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Preliminary Studies Towards the Development of DNA Biosensors for Detection of Cylindrospermopsin – – A Cyanobacterial Toxin

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Abstract

The suitability of polytyramine (PTy) modified electrodes for the development of DNA biosensors is analysed in this work. PTy films were synthesised potentiodynamically on Pt electrode, from acidic aqueous solution. A cylindrospermopsin coding sequence (PKSM4 probe 5'-phosphate modified) was covalently bound on the polytyramine matrix using N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) as coupling agents. Changes of the open circuit potential were registered as a first approach to monitorise the immobilization and hybridization processes.

The modified electrodes have been characterised by cyclic voltammetry and square wave voltammetry, using methylene blue (MB), as electroactive indicator and the successful immobilization and hybridization steps are revealed by the increase of the peak currents. The hybridization has also been confirmed by measuring the fluorescence of the modified electrodes after staining with PicoGreen® DNA dye.

Keywords: cylindrospermopsin, DNA biosensor, PicoGreen®, polytyramine, methylene blue.

Introduction

Biosensors offer simplified analysis for a range of biomedical and industrial applications, being in continuous development for the last couple of decades [1]. Electrochemical DNA hybridization biosensors have been attracting increased attention due to superior properties since electrochemical biosensors enable fast, simple and low-cost detection, and may be miniaturized into portable devices [2].

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Cyanobacteria are photosynthetic prokaryotes that in adequate conditions may develop mass occurrences (water blooms) often toxic and constitute a high potential risk for human health. The development of a method to detect one of the most common and aggressive cyanobacterial toxins, cylindrospermopsin coding sequences (such has the PKSM4 [3]), is of major importance since there are no analytical standards [4]. This cyanobacterial sequence can be immobilized in a conducting polymer matrix to develop an electrochemical DNA hybridization biosensor.

Tyramine (Ty) has been chosen as a monomer because of its pendant amine group. The use of acidic aqueous medium to polymerise the polytyramine (PTy) films allows the formation of thicker films than those obtained from neutral solutions, with improved conductivity properties [6, 7]. Polytyramine presents one primary aliphatic amine per tyramine moiety. This amine functional group can be used for the attachment of organic molecules or biomolecules of interest [5], for instance by the covalent attachment through a carboxamide or a phosphoramidate bond [6]. For this reason, PTy has been employed in immobilization studies of enzymes [8-14], oligonucleotides (ODN) [6, 15, 16] and antibody [17]. Water soluble N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) can be used as activating reagents of the 5'-phosphate group of a single-stranded DNA (ss-DNA) and promote its covalent attachment to primary amino groups of the electrode surface by the formation of a phosphoramidate linkage [18-21].

Electrochemical detection of hybridization is mainly based on the differences in the electrochemical behaviour of compounds that interact in a different way with ss-DNA and double-stranded DNA (ds-DNA). A variety of small molecules may interact reversibly with DNA, either through intercalation or electrostatic binding in well-defined binding sites. For instance, the methylene blue (MB), an electroactive redox indicator which displays a blue colour in the oxidised form and is colourless in its reduced one, is widely used. The MB can establish electrostatic interactions to the negatively charged DNA sugar phosphate backbone [22, 23].

An alternative route for the detection of hybridization relies on the fluorescence measurements. The PicoGreen® reagent is a fluorochrome described as an ultrasensitive fluorescent nucleic acid stain, that selectively binds to double-stranded DNA [24-26] by intercalation in the DNA minor-groove [25], and allows the detection of as little as 25 pg mL^{-1} of ds-DNA and has a minimize fluorescence contribution of RNA and single-stranded DNA (ss-DNA). It has an excitation maximum at 480 nm and an emission peak at 520 nm. When bound to ds-DNA, fluorescence enhancement of PicoGreen® is exceptionally high; and little background occurs since the unbound dye has virtually no fluorescence [24-26].

