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Oligonucleotide Immobilisation on Polytyramine-Modified Electrodes Suitable for Electrochemical DNA Biosensors

A. Tenreiro [a)*], C.M. Cordas [b)], L.M. Abrantes [b)]

[a)] *Laboratório de Bioelectroquímica, ICAT, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749 - 016 Lisboa, Portugal*

[b)] *Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749 - 016 Lisboa, Portugal*

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Abstract

The surface of platinum electrodes was modified by electrochemical polymerisation of tyramine to provide binding sites for covalent specific immobilisation of the nucleotide deoxyguanosine triphosphate (dGTP). The EQCM has been used to monitor the growth of polymeric films, which is clearly demonstrated by the decrease in the frequency, corresponding to a continuous mass increase.

The carbodiimide coupling reaction was used to bind the terminal 5' phosphate groups of the dGTP to the available primary amine functions on the polymer surface. The biomolecule immobilisation process was followed by measuring simultaneously the evolution of QC-frequency and open circuit potential. Intrinsic redox signal of guanine base residues provides evidence of the dGTP grafting.

Keywords: Polytyramine; electrochemical polymerisation; EQCM; covalent immobilisation; DNA biosensor.

Introduction

In recent years, there has been considerable interest in the development of DNA biosensors due to their numerous potential applications that range from health care, medicine, food industries and environmental monitoring [1-6]. An important effort has been devoted to develop techniques for immobilising oligonucleotides on suitable supports. In this context, modified electrodes prepared by electropolymerisation of different monomers aiming the immobilisation of oligonucleotides have had many advances during last decade

* Corresponding author. E-mail address: amtenreiro@fc.ul.pt

[2, 7-9]. Among the advantages concerning this approach for biosensor development are the control of surface properties, thermal and chemical stability, reproducibility and amenability of biomolecules immobilisation. A widely used approach for grafting biomolecules to solid supports is by means of an amine functionality introduced to the surface. The utility of amines stems from their high nucleophilicity and the existence of a wide variety of amine-based coupling reactions suitable for use under aqueous conditions [2, 10].

The electropolymerisation of tyramine (4-hydroxyphenethylamine), which takes place through the hydroxyl function leaving available primary amine groups, not requiring chemical modifications, is demonstrated to be a convenient method for electrode modification aiming the immobilisation of biomolecules [11-13].

The grafting of nucleotides, such as the deoxyguanosine triphosphate (dGTP), can be carried out using a standard procedure in which the terminal 5' phosphate groups of the oligonucleotides are activated using carbodiimide chemistry (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hidroxysuccinimide (NHS) as coupling agents) and subsequently react with amino group on the polymer surface [14].

In the present work, the oligonucleotide immobilisation in polytyramine (PTy) films has been followed up by open circuit potential measurements and by microgravimetry. The electrochemical detection of dGTP immobilised was based on the intrinsic oxidation signal due to the electroactivity of the guanine base [15].

Experimental

Electrochemical experiments were performed with a Potentiostat CH Instruments model 620A. A three-electrode cell configuration consisting of a Pt disk with 0.196 cm^2 geometrical area, a saturated calomel reference electrode (SCE) and a platinum foil auxiliary electrode was used.

Electrochemical quartz-crystal-microbalance (EQCM) experiments were performed with a frequency analyser (CH Instruments model 420), in a single-compartment cell. The working electrode was a 8 MHz AT-Cut quartz crystal

coated with 1 000 Å Pt disk with 0.2 cm^2 geometrical area, the counter electrode a Pt wire and the reference a saturated calomel electrode.

All potentials are reported versus SCE.

Tyramine (Ty), N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were purchased from Sigma. Reagents for characterisation solutions were of analytical reagent grade and obtained from Merck. Deoxyguanosine triphosphate, PCR grade, was purchase from Invitrogen Life Technologies. All reagents were used as received and the solutions were prepared using Milli-Q water (Millipore).

Polytyramine films were grown by potentiodynamic electropolymerisation on platinum electrodes from a solution containing 0.025 $mol.dm^{-3}$ of tyramine (Ty) in 0.1 $mol.dm^{-3}$ H_2SO_4.

