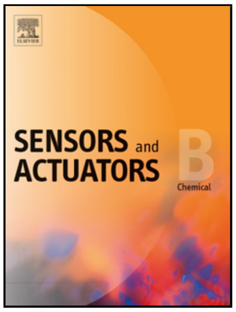

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Construction and evaluation of carbon black and poly(ethylene co-vinyl)acetate (EVA) composite electrodes for development of electrochemical (bio)sensors

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Highlights

- Carbon black (CB) and poly(ethylene co-vinyl)acetate (EVA) as new composite electrode
- EVA/CB rigid composite electrode with high robustness and very good electroanalytical properties
- Electrochemical sensor with high electron-transfer rate constant for hexacyanoferrate(III)/(II) and wide useful potential window
- A simple and alternative composite which can be a useful for preparation of wearable electrodes in the future.

Abstract

The preparation of a new carbon composite electrode based on a mixture of poly(ethylene-*co*-vinyl acetate) (EVA) and carbon black (CB) is described. The resulting EVA/CB electrode combines the excellent electrochemical properties of CB with the advantages brought by the EVA polymer, which allows the preparation of very rigid composite electrodes. The composition of the composite was optimized, that based on 25EVA:75CB (w/w) leading to electrodes with the best performance. The composite electrodes were characterized by surface analysis techniques and by cyclic voltammetry and were applied in different electroanalytical systems. The EVA/CB electrode was employed in a flow injection analysis system as an amperometric detector for ascorbic acid showing a linear dynamic range from 5.0 x 10^{-5} mol L^{-1} to 1.0 x 10^{-3} mol L^{-1} with 63 injections per hour. Prussian Blue (PB) films were prepared on the composite electrode surface and the resulting modified electrode was successfully used for the amperometric determination of hydrogen peroxide. Determination of hydrogen peroxide present in pharmaceutical samples was realized using the proposed methodology and official method and the results found are in agreement at the 95% confidence level. The EVA/CB/PB electrode was also used for the preparation of a glucose biosensor, by immobilizing the enzyme glucose oxidase (GOx) on top, EVA/CB/PB-GOx, with a linear range from 9.99 x 10^{-5} mol L^{-1} to 6.95 x 10^{-4} mol L^{-1}. The proposed composite electrode exhibits favorable features such as high mechanical resistance, low cost, simple preparation, and ease of chemical modification, being versatile for a variety of electroanalytical applications. The results show an excellent combination of good electrode kinetics with small background current and reproducible response.

Keywords: *Carbon black; poly(ethylene co-vinylacetate); composite electrodes; flow injection analysis; amperometric detector; biosensor.*

1. Introduction

Since their discovery by Adams [1], in 1958, composite electrodes have been widely used in electroanalysis in the development of various sensors due to their low background current, wide potential window, good mechanical resistance, and chemical inertness [2]. Almost three years later, Tallman and Petersen [3] described a composite electrode as “a material consisting of at least one conductor phase commingled with at least one insulator phase, the typical case being a material containing one conducting and one insulating phase”. Therefore, the properties of the composite depend on the nature of each individual component and their relative amounts and distribution [3].

A large number of new composite materials has been prepared and their properties and electrochemical applications investigated [2, 4, 5]. The conductive component is normally a carbonaceous material [5-7]. This great interest in carbon conductive phase composites is due to the unique properties of carbon, existing as different allotropes, having different types of graphitization, with a variety of dimensionalities (0 to 3D). It, also exists in different forms (powder, foams, nanotubes, fibers, composites, among others), making it a material with numerous application possibilities [8].

Carbon black (CB) has been extensively studied because it is cheaper than other conductive carbon materials and has offered a new way for developing sensors and biosensors with low cost and excellent electroanalytical performance [9-11]. Recently, Talarico et al. [12-14] reported an electrochemical sensor modified with a dispersion of carbon black for phosphate and phenolic compound detection. The improvement due to the use of CB was attributed to its excellent conductive and electrocatalytic properties which were related to the presence of defect sites on the carbon black surface, similarly to other carbon nanostructures, such as carbon nanotubes and/or graphene [10]. Besides the direct use of carbon black as an electrode modifier, it can also be used as a support for the preparation of other nanostructures, as reported by Hou et al. [15]. They described the use of CB for supporting ferroferric oxide nanoparticles, which demonstrated a high electrocatalytic activity towards the oxidation of bisphenol. A similar approach was proposed by Arduini et al. [16], using gold nanoparticles deposited on the carbon black surface which also showed an electrocatalytic effect on the redox processes of glucose, hydrogen peroxide and ascorbic acid. Although carbon black has been exploited as a modifier for developing sensor architectures due to its advantageous performance/cost ratio, even in comparison with other carbon nanomaterials such as carbon nanotubes, its use as a conductor phase for the development of composite electrodes was not explored until recently, but with different binding phases than those studied here [9, 17].

For the binder phase, polymers have often been employed, such as PVC [18], Teflon [19], poly(ethylene terephthalate) [20], poly(methylmethacrylate) [21], polyurethane [22], polystyrene [23], Nafion [24], cellulose acetate [25], epoxy [26], silicone rubber [27], among others. The main advantage in using polymers is related to their high stability in non-aqueous media and in hydrodynamic conditions (e.g. flow injection analysis procedures), to the possibility of incorporating a variety of modifiers and to the easy adjustment of the shapes and sizes of the fabricated devices [2, 28]. Poly(ethylene-*co*-vinyl acetate) copolymer (EVA) has been reported for membrane preparation in the development of ion-selective electrodes (ISE) [29, 30], its main characteristics being high elasticity, chemical inertness and high thermal and mechanical resistance [31]. It is, therefore, a promising material for the development of rigid composite electrodes.

Solid composites based on a carbon–polymer matrix have been shown to be good voltammetric and amperometric detectors for the determination of various organic and inorganic species [2]. The main approach used in the development of new composite electrodes is based on the procurement of conductive and binder phases that combine a high electrochemical response with a low-cost fabrication process. In general, two types of research are undertaken in the development of a new electrode, one related to its electrochemical behavior [32-34] and the other related to the electroanalytical applicability as a bare or chemically modified electrode [35-37].

In the present work, a new composite electrode prepared using CB and EVA is described as an alternative material for the development of electrochemical (bio)sensors with advantages such as resistance to organic solvents, mechanical resistance and very low cost, due to the choice of carbon black as conductive phase. After characterizing the carbon black nanoparticles, characterization of the composite electrodes was done by scanning electron microscopy. Cyclic voltammetry was employed for the determination of the potential window in different media and for the determination of electroactive areas. The applicability of the composite electrode material as a sensor was evaluated by testing its analytical performance towards some classical electrochemical probes such as ascorbic acid, and as an amperometric detector in flow injection analysis (FIA). The CB/EVA electrode modified with a Prussian Blue (PB) film was used for the amperometric detection of hydrogen peroxide and served as substrate for a glucose oxidase biosensor. Additionally, this new composite brings the possibility of a wide range of applications, when prepared in different formats, for construction of other electrochemical sensor platforms such as: screen-printed electrodes, wearable electrochemical sensors or conductive filaments for 3D-printing.

