Amperometric Response of Hydrogen Peroxide at Carbon Nanotubes Paste Electrodes Modified with an Electrogenerated Poly(Fe(III)-5-amino-phenantroline). Analytical Applications for Glucose Biosensing

Maria L. Lozano,[a] *Marcela C. Rodríguez*,[b] *Pilar Herrasti*,[c] *Laura Galicia*,[a]* *Gustavo A. Rivas*[b]*

[a] Universidad Autónoma Metropolitana Iztapalapa, México
[b] INFIQC, Departamento de Físico Química, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina
*e-mail: grivas@mail.fcq.unc.edu.ar
[c] Departamento de Química Física Aplicada, Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain

Received: July 15, 2009
Accepted: October 3, 2009

Abstract
This work reports the catalytic activity of a polymer electrogenerated from Fe(III)-5-amino-1,10-phenantroline solution at a carbon nanotubes paste electrode (CNTPE) towards the oxidation and mainly the reduction of hydrogen peroxide. The important role of carbon nanotubes on the generation of poly(Fe(III)-5-amino-1,10-phenantroline) is demonstrated through the comparison with the behavior of graphite paste electrode (CPE). The polymer electrogenerated at CNTPE largely improves the amperometric detection of hydrogen peroxide at -0.100 V. The analytical application of the resulting electrode is demonstrated in connection with the design of a glucose biosensor based on the deposition of GOx and diluted Nafion on the top of the polymer-modified CNTPE. The quantification of glucose in human serum samples showed a good correlation with the values obtained by the spectrophotometric technique.

Keywords: Fe(III)-5-amino-1,10-phenantroline complex, Carbon nanotubes paste electrode, Glucose biosensor, Hydrogen peroxide, Glucose oxidase, Polymers, Biosensors, Nanotubes

DOI: 10.1002/elan.200900359

1. Introduction

Carbon nanotubes (CNT), a well-known low-dimensional material with large surface-to-volume ratio, have received great attention in the last years due to their unique mechanical, geometric, electronic and structural properties [1–3]. Since 1996 [4], they have been largely used for the preparation of electrochemical (bio)sensors, with excellent performance [5–8].

The main problem of using CNT for the development of electrochemical sensors is their insolubility in usual solvents. To overcome this problem, different strategies have been proposed. The incorporation within composite matrices using binders like bromoform [4] and Teflon [9] has demonstrated to be highly successful. We have reported the excellent properties of a composite electrode obtained by dispersion of multiwall carbon nanotubes within mineral oil [10, 11], called carbon nanotubes paste electrode (CNTPE), as detector, either in batch or in flow systems [12].

Facilitated electron transfer was obtained at CNTPE for dopamine, ascorbic acid, dopac, uric acid and hydrogen peroxide [10]; guanine, adenine and nucleic acids [13]; phenol, catechol, NADH and hydroquinone [14], amitrole [15]; epinephrine and norepinephrine [12]. The effectiveness of CNTPE as a matrix for the immobilization of redox enzymes like glucose oxidase [10]; lactate oxidase, alcohol dehydrogenase and polyphenol oxidase [14] has been also reported. Wang et al. [16] have proposed the use of CNTPE for the detection of homocysteine. Magno et al. [17] have reported the use of CNTPE as a substrate for the polymerization of 3,4-dihydroxydybenzaldehyde to obtain an amperometric biosensor for fructose. CNTPE modified with copper particles have been used as detector in Capillary Electrophoresis for the determination of carbohydrates with a detection limit of 20 μM for glucose [18]. Rivas et al. have proposed the advantages of CNTPE modified with copper microparticles to enhance the sensitivity of glucose determinations [19] and to allow the highly sensitive and selective quantification of electroactive and non electroactive aminoacids and albumin at physiological pHs and low potentials [20, 21]. A CNTPE modified with 2,2′-[1,2-ethanediylbis(nitriloethylidyne)]-bis-hydroquinone has been also used for the successful discrimination between dopamine and uric acid oxidation signals [22]. CNTPE prepared with paraffin as binder has been used for the determination of copper at pM level [23]. Ye and Li [24]

have reported the influence of the nature of CNT used to prepare the CNTPE on the oxidation of NADH.