In this work, the covalent immobilization of a single-stranded cylindrospermopsin coding sequence bearing a 5'-phosphate modification to a polytyramine film has been performed. Subsequent hybridization assays with complementary and non-complementary sequences were carried out. Several methods were used to confirm the immobilization and hybridization processes.

These included the monitoring of the open circuit potential with time, characterization of the modified electrodes by cyclic voltammetry and square wave voltammetry, using methylene blue, as electroactive indicator and also the fluorescence quantification, after staining with PicoGreen® reagent.

Experimental

Chemicals

All the solutions for the electrochemical studies, were prepared from ultra-pure water, obtained from a Millipore purification system (nominal resistivity 18.2 MΩ cm at 25 ºC) and deoxygenated directly in the cell with a stream of nitrogen (purity > 99.99997%) for at least 30 min before use. Unless specified, all experiments were performed at room temperature.

The following chemicals, used in this research, were from analytical grade and utilised without further purification: H_2SO_4 (*BDH AnalaR*), Na_2HPO_4 and $NaH_2PO_4.H_2O$ (*Merck*), 4-hydroxyphenethylamine (Tyramine, *Sigma*), 2-morpholinoethanesulfonic acid (MES, *Fluka*), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC**,** *Sigma-Aldrich*), N-hydroxysuccinimide (NHS, *Fluka*), SSC buffer, pH 7.0 (20x: 3 mol dm^{-3} sodium chloride + 0.3 mol dm^{-3} sodium citrate, *Invitrogen*), 3,9-bisdimethyl-aminophenazithionium chloride (methylene blue, *Merck*), 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl, *Duchefa*), ethylenediaminetetraacetic acid disodium salt (EDTA, *Duchefa*), and PicoGreen® (*Invitrogen*).

Oligonucleotides, PCR grade were purchased from Invitrogen Life Technologies:

- Probe modified with 5' phosphate (PKSM4-5'P): 5'-P-GAAGCTCTGGAATC-CGGTAA-3'
- Complementary sequence (PKSM4comp): 5'-TTACCGGATTCCAGAGC-TTC-3'
- Non-complementary sequence (DSR737F): 5'-ACTGCATMAATAAGAT-GCC-3

Stock solutions of the oligonucleotides were prepared with autoclaved Milli-Q water and kept frozen until use.

Apparatus

Experiments were carried out in a three-electrode compartment glass cell, with a Pt foil counter electrode and a saturated calomel reference electrode (SCE), and the working electrode consisted of a Pt disk, 0.196 cm^2 geometrical area. All potentials are reported versus SCE.

Prior to all measurements, the glassware, and cells were soaked overnight in concentrated chromosulphuric solution, followed by rinsing with purified water.

Cyclic voltammetry (CV) experiments were performed using a standard potentiostat - Wenking ST 72 (Bank Elektronik), a voltage scan generator - Wenking Model VSG 72, and an XY recorder - Servogor 790 (Goerz). Square wave voltammetry (SWV) was conducted with an Electrochemical Workstation CH 420 (CH Instruments, Inc). Scanning electron microscopy (SEM)

instrumentation consisted on an analytical SEM (Hitachi S-2400) with tungsten filament, using 25 kV acceleration tension, 20,000x magnification, and a sputter coater of gold (Polaron) for SEM uniform and thickness-controlled coating of the PTy film. The fluorescence measurement of the different modified electrodes after staining with PicoGreen® dye was performed with an Olympus IX-50 inverted microscope using a 20x (0.75NA) Plan Apo objective, Ludl BioPoint filter wheels and a PCO Sensicam cool CCD. The camera has a frame transfer architecture and requires no shutter. Integrated control of filter wheel and image acquisition is achieved by Image-Pro Plus 4.0 and Scope-Pro 3.1 (Media Cybernetics). Settings for image acquisition (camera exposure time, filters, time interval and storing modes) are determined by custom-made macros. Fluorescence intensity was measured using Image-Pro Plus 5.0 software (Media Cybernetics, Leiden, Netherlands).