The immobilisation of the dGTP onto synthesised PTy films was performed at room temperature from solutions containing 0.001 mol dm^{-3} of the nucleotide, 0.03 mol dm^{-3} NHS and 0.01 mol dm^{-3} EDC in water.

The morphology of the PTy films was observed by scanning electron microscopy (SEM – JEOL.JSM – 5200 LV).

Results and Discussion

Electropolymerisation and characterisation of polytyramine films

Cyclic voltammograms for electropolymerisation of tyramine are presented in Fig. 1. The potential was cycled between 0 and 1.15 V at 20 $mV.s^{-1}$. In the first cycle, oxidation currents are detectable at *ca.* 0.8 V and a well defined current peak is observed at 1.05 V. During the reverse potential sweep, the nearly zero current shows no indication of polymer reduction. During the second and third cycles (Fig. 1a) the anodic peak current drops significantly, whereas for the 18 subsequent cycles, an increase in anodic current (seen in Fig. 1b) suggests that the oxidation of the monomer continues, thereafter a decrease in the peak current is observed again (Fig. 1c).

The electrodeposition of polytyramine films on the electrode surface has also been monitored by the EQCM, which helps in elucidating the changes observed

on the growth pattern. As expected, essentially identical results were obtained (Fig. 2a). When the potential is cycled in the – 0.1 V to 1.05 V domain a gradual decrease of the anodic current is observed during the first 10 cycles, followed by an intermediate period where the current remains constant (from the 10^{th} to 14^{th} cycle), and finally by a current increase until the 25^{th} cycle. Notwithstanding, the polymer growth on the electrode surface is clearly demonstrated by the decrease in the frequency observed by EQCM (Fig. 2b), corresponding to a continuous mass increase.

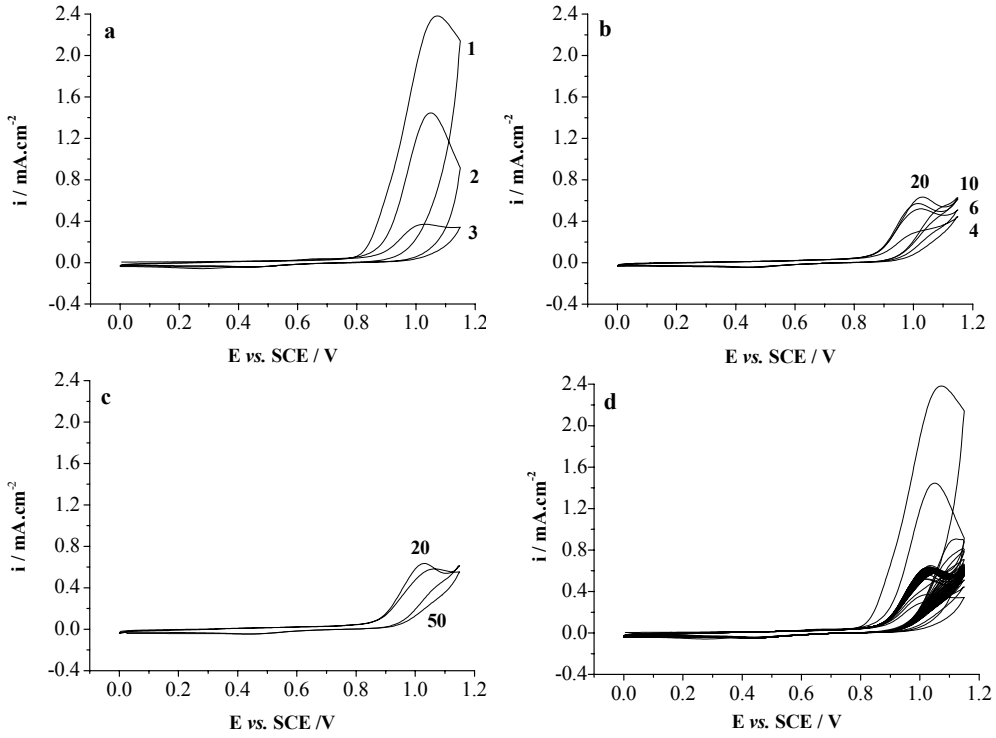

Figure 1. Cyclic voltammograms for the potentiodynamically growth of PTy on Pt electrode from 0.025 mol dm^{-3} Ty/0.1 mol dm^{-3} H_2SO_4; ν=20 $mV.s^{-1}$. (**a)** cycles 1 to 3; (**b)** cycles 4, 6, 10 and 20; (**c)** cycles 20 and 50; (**d)** 50 cycles. The numbers on the curves indicate the cycle number.