This article reports for the first time the use of CNTPE as substrate for the polymerization of Fe(III)-5-amino-1,10-phenantroline and the advantages of the resulting electrode (CNTPE/poly-Fe-Aphen) for the catalytic oxidation and mainly reduction of hydrogen peroxide in aqueous medium. The 5-amino-1,10-phenantroline complex immobilized at different electrodes has been used for the development of pH and alkaline metals sensors [25, 26]. Complexes of this ligand with transition metals like iron, ruthenium and cobalt in a 1:3 stoichiometric ratio [27, 28] have demonstrated to be able to polymerize either in aqueous or organic media [29]. Carbon paste electrodes (CPE) modified with poly 5-amino-1,10-phenantroline have been used for the catalysis of the oxygen and hydrogen peroxide reduction [30]. Herrasti et al. [31] have recently proposed the electrocatalytic activity of CPE and CNTPE modified with poly(Fe(III)-5-amino phenantroline) towards the electroreduction of oxygen in acidic medium.

In this work we demonstrate the importance of CNT for the electrogeneration of the polymeric layer at CNTPE and for the electrocatalytic reduction of hydrogen peroxide and glucose biosensing previous modification with glucose oxidase (GOx). The selection of the best GOx immobilization conditions to obtain a selective and sensitive glucose biosensor is discussed in the following sections.

2. Experimental

2.1. Reagents

Hydrogen peroxide (30% v/v aqueous solution), ascorbic acid (AA) and $FeSO_4 \cdot 7H_2O$ were purchased from Baker. Glucose oxidase (GOx) (Type X-S, *Aspergillus niger*, (EC 1.1.3.4), 157,500 Units per gram of solid, Catalog number G-7141) was obtained from Sigma. Uric acid (UA) and glucose were acquired from Merck. 5-amino-1,10-phenantroline (5-Aphen) was obtained from Polysciences Inc. Standard blood human serum (Standatrol S-E level 1, Lot. 002740) was obtained from Wiener Lab. and it was reconstituted with ultrapure water before using. Other chemicals were reagent grade and used without further purification.

Ultrapure water ($\rho = 18\ M\Omega\ cm$) from a Millipore-MilliQ system was used to prepare all the solutions.

2.2. Apparatus

The measurements were performed with a TEQ-02 potentiostat. The electrodes were inserted into the cell (BAS, Model MF-1084) through holes in its Teflon cover. A platinum wire and Ag/AgCl, 3 M NaCl (BAS, Model RE-5B) were used as counter and reference electrodes, respectively. All potentials are referred to the latter. A magnetic stirrer provided the convective transport during the amperometric measurements.

2.2.1. Preparation of the CNTPE

The carbon nanotubes paste electrode (CNTPE) was prepared by mixing in an agata mortar multiwalled carbon nanotubes powder (30 ± 15) nm diameter, (1 – 5) μm length, 95% purity, NanoLab, U.S.A.) and Nujol oil (Fluka) in a ratio 70.0% w/w nanotubes powder and 30.0% w/w Nujol for 30 min. The classical carbon (graphite) paste electrode (CPE) was prepared in a similar way by mixing graphite powder (Fisher grade # 38) with Nujol oil. A portion of the resulting paste was packed firmly into the cavity (3.0 mm diameter) of a Teflon tube. The electric contact was established via a stainless steel screw. A new surface was obtained by smoothing the electrode onto a weighing paper.

2.2.2. Preparation of CNTPE Modified with Poly-Fe-Aphen (CNTPE/poly-Fe-Aphen)

The Fe(III)-5-amino-1,10-phenantroline (Fe-5-Aphen) complex was obtained by mixing in a 3:1 ratio 5-Aphen with $Fe_2(SO_4)_3$ in an O_2 free sulfate solution (pH 6.25). The polymerization of the Fe-5-Aphen complex was performed according to the optimized conditions [31]. Briefly, the CNTPE was immersed in a 2.5×10^{-4} M solution and the potential was cycled between − 1.200 and 0.800 V at 0.100 V/s (70 cycles).

