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Preparation, characterization, and application in biosensors of functionalized platforms with poly(4-aminobenzoic acid)

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Abstract Electropolymerization of 4-aminobenzoic acid (4-ABA) on graphite electrodes (GEs) was investigated for the development of electrochemically functionalized platforms applied to the immobilization of biomolecules. The electrogeneration of 4-ABA was carried out in perchloric acid solutions using cyclic voltammetry (CV) and chronoamperometry (CA) techniques. In the case of CV studies, the GEs were modified by applying 100 consecutive potential cycles, while, in the case of CA studies, the electrodes were modified at different potentials (*E*/V vs. Ag/AgCl): 0.95, 1.05, and 1.15. The modified GEs were characterized in $HClO_4$ solutions in the presence and absence of the ferricyanide/ferrocyanide redox couple (redox probe) using the CV and electrochemical impedance spectroscopy techniques. Scanning electron microscopy was used for morphological characterization. In the case of CA, the best electrochemical activities for the electropolymerization reaction are in the following order of performance: $1.05 > 1.15 > 0.95$ V. The poly(4-ABA) platforms were investigated for the immobilization and direct detection of purine bases (adenine and guanine), where higher values of the anodic peak current ($I_{p,a}$) were observed for the transducers electroformed using CV. In the case of immobilization of poly(GA) oligonucleotides, as well as for the recognition of the hybridization event with the complementary target poly(CT), methylene blue (MB) and ethidium bromide (EB) were used as the indicator and intercalator, respectively. MB was reduced at -0.26 V resulting in the cathodic peak current ($I_{p,c}$) for the *ss*DNA, while EB was oxidized at $+0.58$ V yielding the higher anodic peak current ($I_{p,a}$) for the *ds*DNA. The platforms were also evaluated for immobilization of the DD K peptide, with the antibacterial activity and biological recognition being verified using the complementary (phospholipid 1-palmitoyl-2-oleoyl phosphatidylcholine—POPC) and noncomplementary (phospholipid POPC + cholesterol) targets. The recognition mechanism was monitored from impedance measurements, with a good interaction of the DD K peptide with the POPC mimetic membrane being verified. In addition, the interaction was affected by the presence of cholesterol, revealing that the use of poly(4-ABA) platforms is very promising for the development of biosensors.

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Introduction

One of the major challenges in the bioanalytical analysis is mainly concerned with the development of a method that is rapid and permits the simultaneous detection of several different types of analytes present in different matrices (e.g., biological fluids, foods and environmental samples) [1]. Therefore, the search for better bioanalytical

techniques comprises an important issue in the improvement of clinical diagnoses of several different diseases [2]. One way to obtain a biosensor with the appropriate characteristics for a target analyte is mainly based on the use of electrochemical methods, where the modification of a solid electrode may allow the development of various types of biosensors [3].

It is currently known that a wide variety of living organisms are capable of producing molecules of peptides that act in the first line of defense. Although these studies are relatively recent, thousands of peptides have been isolated, and their biological activities have been proven, i.e., in several cases, the peptides have demonstrated antimicrobial activity of great importance for the body's defense system [4]. Unlike the classical mechanism of action of the antibiotics, which is commonly slow, in the case of antimicrobial peptides, the interaction mechanism is more rapid since it occurs at the bacterial cell surface. Some studies with amphibians have demonstrated that the secretion of antimicrobial peptides increases with the exposure of animals to pathogens and microorganisms, thus acting as an initial barrier to these organisms [5].

According to the literature [6, 7], the search for methods for the identification of genes associated with diseases is of current interest due to its importance for the development of new drugs and new methods for clinical diagnosis, as well as for the premature control of several types of maladies. From this viewpoint, the development of new biological sensors for applications in real-time detection and decentralized tests is of paramount importance. At present, direct and indirect methods are used to detect immobilization of the probe and the target on a specific type of DNA sequence. For instance, the so-called single-stranded (*ss*DNA) obtained after interaction with the complementary target regenerates the double-stranded (*ds*DNA) during a process of hybridization. The signal associated with this process can be detected directly, by oxidation of the nitrogenous bases present in the DNA, or indirectly, through the use of an indicator/intercalator such as methylene blue (MB), ethidium bromide (EB), etc. [8, 9].

Research in the field of conducting polymers for biomedical applications has suffered a great expansion since the discovery in the 1980s that these materials are compatible with many biological molecules [10]. Furthermore, functionalized polymer films can be obtained using electrochemical techniques [e.g., cyclic voltammetry (CV)] by means of the oxidation/reduction of an appropriate monomer. The use of electrochemistry for obtaining polymeric films presents several advantages over other methods, such as a low production cost, a facile synthesis in aqueous solutions at ambient temperature, as well as the formation of conductive thin films whose physical properties can be tuned by adjusting the electropolymerization conditions (e.g., pH, potential scan rate, number of the potential cycles, supporting electrolyte, etc. [11, 12]).

According to the literature [13], the dermadistinctina K antimicrobial peptide (DD K), which can be isolated from the glands of *Phyllomedusa distincta anuran* species found in the Atlantic Forest [14], belongs to the class of cationic dermaseptin molecules and contains 28–34 residues. This peptide is capable of penetrating through the bacterial membrane where it exerts its biological activity and, therefore, is very interesting from the theoretical viewpoint for the development of biosensors.

The main objective of this work is the study of the electropolymerization and characterization of 4-aminobenzoic acid (4-ABA) electroformed on graphite electrodes (GEs) using the CV and chronoamperometry (CA) electrochemical techniques. The platforms obtained were applied in the immobilization of the purine bases and also in the development of genosensors and microbial electrochemical biosensors containing the DD K peptide.

Materials and methods

Chemicals and materials

All chemical reagents were of analytical grade and used without further purification: monomer 4-ABA (Alfa Aesar, 99 %); $HClO_4$ (Vetec, 70 %); $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (Sigma-Aldrich, 99 %); KCl (Vetec, 99 %); purine bases adenine and guanine (Sigma-Aldrich, 99 %); CH_3COOH (Vetec, 99 %) and CH_3COONa (Isofar, 99 %); Na_2HPO_4 and NaH_2PO_4 (Vetec, 99 %); MB (Vetec, 98–103 %); EB (Vetec, 97 %); NaCl (Vetec, 99 %); and chloroform (Vetec, 99 %). The oligonucleotides poly(GA) (5′-GGG GGG GGA AAA AAA A-3′) and the complementary target poly(CT) (3′-CCC CCC CCT TTT TTT T-5′) were obtained as lyophilized powder from Invitrogen®, containing a sequence of 16 base pairs. All solutions were freshly prepared, using ultra-pure water (18.2 MΩ cm) obtained from a Classic DI PURELAB purification system from ELGA. For electrochemical measurements, when necessary, the solutions were degassed with ultra-pure $N_{2(g)}$ for 15 min.

All experiments comprising the electropolymerization and characterization of the electroformed polymeric thin films were carried out using an all-glass three-compartment electrochemical cell ($V = 25.0$ cm^3). In the particular case of electrochemical analysis with biosensors, measurements were carried out using an all-glass one-compartment electrochemical cell ($V = 1.00$ cm^3). In all cases, a Pt foil ($A = 1.00$ cm^2) was used as the counter electrode, while the Ag/AgCl (3.00 M KCl) electrode was used as the reference.

Electropolymerization of 4-ABA

The electropolymerization of 4-ABA was carried out using the CV and CA electrochemical techniques. In the case of CV studies, the GEs were modified by carrying out 100 consecutive potential cycles in the interval from 0.00 to +1.20 V at a scan rate of 50 mV/s. In the case of the experiments carried out using the CA technique, the GEs were modified using three different potentials (*E*/V vs. Ag/AgCl: 0.95, 1.05 and 1.15) that were applied over 4800 s. A geometric surface area of the GEs equal to 29.70 mm^2 was used throughout.

