Graphite Electrodes Modified with poly(3-hydroxybenzoic acid) for Oligonucleotides Sensors

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Poly(3-hydroxybenzoic acid) (poly(3-HBA)) was produced by the electropolymerization of 3-hydroxybenzoic acid on graphite electrode and characterized by spectroscopic and electrochemical methods. This polymer was used for incorporation of an oligonucleotide as well as detection of the complementary target, by differential pulse voltammetry. The results suggest that graphite electrodes recovered with poly(3-HBA) can be an efficient matrix for immobilization of oligonucleotides in biosensors for DNA.

1. INTRODUCTION

Conducting polymers (CPs) have attracted much interest as suitable matrices of biomolecules and have been used to enhance the stability, speed and sensitivity of various biomedical devices [1,2,3].

Moreover, CPs are inexpensive, easy to synthesize and versatile because their properties can be readily modulated by (i) surface functionalization techniques and (ii) the use of a wide range of molecules that can be entrapped or used as dopants [4].

Some monomers can be electropolymerized directly over a conductive surface, with the goal of improving the properties of these polymers so as to use them as electrodes, permitting the characterization *in situ* of the polymer matrix by using electrochemical and spectroscopic methods [5].

Functionalization of electrode surfaces with conducting polymer films is usually applied in electroanalytical techniques aiming to optimize the electrochemical detection of analytes, non-ionic redox immobilization of biomolecules, particularly enzymes, antibodies and DNA for the development of biosensors [6-10]

Details studies on different aspects of biomolecule immobilization techniques on conducting polymers (CP) for applications in biosensors are presented in the literature [11] and comparative studies were realized between the different mode of biomolecule immobilization techniques, viz. physical, covalent and electrochemical immobilization onto the conducting polymer films for the fabrication of electrochemical biosensors for clinical, food and environmental monitoring applications [12]. In this case, a way as to complement and support the experimental data about molecular structure of polymeric films, methods from quantum mechanics have contributed to the elucidation of the mechanisms related to electropolymerization, through a better understanding of the molecular interactions, properties of polymeric films, also contribute to the understanding of the processes related to the immobilization of biomolecules on the sensing surface [10,13].

In present work, we study the electrochemical polymerization of 3-hydroxybenzoic acid (3-HBA) on a graphite electrode, and application of this matrix for immobilization of oligonucleotides to evaluate of this probe-target complex interaction involving a complementary strand. To the best of our knowledge, this is the first work of polymerization of 3-hydroxybenzoic acid (3-HBA). The electrochemical and spectroscopic characterization of the electro-generated material provided subsidies for the proposition of a mechanism for electropolymerization of 3-HBA.

2. EXPERIMENTAL METHODS

2.1 Chemicals

All reagents were of analytical grade and used as received. Ultrapure water (Millipore Milli-Q system) was used in the preparation of the solutions. Solutions of 3-HBA (2.5 $mmol \cdot L^{-1}$) were prepared in $HClO_4$ solution (0.5 $mol \cdot L^{-1}$) immediately before use. For the electrochemical characterization of the polymer, solutions of potassium ferrocyanide/potassium ferricyanide (5.0 $mmol \cdot L^{-1}$, containing 0.10 $mol \cdot L^{-1}$ KCl) and a solution of hexaaminruthenium (II) (5.0 $mmol \cdot L^{-1}$, containing 0.10 $mol \cdot L^{-1}$ KCl) were used. The following sequences (16mer): probe 5'-GGG GGG GGA AAA AAA A-3' [poly(GA)] and target 3'-CCC CCC CCT TTT TTT T-5' [poly (CT)] of oligonucleotides synthesized by Invitrogen Life Technologies Inc., were used. Stock solutions of the probe (6.3 $\mu mol \cdot L^{-1}$) and target oligonucleotides ($5.86 \mu mol \cdot L^{-1}$; 29 $\mu mol \cdot L^{-1}$; 58.1 $\mu mol \cdot L^{-1}$) were prepared using Milli-Q water. The components for the preparation of pH 4 buffer (CH_3COOH and CH_3COONa) were purchased from Sigma–Aldrich Chemical Company, USA (ACS purity). All essays were conducted at room temperature (25 ± 1ºC).

2.2 Apparatus

Electrochemical polymerization was performed in a three-compartment cell using a CH Instruments model 420A potenciostat with a 6 mm diameter graphite disk, prepared from a graphite rod (99.9 %, Alfa Aesar), as working electrode. Platinum was used as counter electrode. All potentials were referred to the silver/silver chloride reference electrode (Ag/AgCl, KCl 3.0 mol L^{-1}). Prior to electropolymerization, the graphite surface was mechanically polished with alumina slurry (0.3 μm diameter), ultra-sonicated, washed with distilled water, and dried in the air. All solutions were degassed by nitrogen bubbling. The FTIR spectra of the monomer and polymer were obtained on KBr pellets, in the range between 400 and 4500 cm^{-1}, after 20 cycles at a resolution of 4 cm^{-1}, and carried out in a Shimadzu Prestige-21 IR spectrometer.