Procedure

Before each assay, the working electrode was hand-polished with successively finer grades of 5, 1 and 0.3 μm alumina suspension (Al_2O_3, Buehler) until a fresh mirror-finish surface has been generated followed by consecutive potential cycling, from the hydrogen evolution region to the oxygen evolution domain, -0.250 to +1.050 V *vs*. SCE, in 0.1 mol dm^{-3} H_2SO_4 aqueous solutions.

Polytiramine electropolymerisation and characterisation

In this study, the potentiodynamic polymerisation of tyramine (Ty) was carried out as described in Tenreiro *et al*. [7]. The polytyramine (PTy) films were grown on Pt electrode, from acidic aqueous solution of 0.1 mol dm^{-3} H_2SO_4 containing 0.025 mol dm^{-3} tyramine. The polymers were synthesised potentiodynamically by cycling the potential between –0.100 and +1.050 V *vs*. SCE for 25 potential cycles at 0.050 V s^{-1} (designated PTy25/50).

The obtained films were electrochemically characterised by CV in 0.1 mol dm^{-3} H_2SO_4 and 0.1 mol dm^{-3} phosphate buffer, pH 7 solutions. Their morphological characterisation was performed by SEM.

Probe immobilization

Buffered solutions of 0.01 mol dm^{-3} MES pH 6, containing 0.04 mol dm^{-3} EDC, 0.01 mol dm^{-3} NHS and 0.7 μmol dm^{-3} PKSM4-5'P probe were prepared immediately before use. The PTy modified electrode was immersed in this solution at room temperature and after 7200 s, films were thoroughly washed with Milli-Q water prior to its voltammetric characterisation, to remove coupling agents and non-covalently bound oligonucleotides.

Hybridization

Hybridization tests consist in soaking these probe-modified electrodes in a solution containing 0.7 μmol dm^{-3} of the complementary (complementary hybrid formation) or non-complementary sequence (non-complementary hybrid formation) in 2X SSC buffer pH 7, for 7200 s at room temperature.

Monitoring of the open circuit evolution with time
During the immobilization and hybridization course the evolution of the open circuit potential with time was measured.

Voltammetric studies
In cyclic voltammetry characterisation, the modified electrodes were immersed in a 250 μmol dm^{-3} methylene blue in 0.1 mol dm^{-3} phosphate buffer, pH 7 solution and the potential was scanned from +0.150 V to -0.350 V at sweep rate, $\nu = 0.100$ V s^{-1}. Detection of the electrodes modification was also monitorised by measuring the cathodic peak current of MB with SWV. In SWV experiments the parameters used were 0.002 V step, 0.025 V pulse amplitude, 5 Hz frequency, and the initial potential was +0.150 V. All the MB solutions were prepared immediately before use.

Fluorescence measurements
Immediately before use, a 200-fold dilution of PicoGreen® reagent was prepared in TE buffer pH 7.5 (0.01 mol dm^{-3} Tris-HCl + 0.001 mol dm^{-3} EDTA) in a plastic container, since the reagent may adsorb to glass surfaces. The modified electrodes were stained (by immersion) for 10 min with DNA dye ligand PicoGreen® protected from light by covering it with aluminium foil. Thereafter, the electrodes were observed using an inverted microscope and the fluorescence measurements correspond to fluorescence intensity at an excitation wavelength of 485 nm with 530 nm emission. Fluorescence intensity was determined using Image-Pro Plus 5.0 software.