The acidic solution (0.50 M $HClO_4$) of 4-ABA monomer was prepared in a concentration of 2.50 mM. After electropolymerization, the modified electrodes containing the polymeric films of poly(4-ABA), denoted as GEs/poly(4-ABA), were copiously washed with ultra-pure water and dried using a stream of $N_{2(g)}$.

After that, the modified graphite electrodes (GEs/poly(4-ABA)) were immersed in a solution containing only the supporting electrolyte (0.50 M $HClO_4$) and then 5 cycles of the electrode potential were registered for the same potential interval used in the electropolymerization in order to remove the "*residual monomer*." In addition, the electrochemical spectra of GEs/poly(4-ABA) were analyzed to check the anchoring of the polymeric thin film on the graphite substrate, as well as to verify its electrochemical activity using a redox probe.

Ion exchange properties of poly(4-ABA)

The ion exchange properties for GEs and GEs/poly(4-ABA) were investigated by means of the outer-sphere electron transfer reaction occurring at the electrode/solution interface using a 0.10 M KCl solution containing 5.00 mM of the $K_4Fe(CN)_6/K_3Fe(CN)_6$ redox couple. Voltammetric curves were registered in the presence of the redox couple for the −0.25 to +0.80 V interval at a scan rate of 100 mV/s. Blank experiments were carried out using only the 0.10 M KCl solution, i.e., in the absence of the redox couple.

Electrochemical impedance spectroscopy (EIS)

After verification of the steady-state for the voltammetric profile, the EIS experiments were carried out at the open circuit potential (OCP) obtained in the 0.10 M KCl solution containing 5.00 mM of the $K_4Fe(CN)_6/K_3Fe(CN)_6$ redox couple. The frequency range was swept from 100 kHz to 10 mHz using the single-sine mode. In order to ensure linearity to the impedance response, the RMS amplitude of the senoidal perturbation was 10 mV (p/p). The Kramers–Krönig transforms were applied, and the linearity was verified in all cases ($\chi^2 \leq 10^{-5}$).

All EIS measurements were carried out using a model 128N Potentiostat/Galvanostat from AUTOLAB equipped with the FRA32M module. The quantitative analysis of the impedance data was carried out using the well-known equivalent circuit models (ECs) available in the literature for the study of conducting polymers [15]. The simulation and fitting routine procedures were carried out using the software furnished by AUTOLAB. In all cases, statistical analysis using the CNLS method revealed a good correlation ($\chi^2 < 10^{-4}$) between the EC model and the experimental findings.

Scanning electron microscopy (SEM)

The morphology of GEs/poly(4-ABA) was verified from the SEM images obtained using a model TM-3000 tabletop microscope from Hitachi. Images were obtained at 15 kV using the backscattered electrons detected using the composed mode.

Immobilization of purine bases: adenine and guanine

The immobilization of purine bases on the electrode was carried out by adsorption in 18 μL of an aqueous solution containing 20 mM of adenine and guanine. After this procedure, the electrode was maintained at 42 °C for 20 min. Thereafter, the electrode was rinsed in acetate buffer solution for 6 s and dried in a stream of N_2. The direct detection of purine bases was carried out by differential pulse voltammetry (DPV) using the following conditions: $\Delta E = 25$ mV, pulse period = 0.20 s, and scan rate = 20 mV/s.

Investigation of poly(4-ABA) platforms as genosensors

The immobilization of the poly(GA) probe was conducted by adsorption using 18 μL of the solution containing poly(GA) (0.06 mM in phosphate buffer, pH 7.40) directly on the surface of GEs/poly(4-ABA) at 42 °C for 20 min. This electrode was designated *ss*DNA/GE/poly(4-ABA). The hybridization procedure was performed by adding the target (18 μL of the 0.13 mM poly(CT) solution in the phosphate buffer, pH 7.40) on the surface of *ss*DNA/GE/poly(4-ABA), keeping the electrode in an oven at 42 °C for 20 min. This electrode was designated *ds*DNA/GE/poly(4ABA). For each run, the electrodes were rinsed with 0.10 M of the phosphate buffer solution for 6 s and dried in a stream of $N_{2(g)}$.

In the cases where the indicators/intercalators MB or EB were used, 18 μL of the solution (MB or EB, 0.50 mM containing 20 mM of NaCl) was placed in contact with the

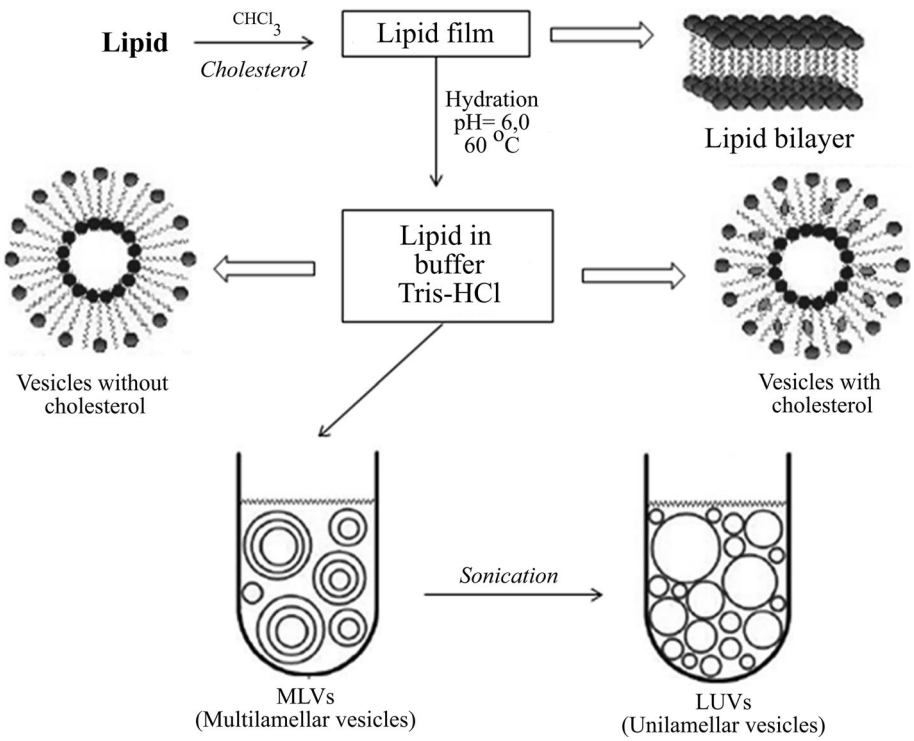

Fig. 1 Schematic representation of the stages of preparation of the liposomes

surface of the *ss*DNA/GE/poly(4-ABA) and *ds*DNA/GE/poly(4-ABA) electrodes for 5 min. After that, the electrodes were rinsed in 0.10 M phosphate buffer solution for 6 s and dried in a stream of $N_{2(g)}$. This procedure was also repeated before each experimental run.

The detection of probing and hybridization events was conducted by monitoring the electroactivity of the indicator/intercalator using the DPV technique. In the case of MB, the potential range of −0.70 to +0.30 V was used, while in the case of EB, the potential range was +0.30 to +0.90 V. The conditions in the DPV experiments were $\Delta E = 25$ mV, pulse period = 0.20 s, and scan rate = 20 mV/s.

Investigation of poly(4-ABA) platforms for immobilization and impedimetric detection of the DD K peptide

Samples of the DD K peptide were synthesized according to the standard peptide synthesis method in solid phase (SPSSP) using the F_{moc} strategy [16]. A Tenta GEL-SRAM resin (Iris Biotech, Marktredwitz, Germany) was used with a degree of substitution of 0.27 mmol/g, thus, resulting in the amidated peptide as the final product. Figure 1 shows a schematic representation of the steps for preparing the liposomes.