2.2.3. Preparation of the CNTPE Modified with Poly-Fe-Aphen and GOx

The electrodes containing GOx were prepared in different ways: by incorporating 5.0 or 10.0% w/w GOx within CNTPE before the polymerization (CNTPE + GOx/poly-Fe-Aphen) (Scheme 1a), or by deposition of 10 μL 50 mg/mL GOx on the top of CNTPE either before (CNTPE/GOx/poly-Fe-Aphen) (Scheme1b) or after (CNTPE/poly-Fe-Aphen/GOx) (Scheme 1c) the polymer electrogeneration.

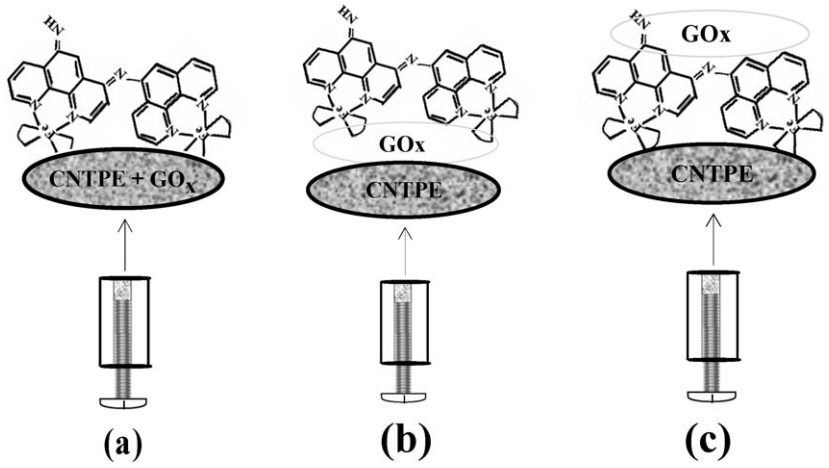
2.3. Procedure

The amperometric experiments were carried out in a phosphate buffer solution (0.050 M, pH 7.40) by applying the desired potential and allowing the transient current to decay to a steady-state value prior to the addition of the analyte and the subsequent current monitoring. All the experiments were conducted at room temperature.

3. Results and Discussion

3.1. Electropolymerization of Fe(III)-5-Aphen

Previous studies demonstrated that the polymer electrogenerated at a CNTPE after cycling the potential in a Fe-5-Aphen solution behaves different from the polymer grown under identical conditions at CPE [31]. In fact, Koutechy – Levich plots for oxygen reduction at rotating CPE and



Scheme 1. Schematic representation of the enzymatic biosensor according to the GOx immobilization procedure: within the composite (a), on the top of the composite electrode before (b) and after the polymer formation (c).

CNTPE modified by polymerization in a Fe-5-Aphen solution, confirmed that this process occurs via four electrons at CPE/poly-Fe-Aphen, while at CNTPE/poly-Fe-Aphen, it occurs via hydrogen peroxide through two steps of two electrons. This interesting difference has been the starting point of this work.

Figure 1a shows the last cycle (number 70) obtained in the polymerization solution (2.5×10^{-4} M Fe-Aphen solution, pH 6.25) during the electrogeneration of poly-Fe-Aphen film on CNTPE. There are two oxidation peaks, at -0.569 V (Ia) and -0.375 V (IIa), and two reduction peaks at -0.829 V (Ic) and -0.537 V (IIc). The pair Ia/Ic is due to the reduction/oxidation of the ligand while the IIa/IIc pair is associated with the Fe(II)/Fe(III) system. This information was obtained from cyclic voltammetry experiments performed separately only with Fe(III) or 5-Aphen ligand solutions (not shown). Figure 1b shows the corresponding voltammogram for CNTPE/poly-Fe-Aphen in a 0.050 M phosphate buffer solution pH 7.40. The CV shows some shifting in the peak potentials and a decrease in the associated currents of the processes described above (see inset).