2. Experimental

2.1. Reagents and Chemicals

All reagents were of analytical grade and were used as received. Carbon black pearls and poly(ethylene-*co*-vinyl acetate, vinyl acetate 18 wt. %) copolymer were acquired from Cabot (Schaffhausen, Switzerland) and Exxon-Mobil Chemical (Meerhout, Belgium), respectively. Potassium hexacyanoferrate(III), ascorbic acid, bovine serum albumin, α-D(+)-glucose and glutaraldehyde 25% (v/v) were from Sigma-Aldrich (Steinheim, Germany). Potassium chloride and sulfuric acid were from Merck (Darmstadt, Germany). Hydrogen peroxide was from Neon (São Paulo, Brazil), potassium phosphate dibasic trihydrate from Synth (Diadema, Brazil), potassium phosphate monobasic monohydrate from Carlo Erba (Milan, Italy), sodium hydroxide from Cromato (São Paulo, Brazil), glacial acetic acid from Isofar (Duque de Caxias, Brazil), anhydrous sodium acetate from J. T. Baker (Phillipsburg, U.S.A.) and glucose oxidase (GOx, EC 1.1.3.4, from Aspergillus niger, 24 units/mg) was from Fluka (Buchs, Switzerland). All solutions were prepared with deionized water obtained with a Millipore Milli-Q system.

2.2. Apparatus

Cyclic voltammetry (CV) and fixed-potential amperometric detection were done using a µAUTOLAB Type III (Metrohm-Autolab, Netherlands) potentiostat with GPES 4.9 software for experimental control and data acquisition. The measurements were performed in a conventional electrochemical three-electrode configuration cell of 10.0 mL capacity, containing the EVA/CB composite electrode together with the auxiliary and reference electrodes, Pt wire and Ag/AgCl in 3.0 mol L^{-1} KCl (or SCE), respectively. The pH measurements were carried out using a Metrohm pH-meter with a Metrohm combined pH reference electrode. All experiments were performed at a temperature of ≈25 °C.

FIA experiments were carried out using a flow cell with an effective volume of 95 μL. The working electrode (EVA/CB electrode), reference electrode (Ag/AgCl/3.0 mol L^{-1} KCl) and the platinum auxiliary electrode were introduced through holes in its cover. The electrochemical flow cell, schematically represented in Figure 1, was inserted in a one-channel FIA system. The system was assembled with a peristaltic pump (Ismatec, model IPS12, Switzerland) and a manual homemade injector of polyurethane. The manifold connections were made with tygon tubing (0.5 mm i.d.). Phosphate buffer solution, 0.10 mol L^{-1} pH 7.0, was used as the carrier solution. The analytical path was 30 cm long and the entire flow injection system was kept at room temperature.

Scanning electron microscopy (SEM) was performed with a JEOL JMS 6360-LV microscope, operated at 20 kV. Transmission electron microscopy (TEM) was performed with a JEOL JEM 1200EX-II microscope, operated at 80 kV. X-ray diffraction patterns (XRD) were recorded by a Shimadzu XRD-6000 diffractometer under the conditions: 40 kV, 30 mA, Cu Kα radiation ($\lambda = 0.154056$ nm) in step-scanned mode with steps of 0.04°/2θ in the range from 3 to 80°, and a count time of 10 s/step.

Gas adsorption measurements were performed in N_2 atmosphere at 77 K using a Quantachrome Autosorb Automated Gas Sorption System NOVA 1200. The specific surface area (SSA) was calculated by the Brunauer, Emmet e Teller (BET) method [38].

2.3. Preparation/modification of the EVA/CB composite electrodes

The EVA/CB composite was prepared by mixing appropriate amounts of EVA and CB. A solution containing 25% EVA w/v in toluene was prepared under heating and constant magnetic stirring. CB was slowly added to this solution to obtain 30-70% w/w suspensions. After homogenization, the mixture was drained into a cylindrical Teflon mold (1.0 cm high) of internal diameter 0.4 cm. After a 24 h drying period, the "dried" composite cylinders were inserted in a plastic tube (micropipette tip) and fixed with epoxy resin (Araldite), leaving the outer surface of the cylinder free. The electrodes were left to cure for 24 h at room temperature. An external electrical contact was then made with a copper wire and silver ink and a further 24 h for drying was necessary. After the drying period, the surfaces of the electrodes were polished using abrasive papers of 360 and then 1200 grit.

A glucose biosensor using the enzyme glucose oxidase (GOx), EVA/CB/PB-GOx, was prepared in two steps, based on literature methods [39]. First, the electrochemical deposition of Prussian Blue mediator was done by cyclic voltammetry from a solution containing 2.5 mmol L^{-1} $K_3[Fe(CN)_6]$, 2.5 mmol L^{-1} $FeCl_3$ and 1.0 mmol L^{-1} HCl in 0.1 mol L^{-1} KCl, by cycling the potential at a sweep rate of 10 mV s^{-1} between −1.0 and +0.5 V *vs.* SCE for 25 cycles. The second step consisted in the immobilization of GOx onto the surface of the PB modified electrode by cross-linking with glutaraldehyde (GA). A solution containing 1.0% w/v GOx and 4.0% w/v bovine serum albumin (BSA) was prepared in 0.1 mol L^{-1} sodium phosphate buffer saline (0.1 mol L^{-1} phosphate buffer + 0.05 mol L^{-1} NaCl, pH = 7.0). A volume of 10 µL of enzyme solution was then mixed with 5 µL GA (2.5% v/v diluted in water), of which 10 µL was dropped onto the electrode surface and left to dry at room temperature during at least 4 h. When not in use, the prepared biosensor was kept in sodium phosphate buffer saline (0.1 mol L^{-1} phosphate buffer + 0.05 mol L^{-1} NaCl, pH = 7.0) at 10 °C.

2.4. Cyclic voltammetry

A solution of 3.0 mmol L^{-1} potassium hexacyanoferrate(III) in 0.1 mol L^{-1} KCl supporting electrolyte was used for the voltammetric characterization of each electrode composition, in particular the determination of electroactive area, capacitance and heterogeneous electron transfer rate constant.

2.5. Flow injection analysis

Flow injection experiments were carried out using an electrode with 25:75 (w/w) of EVA/CB, respectively, as amperometric detector. The influence of all experimental parameters (flow rate, volume of injection and applied potential) was studied by analyzing the sensor response to ascorbic acid (AA) in 0.10 mol L^{-1} phosphate buffer solution, pH 7. The optimized analytical response was obtained by injecting 100 µL ascorbic acid (AA) into the carrier solution, at a flow rate of 4.3 mL min^{-1}, monitoring the amperometric signal at +600 mV *vs.* Ag/AgCl. The percentage content of ascorbic acid (AA) in orange juice samples was determined by the standard addition method and compared with that given by iodometric titration (methodology recommended by the Brazilian Pharmacopeia) [40].

2.6 Determination of hydrogen peroxide

Pharmaceutical sample containing hydrogen peroxide (*água oxigenada* 10 v/v, Catarinense Farma) was purchased from local market and submitted to analysis using the proposed sensor and permanganometric titration was chosen as comparative methodology. The procedure was based on direct dilution of sample portion (500 µL) in 20 mL of solution 2.0 mol L^{-1} H_2SO_4 following of titration using previously standardized solution of 0.02 mol L^{-1} MnO_4^- (triplicate). The end point was reached when a persistent purple color was observed.

Hydrogen peroxide determination employing EVA/CB/PB was performed by amperometry at a fixed potential of - 400 mV *vs.* SCE under hydrodynamic conditions using standard addition method. An aliquot of 50 µL of diluted sample (1:10) was added directly into electrochemical cell following of four successive additions of hydrogen peroxide stock solution in order to reach final concentration added varying from 1.0×10^{-4} mol L^{-1} to 4.0×10^{-4} mol L^{-1}.

2.7 Biosensing of glucose

The detection of glucose at the EVA/CB/PB-GOx mediated biosensor was performed by amperometry at a fixed potential of - 400 mV *vs.* SCE, in order for the PB mediator to respond to H_2O_2. The analytical curve was obtained by the addition of 0.10 mol L^{-1} glucose in 0.10 mol L^{-1} phosphate buffer saline solution pH 7 under constant magnetic stirring. The stock solution of glucose was prepared in sodium phosphate buffer saline at least one day before use, to permit equilibration of α and β anomers of D-glucose, was kept in the refrigerator and was used within one week.

