Feature Article

Investigation of the Effect of Different Glassy Carbon Materials on the Performance of Prussian Blue Based Sensors for Hydrogen Peroxide

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Abstract
Three different kinds of glassy carbon (GC-R, GC-K, GC-G) were equally pretreated, further modified with electrochemically deposited Prussian Blue and used as sensors for hydrogen peroxide at an applied potential of − 50 mV (vs. Ag | AgCl). Their performance was evaluated with respect to the following parameters: the coverage and electrochemistry of the electrodeposited Prussian Blue, the sensitivity and the lower limit of detection for hydrogen peroxide, and the operational stability of the sensors. GC-R showed the best behavior concerning the surface coverage and the operational stability of the electrodeposited Prussian Blue. For this electrode the sensitivity for hydrogen peroxide (10 μM) was 0.25 A/M cm^2 and the detection limit was 0.1 μM. Scanning electron microscopy was used to study the surfaces of the three electrodes before and after the electrodeposition of Prussian Blue and to search for the reason for the three different behaviors between the different glassy carbon materials. The Prussian Blue modified GC-R was also used for the construction of a glucose biosensor based on immobilizing glucose oxidase in Nafion membranes on top of electrodeposited Prussian Blue layer. The operational stability of the glucose biosensors was studied in the flow injection mode at an applied potential of − 50 mV (vs. Ag | AgCl) and alternatively injecting standard solutions of hydrogen peroxide (10 μM) and glucose (1 mM) for 3 h. For the GC-R based biosensor a 2.8% decrease of the initial glucose response was observed.

Keywords: Glassy carbon, Prussian Blue, Hydrogen peroxide, Glucose, Nafion

1. Introduction

Since it was announced almost 25 years ago [1], that Prussian Blue or ferric ferrocyanide, electrodeposited onto an electrode surface, could act as an electrocatalyst for hydrogen peroxide reduction, many attempts were made to achieve a suitable catalytic surface for the amperometric determination of hydrogen peroxide [2, 3]. Both the electrodes material (Pt [4], Au [5], graphite [5 – 7], carbon paste [8], glassy carbon [9, 10]) and the techniques of immobilization of Prussian Blue (mechanical immobilization, electrodeposition [11, 12] etc.) were tested.

The main advantage of electrodeposited Prussian Blue relies on the fact that hydrogen peroxide can be detected selectively through electrocatalytic reduction in the presence of molecular oxygen [13], at a low electrode potential (− 0.05 V vs. SCE), where the influence of the so-called reductants (ascorbate, urate, acetaminophen) on the electrochemical response can be largely avoided [14 – 16], which is always a common problem for systems based on the electrochemical oxidation of hydrogen peroxide. Moreover, it is known that the detection of hydrogen peroxide plays a very important role for the construction of many electrochemical biosensors [15 – 18], since the hydrogen peroxide producing oxidases such as, glucose, lactate, alcohol, glutamate oxidase, etc., commonly used for biosensor construction, in their reaction sequence starting with the oxidation of their substrate produce hydrogen peroxide as an end product that in turn is measured. A glucose biosensor, using glassy carbon as basic electrode material, can thus be obtained by initially electrodepositing Prussian Blue followed by immobilizing glucose oxidase. These kind of glucose biosensors have been found exhibiting high sensitivity and linear behavior in a broad concentration range [2, 19, 20]. Similarly ethanol, glutamate, oxalate, choline, D-alanine, and L-lactate biosensors have been produced [2, 3].

The aim of this work was to investigate and compare the effects of different glassy carbon materials on the sensor performance. The reason for focusing on glassy carbon is that it is a cheaper electrode material than the noble metals and it has lower background current and noise levels than other carbon materials, e.g., graphite, and compared with both platinum and graphite it is less prone to catalyze possible interfering electrochemical reactions. Additionally, previous reports on Prussian Blue modified glassy carbon electrodes reveal promising properties as selective sensors for hydrogen peroxide. Three different kinds of glassy carbon were used for the construction of Prussian Blue modified glucose biosensors. The electrodeposition of

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Prussian Blue was obtained according to a recent protocol [20, 21]. Glucose oxidase was immobilized on top of the Prussian Blue modified glassy carbon electrodes in a Nafion film [22], a method that was previously shown to yield high sensitivity, long term operational stability, and also providing a boundary removing electrochemically active interfering compounds such as ascorbate, urate and acetaminophen [20, 23].