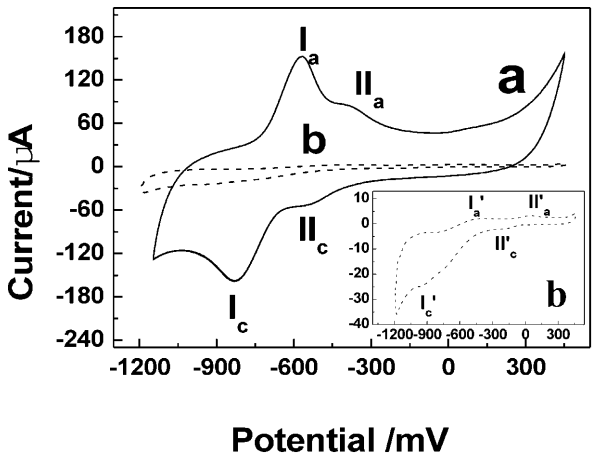


Fig. 1. a) Cyclic voltammograms for the last cycle during the electropolymerization of 2.5×10^{-4} M Fe(III)-5-Aphen in a sulfate solution pH 6.25. b) Cyclic voltammogram obtained for a fresh CNTPE/poly-Fe-Aphen in a 0.050 M phosphate buffer solution pH 7.40. Inset: cyclic voltammogram b in a more sensitive scale. Initial potential: -1.200 V; Final potential: 0.800 V. Scan rate: 0.100 V/s.

3.2. Electrochemical Behavior of Hydrogen Peroxide

Hydrodynamic voltammograms for hydrogen peroxide obtained at CNTPE/poly-Fe-Aphen showed a slight decrease in the overvoltages for the oxidation and the reduction of hydrogen peroxide compared to bare CNTPE, although the associated currents largely increased as a consequence of a synergistic effect resulting from the combination of the catalytic activity of polyFe-Aphen and CNTs (not shown).

Figure 2A and 2B show amperometric recordings at CNTPE (a) and CNTPE/poly-Fe-Aphen (b) obtained at -0.100 V (A) and 0.700 V (B) after successive additions of 2.0 mM hydrogen peroxide. A fast, sensitive and well-defined response is obtained at CNTPE/poly-Fe-Aphen, either at -0.100 V or at 0.700 V. The insets show the amperometric responses obtained at CNTPE in a more sensitive current range. Figure 2C and D display the corresponding calibration plots at -0.100 V and 0.700 V, respectively, while the insets depict the calibration plots obtained at bare CNTPE in a more sensitive scale. At CNTPE/poly-Fe-Aphen there is an enhancement of 185-fold in the sensitivity at -0.100 V (sensitivities of (0.7 ± 0.1) μA M^{-1} and $(1.3 \pm 0.3) \times 10^2$ μAM^{-1} at CNTPE and CNTPE-poly-Fe-Aphen, respectively). Regarding the sensitivity for hydrogen peroxide at 0.700 V, it increases from (1.8 ± 0.3) μAM^{-1} at CNTPE up to $(2.7 \pm 0.3) \times 10^2$ at CNTPE/poly-Fe-Aphen. These results clearly demonstrate that the association of the catalytic activity of CNT with that of the polymeric layer towards the oxidation and reduction of hydrogen peroxide allows obtaining significantly more