For each GE/poly(4-ABA) electrode, 20 µL of the DD K peptide solution (0.60 mM in 0.10 M Tris–HCl buffer, pH 8.00) was placed on the electrode surface and then kept at 25 °C for 20 min. After that, 20 µL of the phospholipid POPC solution (20 mM in 0.10 M Tris–HCl buffer, pH 8.00) used as a specific target for the electrode containing the DDK peptide was added. The same procedure was repeated, but with a solution containing the nonspecific target (phospholipid POPC containing 43 % of cholesterol). After these procedures, the biosensors were incubated for a period of 20 min at 25 °C, washed for 6 s in Tris–HCl buffer solution, and finally dried in a stream of ultra-pure N_2 for each step of the procedure.

Detection of the effects arising from the interaction of the DD K peptide with the specific and nonspecific targets was assessed by means of the EIS technique using the same conditions as specified in "Electrochemical Impedance Spectroscopy (EIS)" section.

Results and discussion

Scanning electron microscopy analysis (SEM)

The surface morphology of GE/poly(4-ABA) was examined using the SEM technique. As can be seen from the SEM images in Fig. 2, it is apparent that the EG substrate was not completely covered by a homogenous polymeric film in all cases. The dark regions represent the GE surface, while the white spots represent the polymeric material.

In addition, it was verified that the surface coverage by polymeric films is affected by the method of synthesis (CV

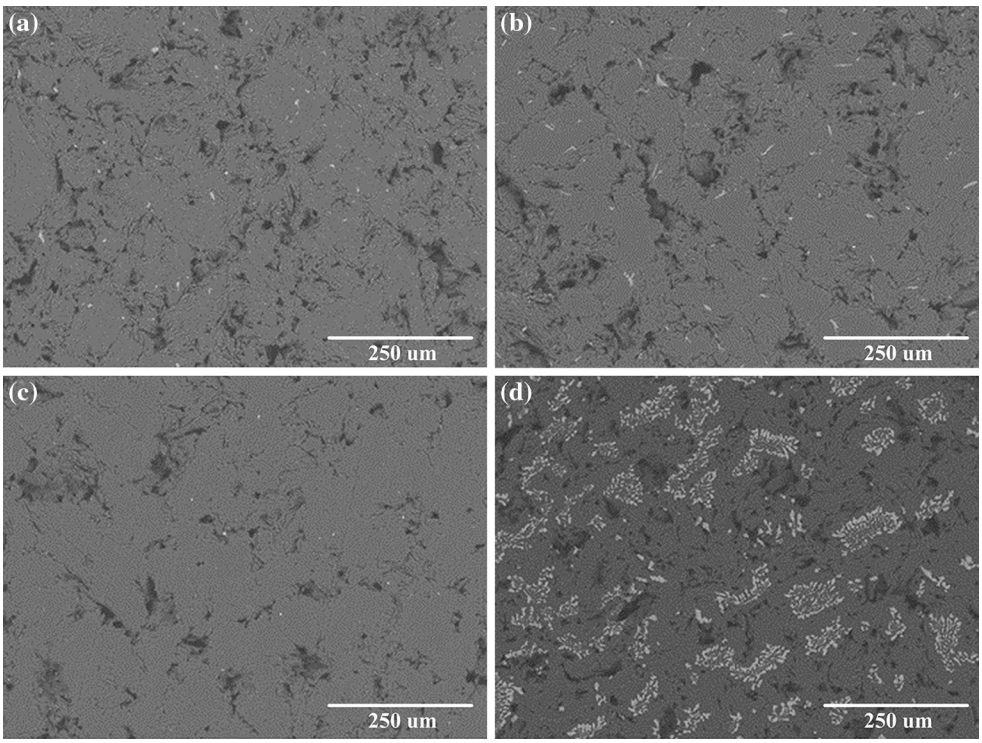

Fig. 2 SEM images obtained for GE/poly(4-ABA) electroformed using the CA **a** 0.95 V; **b** 1.05 V; **c** 1.15 V; and CV (**d**) techniques. Magnification of ×200

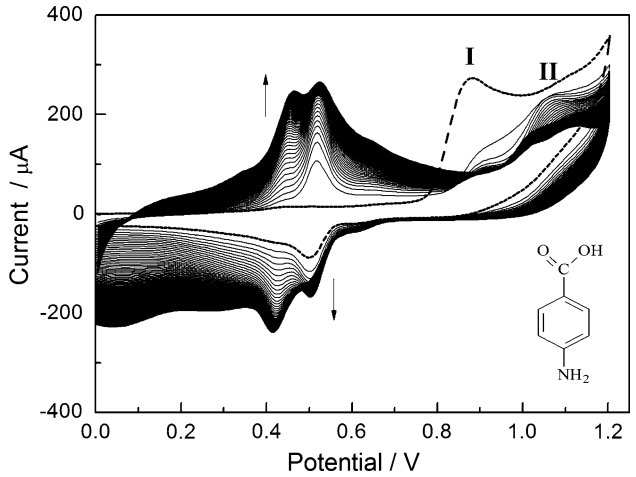

Fig. 3 Cyclic voltammograms obtained for the electropolymerization of 4-ABA (2.50 mM) on the GE substrate. Supporting electrolyte: 0.50 M $HClO_4$. Number of cycles = 100; $v = 50$ mV/s. Curve I (*dashed line*): first cycle of potential. The *arrows* indicate the direction of the current increase as a function of the potential cycle. The *inset* shows the chemical structure of 4-ABA

and CA). As seen, for films electroformed by CA, there was a greater presence of material deposited onto the platform developed at 1.05 V when compared to platforms obtained for the other potentials, thus, indicating a higher electrochemical activity for the polymerization reaction at this potential. In addition, it can be seen that in the case of the modified electrode obtained by CV, its surface morphology contains a high concentration of white spots which are indicative of a more intensive growth of the polymeric material. On the whole, these results have shown the formation of good surface coverage by the polymeric material where the film was randomly distributed on the GE surface.

Electropolymerization and electrochemical characterization of poly(4-ABA)

Electropolymerization using cyclic voltammetry (CV)

Figure 3 shows cyclic voltammograms accounting for the electropolymerization process on the GE substrate, where 100 voltammetric curves were registered for the solution containing the 4-ABA monomer.

As seen, the monomer was irreversibly oxidized at about +0.87 V during the first cycle (see peak I). However, during the consecutive cycles, there was a shift of the irreversible oxidation process to more positive potentials (see peak II). The occurrence of two irreversible peaks for the oxidation of the monomer suggests the formation of highly reactive positively charged radicals, i.e., the oxidation observed for peak I indicates the initiation of the