Evaluation of analytical performance of proposed biosensor was performed by addition and recovery experiment using an artificial tears sample (Lacrima Plus®, Alcon). An aliquot of the sample was spiked with known amount of glucose (6.0×10^{-4} mol L^{-1}, value typical found in this kind of sample) and diluted 1:1 with phosphate buffer solution. Amperometric determination (triplicate) was achieved at a fixed potential of - 400 mV vs. SCE under constant magnetic stirring using standard addition method.

3. Results and Discussion

3.1. Surface characterization of carbon black and of CB/EVA composite electrodes

It is highly important to understand the structure and morphology of the materials involved in the development of an electroanalytical sensor, especially when dealing with materials such as CB, which have properties, e.g. surface area and structure, that are extremely dependent on the variables used in the production process, such as reaction time, temperature etc. For this reason, BET, TEM and XRD were conducted to characterize the CB used in this work.

The BET specific surface area determined for carbon black of 103 $m^2\ g^{-1}$ is in agreement with the values reported in the literature, which range from 6 to 1200 $m^2\ g^{-1}$ [41]. TEM images in Fig. 2 (A) reveal the typical CB aggregates formed by Van der Waals attraction forces [42]. The internal structure of these aggregates, still poorly understood in the literature [40, 41], presents some quasi-crystalline features, very similar to those of graphite. Donnet and Voet identified graphite basal planes in the CB structure but angularly distorted and with a different spacing between layers [42]. These deductions were confirmed by XRD analysis, Fig. 2 (B), in which a XRD pattern similar to that of graphite can be observed, with peaks located at 26.5°, 44.3°, and 54.5° corresponding to the planes (002), (101), and (004), respectively [43]. Moreover, the main peak of the (002) phase at ca. 26.4° corresponds to the carbon black pattern [44].

Micrographs of the 30–75% (CB, w/w) composite surfaces, after polishing on 360 followed by 1200 grit abrasive paper, are presented in Fig. 3. As can be seen, the 30–50% (CB, w/w) presents a smoother surface than those of 60 and 75%, which showed higher roughness. Mendes et al. [45] described similar morphologies for a polyurethane/graphite composite and a high roughness was observed for composites prepared with a greater amount of conductive phase material. Pacios et al. [33] also correlated roughness of the composite electrode surface with better electrochemical response, since the roughness is related to the increased surface area of the electrode.

3.2. Voltammetric characterization of the composite electrodes

The composition of the composite material has a big influence on its electrochemical properties. In order to optimize the mixture, electrodes containing different compositions of CB (30–75 CB, w/w) were evaluated by cyclic voltammetry. Important parameters, namely capacitance, electroactive surface area and heterogeneous electron-transfer rate constant (k^0) were determined and compared.

Capacitance values were calculated from the background current recorded at different scan rates in 0.1 mol L^{-1} KCl solution (not shown) [46]. The estimated capacitances of the electrodes are summarized in Table 1. The capacitance value is very high at 75% (CB, w/w), decreasing upon reducing the CB content. Similar results were observed by Fagan-Murphy and Patel [26] and Klatt et al. [47] for other composite electrodes. The increase of capacitance on increasing the proportion of CB can be attributed to the higher quantity of conductive phase exposed on the surface of the composite electrode.

The determination of the electroactive surface areas and heterogeneous electron-transfer rate constants (Table 1) was done from cyclic voltammograms using hexacyanoferrate(III) as redox species. Fig. 4A displays cyclic voltammograms obtained at 50 mV s^{-1} in 3.0 mmol L^{-1} $[Fe(CN)_6]^{3-}$ in 0.1

mol L^{-1} KCl solution using composite electrodes of different compositions (30–75 CB, w/w). As expected, the voltammetric response of the composite electrodes is highly dependent on the percentage of carbon black. Compositions of 60 and 75 % (CB, w/w) presented the best results while 30, 40 and 50 % (CB, w/w) showed the effect of their higher electrical resistance and the hexacyanoferrate(III)/(II) redox couple response was not clearly defined.

The electroactive surface area of the EVA/CB with 50-75 % (CB, w/w) electrodes was evaluated from cyclic voltammograms recorded in 3.0 mmol L^{-1} $[Fe(CN)_6]^{3-}$ in 0.1 mol L^{-1} KCl solution at scan rates from 10 to 200 mV s^{-1} and applying the Randles–Sevčik equation. The values of calculated electroactive area (*A*) and the percentages of the electroactive area versus geometric area (*A* %) of the EVA/CB composite electrodes are also presented in Table 1. Electroactive areas of the composite electrodes increase with CB content, being highest for 75 % (CB, w/w) as expected, and are smaller than the geometric area, due to the EVA non-conductive phase.

The same cyclic voltammograms were used to estimate values of the standard electron transfer rate constant, k^0, (Table 1) [46], for the same CB 50-75 % (CB, w/w) electrodes. The EVA/CB composite electrodes presented similar values to those in [48] for carbon paste electrodes (1.0 x 10^{-4} cm s^{-1}). Cinti et al [14] studied screen printed electrodes modified with carbon black and they found values of k^0 of 4.5 x 10^{-3} cm s^{-1} using CB, higher than that of unmodified SPCE. It was shown that different carbon black samples also lead to different k^0 values [11].

Although the larger the amount of conductive phase and the lower the amount of EVA, the higher the electroactive surface area of the composite, lowering the amount of EVA below 25%, leads to a decrease in the mechanical resistance of the composites, resulting in easily-breakable and hard to manage materials. Therefore, lower percentages of EVA were not employed, the material chosen for further studies being that with 75% w/w CB and 25 % EVA, which had the highest percentage of electroactive area, and the highest value of k^0 [49].

The influence of scan rate (10–200 mV s^{-1}) on peak current in the voltammetric response was evaluated and a linear relation between the peak current and the square root of scan rate revealed a diffusion-controlled process occurring at the electrode surface [46].

The potential window of the 25EVA/75CB composite electrode in electrolytes of different pH of concentration 0.1 mol L^{-1}, namely H_2SO_4 pH 1.0, acetate buffer pH 4.0, phosphate buffer pH 7.0, ammonium buffer pH 11.0 and NaOH pH 13.0, was determined from the cyclic voltammograms displayed in Fig. 4B. The 25EVA/75CB composite electrode has a broad potential window and a low background current. There is very little dependence of the hydrogen evolution potential on pH (~ -0.80 V), becoming slightly more negative at higher pH values. In neutral phosphate buffer solution the potential window is around 2.5 V.

3.3 Analytical evaluation of the proposed CB/EVA composite electrode

3.3.1 Amperometric detector in flow injection analysis

The analytical performance of the EVA/CB composite electrode as an amperometric detector for ascorbic acid (AA) was investigated under hydrodynamic conditions using flow injection analysis (FIA). Experimental parameters were optimized by analyzing the sensor response [50].

The effect of the applied potential was evaluated between 400 and 700 mV vs Ag/AgCl using a sample volume of 200 µL and a flow rate of 4.3 mL min^{-1}. These values of applied potential were chosen based on cyclic voltammograms obtained in the presence of AA, see supplementary material, Fig. S1. A more positive potential led to a higher sensor response, Fig. S2, but there is no significant increase above 600 mV. Thus, 600 mV was chosen for further studies, since it also presented a lower standard deviation between successive measurements.