2.3 Electropolymerization of the 3-HBA

The growth of poly(3-HBA) were conducted on graphite electrodes (100 successive scans of potential, between 0.0 and 1.2 V vs. Ag/AgCl, at 50 mV s^{-1}), in the different pHs values (0.5; 7.0 and 12.0) from 3-HBA solution (2.5 mmol·L^{-1}), prepared in HClO$_4$ (0.5 mol·L^{-1}). The pH adjustment was done using an aqueous solution of NaOH.

2.4 Oligonucleotide probe immobilization on a graphite electrode containing poly(3-HBA)

The biomolecules immobilization was carried out by applying 18 μL of the stock solution (6.3 μmol L^{-1}) of poly(GA) on the surface of the modified electrode and dried at room temperature (25 ± 1 °C) during 60 min. After that, the electrode was immersed for 6 s in acetate buffer (0.1 mol L^{-1}, pH 4.0). Differential pulse voltammetry measurements were conducted using the same buffer as electrolyte.

2.5 Hybridization of the oligonucleotide immobilized on poly(3-HBA)

After the probe immobilization, 18 μL of poly(CT) (5.86 μmol L^{-1}, 29 μmol L^{-1}, 58.1 μmol L^{-1}) were applied on the modified electrode. The electrode was then rinsed by immersion in acetate buffer (0.1 mol·L^{-1}, pH 4.0). In sequence, the hybridization was evaluated by differential pulse voltammetry. Hybridization was carried out at 37 °C for 15 min.

3. RESULTS AND DISCUSSION

3.1 Electrochemical behavior of 3-HBA

Figure 1 shows the cyclic voltammetric behavior of 3-HBA at pH 0.5, 7.0 and 12.0, as function of continuous scanning of potential.
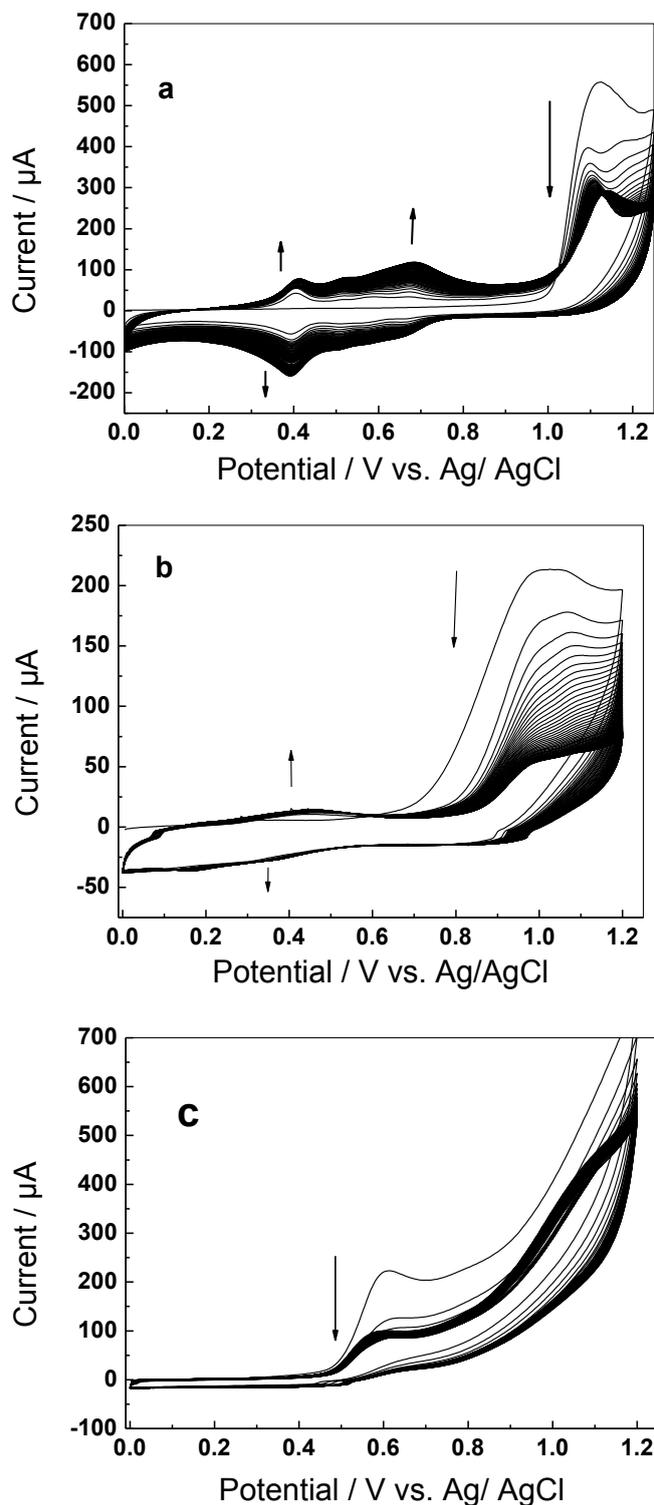
Figure 1. Consecutive cyclic voltammograms (100 cycles) of graphite electrode in solution containing 3-hydroxybenzoic acid (2.5 mmol L^{-1}), at pH 0.5 (a), pH 7.0 (b), and pH 12.0 (c). The arrows indicate the behavior of the current with consecutive scans of potential.