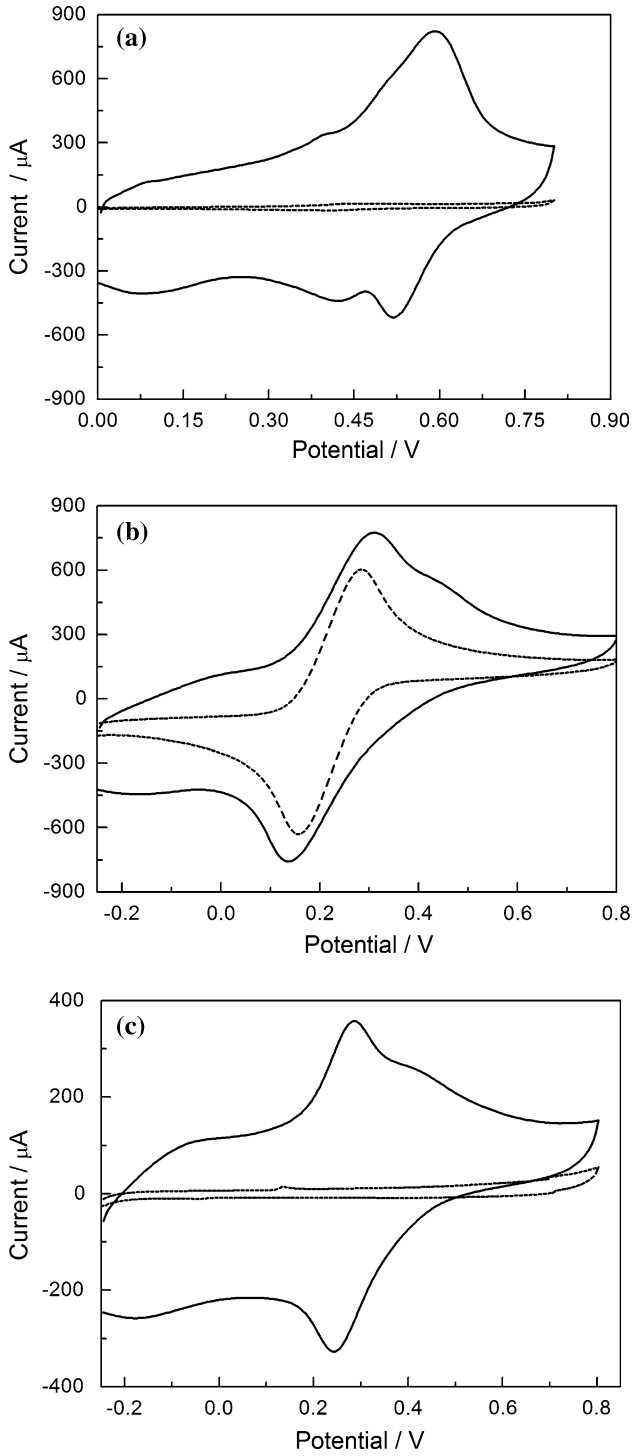

Fig. 4 Voltammetric curves obtained for the unmodified (GE) and the modified (GE/poly(4-ABA)) electrodes after electropolymerization: Electrolyte conditions: **a** 0.50 M $HClO_4$ in the absence of the monomer; **b** anodic redox probe (5.00 mM) in KCl (0.10 M) and **c** 0.10 M KCl. Symbols: (*dashed line*) GE and (*solid line*) GE/poly(4-ABA). $v = 100$ mV/s

electropolymerization of 4-ABA, whereas the other oxidations seen in peak II indicate the continuation of the electropolymerization on the GE substrate until a critical current density where a stationary thickness of the polymer is obtained. At this point, the CV profile is no longer affected by the potential scan.

Also, the decrease in values of the anodic peak current ($I_{p,a}$) upon increasing the number of the potential cycles was verified. This behavior is a result of the progressive consumption of the monomer at the vicinity of the active centers of the electrode surface where the electropolymerization occurs. As seen in Fig. 3, during the earlier stages of the polymerization, after the second cycle, there is the appearance of a reduction band that resulted in formation of two bands located around +0.50 and +0.41 V (potential 100th cycle) in the later stages of the polymerization process. As the successive potential cycles proceeded, these bands were displaced to potential values of about +0.46 and +0.52 V.

The kinetics for the transport of ions at the poly(4-ABA)/solution interface was studied by means of the voltammetric curves registered in aqueous solution containing the $K_4Fe(CN)_6/K_3Fe(CN)_6$ redox couple (anionic probe). The nature of the interaction of poly(4-ABA) with the redox probe yields important information since the electrostatic interaction and the charge transfer (outer-sphere) reaction that can exist between the redox probe and the polymer are very important for studying the electronic properties of poly(4-ABA).

Figure 4 shows the electrochemical profiles for the unmodified substrate (GE) and modified electrode (GE/poly(4-ABA)) obtained in the supporting electrolyte, as well as in the electrolyte containing the redox probe.

As seen in Fig. 4a, there was a change in the electrochemical response of GE after the electropolymerization process, since the modified electrode exhibited a high electroactivity at about +0.51/+0.59 V. As can be seen in Fig. 4b, for the case of the solution containing the redox probe, the voltammetric profile of GE/poly(4-ABA) exhibited a potential difference for the separation of the anodic and cathodic peaks of $\Delta E = 170$ mV. This value is high when compared to the unmodified GE ($\Delta E = 120$ mV), thus, indicating that the charge transfer reaction involving the redox probe is affected by the presence of poly(4-ABA).

Figure 4c shows the electrochemical activity of the polymeric film in the supporting electrolyte (0.10 M KCl). A comparison of Figs. 4b, c clearly reveals that the major contribution for the electrochemical spectrum of the conducting polymer arises from the electronic activity of the polymer itself.

The rationalization of the above findings permits the proposal that the redox process in the present system is a result of a contribution of the electroactivity of the conducting polymer film in conjunction with the response obtained in the presence of the redox probe. Therefore, it

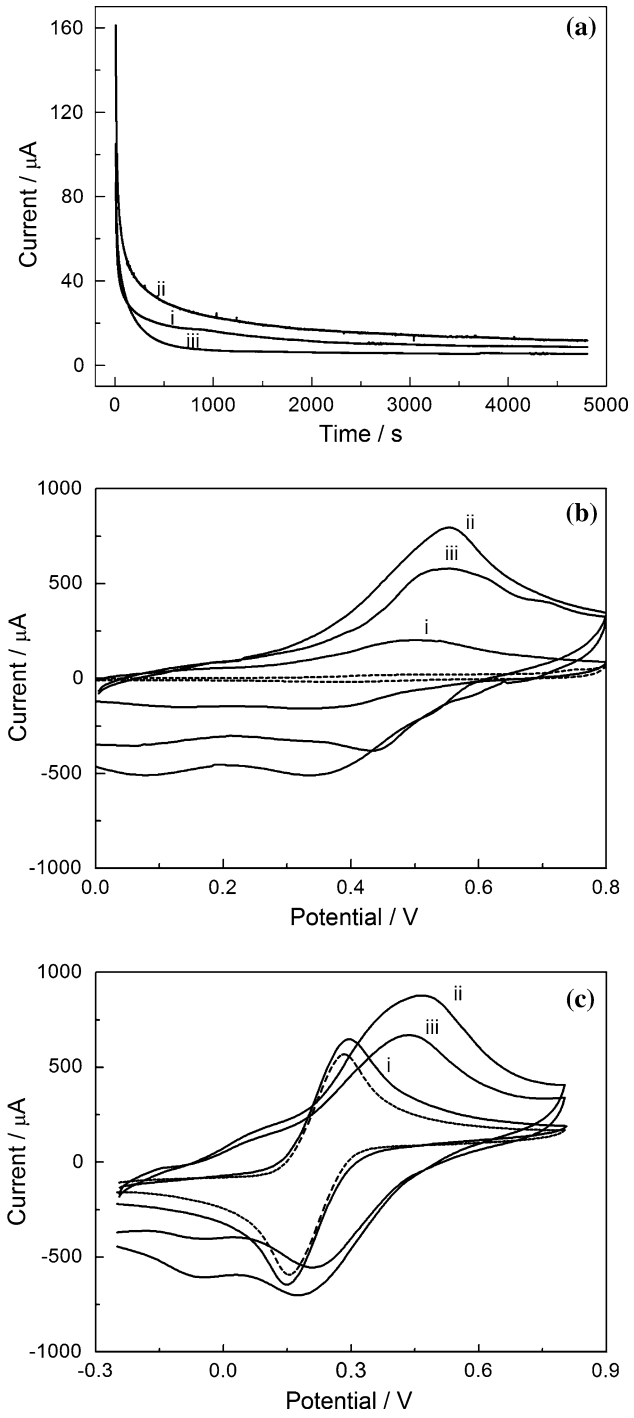

Fig. 5 **a** Chronoamperograms obtained in a solution of 2.50 mM 4-ABA. Supporting electrolyte: 0.50 M $HClO_4$. Electrode potential: (*i*) 0.95 V, (*ii*) 1.05 V, and (*iii*) 1.15 V. Voltammetric curves obtained after the electropolymerization using CA: **b** 0.50 M $HClO_4$ and **c** 5.00 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ + KCl 0.10 M. CVs obtained for: (*i*) 0.95 V, (*ii*) 1.05 V, (*iii*) 1.15 V and (*dashed line*) GE. $v = 100$ mV/s

can be proposed that poly(4-ABA) has an anionic character that strongly inhibits the outer-sphere charge transfer reaction with the anionic redox probe.