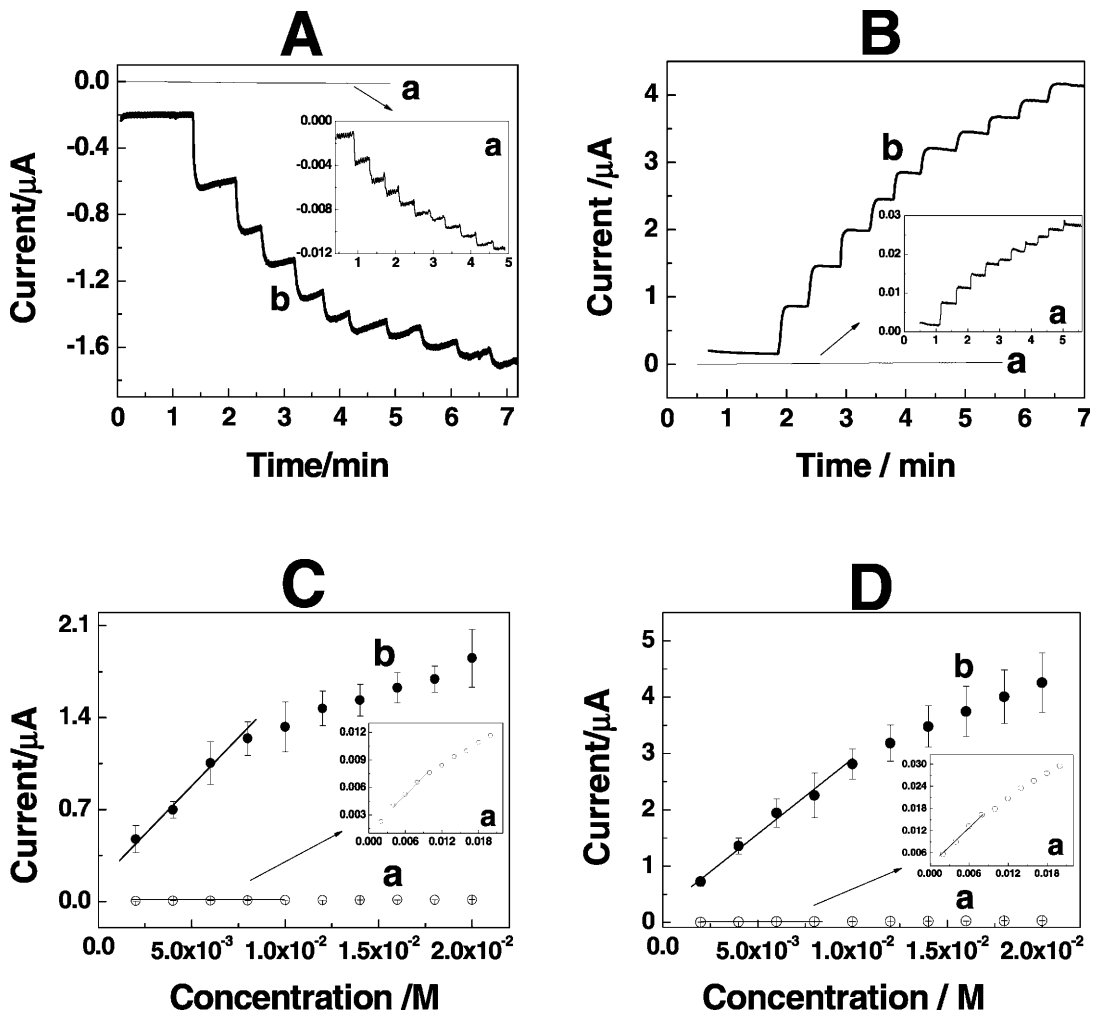


Fig. 2. Amperometric recordings obtained at −0.100 V (A) and 0.700 V (B) for successive additions of 2.0 mM hydrogen peroxide at CNTPE (a) and CNTPE-poly-Fe-Aphen (b). (C) and (D) Calibration plots for hydrogen peroxide obtained from the recordings shown in (A) and (B), respectively. Insets show the amperometric recordings at bare CNTPE (A and B) and the corresponding calibration plots (C and D) in more sensitive scales. Supporting electrolyte: 0.050 M phosphate buffer solution pH 7.40.

sensitive amperometric signals than at bare CNTPE. Similar amperometric experiments performed at −0.100 V using the graphite composite electrode (CPE/poly-Fe-Aphen), show a very poor response for hydrogen peroxide, with a sensitivity of $(0.65 \pm 0.07)\ \mu AM^{-1}$ confirming that the catalytic activity is really connected to the polymer that grows at the CNT composite, in agreement with the behavior previously reported for oxygen reduction at CPE/poly-Fe-Aphen and CNTPE/poly-Fe-Aphen [31].

In order to confirm if the catalytic activity was really due to the polymer, we evaluate the amperometric response of hydrogen peroxide at −0.100 V at CNTPEs treated under the 'polymerization conditions' using 5-Aphen or iron (III) sulfate solutions instead of 5-Fe-Aphen. In all cases the sensitivities were considerably smaller than those obtained at CNTPE/poly-Fe-Aphen. In fact, the sensitivity was $(0.7 \pm 0.1)\ \mu AM^{-1}$ at CNTPE, and increased to $((8.0 \pm 0.9)\ \mu AM^{-1})$ and to $((20 \pm 5)\ \mu AM^{-1})$ when the potential was cycled in the presence of Fe(III) or 5-Aphen solutions, respectively. These significant differences with the sensitivity obtained at CNTPE/poly-Fe-Aphen $((1.3 \pm 0.3) \times 10^2\ \mu AM^{-1})$ confirm that the polymer is the responsible for the catalytic effect observed for hydrogen peroxide reduction.