Figures 1a, 1b and 1c show current peaks at 1.12 V, 0.98 V and 0.62 V, respectively, attributed to the oxidation of the monomer (formation of radical-cation) at different pH values. Based on the

carboxylic/carboxylate and the phenol/phenolate equilibria and taking into account the pKa values (pKa$_1$ = 4.08 and pKa$_2$ = 9.92) attributed to 3-HBA, the species present at pH 7.0 and 12.0 are 3-hydroxybenzoate and 3-oxidobenzoate anions, respectively, indicating that the oxidation peaks in neutral and basic medium were shifted to potentials more cathodic, with respect to acid medium, due to the higher electronic density of these species present in these media, resulting in an easier electrooxidation. During the subsequent scans of potential, it is observed that the current peaks decrease, indicating the consumption of monomer.

　　　Figure 1a shows, after the first cycle, a gradual increase in the current peak. It was observed at 0.44 and 0.68 V (oxidation peaks) and 0.43 and 0.60 V (reduction peaks), suggesting the coverage of the graphite electrode by an electroactive material. This is not so remarkable at neutral pH (Figure 1b). At basic pH (Figure 1c), no oxidation/reduction peaks were detected, suggesting electrode passivation. Similar behavior was observed for the isomer 4-hydroxybenzoic acid [14].

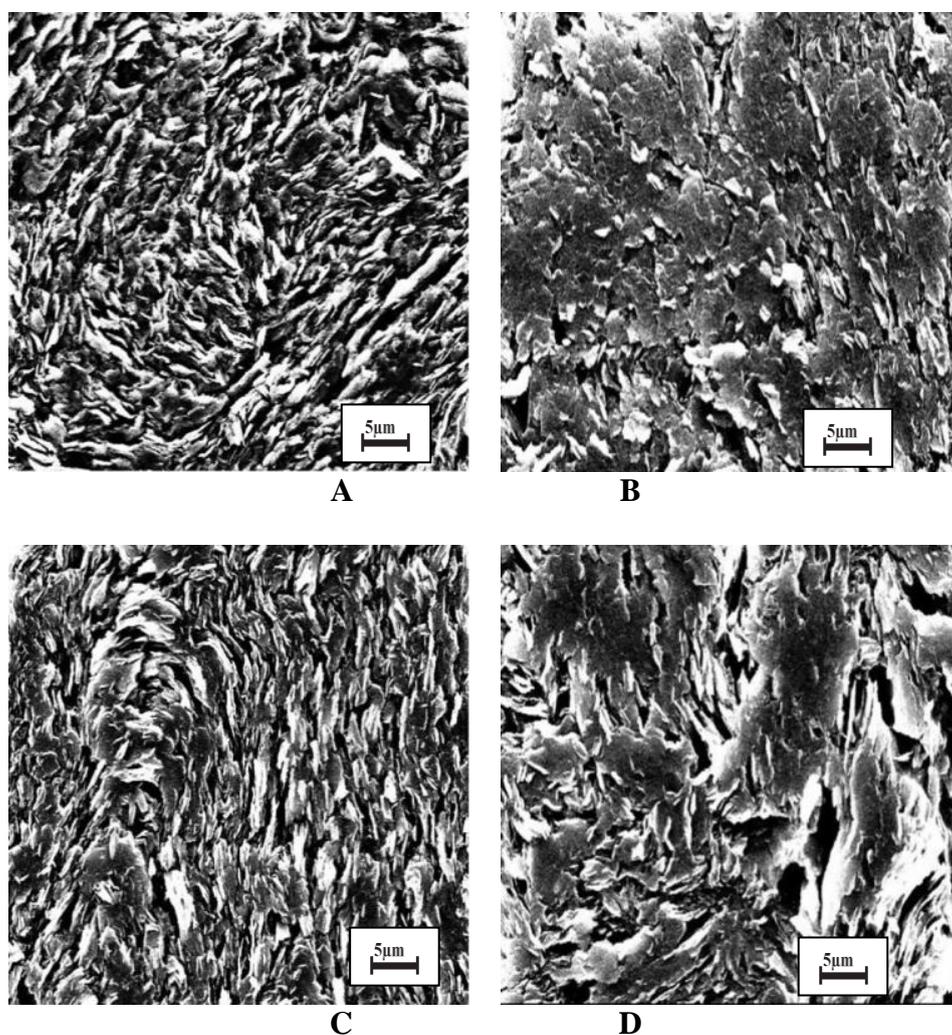*3.2 Morphological characterization of the graphite electrodes modified with* poly(3-HBA)