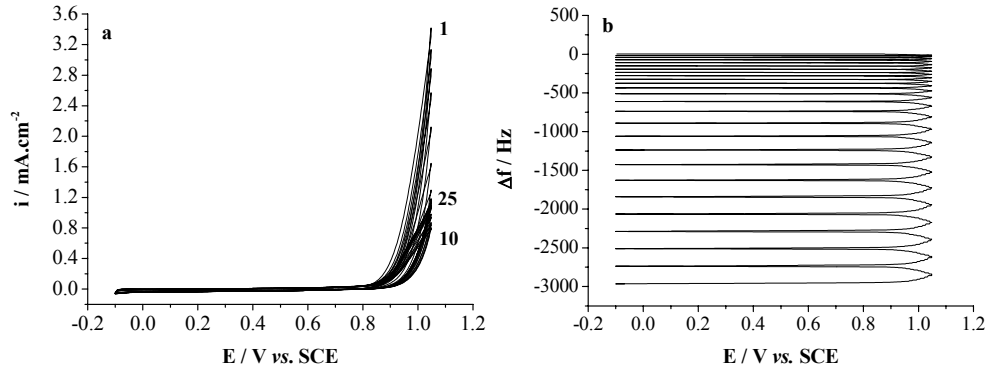

Figure 2. Cyclic voltammograms (**a**) and simultaneously recorded frequency variation (**b**) for the potentiodinamically growth of PTy (25 cycles, between –0.1 V and 1.05 V) on Pt electrode from 0.025 mol dm^{-3} Ty/0.1 mol.dm^{-3} H_2SO_4; ν=50 $mV.s^{-1}$. The numbers on the curves indicate the cycle number.

The overall frequency change due to the electrodeposition of polytyramine film is about 2 818 Hz, corresponding to a mass increase of approximately 18.1 μg [16, 17].

It must be pointed out that the frequency decrease during the polymerisation process is not monotonic. For the first 10 cycles the change in frequency is about 51.6 Hz, which yields a mass increase of 0.34 μg per polymerisation cycle; from the 10^{th} to the 14^{th} cycle, an average mass increase of 0.41 μg (Δf = 64.4 Hz) can be estimated, but the largest mass variation is achieved during the last 11 cycles, corresponding to an increase of 1.19 μg (Δf = 185.87 Hz) per cycle. This behaviour is in agreement with the observed electrochemical responses where three distinct growth regimes appear to occur.

The EQCM data also confirm that the polymer film is not reduced within the considered potential domain. In fact, the frequency pattern is remarkably constant below 0.8 V indicating the lack of participation of electrolyte ionic species.

The morphology of the PTy film, observed by scanning electron microscopy (Fig. 3) shows an irregular polymer film, which is likely related with the different polymerisation regimes.

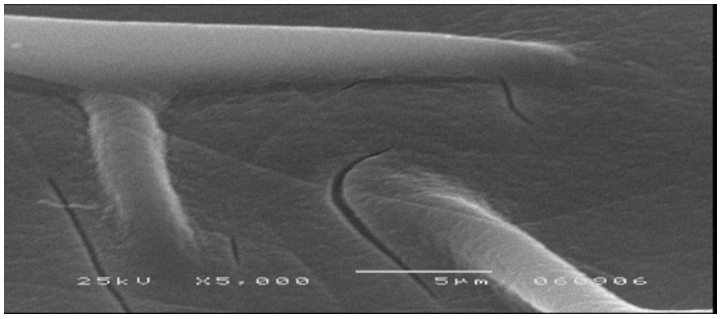

Figure 3. Scanning electron micrograph of PTy modified electrode. Magnification: 5 000x