The effect of the injection volume on the sensor signal was investigated for volumes in the range 25 to 400 µL at a flow rate of 4.3 mL min^{-1} and applied potential of 600 mV. It was found that the sensitivity of the amperometric system gradually increased with increase of sample volume up to 100 µL, Fig. S3. This behavior is attributed to a good dispersion of the sample in the FIA system channel. The stabilization of the response indicates minimal dilution of the sample, with a maximum in the current transient signal for 100 µL. For volumes higher than 100 µL, an increase in the time of washing occurs with concomitant decrease in the analytical signal. Thus, a volume of 100 µL was selected.

The effect of flow rate on the peak current was also evaluated, in the range 1.7 to 10.7 mL min^{-1} at an applied potential of 600 mV and a sample volume of 100 µL, see Fig. S4. The sensitivity of the system increased continuously up to 7.3 mL min^{-1}, due to the increase in mass transport. For flow rates higher than 7.3 mL min^{-1}, an increase in the internal pressure of the system occurred, which generated turbulent flow, leading to higher background noise, and which increases the possibility of leaks in the pipe connections. Thus, a flow rate of 4.3 mL min^{-1} was chosen for further studies since, above this flow rate, the increase in the current signal is not high enough to justify the larger reagent consumption and waste generation.

Under optimized operating conditions for the FIA procedure, amperometric measurements were carried out in 0.10 mol L^{-1} phosphate buffer solution (pH 7.0) containing different AA concentrations in order to obtain the analytical curve. The results are presented in Fig.5 (A), for concentrations ranging from 5.0 x 10^{-5} to 1.0 x 10^{-3} mol L^{-1}; the analytical parameters obtained from the calibration plot are presented in Table 2. No cleaning or pretreatment of the EVA/CB composite electrode surface was necessary between successive injections. The standard deviation for ten consecutive injections of 5.0 x 10^{-4} mol L^{-1} of AA was 4.2% and the analytical frequency was 63 injections per hour.

The applicability of the method developed using the EVA/CB composite electrode as FIA amperometric detector for the determination of ascorbic acid was tested by analyzing a commercial sample of orange juice. The solution of the commercial sample was prepared by simple dilution of the juice in 0.10 mol L^{-1} phosphate buffer (pH 7.0), corresponding to a final concentration of 2.6 x 10^{-4} mol L^{-1}, according to the manufacturer's label. The amounts of AA found in the sample using the proposed method (88.6 ± 4.7 mg/200 mL) were in good agreement with those obtained by iodometric titration (82.4 ± 4.1 mg/200 mL) at a 95% confidence level (n = 5), the labeled value being 72.0 mg/200 mL. It should be noted that the proposed method has the advantage of using a FIA system combined with electrochemical detection that ensures good reproducibility in the results obtained without difficulties and at very low cost.

3.3.2 Glucose biosensor

The new composite electrode was also tested as a support for the preparation of a mediated glucose enzyme biosensor with glucose oxidase (GOx) immobilized on a Prussian Blue (PB) modified EVA/CB composite electrode. The procedure used to prepare the EVA/CB/PB-GOx biosensor involves two steps: (1) PB film formation on the electrode surface under potentiodynamic conditions as detailed in the experimental section followed by (2) GOx immobilization on the electrode surface previously modified with the redox mediator.

Fig. 6A illustrates typical cyclic voltammograms obtained at the EVA/CB/PB electrode in 0.10 mol L^{-1} KCl solution. Two redox couples can be observed in the cyclic voltammogram, the first related to reduction/oxidation of PB ($Fe^{II}Fe^{III}$)/Prussian White (PW: $Fe^{II}Fe^{II}$) (around 0.15-0.20V) and the second couple to PB($Fe^{II}Fe^{III}$)/Berlin Green (BG: $Fe^{III}Fe^{III}$) transition (around 0.80-0.90V) [51].

The PB-film modified composite electrode surface was evaluated as a sensor for the determination of hydrogen peroxide in batch analysis. Fig. 6 (B) displays the amperometric responses of the EVA/CB/PB modified electrode to successive injections of H_2O_2 at −400 mV, a value chosen from cyclic voltammograms to ensure diffusion-controlled reduction to water. Hydrogen peroxide can also promote the chemical oxidation of PW to PB and at −400 mV, PB is reduced to PW again, resulting in an increase in the cathodic current. This behavior is a classic electrochemical-chemical reaction (EC´) mechanism with regeneration of the initial catalytic species (PW). The analytical curve was linear over the entire interval of H_2O_2 concentrations tested (9.99×10^{-5} to 8.91×10^{-4} mol L^{-1}) with a limit of detection of 3.33×10^{-5} mol L^{-1}. Commercial sample containing hydrogen peroxide (3.00% - labeled value) was evaluated using the proposed composite electrode (3.08%) and results compared with those provided by permanganometric titration (3.05%). The obtained results are in statistical agreement based on t-test at 95% confidence. These results have demonstrated the analytical applicability of the sensor proposed for H_2O_2 determination in pharmaceutical samples.

Since the composite electrode modified with PB can be employed for the determination of H_2O_2, it can be used in biosensor platforms based on oxidoreductase enzymes, where the biosensing usually involves the detection of H_2O_2 formed by the enzymatic reaction. Thus, glucose oxidase enzyme was immobilized on the electrode surface previously modified with PB redox mediator, forming the EVA/CB/PB-GOx biosensor. The biosensors were evaluated by measuring the current response at a fixed potential (- 400 mV) with the addition of the analyte in the measuring solution. Fig. 7(A) illustrates the chronoamperogram obtained using EVA/CB/PB-GOx composite biosensor in absence and presence of different concentrations of glucose in a 0.10 mol L^{-1} phosphate buffer solution (pH 7.0) under the optimized conditions. A linear response was obtained for glucose concentrations in the range 9.9×10^{-5} to 7.0×10^{-4} mol L^{-1}. The relationship between the change in current and glucose concentration is represented by the equation: I (A) = 7.12 10^{-3} (A L mol^{-1})*$C_{glucose}$ – 1.14 10^{-8} (A) with a limit of detection of 7.8×10^{-6} mol L^{-1}. The apparent Michaelis–Menten constant (K_M^{app}), which gives an indication of the enzyme-substrate kinetics for a biosensor, obtained from the Lineweaver–Burk plot [52], had a value of K_M^{app} 0.38 mM, which is similar to other mediated GOx-based electrodes presented in the literature [53, 54]. The biosensor was stored at 4 °C in PBS solution and its response was stable at least for one week.

Table 3 presents the analytical performance of the biosensor in comparison with others reported for glucose determination using different composite electrode supports. As can be observed, the limit of detection of 7.8 µmol L^{-1} and the upper limit of the linear range are very close to the best results for cited works. From the results presented, it can be seen that the EVA/CB composite electrode can provide a good platform for the fabrication of enzyme-based biosensors.

Finally, measurements of glucose in spiked artificial tear were performed according to the proposed method employing EVA/PB/GOx under amperometric detection mode. Values (recoveries) ranging from 96.5 to 105 % were found and they were in agreement with those levels spiked demonstrating the applicability of the proposed biosensing.

4. Conclusions

A new and attractive electrode composite material for electroanalytical application has been developed by using carbon black and EVA co-polymer. The 75% (carbon black, w/w) composite showed high robustness and very good electroanalytical properties making it useful for diverse applications in electrochemical sensors and biosensors. The proposed electrode can be used as a simple transducer (unmodified) with good electrochemical performance, demonstrated by its high electron-transfer rate constant for hexacyanoferrate(III)/(II) and wide useful potential window. In addition, excellent stability of the amperometric response for ascorbic acid oxidation was found in flow injection analysis with good analytical performance. Prussian Blue was successfully anchored on the composite electrode and enabled the reduction of hydrogen peroxide, leading to the development of a stable and sensitive biosensor for glucose determination. The results obtained in the present investigation, together with examination of the mechanical properties of EVA/CB cast as thin layers suggest that EVA/CB can be a useful composite for the preparation of flexible electrodes in the future.