Electropolymerization using chronoamperometry (CA)

The electropolymerization of 4-ABA was also carried out using CA for comparison with the experimental findings obtained using CV. In order to ensure consistency between findings obtained with these two techniques, the electropolymerization was carried out at a given potential applied during 4800 s (leveling procedure), i.e., for the same time interval spent for registering the consecutive voltammograms ($n = 100$). In the present case, poly(4-ABA) was obtained at three different potentials: 0.95, 1.05, and 1.15 V. These potentials correspond to the oxidation peak potentials obtained for 4-ABA in the CV studies considering a standard deviation of about ±10 mV.

Figure 5a shows the chronoamperometric curves obtained for the electropolymerization of 4-ABA as a function of the electrode potential.

As can be seen in Fig. 5a, the magnitude of the current density for the electropolymerization reaction increased as a function of the electrode potential in the following order of performance: $j(1.05\ V) > j(1.15\ V) > j(0.95\ V)$. The modification of the GE was confirmed from a comparison of the electrochemical profiles obtained for GE and GE/poly(4-ABA) in the supporting electrolyte (see Fig. 5b) with the profiles obtained in the presence of the anionic redox probe (see Fig. 5c).

From the comparison of Figs. 4a and 5b, it can be verified that the electrochemical activity (e.g., profile of the voltammetric curve) presented by GEs modified using CV and CA is very similar for these specific conditions.

From the analysis of Fig. 5c, a small increase in current density for the outer-sphere reaction using the redox probe can be verified for the polymeric films electroformed at 0.95 V. In addition, for the polymeric films electroformed at 1.05 and 1.15 V, the occurrence of a great shift in the oxidation potential of the redox probe was observed. However, as shown in Fig. 4c, there is a contribution of the redox process exhibited by the polymeric film located in the same potential region of the redox probe.

Electrochemical impedance spectroscopy studies: EIS

The EIS is one of the most powerful and sensitive techniques for investigating the electrical properties of surface-modified electrodes [17]. The EIS technique permits that different bulk and interfacial processes characterized by different time constants might be identified and/or distinguished in the frequency domain. As a result, it is possible to characterize the electrode system by means of the simulation/fitting procedure using an appropriate equivalent circuit model containing resistors, capacitors and inductors. In some cases, the Warburg element is also used if the system is characterized by semi-infinite linear diffusion of

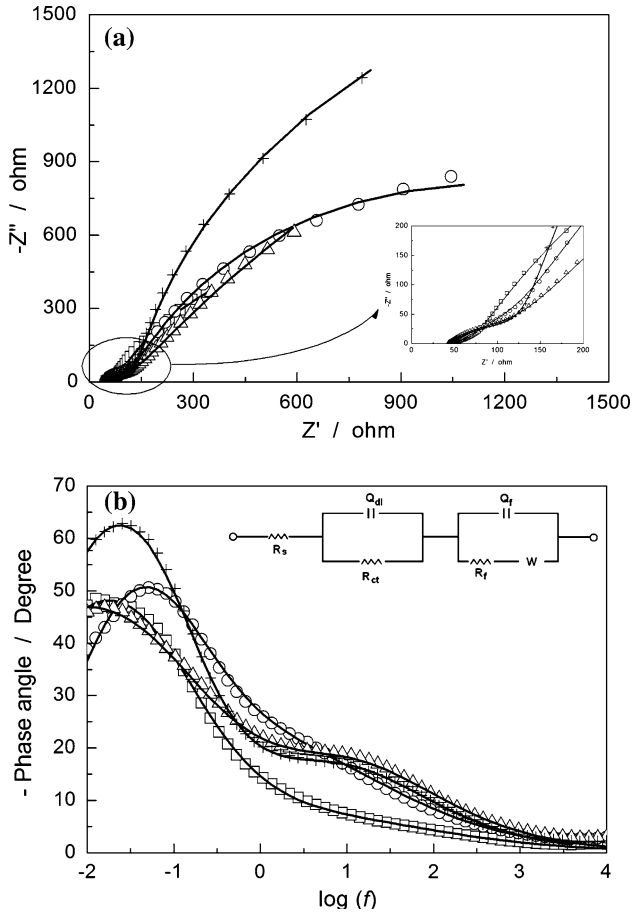

Fig. 6 Impedance diagrams obtained for the unmodified (GE) and modified (GE/poly(4-ABA)) electrodes. Electrolyte: 5.00 mM $K_4Fe(CN)_6/K_3Fe(CN)_6 + 0.10$ M KCl. Electrosynthesis potentials: (*white square*) 0.95 V, (*white circle*) 1.05 V, (*white up-pointing triangle*) 1.15 V, and (*plus symbol*) VC. Conditions for the impedance study: $E = 0.24$ V; $\Delta E = 10$ mV (RMS); Frequency range: 10^6–10^{-2} Hz. The inset shows the equivalent circuit used in the simulation/fitting procedure. *Solid lines* represent the fit of the equivalent circuit to the experimental findings

the charge carriers across the electrode/solution interface, as well as inside the electrode itself.

In the present study, the EIS measurements were carried out for better understanding of the electrical properties of the poly(4-ABA) thin films electroformed at 0.95, 1.05, and 1.15 V ($t = 4800$ s). These experimental findings were compared to those obtained using the CV technique.

Figures 6a, b show the complex-plane (Z' vs. $-Z''$) and Bode ($-\phi$ vs. log(f)) plots, respectively, obtained for the GE and GE/poly(4-ABA) electrodes. These two types of graphical representation are complementary in order to identify and quantify events in the frequency domain.

All impedance data were obtained at the open circuit potential (OCP) of +0.24 V in the KCl solution containing the redox probe. In agreement with the previous findings reported in "Electropolymerization using cyclic voltammetry (CV)" and "Electropolymerization using chronoamperometry (CA)" sections, it was verified that the impedance of the electrode system is governed by the faradaic processes occurring in the polymeric structure, as well as for the outer-sphere reaction involving the Fe^{2+}/Fe^{3+} redox couple.

Table 1 Parameters obtained from the impedance study carried out for GE and GE/poly(4-ABA) electropolymerized by CV and CA

Parameter	CA/0.95 V	CA/1.05 V	CA/1.15 V	CV
$R_s/\Omega\ cm^2$	47.5	46.4	42.8	46.1
$R_{ct}/\Omega\ cm^2$	91.3	157.3	77.2	104.6
$Q_{dl}/m\Omega^{-1}\ s^n\ cm^{-2}$	11.8	3.85	1.71	1.92
n_{dl}	0.35	0.48	0.58	0.56
$R_f/k\Omega\ cm^2$	0.20	1.39	3.53	2.26
$Q_f/m\Omega^{-1}\ s^n\ cm^{-2}$	18.5	6.67	7.31	7.87
n_f	1.00	0.95	0.67	0.96
$W/m\Omega^{-1}\ s^{0.5}\ cm^{-2}$	7.64	8.76	2.76	2.94
$\chi^2 \times 10^{-3}$	1.00	5.47	3.52	1.48

On the whole, the impedance spectra were characterized by two different behaviors distributed in the low- and high-frequency regions. In the high-frequency region, the semicircle is not as well developed for the polymeric films electroformed at 0.95 V. These findings indicate a low Coulombic efficiency for the electropolymerization reaction, as well as a less conductive behavior (see Fig. 5b).

The impedance behavior verified in the low-frequency region was characterized by the intrinsic properties of the polymer/solution interface (e.g., electric double layer) in conjunction with the mass transport by diffusion of the redox probe across the diffusion layer, which is commonly represented by the Warburg element.