Figure 2. Scanning electron microscopy of the graphite electrode (A) and of the graphite electrode modified with poly(3-HBA), prepared at pH 0.5 (B), pH 7.0 (C) and pH 12.0 (D).

Scanning electron microscopy was used to evaluate the surface modification of the graphite electrode (Figure 2), before and after electrochemical treatment in 3-HBA solution.

Figure 2 indicates that the graphite electrode surface is quite porous (Figure 2A), and that after electrochemical treatment a product was deposited, modifying the electrode surface (Figures 2B, 2C and 2D). This effect was predominant in acidic medium (Figure 2B), confirming the results obtained by cyclic voltammetry, Figure 1.

3.3 Electrochemical characterization of the modified electrodes

After 100 scans of potential in 3-HBA solution, the graphite electrode was effectively modified by electropolymerization. The modified electrode was compared to the original graphite electrode using two pairs of redox probes: $K_3Fe(CN)_6/K_4Fe(CN)_6$ (Figure 3b) and $Ru(NH_3)_6Cl_2$ (Figure 3c).
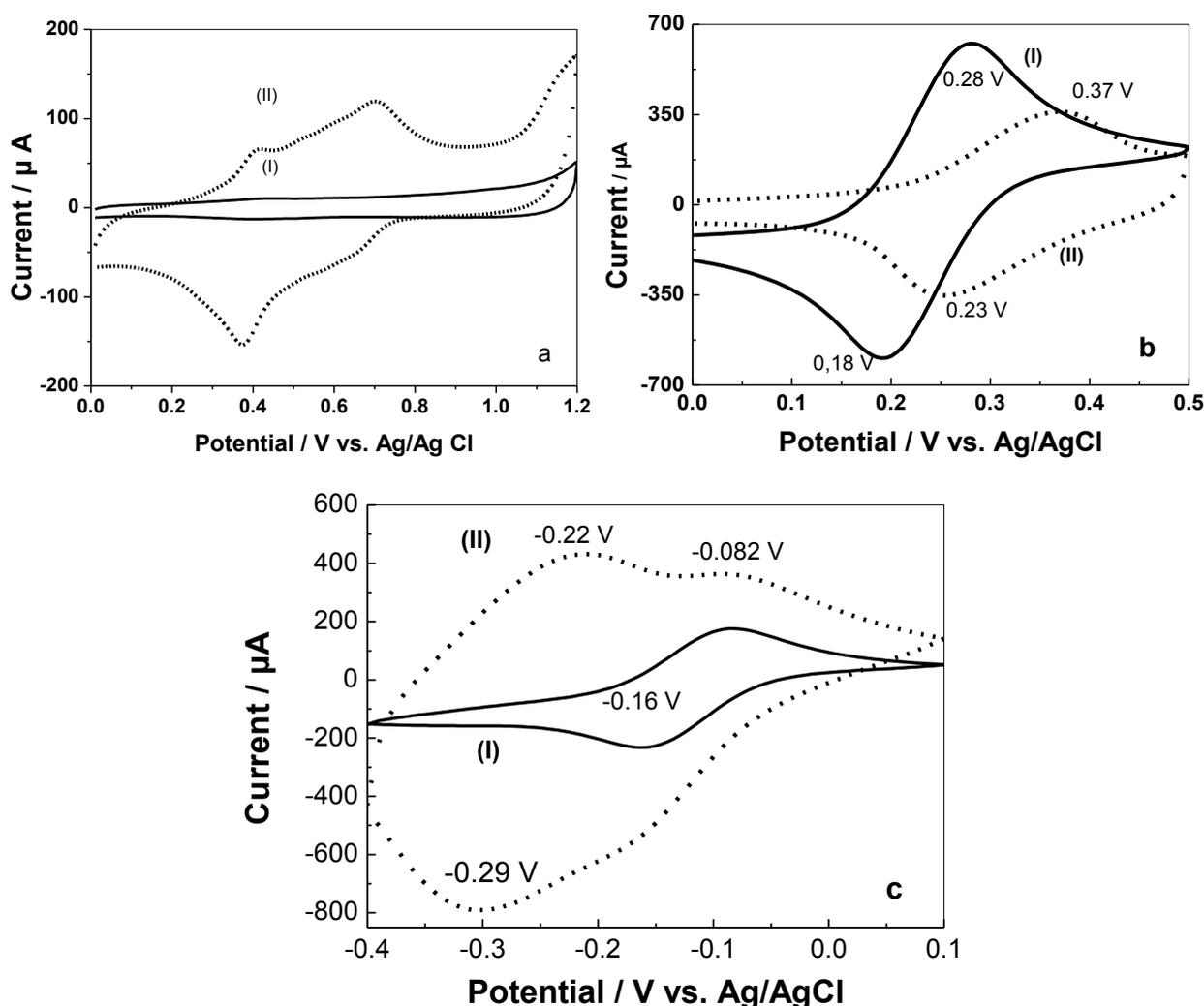