In addition to the obvious analytical application of this sensor for hydrogen peroxide quantification, this excellent behavior of CNTPE/poly-Fe-Aphen towards the oxidation/reduction of hydrogen peroxide represents a new alternative for the development of electroanalytical devices based on the quantification of hydrogen peroxide, like enzymatic biosensors containing some oxidase as biorecognition element. Considering this possibility, and taking into account that the usual interferents when working with biological samples like human serum are ascorbic acid (AA) and uric acid (UA), we evaluate the voltammetric response of these compounds at CNTPE and CNTPE/poly-Fe-Aphen (not shown). At CNTPE/poly-Fe-Aphen the oxidation currents for 1.0×10^{-3} M AA and 1.0×10^{-3} M UA largely decrease (51.0% for AA and 77.0% for UA, respectively) compared to the response obtained at CNTPE. These

results suggest that, in addition to the catalytic effect towards the redox behavior of hydrogen peroxide, the polymer rejects negatively charged compounds such as ascorbate and urate.

3.3. Glucose Biosensing at CNTPE/poly-Fe-Aphen Modified with GOx

The excellent catalytic activity demonstrated by CNTPE/poly-Fe-Aphen towards the reduction of hydrogen peroxide has been the initial point for the design of a glucose biosensor based on the incorporation of GOx in a CNTPE/poly-Fe-Aphen. Glucose oxidase is the most widely used biorecognition element in electrochemical enzymatic glucose biosensors and catalyzes the oxidation of glucose to gluconolactone in the presence of oxygen which, in turn, is converted into hydrogen peroxide.

In order to obtain the best conditions for the biorecognition/transduction processes, we investigate different alternatives to immobilize GOx at CNTPE/poly-Fe-Aphen including within the composite (CNTPE + GOx/poly-Fe-Aphen, Scheme 1a); deposition on top of the CNTPE before the polymerization (CNTPE/GOx/poly-Fe-Aphen, dispersion Scheme 1b), and deposition on CNTPE once the polymer was electrogenerated (CNTPE/poly-Fe-Aphen/GOx, Scheme 1c).

I) Immobilization of GOx by dispersion within the composite (CNTPE + GOx/poly-Fe-Aphen): Amperometric recordings obtained at -0.100 V at a CNTPE containing 5.0% w/w GOx and modified by electropolymerization in 5-Fe(III)-Aphen solution under the optimum conditions for the polymer formation, gave a sensitivity of (0.20 ± 0.01) μAM^{-1} for successive additions of glucose. Similar experiments obtained with an electrode prepared by incorporation of 10.0% w/w GOx instead of 5.0% w/w, gave no response.

In order to evaluate if this small or null response of the bioelectrode towards glucose was due to a blockage of GOx active site and/or to a poor polymer formation (and hence, poor catalytic activity), similar amperometric experiments using hydrogen peroxide instead of glucose were performed at -0.100 V. A sensitivity of (4.0 ± 0.9) μAM^{-1} was attained for hydrogen peroxide at the electrode containing 5.0% w/w GOx (CNTPE + 5.0% w/w GOx/poly-Fe-Aphen), while no response was obtained at the composite containing 10.0% w/w GOx (CNTPE + 10.0% w/w GOx/poly-Fe-Aphen). Considering that the sensitivity for the reduction of hydrogen peroxide at CNTPE/poly-Fe-Aphen is $(1.3 \pm 0.3) \times 10^2$ μAM^{-1}, the significant decrease obtained at CNTPE + GOx/poly-Fe-Aphen indicates that the presence of the protein within the composite prevents the polymer formation. For this reason, the incorporation of the enzyme within the CNTPE was not selected as strategy to prepare the glucose biosensor.

II) Immobilization of GOx by deposition on the top of the composite: The immobilization of GOx was also evaluated by depositing 10 μL of 50 mg/mL GOx solution at CNTPE before (CNTPE/GOx/poly-Fe-Aphen) and after (CNTPE/poly-Fe-Aphen/GOx) the polymer formation (see Schemes 1 b and c). In order to select the most appropriate scheme for GOx immobilization, the performance of the resulting electrodes was evaluated from three parameters, sensitivity to glucose, short-term stability, and interference of easily oxidizable compounds like AA and UA.

