J Mater Sci (2010) 45:475–482
DOI 10.1007/s10853-009-3964-z

Electrochemical and morphological studies of an electroactive material derived from 3-hydroxyphenylacetic acid: a new matrix for oligonucleotide hybridization

**Ronalt M. L. Oliveira · Sabrina N. Vieira · Heden C. Alves ·
Erick G. França · Diego L. Franco · Lucas F. Ferreira ·
Ana G. Brito-Madurro · João M. Madurro**

Received: 5 July 2009 / Accepted: 5 October 2009 / Published online: 22 October 2009

Abstract This paper describes the formation of polymeric films derived from 3-hydroxyphenylacetic acid electropolymerized onto graphite electrodes through cyclic voltammetry. We observed the formation of an electroactive material over the electrode surface. The modified electrode showed significant blocking behavior to electron transfer reaction of the pair redox ferricyanide/ferrocyanide, indicating repulsion electrostatic with the negatively charged carboxylate groups of the polymer. The quasi-reversible behavior to $Ru(NH_3)_6Cl_2$ suggests electrostatic attraction, facilitating the charge transfer. The modified electrode was studied through electrochemical quartz crystal microbalance, electrochemical impedance spectroscopy, and atomic force microscopy. These analyses indicate modification of the graphite electrode. Surface analysis by AFM showed that the morphology of the modified electrode surface presents globular form, randomly distributed, and formed by lower globules with diameter near 100 nm. Immobilization and hybridization of oligonucleotide onto the modified electrode were successfully carried out by using both direct electrochemical oxidation of nitrogenated bases and the redox electroactive indicator methylene blue.

R. M. L. Oliveira · S. N. Vieira · H. C. Alves ·
E. G. França · D. L. Franco · L. F. Ferreira · J. M. Madurro (✉)
Institute of Chemistry, Federal University of Uberlândia,
Uberlândia 38400-902, Brazil
e-mail: jmadurro@ufu.br

A. G. Brito-Madurro
Institute of Genetics and Biochemistry, Federal University
of Uberlândia, Uberlândia 38400-902, Brazil

Introduction

The modification of electrodes surface by deposition of functionalized materials has received considerable attention over the last two decades, due to the possibility of generating devices for diverse applications, including electrocatalysis [1–3], membranes [4], and biosensors [5–7] of high sensitivity and selectivity.

The electrochemical technology has been significantly applied to the production of modified electrodes because of the simplicity and advantage of obtaining conducting polymers being simultaneously doped [8–11]. Previous studies of electropolymerization of aromatic compounds indicate that monomers containing aromatic groups directly bonded to oxygen are easier to polymerize, presenting high reproducibility and mechanical resistance of the obtained film, allowing higher stability of the modified electrode [12, 13].

Conducting polymers present π-electrons conjugated that can produce single and double bonds alternating along the polymer chain, conferring properties to the polymer formed, such as: electrical conductivity, low ionization energy, low optical energy of transition, and high electron affinity, properties found only in inorganic materials [14–19]. These polymers show mixed ionic and electronic conductivity, and therefore, act as ion-to-electron transducers. For this reason, conducting polymers are greatly promising to be robust, reliable electrochemical sensors and to offer low manufacturing cost with the possibility of easy miniaturization and automation [20].

Polymers functionalized with carboxyl, amino, aldehyde or succinimidyl carbonate groups favor the formation of covalent attachment with biomolecules for potential applications in catalysis, sensors, and others [6].

An intensive research effort has been performed in the field of analytical electrochemistry seeking for designs of electrochemical biosensors capable to provide better analytical characteristics in terms of sensitivity, selectivity, reliability, easy manufacturing, and use, as well as low cost [21]. There is an increased interest in the application of DNA to biosensors because of its interesting electrical conducting electrochemical properties and unique three-dimensional structures.

Immobilized DNA on the surface of a substrate electrode not only provides a biocompatible microenvironment for biomolecules, but also greatly increases the coverage of biomolecules on the electrode surface [22, 23].

In electrochemical biosensors, a single-chain of DNA is immobilized onto an electrically active surface (electrode), being measured changes in electrical parameters (e.g., current, potential, conductance, impedance, and capacitance) caused by the hybridization reaction. The emergence of solid electrodes has improved the applicability of electrochemical methods for nucleic acid analysis [24].

Recently, we investigated the electropolymerization of 4-hydroxyphenylacetic acid on the surface of graphite electrodes and their applications in incorporation and electrooxidation of nitrogenated bases of DNA [25], oligonucleotides or conserved genomic sequence of the dengue virus [7]. These electrodes modified with poly(4-hydroxyphenylacetic acid) were found to be more efficient in immobilizing purine bases when compared to bare graphite electrodes. This parameter is very important to guarantee the sensibility of the DNA biosensor [26].