Figure 3. Cyclic voltammograms of the graphite electrode (I) and modified graphite electrode with poly(3-HBA) (II) after 100 scans of potential in acidic solutions of 3-HBA: (3a) $HClO_4$ solution at pH 0.5, (3b) KCl (0.1 mol L^{-1}) containing $K_3Fe(CN)_6$ 6.5 mmol $L^{-1}/K_4Fe(CN)_6$ 5.0 mmol L^{-1}, and (3c) KCl (0.1 mol L^{-1}) containing $Ru(NH_3)_6Cl_2$, 5.0 mmol L^{-1}; 100 mV.s^{-1}.

The modified electrode in acidic medium presents a gradual increase of the peak current at 0.44 V and 0.68 V (oxidation peaks), and 0.43 and 0.60 V (reduction peaks), vs. Ag/AgCl, suggesting the electrodeposition of the electroactive material with reversible behavior. Figure 3b shows that the graphite electrode modified with poly(3-HBA), features a significant block to the charge transfer when in the presence of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox pair. This redox pair is sensitive to the charge on the electrode surface, because it is negatively charged in both redox states. In aqueous solutions of KCl at pH 7.0, the carboxylic groups of the polymer must be deprotonated ($pKa_{1(3\text{-HBA})}= 4.06$), resulting in electrostatic repulsion between the negative charges of the carboxylate groups and the redox probe, causing a significant reduction of the electron transfer reaction between the redox pair $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and the modified electrode. Similar effect was observed in glassy carbon electrodes modified with poly(4-hydroxyphenylacetic acid) [15]. On the other hand, the redox reaction involving Ru(II) through the modified electrode (Figure 3c) shows a reversible behavior, indicating that the positively charged Ru(II) complexes establish an electrostatic attraction with the negatively charged carboxylate groups of the polymer, facilitating the charge transfer [14,15]. The increase in current values and charge related to the Ru(II) complex suggests that the modified electrode presents higher superficial area when compared to the bare graphite electrode, increasing the electron transference. These same analysis by electrochemical characterization of the graphite electrode modified with poly(3-HBA) were also performed at the pHs 7.0 and 12.0.

The graphite electrodes modified with the polymer prepared at pH 7 and pH 12 are probably anionic, since the carboxylic groups present in these polymers must be de-protonated. There are a gradual increase in the peak current at 0.61 and 0.69 V (neutral and basic oxidation peaks), and 0.29 and 0.35 V (neutral and basic reduction peaks), vs. Ag/AgCl, indicating the electrodeposition of the electroactive material, with a semi-reversible behavior. The electrochemical behavior of the formed polymer in neutral and basic media is similar to the material electrogenerated in acidic medium (Figure 3), except for higher values of anodic and cathodic currents, observed in acidic medium (Figure 3a). This can be an indicative of formation of a large amount of material at pH 0.5 (Figure 1a) or that the electrogenerated material in the neutral and basic media has a major character of passivation. A similar behavior was observed with the isomer 4-hydroxybenzoic acid, confirmed by electrochemical quartz crystal microbalance and impedance analyses [14]. The formed polymer at pH 7.0 and 12.0 presents higher charge transfer resistance leading to a lower yield than the polymer prepared at pH 0.5. The lowest value for the oxidation potential of the monomer was attributed to the increased electron density of the ionic species, HBA⁻ and HBA^{2-}, formed in these two media.

3.4 Spectroscopic characterization

The experimental infrared spectra of 3-HBA and poly(3-HBA) are presented in Figure 4.