Calibration plots obtained from amperometric experiments for glucose at -0.100 V using CNTPE/GOx/poly-Fe-5-Aphen and CNTPE/poly-Fe-5-Aphen/GOx gave sensitivities of (7.5 ± 0.5) μAM^{-1} ($r = 0.997$) and (6 ± 1) μAM^{-1} ($r = 0.996$), respectively. In both cases, the linear range was from 1.0 to 4.0 mM. The short-term stability was evaluated from the sensitivities obtained from successive amperometric experiments at -0.100 V using four different electrodes. At CNTPE/GOx/poly-Fe-Aphen, the sensitivity decreased in a significant way after the forth calibration, while at CNTPE/poly-Fe-5-Aphen/GOx, at least eight successive calibration plots were successfully performed without appreciable decrease in sensitivity. The third parameter evaluated was the interference of easily oxidizable compounds, such as AA and UA, on the performance of both bioelectrodes. At -0.100 V, the interference of AA is 0.0% either at CNTPE/GOx/poly-Fe-Aphen or at CNTPE/poly-Fe-Aphen/GOx. A different behavior was observed in the case of UA, since this compound interferes at both electrodes. The largest interference was registered at CNTPE/GOx/poly-Fe-5-Aphen (83.4% interference at CNTPE/GOx/poly-Fe-Aphen versus 46.2% interference at CNTPE/poly-Fe-Aphen/GOx referred to 4.0 mM glucose). Therefore, based on the compromise between the three parameters investigated, the selected strategy for immobilizing GOx, was the deposition of 10 μL of 50 mg/mL GOx solution at CNTPE/poly-Fe-Aphen (CNTPE/poly-Fe-Aphen/GOx).

According to the results previously shown, it is evident that a selective layer is necessary to include in the biosensor scheme to gain selectivity. To obtain more realistic information about the selectivity of the proposed biosensor, we challenged it with human blood serum samples using Nafion on the top of the biosensing platform as permselective barrier (CNTPE/poly-Fe-Aphen/GOx/Naf). Table 1 shows the relative error between the glucose concentration informed by the laboratory and the one obtained with our biosensor (CNTPE/poly-Fe-Aphen/GOx/Naf) prepared with Nafion of different concentrations (by dilution of the commercial Nafion with 0.050 M phosphate buffer solution pH 7.40). The interference obtained in the absence of Nafion was very high, with relative errors in glucose concentration higher than 100%. The concentration of Nafion solution deposited on the top of the bioelectrode demonstrated to have a crucial effect on the whole performance of the biosensor. When the electrode is prepared with the most concentrated Nafion solution, there is not only a decrease in the interference signal due to its permselective properties, but also a decrease in the analytical signal as a consequence of the physical barrier effects of the Nafion layer. The best compromise was obtained with

Table 1. Effect of the dilution of Nafion that covers the electrode (CNTPE/poly-Fe-Aphen/GOx/Naf) on the relative error in the determination of glucose concentration compared to the values provided by the laboratory.

Nafion /phosphate buffer [a] v/v	Relative error (%)
50:50	>100
60:40	(71 ± 3)
65:35	(71 ± 1)
70:30	(15 ± 1)
75:25	(66 ± 2)
80:20	(35 ± 2)

[a] 0.050 M phosphate buffer pH 7.40.

70/30 Nafion/buffer (error in glucose concentration = 15%). Even when this result does not show a perfect agreement with the reported value, it is within the acceptable range suggested by the laboratory.

In order to evaluate if the selected Nafion layer was really efficient in eliminating the interference of UA, amperometric recordings at −0.100 V were obtained at CNTPE/poly-Fe-Aphen/GOx/Naf(70/30) after addition of 4.0 mM glucose followed by additions of UA up to the maximum physiological levels (3.6×10^{-4} M). Under these conditions, the interference decreases from 46.2% to just 7.2%, demonstrating the efficiency of the resulting bioelectrode. No interference was observed for AA. Therefore, the small interference observed when the electrode was challenged with human blood serum samples could be due to the presence of additional easily oxidizable compounds in such a complex matrix.