In this work, we investigated the electropolymerization of 3-hydroxyphenylacetic acid, onto graphite electrodes surface, the electrochemical and morphological properties of the modified electrode, and also the immobilization and detection of oligonucleotide, using the modified electrode. The monomer 3-hydroxyphenylacetic acid was studied to electropolymerization because the *ortho* and *para* positions, relative to hydroxyl group, are free to couple.

As far as we know, this is the first report in the literature about the synthesis, electrochemical, and morphological studies and application to immobilization and detection of oligonucleotide, of polymer derived from 3-hydroxyphenylacetic acid.

Experimental

Chemicals

All reagents used were of analytical grade. Ultra high pure water (Millipore Milli-Q system) was used in the preparation of the solutions. Oligonucleotide probes, poly(A) and target, poly(T), were synthesized by Invitrogen Life Technologies with the following sequences: poly(A) 5′-AAAAAAAAAAAAAAAA-3′, poly(T)5′-TTTTTTTTTTTTTTT-3′. Stock solutions of the probe (6.4×10^{-2} mmol L^{-1}) and target oligonucleotides (1.8×10^{-1} mmol L^{-1}) were prepared in deionized water and stored at −20 °C until use. Buffer components (CH_3COOH and CH_3COONa) were purchased from Sigma–Aldrich Chemical, USA (ACS purity) and prepared at pH 4.7. Monomer solutions, 3-hydroxyphenylacetic acid (2.5 mmol L^{-1}), were prepared in $HClO_4$ solution (0.5 mol L^{-1}), immediately before their use. All reagents were used as received. The experiments were conducted at room temperature (25 ± 1 °C).

Apparatus

Electrochemical polymerization was performed in a three-compartment cell using a using a potentiostat from CH Instruments model 420A, with a graphite disk (6 mm diameter) cut from a graphite rod (99.9995%, Alfa Aesar) as working electrode. Platinum was used as counter electrode. All potentials are referred to the silver/silver chloride reference electrode (Ag/AgCl, KCl 3 mol L^{-1}). The graphite surface, prior to electropolymerization, was mechanically polished with alumina slurry (0.3 μm diameter), ultrasonicated, washed with distilled water, and dried in the air. All solutions were degassed by nitrogen bubbling. The impedance spectra were collected using an AUTOLAB system (PGSTAT302N and FRA2 module) from Eco Chemie BV.

The electrochemical studies were performed using an electrochemical quartz crystal microbalance 420A from CH Instruments. Morphology of the graphite and modified electrodes was assessed by atomic force microscopy (AFM) (Nanoscope IIIa, Digital Instruments).

Preparation of poly(3-hydroxyphenylacetic acid) films

The growth of poly(3-hydroxyphenylacetic acid) [poly(3-HPA)] on graphite electrodes was performed by potentiodynamic electropolymerization (100 successive potential scans between −0.7 and 1.2 V vs. Ag/AgCl, at 50 mV s^{-1}) from 3-HPA solution (2.5 mmol L^{-1}). $HClO_4$ solution (0.5 mol L^{-1}) was used in all experiments as supporting electrolyte. After electropolymerization, the modified electrode was rinsed with deionized water to remove unreacted monomer.

Electrochemical impedance analysis

Electrochemical impedance spectra were measured, in $Ru(NH_3)_6Cl_2$ solution (5.0 mmol L^{-1}) containing KCl 0.10 mol L^{-1}, for graphite electrodes without film and modified with poly(3-HPA) films prepared using 100 potential scans. This solution was deaerated with ultra pure nitrogen for ca. 40 min before and during measurements. A conventional three-electrode cell was used containing a platinum plate as counter electrode and Ag/AgCl as reference electrode. The measurements were carried at ambient temperature (25 ± 2 °C). All measurements were obtained at −0.15 V, after stabilization of current. The frequency range accessed covered the 10^5–10^{-2} Hz interval. A sinusoidal excitation amplitude of 5 mV (p/p) was used. Fitting of the impedance spectra to a model equivalent circuit was carried by using the Autolab PGSTAT 302N software, Version 4.9.007.

Oligonucleotide probe immobilization on graphite electrode/poly(3-HPA)

The biomolecules immobilization was carried out by applying 15 µL of stock solution of poly(A) probe (6.4×10^{-2} mmol L^{-1}) to the modified electrode surface and dried at room temperature (25 ± 1 °C) during 15 min. After this procedure, the electrode was immersed for 6 s in acetate buffer. Differential pulse voltammetry measurements were conducted using the same buffer (0.1 mol L^{-1}, pH 4.74) as electrolyte.