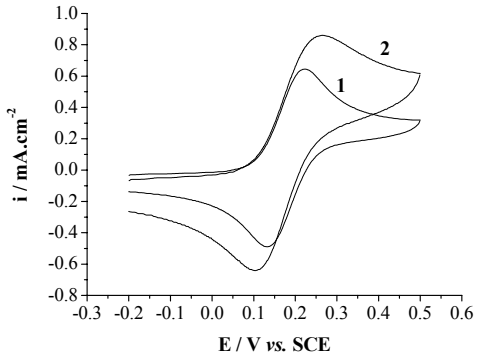

Figure 4. Cyclic voltammograms obtained at PTy modified electrodes using 0.04 mol.dm^{-3} $K_3[FeCN_6]$/ 0.04 mol.dm^{-3} $K_4[FeCN_6]$ in 0.1 mol.dm^{-3} phosphate buffer pH 7; ν=50 mV.s^{-1}; (1) Pt bare electrode; (2) PTy modified electrode.

The PTy modified electrode response in a ferro/ferricyanide solution (0.04 mol.dm^{-3} $K_3[FeCN_6]$/0.04 mol.dm^{-3} $K_4[FeCN_6]$ in 0.1 mol.dm^{-3} phosphate buffer pH 7) is displayed in Fig. 4, pointing to the presence of PTy film.

When contrasted with the Pt signal, the peaks current intensity increases and a significant shift in E_{pa} to more positive values is observed, suggesting a larger electrode surface area and a slower electron transport across the polymer.

Nucleotide immobilisation and detection

dGTP immobilisation was tried by immersion and casting based approaches, both involving a pre-treatment of the modified electrode consisting on a polarisation at –0.1 V during 900 s for the expulsion of incorporated anions, and then a wash with Milli-Q water to remove any free monomers from the film surface.

During a microgravimetric procedure the modified electrode was immersed in the solution containing the dGTP and the coupling agents, and the immobilisation process monitored by measuring simultaneously the frequency variation (Δf) and the open circuit potential (E_{oc}) for three hours.

As can be seen in Fig. 5a the frequency change for dGTP immobilisation in polytyramine film is about 251 Hz (curve 2) whereas it represents only 108 Hz (curve 1) for the adsorption of coupling agents. These decreases of frequency can be attributed in the first case to the immobilisation of dGTP on the film, by both specific binding and non-specific adsorption. In the second case, the frequency shift is only due to the non-specific adsorption of coupling agents on the film surface.

Consequently, the frequency variation due to the dGTP immobilisation (specific binding and non-specific adsorption) was estimated to be 143 Hz. This variation corresponds to an increase of mass of approximately 0.92 μg. From this result, we estimated that the PTy modified electrode could bind up to 1.81 nmol dGTP (0.1 mmol dGTP/g polymer).

At the end of the immobilization process (Fig. 5b), the shift of the open circuit potential observed (0.35 V in the presence of dGTP *vs.* 0.089 V in its absence) is in agreement with EQCM results, suggesting an alteration in the interface, which can be due to the settling of covalent bounds.

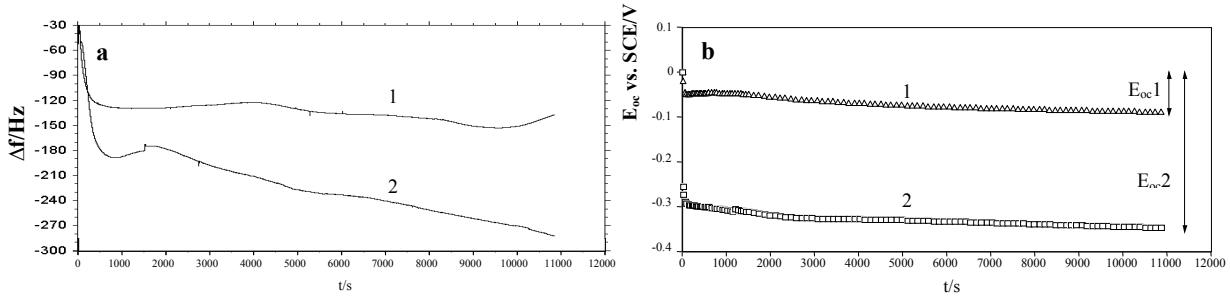

Figure 5. Frequency variation (**a**) and evolution of open circuit potential (**b**) during the dGTP immobilisation in PTy film, grown with 25 cycles between –0.1 V and 1.15 V; v = 50 mV.s^{-1}; immersion of the PTy modified electrode in a solution containing 10 mM EDC + 30 mM NHS (curve 1) and 1 mM dGTP + 10 mM EDC + 30 mM NHS (curve 2).