Results and discussion

Polytyramine electropolymerisation and characterisation

Fig. 1 illustrates the cyclic voltammograms obtained on a Pt electrode during tyramine electropolymerisation from acidic aqueous solution of 0.1 mol dm^{-3} H_2SO_4 containing 0.025 mol dm^{-3} tyramine. The recorded data are identical to the already described in the literature [7]: in the first oxidation cycle, tyramine oxidation currents are detectable at about 0.8 V; during the subsequent cycles, a gradual decrease of the anodic current is observed for the first 13 cycles, then the current starts to increase slowly until the 22^{nd} cycle and finally it remains approximately constant, indicating that the electrode is not passivated; throughout the reverse potential sweep, there is no observation of the appearance of a cathodic peak correspondent to the polymer reduction.
The electrosynthesised films were washed with Milli-Q water and subsequently discharged by applying a potential of -0.100 V *vs*. SCE for 300 s to assure the egress to the solution of species present in the polymer film and thus to prevent their possible interference during subsequent assays.

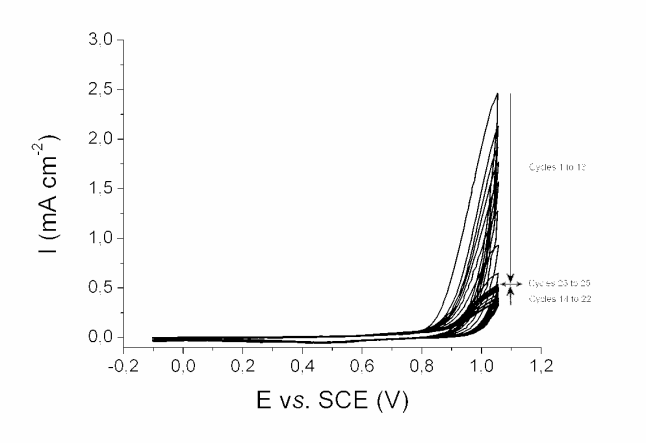

Figure 1. Cyclic voltammogram for the potentiodynamic growth of PTy (25 potential cycles from -0.10 to +1.05 V) on Pt electrode from 0.025 mol dm^{-3} Ty in 0.1 mol dm^{-3} H_2SO_4; $\upsilon = 0.05$ V s^{-1}.

The PTy films were morphologically characterised by scanning electron microscopy. The image, Fig. 2, shows that homogeneous deposits have been obtained.

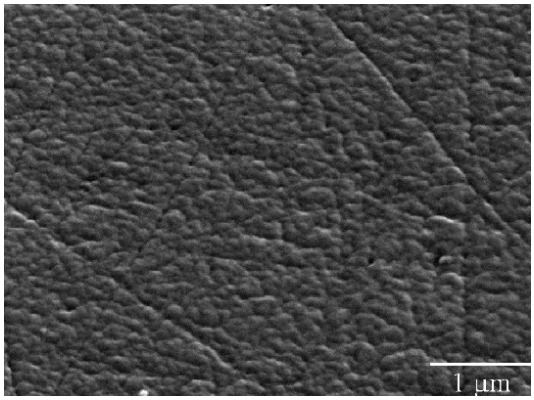

Figure 2. SEM microphotograph of a PTy 25/50 film using 20,000X magnification.

The electrochemical characterisation of the PTy modified electrodes has been performed in 0.1 mol dm^{-3} H_2SO_4 (Fig. 3A) and also in 0.1 mol dm^{-3} phosphate buffer pH 7, a medium compatible with the presence of biological species) (Fig. 3B), where for comparison the bare Pt electrode response is also indicated. As expected [5, 8] the voltammograms are featureless and denote the PTy poor conducting properties.

Detection of probe immobilization and hybridization

An expedite method to detect the immobilization process is the monitorisation of the open circuit potential (OCP) evolution with time. This measurement provides an indication on the feasibility of the processes leading to the effective binding between 5' phosphate ss-DNA probe (activated by EDC and NHS) and the PTy amino terminal groups. As can be seen in Fig. 4, during the immobilization process, a rapid initial negative shift of OCP is verified, indicative of an