The infrared spectrum of 3-hydroxybenzoic acid, Figure 4a, presents a very broad band centered near 3000 cm^{-1}, related to O-H stretching, characteristic of carboxylic acid, superimposed to phenolic O-H stretching, which occurs at 3350 cm^{-1}. Additionally, the C=O stretching due to carboxylic acid can be seen at 1696 cm^{-1}. The bands at 1577 and 1468 cm^{-1} are related to C-C

aromatic stretching. At 1391 cm^{-1}, it is observed a combination of O-H bending and C-O stretching, typical of phenolic compounds. Signals related to bending out-of-plane in aromatic C-H can be seen at 877 and 714 cm^{-1}.
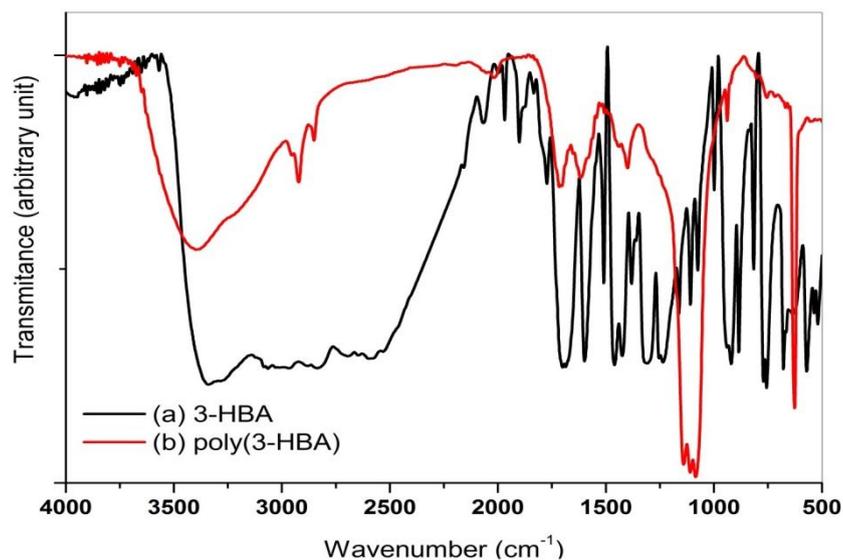

Figure 4. FTIR spectra obtained using KBr pellets for (a) 3-HBA and (b) poly(3-HBA), with 20 consecutive cycles, at a resolution of 4 cm^{-1}.

The spectrum of poly(3-HBA), Figure 4b, presents a band at 3400 cm^{-1}, attributed to OH stretching of phenols superimposed to a very broad band centered near 3000 cm^{-1}, related to OH stretching of carboxylic groups. Two bands, at 1706 and 1627 cm^{-1}, related to the axial deformation of C=O of carboxylic acids, are consistent to the OH deformation in carboxylic acids. As in 3-HBA, for poly(3-HBA), stretches attributed to aromatic C=C, centered at 1399 cm^{-1}, are observed. Intense and well defined bands, centered at 1147, 1055 and 1066 cm^{-1}, related to C-O-C symmetrical stretching, characteristic of ethers, are also observed. The occurrence of this kind of deformation supports the proposition of existence of ether groups in the polymer structure and suggests that at least part of the polymerization occurs by linking between aromatic rings involving phenolic hydroxyl groups, as observed for other similar polymers[13,16]. The proposed model for the film produced by electropolymerization is based on cross-linked chains of poly-aryl-ether, with carboxylic groups located on their extremities. A structure like this, besides presenting a vibrational spectrum that possesses similarities with the obtained for poly(3-HBA), is compatible with the expected, with features that facilitate immobilization of oligonucleotides, promoting transduction, facilitated by its conductive characteristic.

3.5 Formation of poly(3-HBA) by electropolymerization

The proposed mechanism for electropolymerization of 3-HBA can be divided into three steps: (a) electrooxidation of the monomer, (b) coupling of the radical cations involving the C4 and phenolic oxygen, and (c) loss of protons to restabilish the aromaticity. The propagation step is given from the

consequent re-oxidation of the oligomers during the potential cycles on the surface of the graphite electrode, under potentials ever lower, with the formation of the electroactive material. Furthermore, the spectral profiles presented, suggest the predominance of poly-(aryl ether) structures for the polymer obtained under acidic medium.