The experimental impedance data were simulated and fitted using a modified version of the Randles' circuit in order to take into account the electrochemical behavior of the polymeric film in addition to the diffusional process (see the inset of Fig. 6b). The elements in the circuit are as follows: R_s is the uncompensated ohmic resistance exhibited by the electrolyte solution, and R_{ct} and R_f resistances are associated with the charge transfer occurring at the electrode/solution interface and with the ohmic resistance located in the bulk of the film, respectively. Q_{dl} is the constant-phase element describing the pseudo-capacitive behavior of the electric double layer, and Q_f is the constant-phase element representing the pseudo-capacitance of the polymeric film. The diffusional process was described by the Warburg (W) element.

The resulting equivalent circuit model can be represented as $R_s(R_{ct}Q_{d1})(Q_f[R_fW])$ according to the Boukamp's representation (see also the inset of Fig. 6b). The impedance parameters obtained from fitting the model to the experimental data are gathered in Table 1. As can be seen,

the total ohmic resistance R_T (=R_s + R_f) is almost constant ($\approx$ 42–48 Ω cm^2) for the electropolymerization obtained at different electrode potentials.

As seen in Table 1, the charge transfer resistance (R_{ct}) is strongly affected by the electropolymerization conditions, with the lower and higher resistances verified for the polymeric film electroformed using CA at 0.95 and 1.05 V, respectively. A high R_{ct} value was also verified for the film that was electroformed using CV.

Since the R_{ct} measured at the equilibrium electrode potential verified for the redox probe is in fact an indirect measure of the exchange current density ($I_o = RT/nFR_{ct}$) for the outer-sphere redox reaction at the modified electrode/solution interface, the experimental findings in Table 1 indicate that the intrinsic electronic properties of the polymeric films are affected by the electropolymerization conditions.

In agreement with the previous findings obtained using the CV technique, the occurrence of diffusion (W) accompanied by charge transfer (R_{ct}) characterizes the quasi-reversible behavior exhibited by the outer-sphere electrode reaction at the polymer/solution interface. As seen, the diffusion is only slightly affected by the electropolymerization conditions, with the higher resistance for mass transfer being verified for the polymeric film electroformed at 1.05 V. The small values of W indicate that the semi-infinite linear diffusion takes place in a nonrestricted medium, i.e., these findings indicate that the surface morphology of the polymeric film is more flat than porous.

The values of R_f were slightly affected by electropolymerization conditions. Thus, assuming that the intrinsic bulk resistivity of the polymeric films synthesized using different conditions is not a function of the film thickness, it can be proposed that the relative thickness of the polymeric film for the different conditions is 1.15 V(CA) > CV > 1.05 V(CA) > 0.95 V(CA).

Therefore, according to the EIS study, the film thickness can be controlled by using different potentials in the case of CA or by using different potential cycles in the case of CV. The analysis of the Q_f values (n_f = 0.67–1.00), which characterize a capacitive behavior, revealed a good internal consistency with the other impedance parameters, since a higher Q_f value was verified for the polymeric film exhibiting the lower value of R_f (CA: 0.95 V); i.e., from the theoretical viewpoint, the film resistance is a linear function of the film thickness, while the film capacitance is an inverse function of thickness.

Immobilization and detection of purine bases (guanine and adenine) in functionalized platforms

Figure 7 shows the oxidation peaks of adenine and guanine obtained by differential pulse voltammetry (DPV) for GE/poly(4-ABA) electroformed by CV and CA.

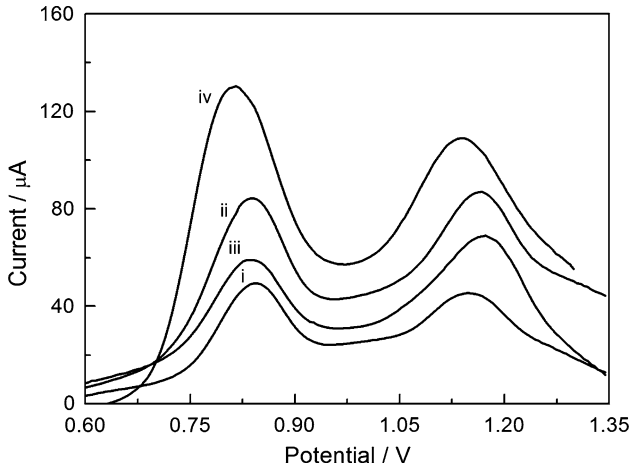

Fig. 7 DPVs obtained from the direct oxidation of adenine and guanine bases immobilized on GE/poly(4-ABA). Synthesis: CA at (*i*) 0.95 V; (*ii*) 1.05 V; (*iii*) 1.15 V and (*iv*) CV. Electrolyte: 0.10 M acetate buffer at pH 4.50. Amplitude: 25 mV; pulse period: 0.20 s; scan rate: 20 mV/s

Table 2 Values of $I_{p,a}$ obtained for detection of adenine and guanine bases immobilized on GE/poly(4-ABA) obtained by CA and CV

$I_{p,a}$/μA	0.95 V	1.05 V	1.15 V	CV
Guanine	48.95	84.17	59.15	129.80
Adenine	45.24	86.71	68.54	108.90

Different values for the anodic peak current ($I_{p,a}$) were obtained for all modified electrodes. The oxidation of guanine occurred at about +0.83 V for all GE/poly(4-ABA) obtained at different potentials (CA). A slightly different behavior was verified in the case of the GE/poly(4-ABA) obtained using CV, since the oxidation of guanine was observed at +0.81 V. The oxidation of adenine occurred at about +1.15 V for the three modified electrodes at a constant potential and at about +1.13 V for the electrode modified by CV. As can be seen, there was a small shift in the oxidation potentials of the purine bases analyzed using the electrodes modified by CA. As seen in Fig. 7, a comparison of the findings obtained for electrodes prepared by CV and CA revealed that the larger peak currents were obtained for the electrode modified by CV. In the case of CA, the larger $I_{p,a}$ values were verified at the following potentials: 1.05 > 1.15 > 0.95 V.

Table 2 shows the $I_{p,a}$ values obtained for the detection of immobilized nitrogenous base on GE/poly(4-ABA) electrodes that were modified by CV and CA.

As seen in Table 2, when the modification of GE was carried out by CV, the amount of purine bases immobilized and detected on the electrode surface increased. However, when comparing the functionalized platform prepared by

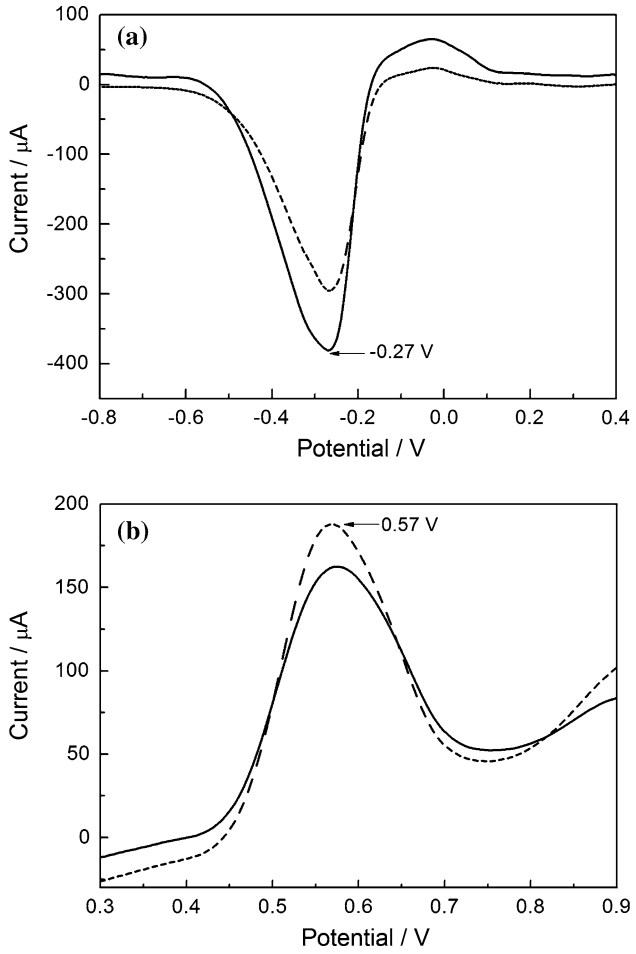

Fig. 8 DPVs obtained for (*solid line*) *ss*DNA and (*dashed line*) *ds*DNA containing 18 μL of **a** 0.50 mM MB and **b** 0.50 mM BE. Electrodes modified with poly(4-ABA) by CV. Electrolyte: 0.10 M phosphate buffer with pH 7.40. Amplitude: 25 mV; Pulse period: 0.20 s; Pulse height: 0.06 s; $\nu = 20$ mV/s. $\text{Time}_{\text{adsorption}} = 5$ min