Hybridization investigation of oligonucleotide immobilized on poly(3-HPA)

After the probe immobilization, 15 µL of poly(T) (1.8×10^{-1} mmol L^{-1}) (target) was applied to the modified electrode. Hybridization was carried out at 42 °C for 15 min. The electrode was then rinsed by immersion in acetate buffer during 6 s. For the electrodes using indicator, 15 µL of methylene blue (50×10^{-5} mol L^{-1}), prepared in NaCl solution (20×10^{-3} mol L^{-1}) was applied to the electrode surface. After 15 min of reaction with MB at room temperature, a final rinsing step was carried out by immersion in acetate buffer during 6 s.

Results and discussion

Electrochemical behavior of 3-HPA

Cyclic voltammograms showing the electropolymerization of poly(3-HPA) onto graphite electrodes, with 100 successive cycles of potential, between −0.7 and +1.2 V are presented in Fig. 1.

Figure 1 shows an irreversible peak in +1.03 V, which is attributed to the monomer oxidation. This value is higher than the oxidation peak of 4-hydroxyphenylacetic acid (+0.95 V) [7]. This difference is related with the higher resonance effect to 4-hydroxyphenylacetic acid. During the subsequent potential scans, it is observed that the peak current decreases, indicating consume of the monomer. After the first cycle, a gradual increase in the peak current is observed between +0.35 and +0.55 V, pointing to the formation of an electroactive material on the graphite electrode.

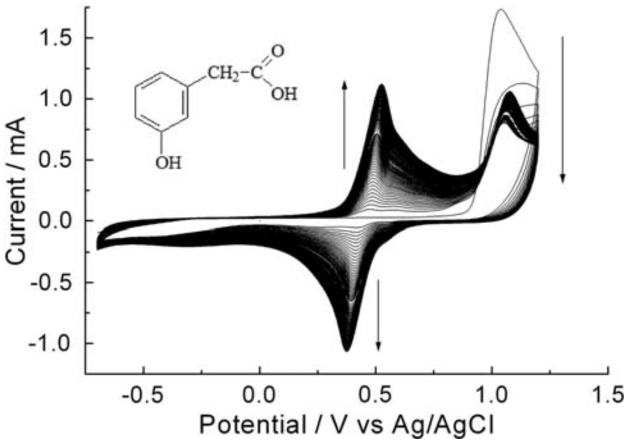

Fig. 1 Cyclic voltammograms of graphite electrode, after successive potential scans, 100 scans, 50 mV s^{-1}, in $HClO_4$ (0.50 mol L^{-1}) + 3-HPA solution (2.5 mmol L^{-1}). The *arrows* indicate the behavior of the current with consecutive potential scans. *Inset* 3-HPA structure

Electrochemical characterization of the modified electrodes

After 100 potential scans in (3-HPA) solution, the graphite electrode was evaluated in $HClO_4$ solution (Fig. 2a), and in KNO_3 solution containing the redox pair $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (Fig. 2b) and $Ru(NH_3)_6Cl_2$ (Fig. 2c), as probe molecules.

Figure 2a shows that the graphite electrode was modified after 100 potential scans in 3-HPA solution. The modified electrode presents well-defined redox peaks, in +0.39 and +0.53 V, vs. Ag/AgCl, indicating electrodeposition of electroactive material, with quasi-reversible behavior ($\Delta E = 140$ mV), in contrast with poly(4-hydroxyphenylacetic acid), that presents redox peaks, in +0.30 and +0.63 V, vs. Ag/AgCl, with irreversible behavior ($\Delta E = 330$ mV).

As shown in Fig. 2b, the bare graphite electrode shows reversible behavior of ferricyanide/ferrocyanide, implying that this redox pair undergoes electron transfer. In contrast, the modified electrode shows significant blocking behavior to electron transfer reaction of this redox pair. The ferricyanide/ferrocyanide redox pair is sensitive to the charge of the electrode surface, because it is negatively charged in both redox states. In KCl aqueous solution, pH 7, the carboxylic groups of the polymer are deprotonated,