2. Experimental

2.1. Materials, Reagents and Standards

Glucose oxidase (GOx, EC type VII-S from *Aspergillus niger*, activity 218.2 U/mg-solid) was obtained from Sigma Chemical Company (St. Louis, MI, USA). Solutions of hydrogen peroxide (30%) and Nafion (5%) were purchased from Aldrich (Steinheim, Germany), ethanol was purchased from Solveco Chemicals AB (Täby, Sweden). All other reagents were prepared with chemicals of analytical-reagent grade and water purified with a Milli-Q system (Millipore, Milford, MO, USA). All solutions of hydrogen peroxide and glucose were prepared daily in phosphate buffer (0.05 M, pH 5.5).

2.2. Apparatus

Electrochemical deposition of Prussian Blue onto the glassy carbon electrodes and electrochemical measurements of the Prussian Blue modified electrodes were performed using an EG&G PAR 273 potentiostat-galvanostat (Princeton, NS, USA). A conventional three-electrode cell containing a platinum net auxiliary electrode and a saturated SCE reference electrode were used.

2.3. Electrodes

Three different kinds of glassy carbon were used as working electrodes. Two were of Sigradur quality (Sigradur-G and Sigradur-K) from Ringsdorff Werke GmbH (Bonn, Germany) [24] and further denoted as GC-G (diameter 3.2 mm, Sigradur-G) and GC-K (diameter 3.1 mm, Sigradur-K). The third glassy carbon was from the Institute of Graphite Materials (Moscow, Russia), denoted as GC-R (diameter 2.9 mm, Type GC-2500). According to the manufacturers' specifications some of the physical properties of the electrode materials can be summarized: GC-G, density: 1.50 g/cm^3, porosity: 0%, resistivity: 44 Ω μm, GC-K, density: 1.55 g/cm^3, porosity: 0%, resistivity: 50 Ω μm, and GC-R, density: 1.44 – 1.50 g/cm^3, porosity: 1.5 – 2.5%, resistivity: 38 – 41 Ω μm.

2.4. Electrodeposition of the Prussian Blue Film

All glassy carbon electrodes were initially mechanically polished with Alumina powder (Al_2O_3, 1 μm, Struers, Copenhagen, Denmark) until a mirror like surface was obtained, then rinsed with Milli-Q water. Electrodeposition of Prussian Blue was achieved by applying a constant potential of + 0.4 V (vs. SCE) to the glassy carbon electrode for 60 s in a carefully deoxygenated (20 min) solution containing 2.5 mM $FeCl_3$, 2.5 mM $K_3Fe(CN)_6$, 0.1 M KCl and 0.1 M HCl [21, 25]. The electrodes were then carefully washed with Milli-Q water and transferred into a supporting electrolyte solution (0.1 M KCl + 0.1 M HCl) and activated through electrochemical cycling between + 350 mV and − 50 mV (vs. SCE) (25 cycles) at a sweep rate of 50 mVs^{-1}, and then washed with Milli-Q water and dried at 100 °C for 1 h. Next the electrodes were dipped into a new solution (phosphate buffer, 0.05 M pH 5.5 + 0.1 M KCl) and electrochemically conditioned through first keeping at a constant applied potential of − 50 mV (vs. SCE) for 600 s followed by electrochemical cycling 10 times between + 350 mV and − 50 mV.

2.5. Preparation of Glucose Biosensors

Enzyme immobilization on the PB-modified electrodes was performed by using the method reported previously by Karyakin et al. [22]. First a layer of glucose oxidase (GOx, dissolved in water, enzyme activity 150 U/mL) was deposited. This was accomplished by syringing 5 μL of the enzyme solution on the PB-modified electrodes surface and allowing the solvent to evaporate for at least 20 min, until the surface was dried. After that, two layers of Nafion (0.3% solution in ethanol, neutralized to pH 5.5 with NH_4OH) were formed. The Nafion film was obtained syringing two portions of 5 μL of Nafion solution onto the dried GOx-PB-modified glassy carbon electrodes. Between the two Nafion depositions a time of 20 min was allowed for the solvent to evaporate.