Acknowledgments

We gratefully acknowledge financial support from Brazilian agencies Fundação Araucária, CAPES and CNPq, Brazil`s program of academic mobility "Science without borders" (Ciência sem Fronteiras) and from Fundação para a Ciência e a Tecnologia (FCT), Portugal projects PTDC/QEQ-QAN/2201/2014, in the framework of Project 3599-PPCDT, and UID/EMS/00285/2013 (both co-financed by the European Community Fund FEDER).

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Table 1

Table 1: General electrochemical features of the proposed composite electrodes

Electrode Composition	Capacitance (μF cm^{-2})	Useful potential window* (V)	Electroactive area (cm^2)	Electroactive area / geometric area (%)	k_o (cm s^{-1})
25EVA/75CB	903.9	-0.80 to 1.30	0.082	63.1	$4.02x10^{-4} \pm 4.98x10^{-5}$
40EVA/60CB	206.1	-0.70 to 1.06	0.016	48.4	$3.22x10^{-4} \pm 1.76x10^{-5}$
50EVA/50CB	46.9	-0.60 to 1.15	0.0089	21.9	$1.51x10^{-4} \pm 1.95x10^{-5}$
60EVA/40CB	39.3	-0.65 to 1.31			

70EVA/30CB	28.9	-0.60 to 1.20

* *vs*. Ag/AgCl/KCl 3.0 mol L^{-1} (in solution of 0.1 mol L^{-1} H_2SO_4)

Table 2

Table 2 – Analytical parameters obtained for the determination of ascorbic acid using the proposed electrode as amperometric detector in FIA system.

Parameter	**Calculated values**
Sensitivity	3.3 μA L mmol^{-1}
LOD	9.8 x 10^{-7} mol L^{-1}
LOQ	3.3 x 10^{-6} mol L^{-1}
Linear dynamic range	0.05 mmol L^{-1} – 1.0 mmol L^{-1}
Analytical frequency	63 injection h^{-1}

Table 3: Analytical performance of recent electrochemical sensors used for glucose determination.

Electrode type	**Technique**	**Sensitivity / µA L mmol^{-1}**	**LDR (mol L^{-1})**	**LOD (µmol L^{-1})**	**Ref.**
EVA-CB/PB-GOx	Amperometry	7.1	9.99×10^{-5} to 6.95×10^{-4}	7.8	This work
graphite-CA composite	Amperometry	5.1	$2.0 \times 10^{-5} – 1.0 \times 10^{-3}$	20.3	[25]
GCE/CoPc-$(CoTPP)_4$ **a**	Amperometry	0.024	2.0×10^{-3} to 1.1×10^{-2}	10	[54]
Carbon Ink/GOx-HRP	Amperometry	2.1	5.0×10^{-5} to 1.0×10^{-3}	25	[55]
SPCE/PB-GOx	Amperometry	2.1	2.5×10^{-4} to 2.0×10^{-3}	10	[56]

CPE/ZnO-GOx	Cyclic Voltammetry	50	4.0 x 10^{-5} to 3.8 x 10^{-3}	8.0	[57]
CPE/Ni-NaA	Amperometry	22	4.0 x 10^{-5} to 6.5 x 10^{-3}	20	[58]
ACF/GOx	Amperometry	0.68	1.0 x 10^{-3} to 2.0 x 10^{-2}	1000	[59]

LDR: Linear dynamic range; LOD: Limit of detection. (CoPc–(CoTPP)$_4$): cobalt(II) phthalocyanine–cobalt(II) tetra(5-phenoxy-10,15,20-triphenylporphyrin); HRP: horseradish peroxidase; SPCR: screen-printed carbon electrode; PB: prussian blue; CPE: carbon paste electrode; NaA: nanozeolite; ACF: activated carbon fibers; CA: cellulose acetate.

List of Captions

Figure 1. Photographs of amperometric cell assembled (and unassembled) used as detector in FIA system where W: working electrode; R: reference electrode; A: auxiliary electrode. All components were inserted in a polyurethane block (PU).

Figure 2. (A) Transmission electron microscopy images and (B) XRD analysis of carbon black.

Figure 3. Scanning electron micrographs for EVA/CB 30, 40, 50, 60 and 75% (w/w). Magnification 100x.

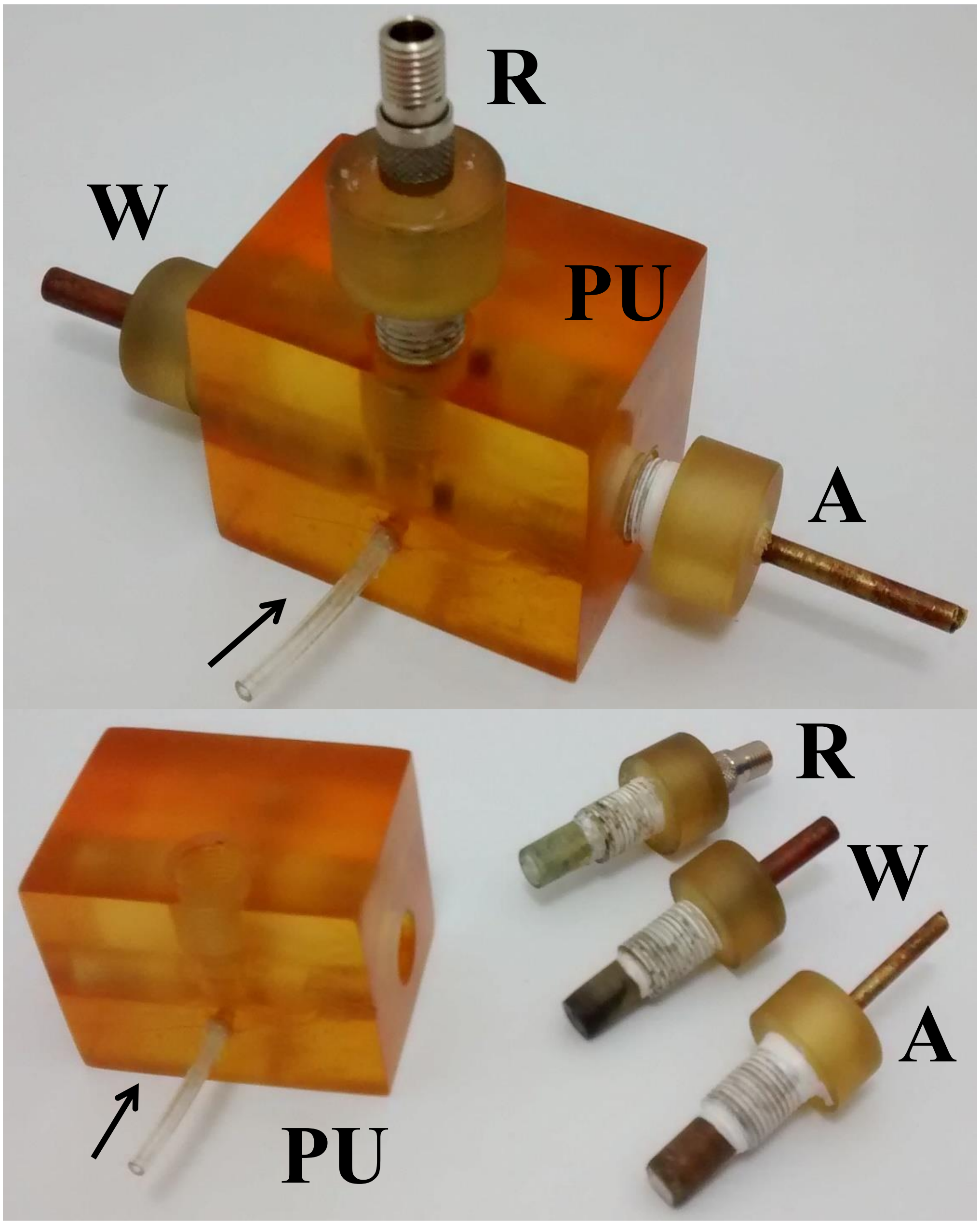
Figure 4. (A) Cyclic voltammograms obtained using composite electrodes: (—) 25EVA/75CB, (—) 40EVA/60CB, (—) 50EVA/50CB, (—) 60EVA/40CB and (—) 70EVA/30CB in 3.0 mmol L^{-1} $K_3[Fe(CN)_6]$ in 0.10 mol L^{-1} KCl solution, ν = 50 mV s^{-1}. (B) Cyclic voltammograms obtained with the 25EVA/75CB composite electrode in different solutions of concentration 0.10 mol L^{-1}: (—) H_2SO_4, (—) acetate buffer (pH 4.0), (—) phosphate buffer (pH 7), (—) ammonium buffer (pH 11.0), (—) NaOH (pH 13.0). ν = 50 mV s^{-1}.