Figure 3 shows an amperometric recording at −0.100 V for successive additions of 0.25 mM glucose at CNTPE/poly-Fe-Aphen/GOx/Naf(70/30) (A) and the corresponding calibration plot (B). A fast response is obtained under these conditions, even in the presence of the Nafion layer, with a linear range up to 1.5 mM and a sensitivity of (14.6 ± 0.8) μA M^{-1} ($r = 0.989$). The biosensors demonstrated to be highly stable, since after five calibration plots obtained from amperometric experiments, that represents around 2.5 hours of continue use, the *RSD* in sensitivities was 4.4%.

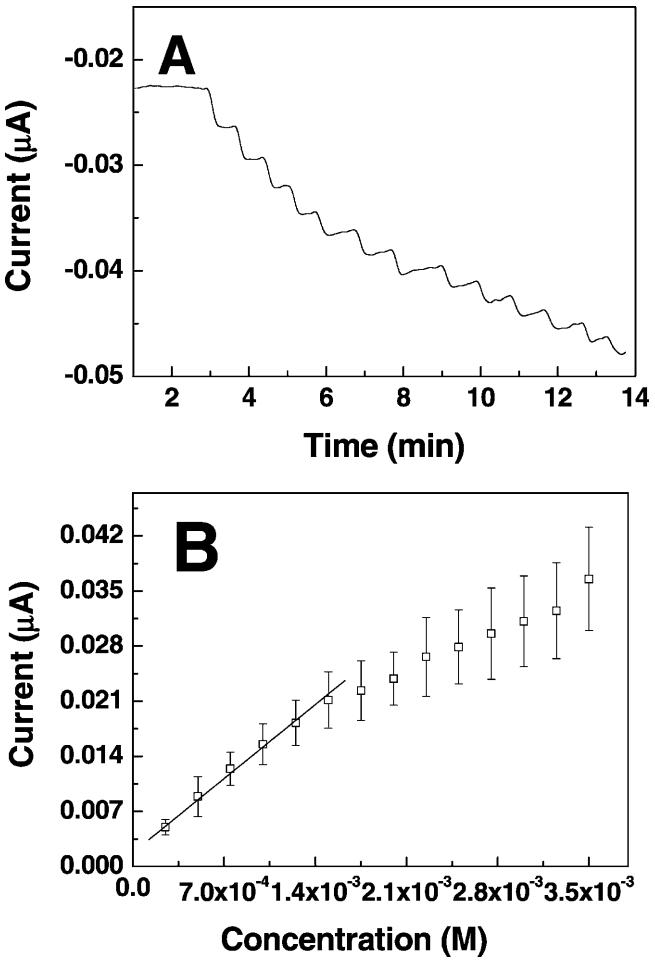


Fig. 3. (A) Amperometric recordings obtained at CNTPE/poly-Fe-Aphen/GOx/Naf(70/30) for successive additions of 0.25 mM glucose. Working potential: −0.100 V. (B) Calibration plots obtained from (A). Supporting electrolyte: 0.050 M phosphate buffer solution pH 7.40.

4. Conclusions

This work confirmed the important role of CNT included within the composite on the generation of poly-Fe(III)-amino-1,10-phenantroline compared to carbon paste electrode. We report for the first time the catalytic activity of this polymer, selectively electrogenerated on a CNTPE, for the oxidation and mainly the reduction of hydrogen peroxide, demonstrating that the combination of CNT with the polymer results in a large improvement of the reduction signals for hydrogen peroxide. The analytical application of the resulting electrode was demonstrating in connection with the development of a selective glucose biosensor. GOx immobilization conditions and Nafion concentration in the permselective layer located on the top of the biosensor have demonstrated to be critical to obtain a good analytical performance. The selected biosensor, CNTPE/poly-Fe-Aphen/GOx/Naf(70/30), has allowed the highly sensitive and selective quantification of glucose even in the presence of large excess of AA and UA. The biosensor was also challenged with human blood serum, without the need of separation step, with highly promising results. Even when the concept of the catalytic activity of the polymer has been demonstrated with the system hydrogen peroxide/GOx, it could be extended to other enzymes that involve hydrogen peroxide, thus offering an interesting alternative for further biosensors designs.

5. Acknowledgements

The authors thank Fundación Antorchas, CONICET, SECyT-UNC, ANPCyT, Becas Banco Santander-Río, Ministery of Science and Technology of Cordoba for the

financial support. MLL acknowledges CONACyT for the scholarship.

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