2.6. Scanning Electron Micrographs (SEM)

Scanning electron micrographs of the three glassy carbon electrodes, before and after the electrodeposition of Prussian Blue, were obtained by using a scanning electron microscope (Philips SEM 515, Eindhoven, The Netherlands).

2.7. The Flow Injection System

Flow injection experiments were carried out using a programmable flow injection equipment (Ismatec SA, Glattburg-Zürich, Switzerland) equipped with two peristaltic pumps and an injection port (50 μL). An in-house made flow through amperometric cell of the confined wall-jet type

[26] was used and connected to the outlet of the flow injection system. The inlet section of the cell contained an Ag | AgCl reference electrode in a circular chamber filled with 0.1 M KCl from an external syringe. The auxiliary electrode was a platinum wire encircling the outlet chamber. A PB modified glassy carbon electrode was used as the working electrode. The electrodes were connected to a three electrode potentiostat (Zäta Elektronik AB, Lund, Sweden) connected to a strip chart recorder (Kipp and Zonen, Delft, The Netherlands). The rate of the flow carrier (phosphate buffer, 0.05 M + 0.1 M KCl, pH 5.5) was at all instances equal to 0.8 mL/min [20].

3. Results and Discussion

3.1. Electrodeposition of Prussian Blue

The three kinds of glassy carbon electrodes were tested to investigate any possible difference between their electrochemical, electrocatalytic, and stability characteristics after being modified with electrodeposited Prussian Blue. In this sense, the two most important parameters that should be compared [11, 21] are the coverage of the Prussian Blue electrodeposited on the electrode surface (Γ), and the difference between the anodic and the cathodic peak potentials (ΔE_p) revealing the electrochemical reversibility of the interconversion between Prussian Blue and Prussian White. Figure 1 illustrates a cyclic voltammogram of a

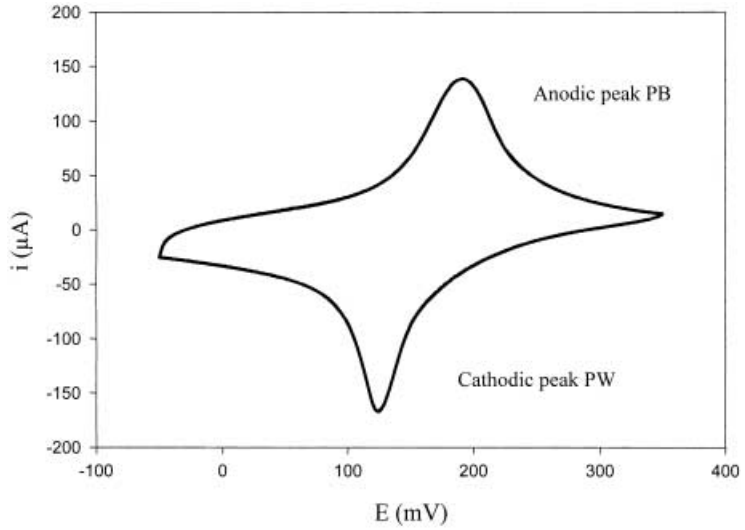

Fig. 1. Cyclic voltammogram of a GC-R electrode after electrodeposition of Prussian Blue registered between −50 and +350 mV (vs. SCE) in phosphate buffer (0.05 M) + KCl (0.1 M) pH 5.5: scan rate 50 mV/s.

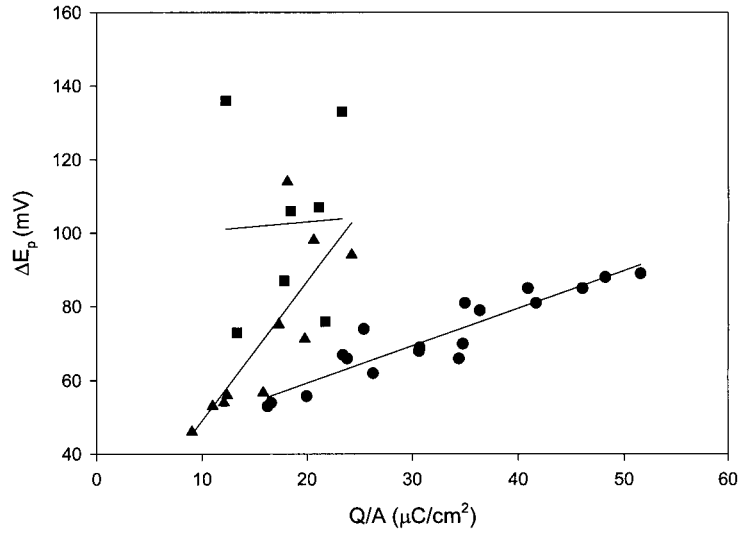

Fig. 2. Variation of ΔE_p with the charge density (Q/A) of the electrodeposited Prussian Blue for the three glassy carbon electrodes, (●) GC-R, (■) GC-K, (▲) GC-G.