The Scheme below represents the mechanism proposed for the oxidative polymerization of poly(3-HBA).
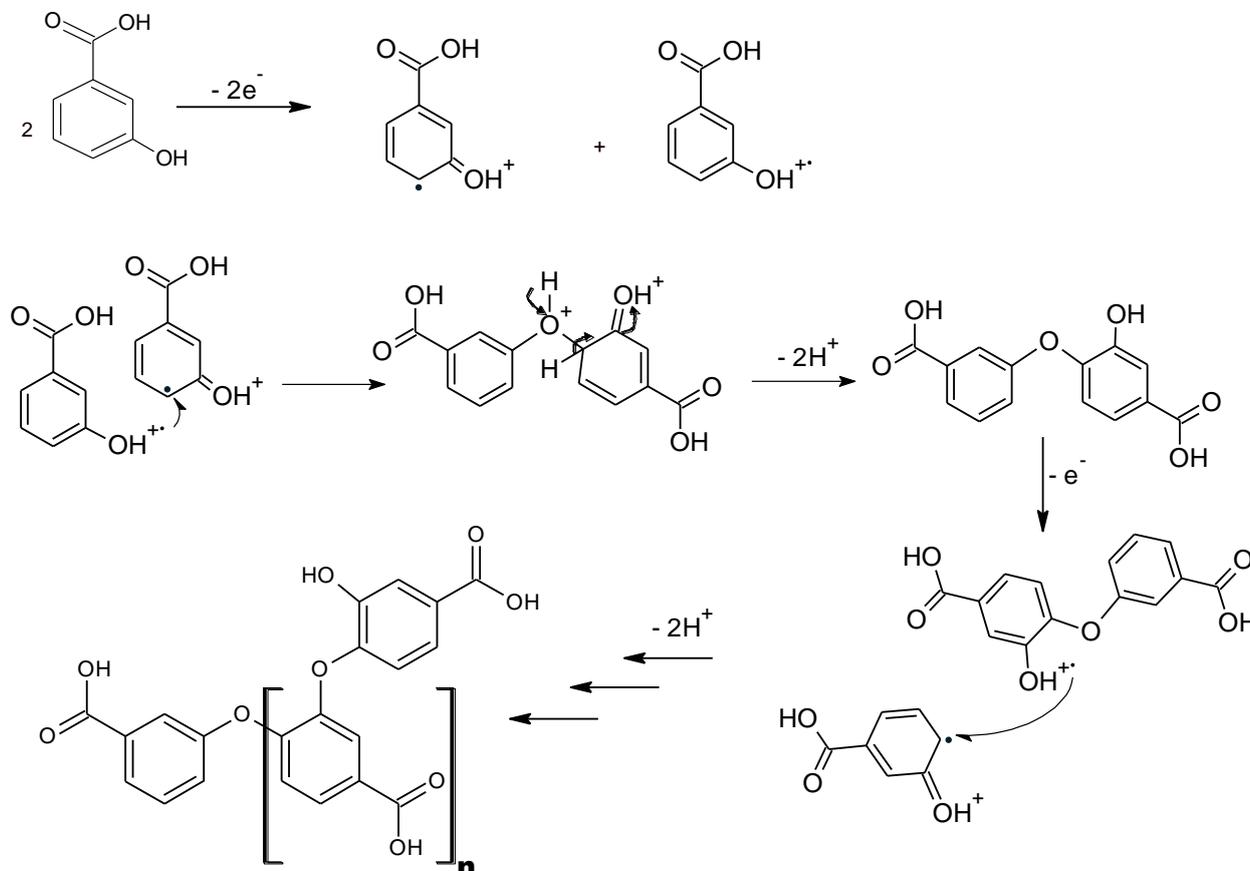
Scheme. Mechanism proposed for the formation of polyethers during the electropolymerization of 3-HBA under acidic medium.

In this scheme the coupling of radical cations based on C4 and the phenolic oxygen results in structures of type poly (aryl ether), with the presence of free carboxyl groups that can interact by hydrogen bonding with biomolecules.

3.6 Immobilization of oligonucleotides on graphite electrodes modified with poly(3-hydroxybenzoic acid)

The hybridization experiments were carried out after incubation with solutions containing the complementary oligonucleotide poly (CT) (3'-CCC CCC CCT TTT TTT T-5') as detection target. Even after washing of modified electrode, two characteristic peaks of oxidation of guanosine and adenosine at 0.9 and 1.28 V were observed, respectively. For quantitative purposes, the information