immediate reaction; the presence of the activating agents (EDC and NHS) and the probe give rise to stronger OCP modifications (0.262 V *vs*. 0.150 V in its absence), suggesting an increase of negative charges (from the ss-DNA) at the surface of the polymeric layer. It must be noticed that the alteration in the potential appears to be mainly due to the presence of the coupling agents, calling for the employment of an independent monitoring method. Notwithstanding, the evolution of the open circuit potential with time was also registered as a first approach to monitorise the hybridization process, as it is depicted in Fig. 5. After immersion in all considered media, the initial OCP of the PTy 25/50 films is approximately +0.315 V *vs*. SCE and an abrupt initial negative shift is observed. The potential decreases continuously and a constant potential plateau is reached after about 1440 s. A clearly noticeable change in the OCP value is only obtained for the hybridization in presence of the complementary sequence, suggesting that this method can be used to distinguish between the presence of a complementary from non-complementary sequence.

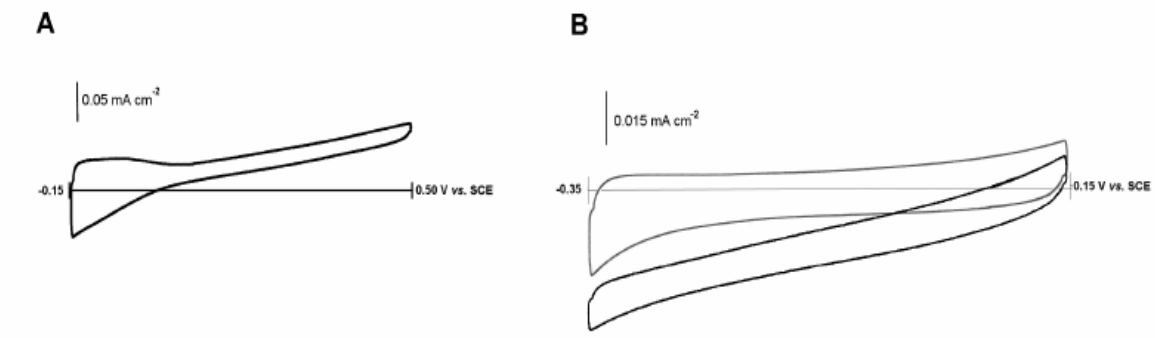

Figure 3. Cyclic voltammogram of the redox behaviour of PTy 25/50 (black) in (A) 0.1 mol dm^{-3} H_2SO_4 and (B) 0.1 mol dm^{-3} PB, pH 7; bare Pt electrode characterisation (gray); $\upsilon = 0.05$ V s^{-1}.

In order to confirm the immobilization and hybridization processes, cyclic voltammetry was used to characterise the modified electrodes in a solution containing a redox indicator (methylene blue) and the results are presented in Fig. 6. Since the prepared solution contains mostly MB in the oxidised form, the voltammetric scan has been performed from the most positive potential limit of (+0.15 V) to the most negative limit (-0.35 V *vs*. SCE), allowing to analyse the indicator reduction reaction. An increase of cathodic peak current density, compared to the PTy alone, was obtained after the immobilization of the ss-DNA probe and the hybridization with non-complementary or complementary sequence. However, the signals of hybridization process with a complementary and non-complementary sequence show only a small difference, a crucial issue for the biosensor testing with total DNA. This can be due to the establishment of some bonds between the immobilized probe and the non-complementary probe, leading to the accumulation of more DNA at the surface of the modified electrode, which gives a current increase when this electrode is characterised in the MB solution.