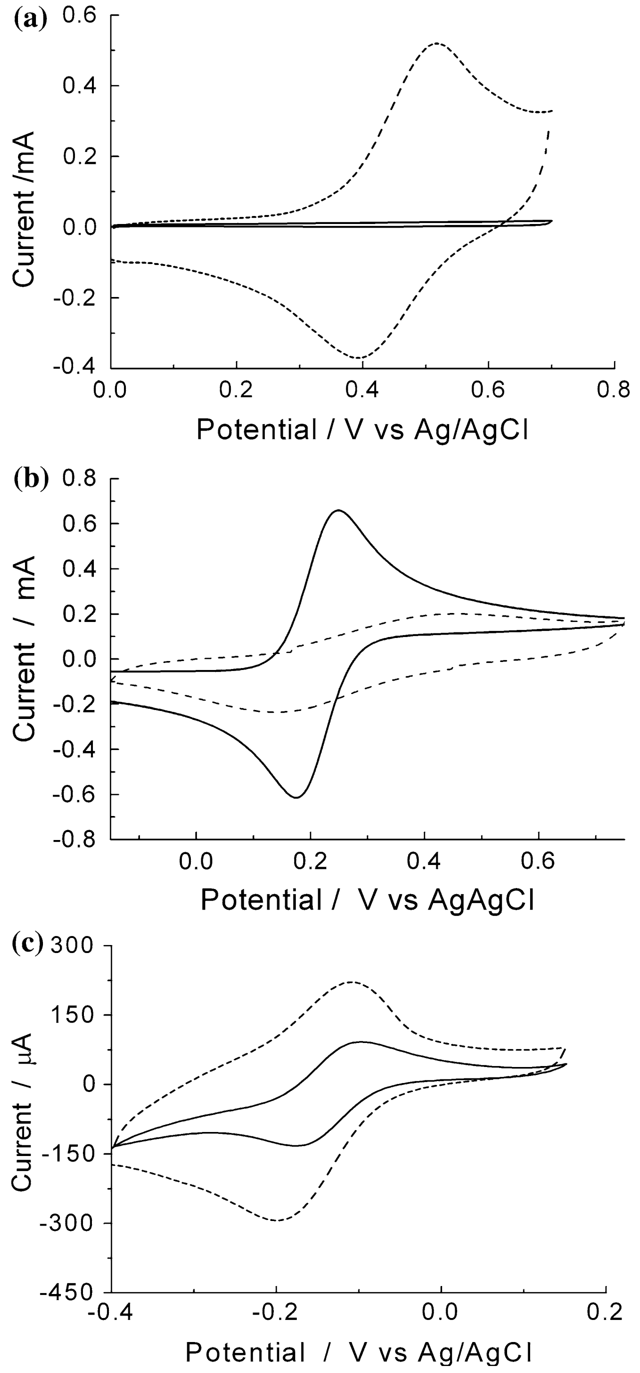

Fig. 2 Cyclic voltammograms of bare graphite electrode (—), and graphite electrode after 100 potential scans in 3-hydroxyphenylacetic acid solution (- - -). **a** $HClO_4$ solution (0.5 mol L^{-1}); **b** KNO_3 solution (0.1 mol L^{-1}), containing $K_3Fe(CN)_6$ (5 mmol L^{-1})/$K_4Fe(CN)_6$ (5 mmol L^{-1}); **c** KNO_3 solution (0.1 mol L^{-1}), containing $Ru(NH_3)_6Cl_2$ (5.0 mmol L^{-1}); 50 mV s^{-1}

resulting in electrostatic repulsion between negative charges of the carboxylate groups and the redox probe, causing significant reduction of the electron transfer reaction between the pair redox ferricyanide/ferrocyanide and the modified electrode. This same effect was observed by Pellissier et al. [27] on glassy carbon electrodes modified with carboxylic acid functional groups.

On the other hand, the ruthenium redox reaction through the modified electrode (Fig. 2c) shows a quasi-reversible behavior, indicating that the positively charged ruthenium complexes establish an electrostatic attraction with the negatively charged carboxylate groups of the polymer, facilitating the charge transfer. The increase in current values and area related to the peaks of this ruthenium complex suggests that the modified electrode presents higher superficial area, when compared with bare graphite electrode, increasing the electron transference.

Study of the polymeric film stability

The graphite electrode modified with poly (3-HPA) shows good stability in $HClO_4$ solution. The experiments of stability show that the proportions between the anodic and cathodic peak currents, as well as between the oxidation and reduction charges, are near the unity, indicating reversibility of the redox process of the polymer. After 100 successive potential scans the polymer keeps 78% of electroactivity, indicating good electrochemical stability of the polymer.

Electrochemical quartz crystal microbalance study

The charge is related to the amount of material deposited. During the deposit, the linear variation of the charge with the frequency decrease indicates that the latter can be related to the mass being deposited. The relation between the frequency and charge can be seen in the equation below:

$$\Delta Q = -F\,\Delta f / MMC_f$$

where F is the Faraday Constant, MM is the monomer molar mass, and C_f is the sensitivity constant derived from the Sauerbrey equation [28].

Figure 3 shows the profile between charge and frequency for the electropolymerization of 3-HPA.