concerning the current responses was also investigated. Therefore, the adenosine peak was chosen as optimal for the rest of the experiments, due to its better reproducibility. Figure 5 presents differential pulse voltammograms as function of the poly (CT) concentration with oligonucleotide (5'-GGG GGG GGA AAA AAA A-3') immobilized on a graphite electrode modified with poly(3-HBA).
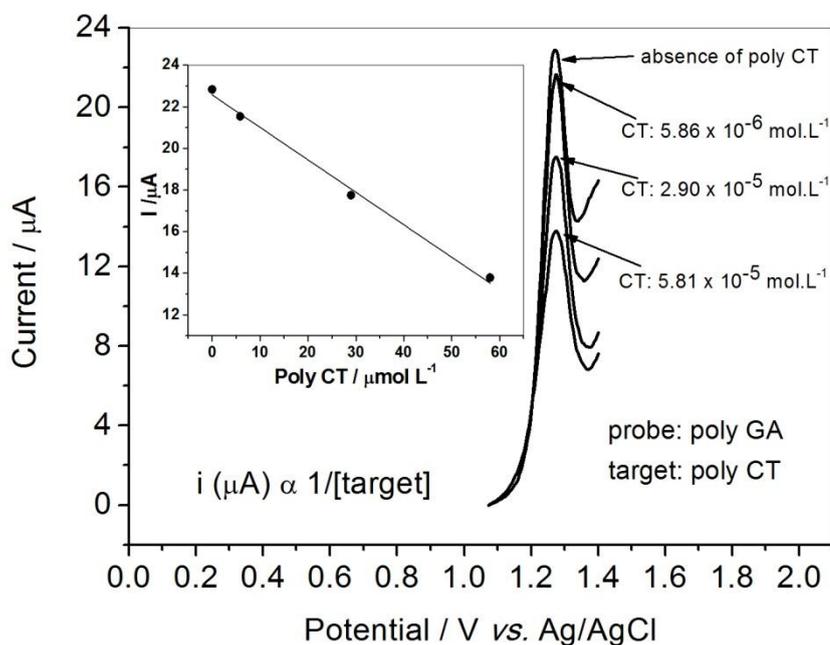

Figure 5. Differential pulse voltammograms of the graphite electrode modified with poly(3-HBA), containing 18 µL of a 6.3 µmol L^{-1} of poly(GA): Absence Poly (CT) and after 15 minutes of incubation with 18 µL of a 5.86 µmol L^{-1}, 29 µmol L^{-1} and 58.1 µmol L^{-1} of the complementary target poly(CT). Electrolyte: acetate buffer (0.1 mol.L^{-1}) at pH 4.00. Experimental conditions: amplitude: 25 mV; pulse width: 0.06 s; sampling width: 0.02 s; pulse period: 0.2 s; 5 mV.s^{-1}.

Figure 5 suggests that the probe poly(GA) was efficiently incorporated on the graphite electrode modified with poly(3-HBA) in acetate buffer (pH 4.0). Studies have shown the interaction of DNA with metal in a graphite platform, where the oxidation peak of guanosine and adenosine were 1.00 V and 1.33 V (vs. Ag/AgCl), respectively in a glassy carbon electrode [17], and 0.95 V and 1.20 V (vs. saturated calomel electrode), respectively, in gold electrodes [18].

The hybridization of the target poly(CT) can be seen where the current peak of guanosine and adenosine decreased after 15 minutes of incubation in acetate buffer. An explanation for this result is that the formation of hydrogen bonds between complementary sequences during the hybridization of oligonucleotides making more difficult to oxidize the nitrogenous bases [8,19, 20]. The reduction in current values observed in hybridization between poly(GA) and poly(CT), when compared to the single strand of poly(GA), due to minor conformational flexibility of hybridized structure (double strand), since the increase in distance between the nitrogen bases and the electro-active surface of the electrode should hinder the charge transfer.

Figure 5 (inset) shows evolution of concentration of Poly (CT) *vs.* I_p. Linear regression analysis of current (μA) versus concentration (μmol L^{-1}) profiles showed a reasonable linearity from 5.86 x 10^{-6} to 5.81 x 10^{-5} mol L^{-1} (r>0.99).

It is important to note that the aim of this work was to show the potential of the new polymeric material used as the immobilization matrix of oligonucleotides in biosensors for DNA.

4. CONCLUSIONS

The 3-hydroxybenzoic acid was electropolymerized on graphite electrode producing an efficient matrix (poly(3-HBA)), suitable for immobilization and detection of oligonucleotides, with potential for the development of biosensors for DNA detection.

The electrochemical characterization showed that the behavior of this electropolymerized material in neutral and basic medium is similar to that observed for the material produced in acidic medium, except by the high values of anodic and cathodic current observed in acidic medium, for being more conductive. This may be an indication that the polymeric materials electrogenerated in neutral and basic media has a larger character of passivation.

The FTIR spectrum of poly(3-HBA) confirming the predicted structure, suggesting the formation of C-O-C bonds during the electropolymerization.

The proposed mechanism for polymerization of 3-HBA occurs basically in three steps: electrooxidation of 3-HBA; coupling between intermediates (involving carbon 4 and phenolic oxygen), and rearrangement with de-protonation, in order to re-establish the aromatic structure, of greater stability. The propagation step is given by the re-oxidation of the oligomers during the cycles of potential on the surface of graphite electrode, on smaller potentials.

Experiments involving the incorporation of poly(GA) and hybridization with the complementary target poly(CT) present good responses that can be detected through differential pulse voltammetry.

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