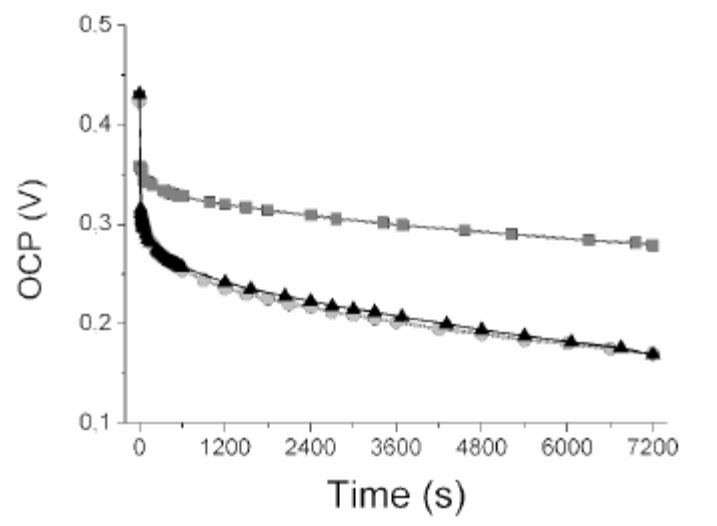

Figure 4. Evolution of the open circuit potential in time of PTy 25/50 modified electrodes after their immersion in (■) 0.01 mol dm^{-3} MES buffer pH 6; (●) 0.01 mol dm^{-3} MES buffer pH 6 with 0.04 mol dm^{-3} EDC and 0.01 mol dm^{-3} NHS; and (▲) 0.01 mol dm^{-3} MES buffer pH 6 containing 0.04 mol dm^{-3} EDC, 0.01 mol dm^{-3} NHS and 0.7 μmol dm^{-3} PKSM4-5'P.

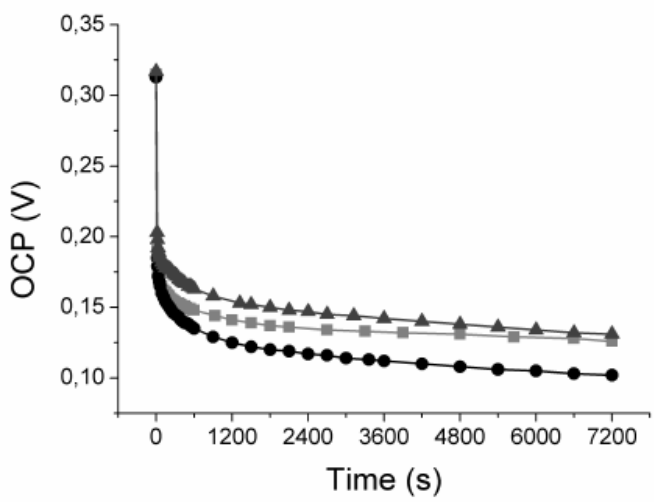

Figure 5. Evolution of the open circuit potential in time of PTy25/50-probe modified electrode after its immersion in (■) 2X SSC buffer, pH 7; (▲) in the presence of 0.7 μmol dm^{-3} non-complementary sequence and (●) in the presence of 0.7 μmol dm^{-3} complementary sequence.

The SWV technique was also used to characterise the modified electrodes in MB solutions, because the capacitive or charging current component, due to the electrical charging of electrode double layer, is largely eliminated, which leads to an increase of the signal to noise ratio [27]. It is known that the optimum pulse amplitude is about 0.050/*n* V, where *n* is the number of electrons in the redox reaction [27]. Thus, the pulse amplitude selected was 0.025 V, since in the methylene blue conversion there are two electrons involved.

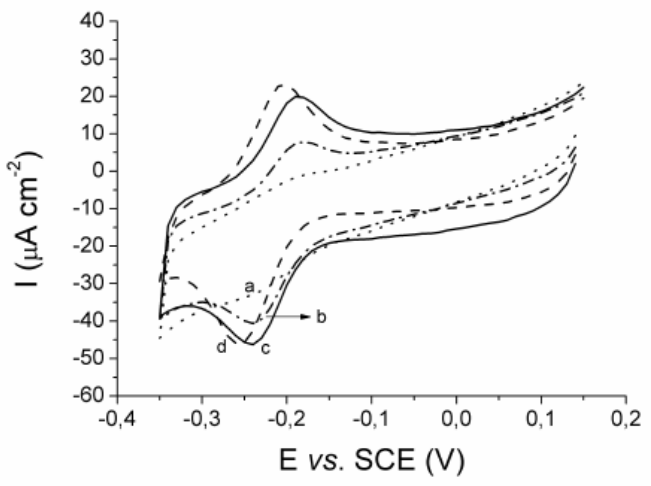

Figure 6. Cyclic voltammogram of modified electrodes characterization (a) PTy; (b) PTy with immobilized probe; (c) PTy with complementary hybrid; and (d) PTy with non-complementary hybrid in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7; υ = 0.1 V s^{-1}.