As shown in Fig. 3, the profile charge/frequency for the electropolymerization of 3-HPA presents linearity with $R = 0.9988$. This means that a film with rigid features is being deposited on the electrode surface and the Sauerbrey equation is valid to the mass calculus.

The first two cyclic voltammograms together with the mass versus potential profile are shown in Fig. 4a. The mass values at the end of each cycle can be seen in Fig. 4b.

Figure 4a shows that, in the first cycle, the mass values keep constant until around +0.90 V, exactly where the electropolymerization process begins. From this potential until +1.0 V there is a drop in the mass values, in a low extension. This must be due to the cations solvated (electrolyte) being expelled of the electrode surface at potentials

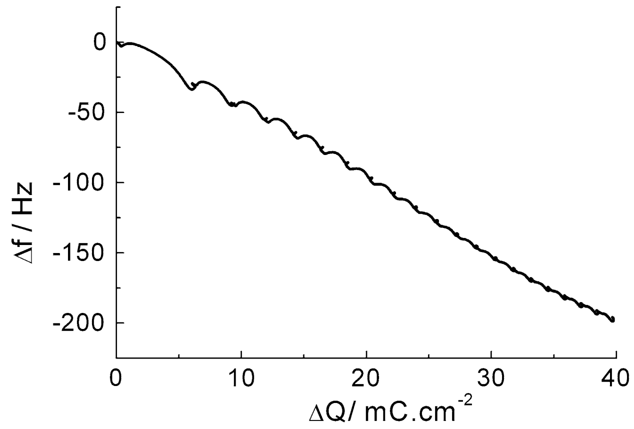

Fig. 3 Frequency vs. charge profile for the electropolymerization of 3-HPA (2.5 mmol L^{-1}) in H_2SO_4 solution (0.2 mol L^{-1}), 20 cycles, 50 mV s^{-1}

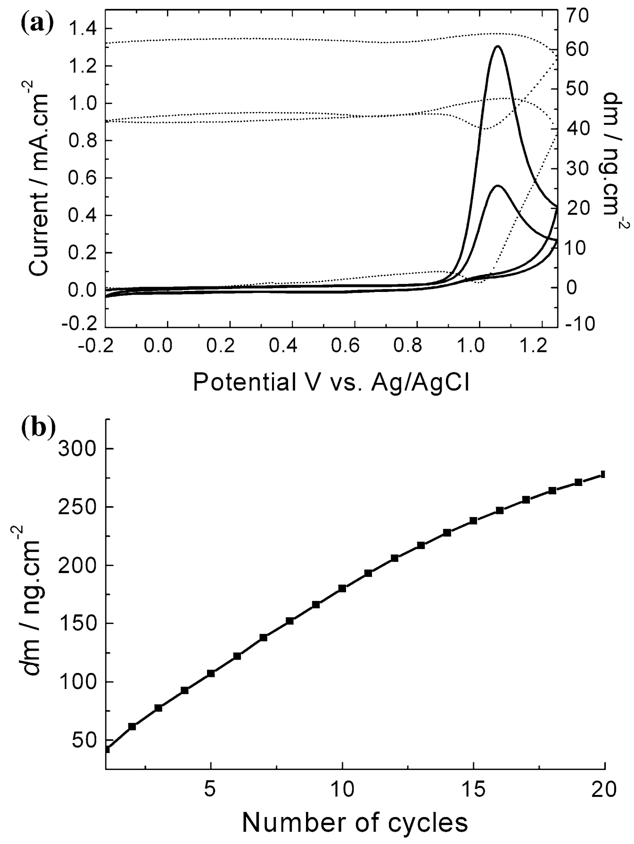

Fig. 4 **a** $j \times E$ (—) and d$m \times E$ (- -) profile of 3-HPA (2.5 mmol L^{-1}) in H_2SO_4 solution (0.2 mol L^{-1}); 2 cycles, 50 mV s^{-1}. **b** dm vs. number of cycles profile, 20 cycles

where non-faradaic current was involved. Near +1.0 V, the monomer is oxidized, producing cation-radical structures, and attacks to neutral molecules, producing oligomers and polymers. These materials adsorb on the electrode surface, as can be seen by the increase of mass values. During the reverse scan, until the same potential (+1.0 V), there is a mass increase due to the oxidation of part of the monomer in this range. From this point on, a little decrease on the mass values is observed, but, maintains constant until the end of the cycle. From the second cycle to the last the same process occurs. Figure 4b shows lack in the linearity at the end of the experiment, pointing less amount of the material being formed due to the electrode surface coverage.

Electrochemical impedance spectroscopy study

The electrochemical impedance spectroscopy (EIS) has been used to study a variety of electrochemical phenomena over a wide frequency range [29].