Figure 5. FIA transient signals obtained at the composite electrode 25EVA/75CB injecting 100 µL of (a) 5.0 x 10^{-5}, (b) 1.0 x 10^{-4}, (c) 2.5 x 10^{-4}, (d) 5.0 x 10^{-4}, (e) 7.5 x 10^{-4} and (f) 1.0 x 10^{-3} mol L^{-1} of ascorbic acid solution into 0.10 mol L^{-1} phosphate buffer pH 7.0 as the carrier solution. Applied potential 600 mV (vs. Ag/AgCl); flow rate 4.3 mL min^{-1}. Inset - corresponding calibration curve.

Figure 6. (A) Cyclic voltammogram of a PB-modified composite electrode (25EVA/75CB-PB) in 0.10 mol L^{-1} KCl solution; ν = 50 mV s^{-1}. (B) Amperometric response for the 25EVA/75CB-PB electrode on successive additions of H_2O_2 solution (final concentration varying from 9.99 x 10^{-5} to 8.91 x 10^{-4} mol L^{-1}) in phosphate buffer pH 7.0 (0.10 mol L^{-1}) under stirring. Applied potential: -400 mV (vs. Ag/AgCl).

Figure 7. (A) Amperograms obtained in PBS solution in absence and presence of different glucose concentration (0.0 to 1.1 mol L^{-1}) using EVA/CB/PB-GOx composite electrode. (B) Calibration curve for the amperometric response for successive additions of glucose. Applied potential -400 mV (vs. Ag/AgCl).

Figure 1



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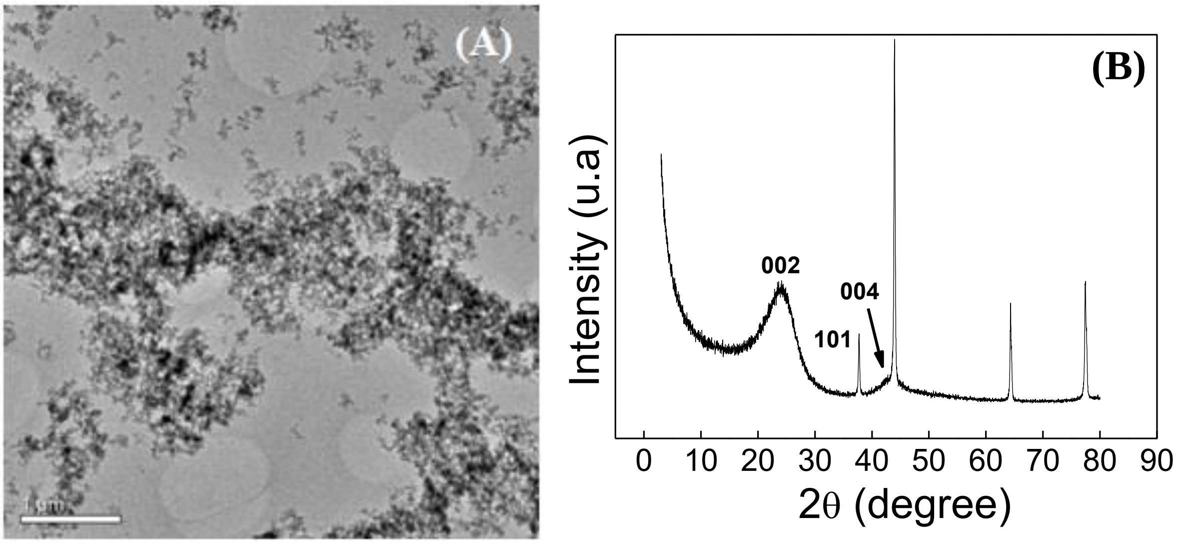
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(B)
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10
20
30
40
50
60
70
80
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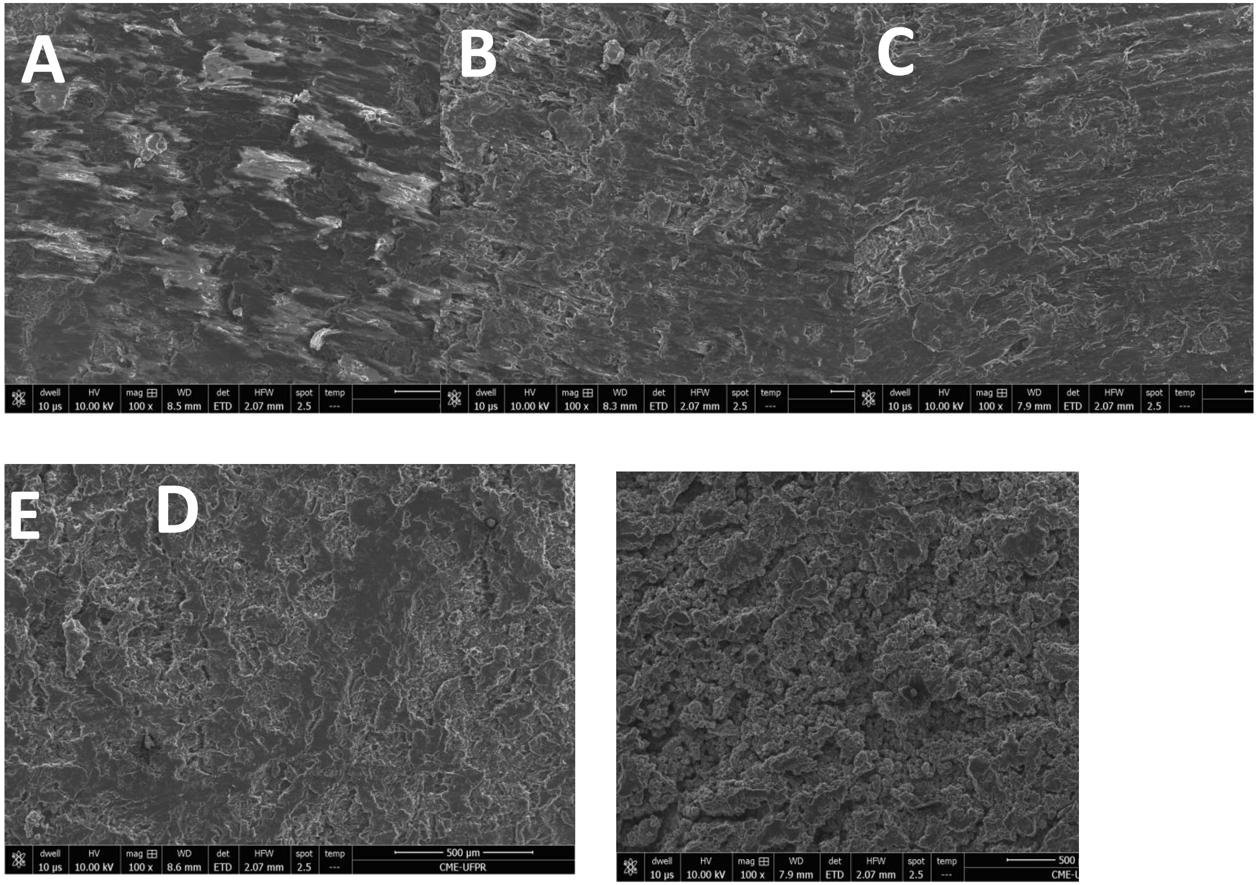
2θ (degree)