CV and at 1.05 V by CA, there was an increase in the oxidation current of about 54 % to guanine and 26 % for adenine, which indicates greater sensitivity of the transducer and capacity of immobilization and detection of the biomarker bases present in the DNA molecule.

In agreement with the previous findings already obtained in this study (see Figs. 4a, 5b), it was verified that the CV technique was more efficient for the generation of poly(4-ABA). In addition, the CV (see Fig. 7) also exhibited the best sensibility for immobilization and detection of purine bases (adenine and guanine).

From the above consideration, all platforms used in the subsequent studies in the present case involving the immobilization, the detection of oligonucleotides and peptides, as well as of its recombinants, were carried out using the modified GEs obtained by CV.

Immobilization and detection of oligonucleotides: genosensors

The immobilization of biomolecules is the most important step in building a biosensor, ensuring sensitivity, selectivity, and stability. The goal of any method of immobilization is to retain the maximum activity of the biomolecule on the surface of the transducer. Investigations were conducted using the platforms functionalized with polymeric films, obtained from the 4-ABA monomer using CV, for the immobilization of oligonucleotides (polyGA, 16 pb), as well as for immobilization and detection of the hybridization event with complementary target (poliCT, 16 pb).

The experimental results to verify the feasibility of the process of immobilization and hybridization were obtained by differential pulse voltammetry, using indirect detection by the use of agents such as the intercalators MB and EB, since, as shown in Fig. 7, the potentials for the direct detection of these bases are high; i.e., this behavior of electrode potential may cause undesirable effects on the detection process, such as the oxidation of other species that have a lower redox potential in relation to purine bases.

Figure 8 shows the detection of the oligonucleotides (probe and probe/target) immobilized on GE/poly(4-ABA) containing MB (Fig. 8a) and EB (Fig. 8b) as intercalating agents.

Figure 8a shows the occurrence of a well-defined voltammetric peak at about −0.27 V for the reduction of MB adsorbed onto GE/poly(4-ABA) containing the probe (*ss*DNA) and hybrid (*ds*DNA). It is well known that MB has a high affinity for the guanine nitrogenous base [18, 19]. Thus, it is possible to recognize the hybridization event, since when the oligonucleotide is hybridized, the access to the MB guanine is reduced, thus resulting in a smaller amount of this compound on the surface of the biosensor, with a consequent drop in the monitored current. As a result, when a difference in the amount of MB is detected, it is possible to identify the hybridization event.

As can be seen in Fig. 8a, after exposure of the probe to the complementary target with the formation of *ds*DNA, there was a decrease in the reduction current, resulting in the adsorption of about 29 % MB. This decrease can be attributed to inhibition by the steric effect of the reducible groups of MB intercalated with the DNA double helix.

Even with the great promise of the use of optical biosensors, many authors have used electrochemistry to study the interactions of DNA with electrochemically active species (e.g., ferrocene, MB, daunomycin, anthraquinone, osmium complex, copper(II) complex, etc.) [20–22]. The EB is electrochemically active, being oxidized at about +0.60 V. This property, in addition to the strong

interaction existing between EB and *ds*DNA, results in an alternative mechanism for the indirect detection of the hybridization, thus permitting the development of genosensors.

Figure 8b shows the effect of adsorption and oxidation of EB on the functionalized platforms containing *ss*DNA and *ds*DNA. The EB has a better interaction with double-stranded DNA, as can be inferred from Fig. 8b. As seen, it was possible to indirectly detect hybridization events from analysis of the electrochemical response obtained for EB. The differences between the current signals are due to the different ways in which the biomolecules interact with EB. In the presence of only the probe *ss*DNA, the current signal (anodic peak) is lower than that obtained for the target *ds*DNA. This is due to the effect of intercalating EB in the native or hybridized DNA, where it can be more easily accommodated between the pairs of the structure. The current resulting from the oxidation of the adsorbed EB was $\approx$188 μA, which is about 16 % greater than that obtained for *ss*DNA. This is very important, since the value of $I_{p,a}$ is derived from the EB connections with the *ds*DNA on the electrode surface.

The analysis of the electrochemical responses obtained for the genosensor reveals very promising conditions for the detection and recognition of oligonucleotides with the complementary target. From these considerations, it can be inferred that the platform functionalized with poly(4-ABA) exhibits very interesting properties for the development and improvement of genosensors, which show strong promise for the quick, cheap, and simple diagnosis of genetic diseases.

Immobilization and detection of interaction of the DD K peptide with phospholipid POPC

A second strategy for application of the poly(4-ABA) platforms in this study was its use as a biosensor for the impedimetric detection of peptides immobilized on the surface of the modified electrode.

Firstly, the DD K peptide was immobilized on the surface of GE/poly(4-ABA) in order to obtain an interaction between DD K and the phospholipid 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC). Afterward, the study investigated the effect of the cholesterol interaction between DD K and the POPC liposomes, as well as the effects incurred from the binding of DD K with large unilamellar vesicles (LUVs of 100 nm). In the latter case, LUVs without cholesterol and cholesterol LUVs were prepared at 43 % per