EIS provides important information on characteristics of an electrochemical system, as the double layer capacitance (Q_{dl}), charge-transfer resistance (R_{ct}), diffusion impedance (W), and resistance of the solution (R_S). It is also an efficient method to evaluate the rate of electron transfer in the electrode in the presence of redox species in solution.

The characterization and investigation of electrical properties of bare graphite electrode and modified with poly(3-HPA) were carried by EIS in $[Ru(NH_3)_6]Cl_2$ solution containing KCl.

Figure 5 shows the EIS spectra known as Nyquist (Fig. 5a) and Bode (Fig. 5b) diagrams for bare graphite electrodes and graphite modified with poly(3-HPA) films, obtained in a potential of −0.15 V. In this potential, the current faradaic is attributed to the redox process of $[Ru(NH_3)_6]^{2+}$, without the influence of electrochemical contribution of polymer (see Fig. 2c).

Figure 5a (inset) shows a semicircle enlargement in high frequency ranges for the modified electrode. For both electrodes, we observed a Warburg (W) behavior which characterizes a system controlled by diffusion. However, the modified electrode presents a second semicircle in low frequency range, which can be seen in Fig. 5b, confirming the graphite-surface modification.

The circuit that describes the electrical properties to the graphite electrode without film and modified with poly(3-HPA) can be represented by $R_S[(R_{ct,1}W)Q_{dl,1}]$ and $R_S[(R_{ct,1}W)Q_{dl,1}](R_{ct,2}Q_{dl,2})$, respectively. The Table 1 gathers the fitting parameters obtained.

The chi-square values of the Kramers–Kronig test of the order of 10^{-2}–10^{-3} attest the good quality of the data. The charge-transfer resistance values in the polymer/solution interface, $R_{ct,1}$, are related to the resistance for the electronic transference process of the redox couple $[Ru(NH_3)_6]^{2+}$. $R_{ct,2}$ values are higher when compared to the $R_{ct,1}$ values, suggesting that the contribution of the electrodic process of the inner region is lower.

Comparing the $Q_{dl,2}$ with $Q_{dl,1}$ values, it is observed that the first is higher. The higher value of double layer capacitance to graphite electrode modified with polymeric film

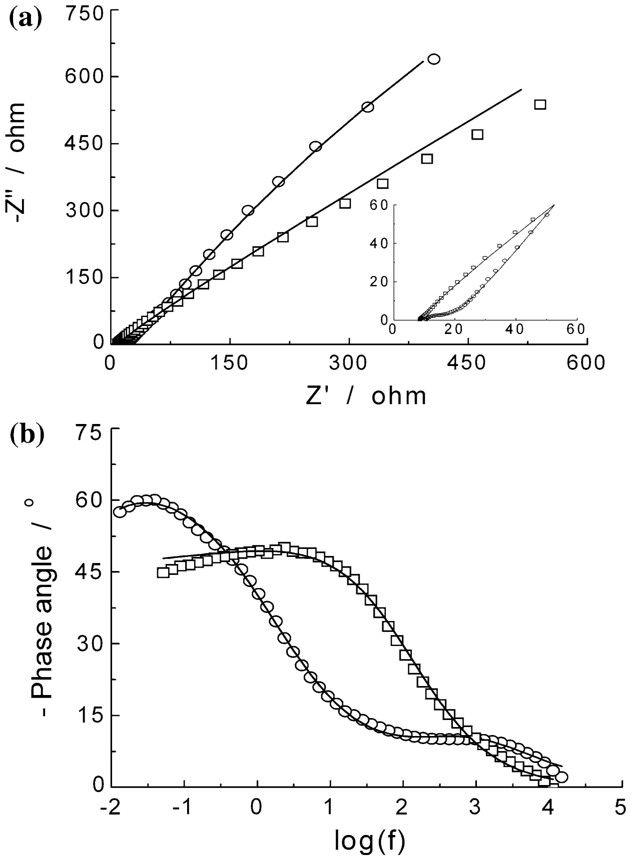

Fig. 5 **a** Nyquist ($-Z''$ vs. Z') and **b** Bode plots in $[Ru(NH_3)_6]^{2+}$ solution (5.0 mmol L^{-1}) containing KCl (0.10 mol L^{-1}) for graphite electrodes without film (*open square*) and modified with poly(3-HPA) (*open circle*). The *continuous lines* represent the fitting to the equivalent circuit. *Inset* amplification of high frequency

Table 1 The fitting values of the equivalent circuit elements for graphite electrode without film (A) and modified with polymeric film (B)

	R_S	$Q_{dl,1}$	n	$R_{ct,1}$	W	$R_{ct,2}$	$Q_{dl,2}$	n	χ^2
A	8.74	0.42	0.81	2.18	0.020	–	–	–	2.52
B	10.11	0.74	0.62	8.94	0.20	3540	12.28	0.81	1.44

R (Ω cm^2) and Q (mF)

indicates the presence of a large number of redox groups on the modified electrode surface which is directly related to the electrochemical process from the polymeric film.

