detection procedure in the presence of 0.1-M PBS (pH=7.0); as seen, 30 min of interaction time increases the electrode response sharply.

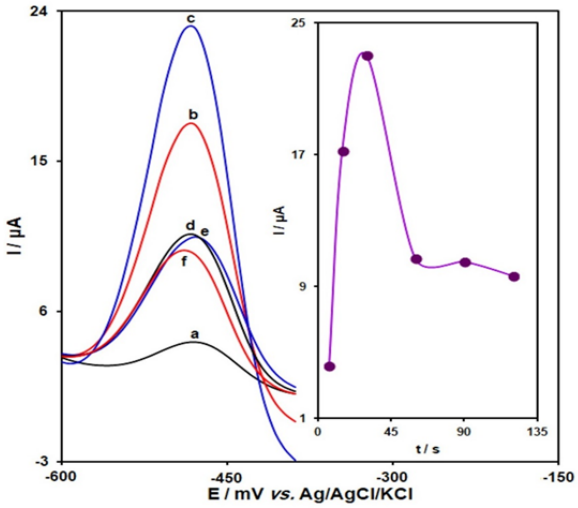

Figure 5. DPVs of SPE-Au Np-Ap-MCH-C-MB with a concentration of 4.0 µM of aptamar in 0.1 M PBS (pH 7.0) containing different times interaction of MB with Cyt C. a-f correspond to 7, 15, 30, 60, 90 and 120 min, respectively. Inset: plot of the peak current as a function of times Interaction in the range of 7-120 min

3.4 Determination of cytochrome C

The Ap /SPE developed under optimized conditions was used to plot the calibration curve for detecting the Cyt C. The Cyt C can be detected by the peak current of Cyt C oxidation on the modified electrode surface. Figure 6 presents different Cyt C concentrations tested by DPV experiments. The peak current of Cyt C oxidation on the modified electrode surface was matched with the Cyt C concentrations (0.05 to 10.0 µM). The Cyt C detection limit (3σ) was 5.0 nM.

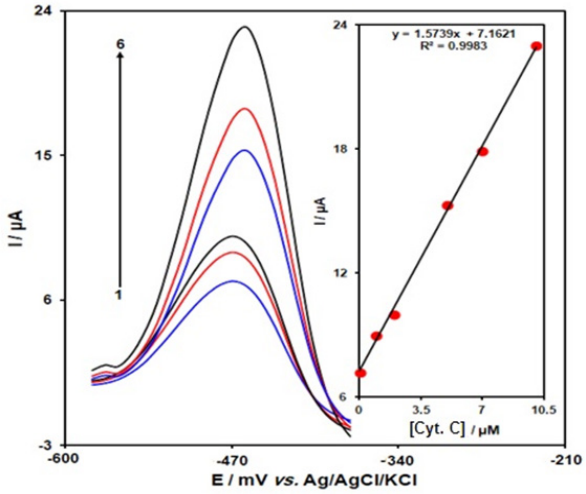

Figure 6. DPVs obtained at aptasensor for Cyt C at different concentrations in 0.1 M PBS (pH 7.0). Numbers 1-6 correspond to 0.05, 0.1, 2.0, 5.0, 7.0 and 10.0 µM of Cyt C. Inset: calibration curve for Cyt C, plot of peak current vs. C Cyt C

3.5 Influence of the scan rate

The voltammograms of the SPE-Au Np-Ap- MCH-C-MB in 0.1 M PBS (pH 7.0) at various scan rates are presented in Figure 7. In CVs, the presence of a pair of almost symmetric anodic and cathodic peaks is evident over the scan rate range from 10 to 100 mV s^{-1} with nearly equal peak currents. In addition, the peak-to-peak separation has increased with the scan rate. There is also a proper linear relationship between the peak current and square root of scan rate; results are shown in Figure 7 (inset). Accordingly, this reaction exhibits the quasi-reversible diffusion controlled behavior with the electron transfer process.

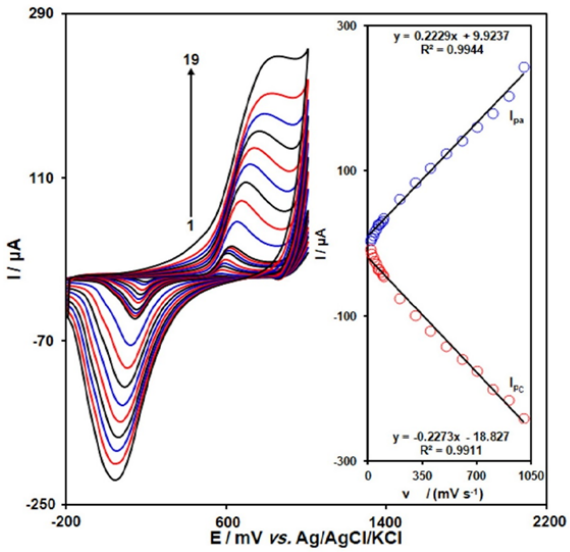

Figure 7. CVs of SPE-Au Np-Ap- MCH-C-MB electrode in 0.1 M PBS (pH 7.0) at various scan rates; numbers 1-19 correspond to 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mV s^{-1}, respectively. Inset: Variation of anodic and cathodic peak current vs. square root of scan rate

4. Discussion

The validation and precision of the present Cyt C electrochemical aptasensor was evaluated to compare with other reported electrochemical techniques. As it is obvious, detection limits are better and linear comparable to those in Table 1. The presence of aptamers in electrodes shows the superiority of this approach in terms of its selectivity.

Table 1. Comparison of Cyt C aptasensor with other reported electrochemical methods for determination of Cyt C

Method	LOD	LDR	Ref.
Voltammetry	0.02 µM	0.08-22.5 µM	[1]
Voltammetry	1.0×10^{-5} M	3.0×10^{-5}-7.0×10^{-4} M	[4]
Voltammetry	3.0×10^{-7} M	5.0×10^{-7}-3.0×10^{-5} M	[6]
Voltammetry	10.0 nM	10.0nM-50.0 µM	[9]
Voltammetry	5.0 nM	0.05-10.0 µM	This work

5. Conclusions

A regenerable aptasensor was developed in the present study aiming for label-free electrochemical detection of Cyt C. The pre-concentration of Cyt C at the modified electrode surface resulted in a significant elevation in the peak current because of interaction with the aptamer. The efficacy of this electrochemical aptasensor was increased analytically following the optimization of the main experimental dimensions. The resulting aptasensor, in contrary to direct methods of detection, is capable of measuring the Cyt C without interferences of thiol-containing amino acids; the advantages of electrochemical approaches are their simplicity, low cost, and multi-usage properties. Hence, the Cyt C in biological samples can be electrochemically detected using this modified electrode as a selective, useful and regenerative aptasensor. Other proteins and small molecules can be detected using this method; however, further research is required to draw definite conclusions.

Conflict of interest

The authors declare that they have no conflict of interest.

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