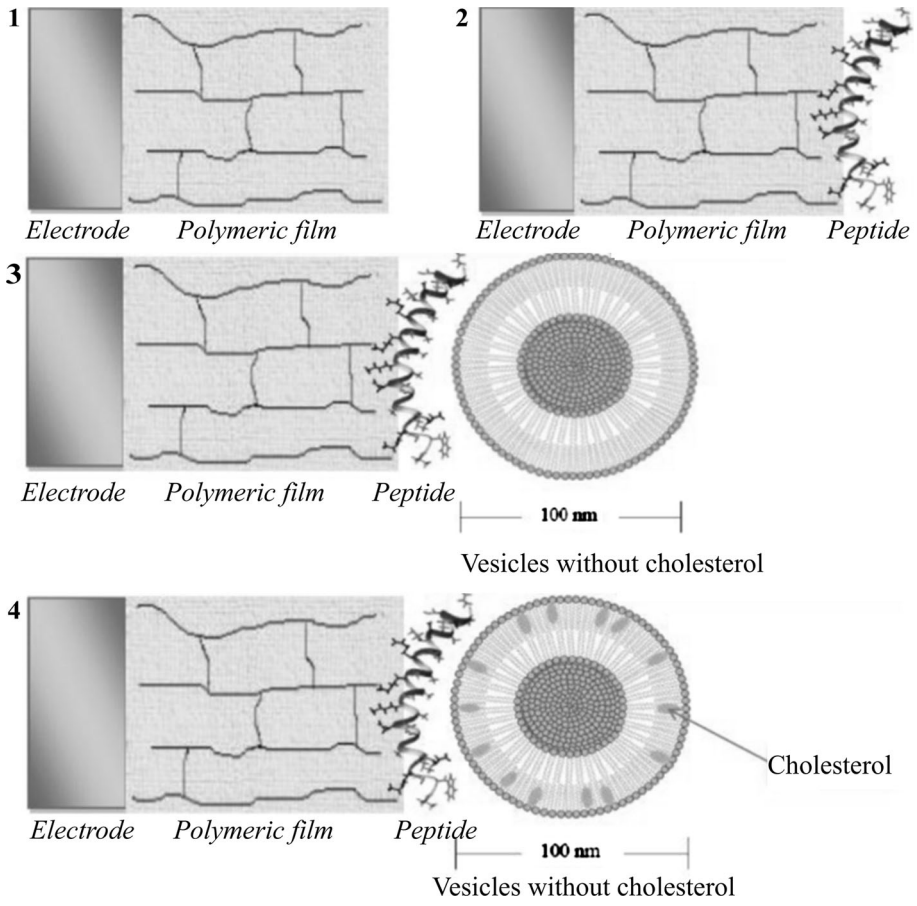

Fig. 9 Scheme representing the immobilization process of peptides and POPC on the GE/poly(4-ABA): **1** GE/poly(4-ABA); **2** GE/poly(4-ABA) + probe; **3** GE/poly(4-ABA) + probe + specific target; and **4** GE/poly(4-ABA) + probe + nonspecific target

mol of cholesterol concentration used in the composition of LUVs. This procedure was adopted in order to replicate the cholesterol/phospholipid molar ratio of 0.84, which is typical for erythrocyte cells [23]. The scheme which represents this procedure is shown in Fig. 9.

Figure 10 shows the complex-plane (Fig. 10a) and Bode (Fig. 10b) plots for the impedance behavior characterizing the immobilization of the peptide and the recognition of the specific (phospholipid POPC) and nonspecific (phospholipid POPC + cholesterol) targets. These impedance spectra were obtained using the same conditions as those already described in "Electrochemical impedance spectroscopy (EIS)" section.

For the present case, a specific impedance model (transfer function) for this type of interaction at the electrode/solution interface is not available in the literature due to the complexity of the particular system. Therefore, the analysis of the impedance data was only qualitative.

DD K is an amphipathic peptide and, hence, the amphipathicity is a key factor for the peptide interaction with the phospholipid membranes. The amphipathic structure of the peptide favors its interaction with the LUVs, while this strong interaction causes disruption of membranes [24]; i.e., loss of the vesicle structure resulting in the release of free phospholipids into solution.

As seen in Fig. 10, for the three investigated systems, there is a presence of two semicircles distributed in the high- and low-frequency regions of the spectrum. In addition, there is a similarity between the Bode plots (Fig. 10b) obtained for the probe and for the probe + specific target. As a result, these findings might indicate a strong interaction occurring at the peptide/LUVs interface.

In the case of the electrode containing the probe + nonspecific target (LUVs with cholesterol), the impedance diagram showed a very distinct profile, irrespective of whether compared to the impedance response obtained for the probe alone. The existence of a weak interaction at the peptide/cholesterol–LUVs interface can be proposed based on these findings, since the presence of cholesterol in LUVs causes a decrease in this type of the interfacial interaction. These results may be helpful for explaining the low hemolytic activity of the peptide, which is due to the presence of cholesterol in eukaryotic cells at high concentrations [23]. However, cholesterol does not exist at the bacterial membrane, thus permitting a strong interaction of the DD K peptide with the surface of the POPC bilayer membrane that results in significant antibacterial activity.

By means of impedimetric measurements with the EIS technique using a redox probe for detection of the electrical signal, it was verified that the platform functionalized with poly(4-ABA) can be effective for the immobilization and sensitivity detection of the interaction between a redox probe and the specific target. In addition, it was verified that the interaction of the DD K peptide with the mimetic membranes of POPC is affected in the presence of cholesterol.

Therefore, it may be argued that the use of a biosensor specifically fabricated for impedimetric detection can constitute an important electroanalytical strategy for studying the interaction of the DD K peptide with the liposomes process.

To the best of our knowledge, there have been no previous studies in the literature dealing with the detection of the interaction between peptides and phospholipid membranes using electrochemical techniques, as well as functionalized polymeric film platforms containing poly(4-ABA).

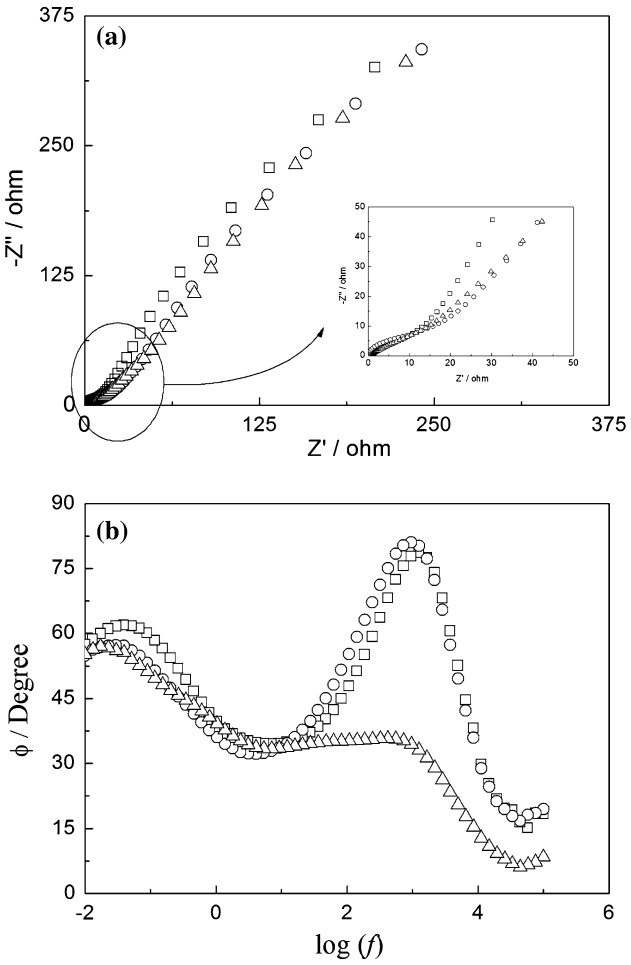

Fig. 10 **a** Complex-plane and **b** *bode plots* obtained from the EIS study for GE/poly(4-ABA) in 5.00 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ + 1.0 M KCl solution. Symbols: (*white square*) peptide; (*white circle*) peptide + phospholipid POPC; and (*white up-pointing triangle*) peptide + phospholipid POPC + cholesterol

Conclusions

The purpose of this study was to contribute to the development of electrochemical sensors, by investigating the properties of electrochemical platforms functionalized with conducting polymeric films and their application as biosensors. It was found that electropolymerization of the 4-ABA monomer by CV showed more promising results for analytical purposes (e.g., immobilization and detection of biomolecules) when compared to polymeric films obtained by CA. It was verified in the latter case (CA) that the growth of the polymeric film was more pronounced at 1.05 V.

The recognition of a hybridization event can be assessed by different experimental approaches making use of direct and indirect detection using the intercalators MB and EB. It was verified that the use of the GE/poly(4-ABA) platform is very promising for the development of new genosensors.

Impedimetric measurements obtained from the EIS technique were carried out using a redox probe for the detection of events taking place at the platform/(probe-analyte)/solution interface. It was verified that the electrochemical platform functionalized with poly(4-ABA) can be rather effective for the immobilization and sensitivity detection of the interaction between a redox probe and the specific targets (analytes) present on the platform's surface.

According to impedimetric measurements, the interaction of the DD K peptide with the mimetic membranes of POPC is affected by the presence of cholesterol. The verification of this type of interaction is very important from the viewpoint of the development of some drugs, as well as for a better understanding of important biochemical effects that are difficult to detect, as in the case of peptide/lipids interactions.

On the whole, the present study revealed that the use of the GE/poly(4-ABA) platform for the fabrication of electrochemical biosensors is very promising.

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