Morphological characterization of the modified electrodes

Atomic force microscopy was used to evaluate the surface modification of the graphite electrode, after electrochemical treatment in 3-HPA solution.

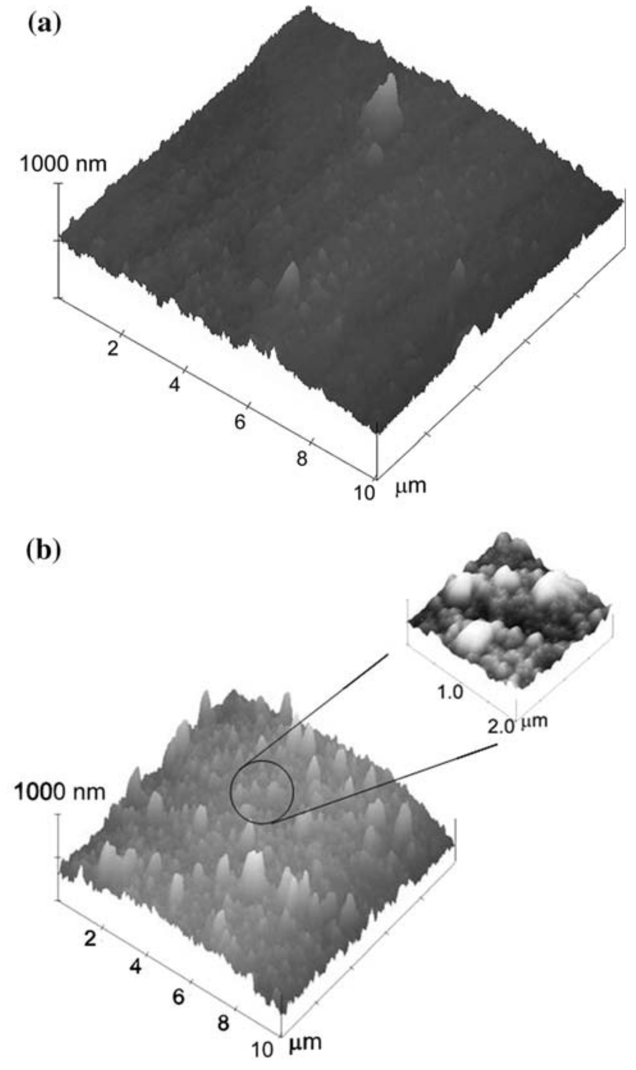

Fig. 6 Topographical images by AFM of bare graphite electrode (**a**) and graphite electrode modified with poly(3-hydroxyphenylacetic acid) (**b**)

Typical 3-D AFM images of graphite electrode and modified graphite electrode are shown in Fig. 6.

Figure 6a shows the graphite surface, after mechanical polishing. As shown in Fig. 6b, the AFM image of the modified electrode reveals material of globular form, randomly distributed, and formed by lower globules aggregated, with diameter near 100 nm (see Fig. 6b, magnifying), with good coverage of the graphite surface.

The roughness values of the graphite and modified electrode, measured through AFM are 496 and 822 nm, respectively. The higher roughness of the modified electrode indicates higher superficial area, enabling higher electron transference.

This observation is in agreement with the results of cyclic voltammetry in ruthenium complex solution (see Fig. 2c).

Investigation of the oligonucleotide immobilization onto graphite electrodes modified with polymeric film

There are two possibilities for the electrochemical detection of a DNA hybridization event: to use electroactive indicators or to detect the guanine moiety signal per se (without indicators).

These two possibilities were explored in Fig. 7. Figure 7a shows the immobilization and detection of a 16-mer DNA sequence on a graphite electrode with film. The hybridization experiments were carried out in incubated solutions containing the two complementary oligonucleotides. Figure 7b shows the voltammetric responses of reduction of electroactive indicator MB accumulated on the modified electrode containing polymeric film in the absence or presence of complementary target.