Figure 3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2.5
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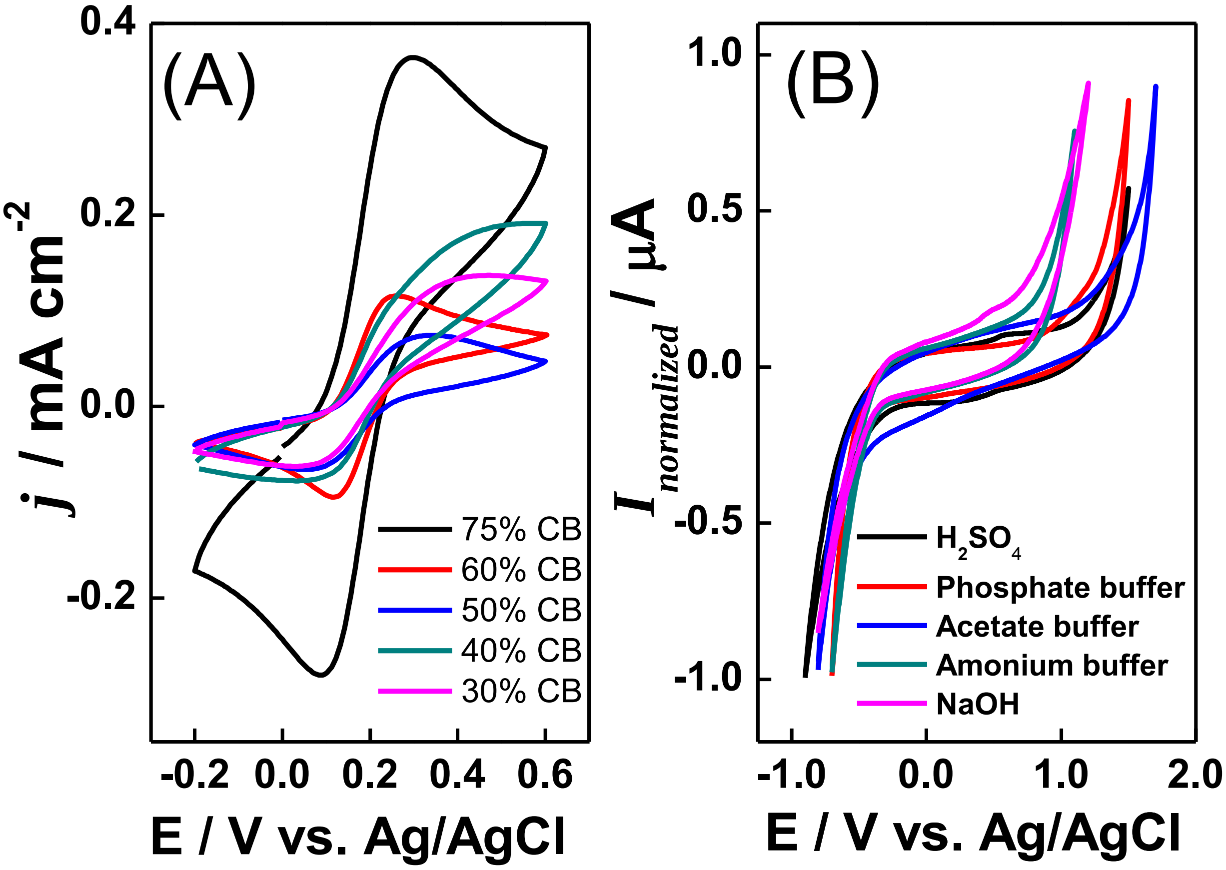
500 µm
CME-UFPR

Figure 4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E / V vs. Ag/AgCl
75% CB
60% CB
50% CB
40% CB
30% CB
(B)
1.0
0.5
0.0
-0.5
-1.0
$I_{normalized}$ / µA
-1.0
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1.0
2.0
E / V vs. Ag/AgCl
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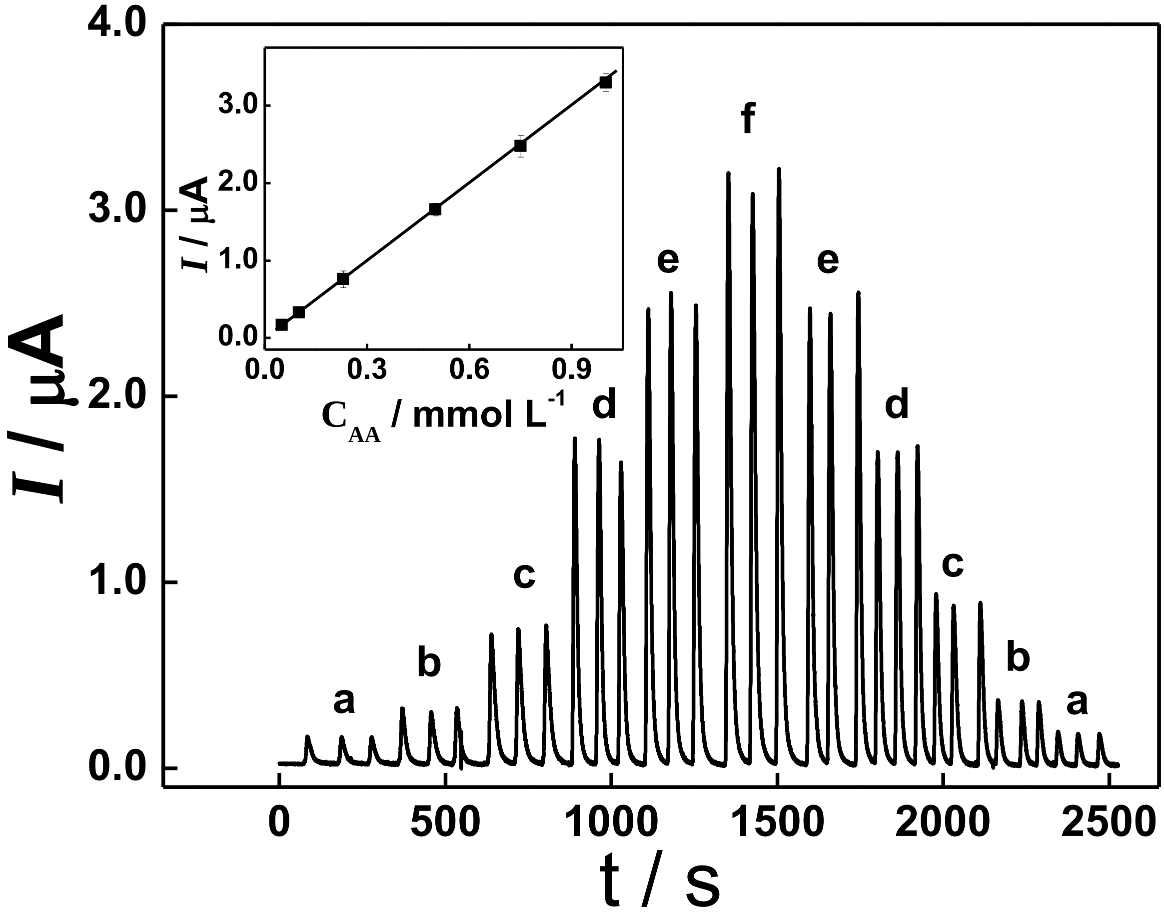
Phosphate buffer
Acetate buffer
Amonium buffer
NaOH