In the casting approach, after washing the PTy-modified electrode, a 50 µL droplet of a solution containing 2.5 µmol dGTP, 0.25 µmol EDC and 0.75 µmol NHS was dispensed onto the electrode surface. After drying with a flow of N_2, the resulting PTy-dGTP modified electrode was rinsed with Milli-Q water prior to its voltammetric characterisation, to remove coupling agents and free nucleotides.

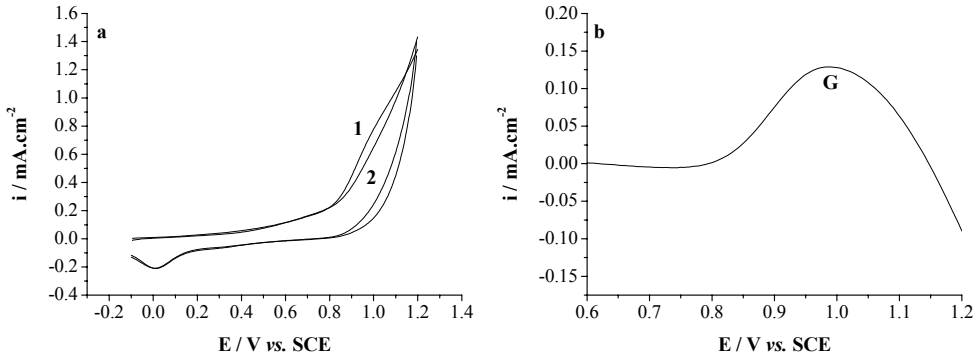

Figure 6. (**a**) Cyclic voltammograms obtained at PTy modified electrodes using 0.1 mol.dm^{-3} phosphate buffer pH 7 ν=50 mV.s^{-1}; curve 1 - after casting of a solution containing 2.5µmol dGTP, 0.25 µmol EDC and 0.75 µmol NHS; curve 2 - after casting of a solution containing 0.25 µmol EDC and 0.75 µmol NHS; (**b**) Anodic guanine signal (**G**) from the current subtraction of curves 2 and 1.

Intrinsic redox signal of guanine base residues in dGTP immobilised at the surface of PTy modified electrode was used as a sign to detect the nucleotide. Guanine oxidation is irreversible and occurs in two consecutive steps, in which the first one is a two-electron/two-proton irreversible process [18]. Fig. 6a shows

the voltammetric responses obtained in 0.1 mol.dm^{-3} phosphate buffer pH 7 for PTy modified electrode after the grafting of dGTP (curve 1) and after casting of the solvent with coupling agents (curve 2).

The results show an increase of current intensity at about 1 V, after dGTP immobilisation. In spite of the weak guanine oxidation signal the subtracted currents (Fig. 6b) make it unequivocal, providing evidence of the dGTP grafting on the polymer surface. The charge associated to the anodic peak (0.1032 mC) allowed estimating an immobilisation of 0.27 nmol dGTP.

Final Comments

This study illustrates the advantages of EQCM for the characterisation of polymer growth and monitorisation of the biomolecules immobilisation, in spite of its non-selectivity between specific and non-specific binding. The variation of the open circuit potential measured in simultaneous with the frequency changes has provided additional information on the immobilisation process.

Tests on the performance of PTy films towards the immobilisation of dGTP revealed promising characteristics: up to 0.1 mmol of dGTP/g polymer.

The PTy-dGTP modified electrodes based on nucleotide immobilisation on an electropolymerised polytyramine matrix display interesting properties, including direct amine functionality, suitable for DNA electrochemical biosensors preparation.

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