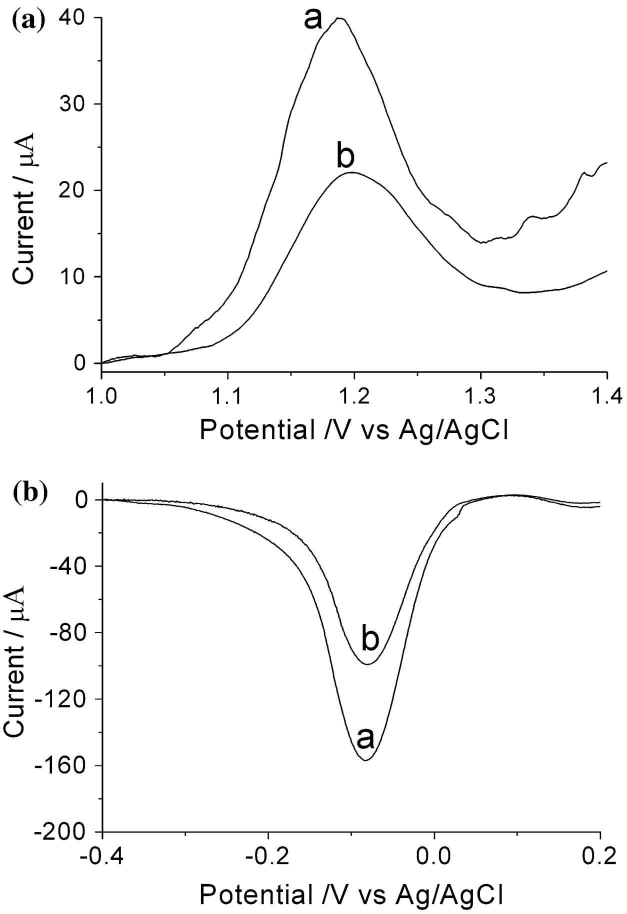

Fig. 7 Differential pulse voltammograms of graphite electrode modified with poly(3-HPA) prepared in pH 0.5, 100 potential scans, containing poly(A). Detection of: (**a**) poly(A); (**b**) methylene blue; (*a*) before hybridization and (*b*) after 20 min incubation with complementary target poly(T). Electrolyte: acetate buffer: (0.1 mol L^{-1}), pH 4.74. Modulation amplitude: 0.05 mV. Pulse interval: 0.2 s; 5 mV s^{-1}, baseline-corrected

The oxidation potential to poly(A) immobilized onto poly(3-HPA) matrix (Fig. 7a) presents minor value (+1.19 V) when compared with immobilization in poly (4-HPA) matrix (+1.29 V) [7], facilitating the electron transfer process. In addition, poly(3-HPA) presents oxidation peak more well-defined to poly(A).

After 15 min of incubation of probe poly(A) with the complementary target poly(T), there is a decrease in the adenine current peak in accordance with the literature [24, 30].

During the oligonucleotides hybridization, hydrogen bonds are formed between the complementary sequences leading to a duplex formation, which is harder to oxidize the bases, decreasing the adenosine oxidation peak current, after hybridization.

Another reason to higher current values obtained for poly(A) is that it presents higher proximity of the electrode surface and higher adsorption degree due to the better conformational flexibility, easing the electron transference between the nitrogenated bases and the electrode.

Single-stranded (ss) and double-stranded (ds) forms of DNA can be distinguished using methylene blue (MB) as redox hybridization indicator [19, 26, 31].

Figure 7b shows a decrease in the reduction current of methylene blue, after hybridization. This may be attributed to lesser MB accumulation on the double-strand DNA caused by the inaccessibility of MB to the DNA nitrogenated bases [32–34], that is, the decrease of the MB signal results from the duplex preventing the interaction of nitrogenated bases with this indicator.

Moreover, the hybridization increases the distance between the mediator and the electrode, decreasing the electron transfer [35, 36]. These effects are in agreement with the literature [23].

Conclusions

This paper presents, for the first time in literature, the electropolymerization of 3-hydroxyphenylacetic acid onto graphite electrode. The electrochemical oxidation of 3-hydroxyphenylacetic acid, investigated by means of cyclic voltammetry, electrochemical quartz crystal microbalance, impedance spectroscopy electrochemical, and AFM images, produced modification onto graphite surface.

The modified electrode presents well-defined redox peaks, indicating electrodeposition of electroactive material.

The profile between charge and frequency, obtained by mean electrochemical quartz crystal microbalance, indicates deposition of film with rigid features on the electrode surface.

AFM images showed that the graphite surface was covered by a material that presents globular form, randomly distributed, and formed by lower globules with diameter near 100 nm.

Incorporation experiments of poly(A) and hybridization with the complementary target poly(T) present good responses and can be detected through differential pulse voltammetry. Additionally, interaction between the oligonucleotides and methylene blue was observed. Based on the indicator response, the hybridization sequence produces a decrease in the current response.

The combination of graphite electrodes with poly(3-HPA) is an interesting strategy for DNA probe immobilization and to other biological elements of recognition.

Acknowledgements The authors are grateful for financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Also, we would like to thank teacher Abílio Borghi for the revision of the English manuscript.

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