Prussian Blue modified GC-R electrode (0.05 M phosphate buffer, pH 5.5, +0.1 M KCl). The coverage of Prussian Blue on the electrodes surfaces, after the electrodeposition, was measured by using cyclic voltammetry between 0 and +650 mV (vs. SCE), through integration of the anodic and cathodic waves. In the cathodic wave the peak potential at about 125 mV corresponds to the reduction of Prussian Blue to Prussian White, and in the anodic wave the peak potential at about 180 mV, to the reoxidation of Prussian White to Prussian Blue. The formal potential ($E^{\circ\prime}$) was estimated as the mean value of the anodic and cathodic peak potentials [27] and was found to be at ca. 150 mV for all the three glassy

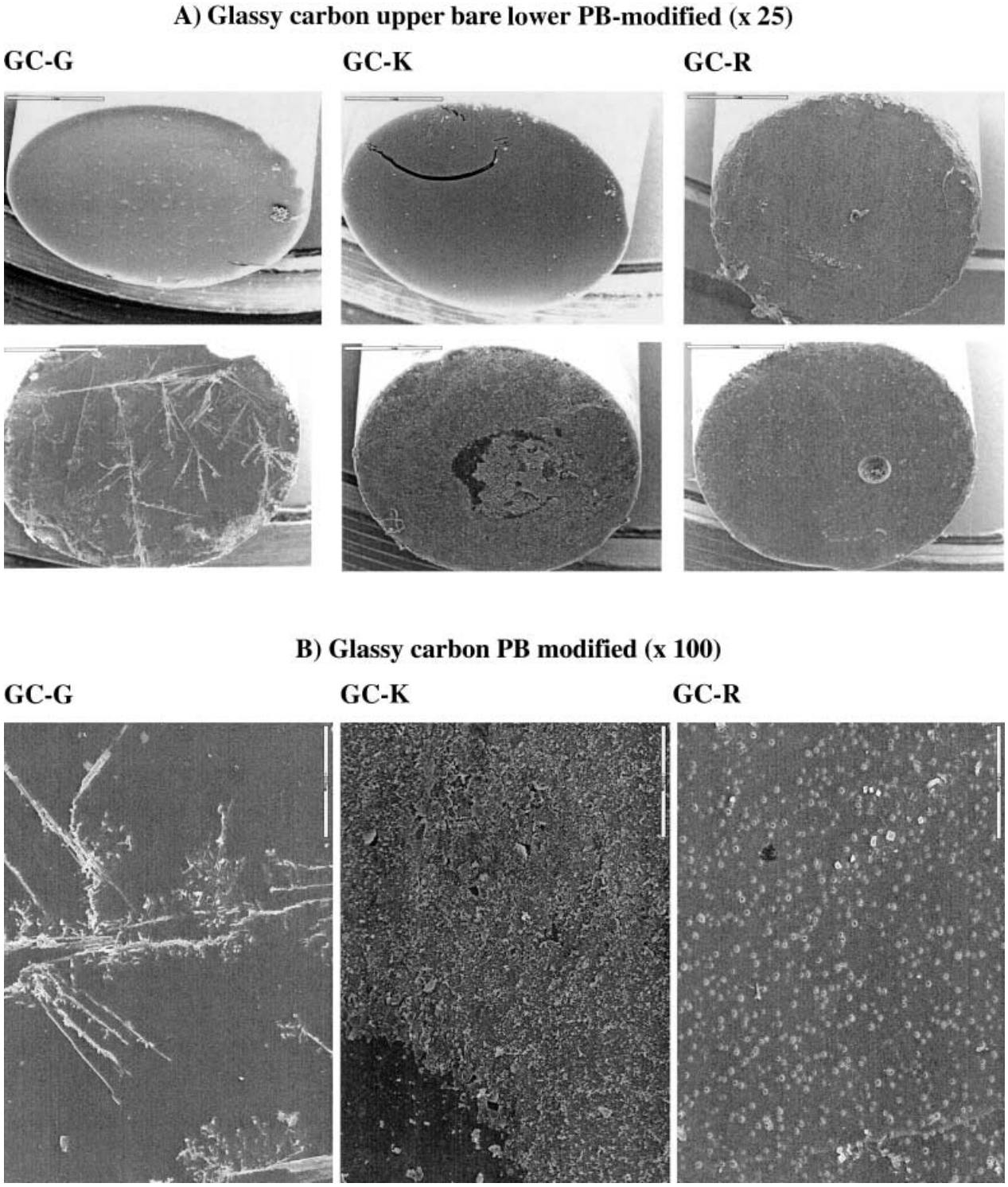

Fig. 3. A) SEM images (×25) of the three different glassy carbons before (upper) and after (lower) Prussian Blue electrodeposition. (B) SEM images (×100) of the three different glassy carbons after Prussian Blue electrodeposition.

carbon types tested, which is in accordance with data previous reported for PB modified glassy carbon electrodes [2, 3, 11].

The charge density (Q), which is directly proportional to the total amount of electrodeposited Prussian Blue, has been calculated dividing by the area of the anodic wave (μC), with the area of the electrode surface (cm^2). From the ΔE_p vs. Q/A plots, it can be generally calculated that the higher the amount of electrodeposited Prussian Blue on the electrode surface is, the larger is the ΔE_p, probably due to the higher impedance of the deposited layer. In Figure 2 the variation of ΔE_p with surface coverage of Prussian Blue is shown for the three different glassy carbon materials using the same procedure of electrodeposition. Even though a linear behavior is not expected from these results, plots of ΔE_p vs. Q/A are relatively linear and their trend can be informative. As is observed in Figure 2 the Prussian Blue on GC-K seems to have the least electrochemical reversible behavior, having a very high ΔE_p and a low coverage of electrodeposited Prussian Blue. The Prussian Blue on GC-G has a better behavior with respect to ΔE_p, but still has a low coverage on the surface and, as the coverage raises, the ΔE_p increases very quickly. Looking at the trend of the Prussian Blue on GC-R it can be said that, even with a larger coverage, the ΔE_p remains low, and, since the charge density is quite high, the Prussian Blue electrodeposition procedure seems to be more suitable for this kind of glassy carbon.