The results obtained for the several modified electrodes allow the detection of the involved processes, as shown in Fig. 7. The probe immobilization originates a marked increase of the MB cathodic peak current, which upon its hybridization with a complementary sequence results in an even more noticeable current increase.

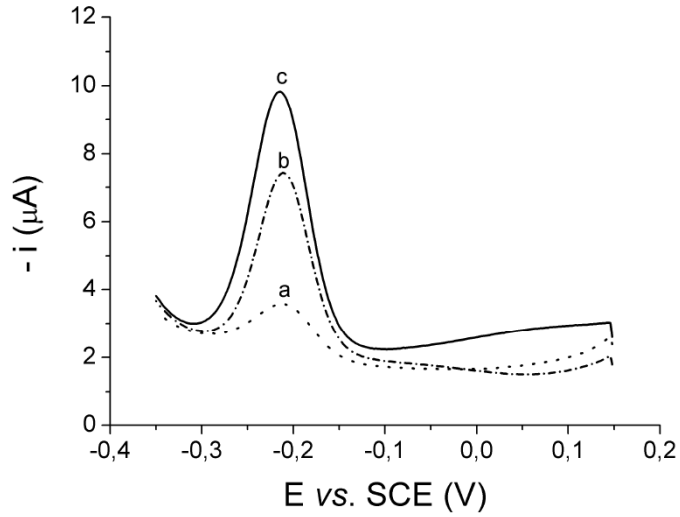

Figure 7. Square wave voltammograms of (a) PTy; (b) PTy with immobilized probe; and (c) PTy with complementary hybrid in 250 μmol dm^{-3} methylene blue in 0.1 mol dm^{-3} phosphate buffer, pH 7 solution. SWV parameters: 0.002 V step, 0.025 V pulse height, and 5 Hz frequency.

An independent analysis of the above mentioned possible interaction among the immobilized probe and the non-complementary sequence was attempted by fluorescence. Presenting different affinity for ss-DNA and ds-DNA, PicoGreen®

reagent can be used to distinguish between the immobilized probe and its hybridization with a complementary sequence (maximum quantum yield) and a non-complementary sequence, that in the case of establishment of some bonds with the immobilized probe, will present an intermediary fluorescence.

The fluorescence (λ = 530 nm) of modified electrodes was measured, after staining with PicoGreen® solution. Thus after the hybridization with the non-complementary or with the complementary sequences, the fluorescence signals increased by 13% and 32%, respectively, in comparison to the signal of the immobilized probe, that was used as the control. The results confirm the hybridization of the immobilized probe with a complementary sequence but also support that the non-complementary sequence and the probe establish some interactions.

Conclusions

The data collected in this study demonstrate that PTy is a suitable matrix for the covalent immobilization of ss-DNA probes with a 5'-phosphate modification.

Methylene blue is an appropriate indicator to discriminate the probe immobilization and the hybridization through the characterization of the modified electrodes by cyclic voltammetry or square wave voltammetry. Immobilization of a cylindrospermopsin probe in PTy modified electrodes and subsequent hybridization have been well succeeded as revealed by the cyclic voltammetry and square wave voltammetry techniques, where a current density increase was obtained upon the immobilization and more after the hybridization process with both methods, in methylene blue containing electrolytes. As expected, the use of square wave voltammetry allows a better discrimination of the involved process and thus it is a more appropriate technique for the characterisation of the systems considered in the present work.

An independent method, based on fluorescence of the modified electrodes after staining with a PicoGreen® solution, also confirmed the hybridization of the covalently immobilized probe.

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