Figure 5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1500
2000
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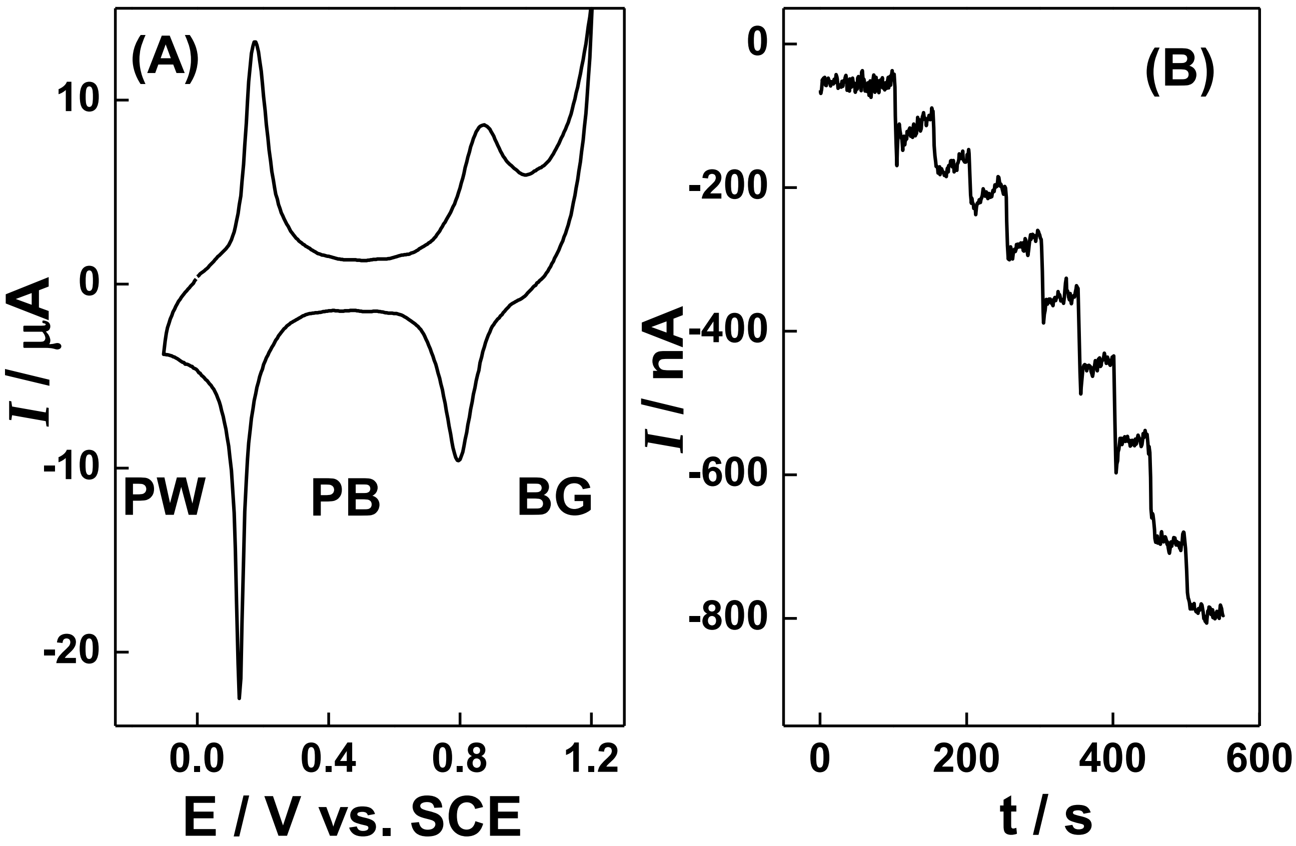
0.3
0.6
0.9
C_{AA} / mmol L-1

Figure 6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I / nA
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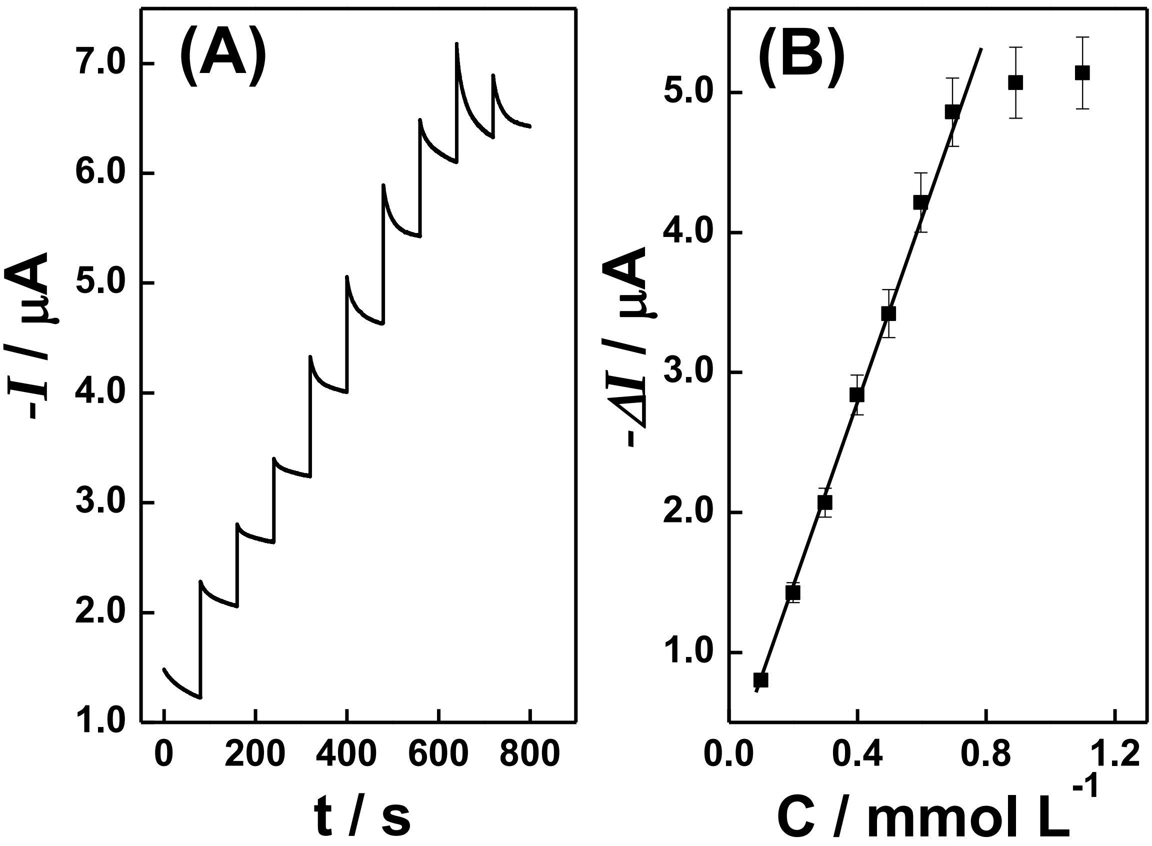
200
400
600
t / s

Figure 7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1.0
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200
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600
800
t / s
(B)
-ΔI / μA
5.0
4.0
3.0
2.0
1.0
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0.8
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