SEM pictures revealing visual differences of the structure of the surface of the three different glassy carbons are shown in Figs. 3A and B. From these pictures it is evident that the surface characteristics of the three electrodes are different both before (Fig. 3A) and after modification with Prussian Blue (Figs. 3A and B). After polishing but before modification with Prussian Blue the surfaces of GC-K and GC-G seem much smoother than that of GC-R, which is expected as the porosity of GC-K and GC-G according to the specifications should be close to zero in contrast to GC-R, having a porosity between 1.5 and 2.5%. However, some porosity of GC-G can be noticed. SEM pictures of the three different glassy carbons after PB electrodeposition are also shown (Figs. 3A and 3B). The surface structures look very different and GC-R seems to provide a much more uniform and homogeneous PB layer, which could possibly be the reason for the improved electrochemical activity. Probably the high porosity of GC-R justifies a larger PB deposition with improved electrochemical characteristics, whereas GC-G (some porosity) and GC-K (non-porous) yield PB-electrodes with less satisfying coverage and electrochemistry of PB.

3.2. Response to Hydrogen Peroxide and Operational Stability of the Prussian Blue Modified Electrodes

The efficiency of the Prussian Blue electrodeposition procedure on the electrodes was also tested in terms of hydrogen peroxide response (10 μM), stability of the hydrogen peroxide response signal during 3 h of continuous injections (1 injection every 5 min) in a flow injection system and the residual amount of electrodeposited Prussian Blue measured with CV after the run. In this work any attempt to further increase the long term stability of the Prussian Blue by other means were deliberately avoided not to obscure the direct influence on the stability caused by the different glassy carbon materials.

Figure 4 shows the average behavior for the three electrodes. As can be seen the highest current density is observed for the GC-R and the lowest for the GC-K, as expected from the results shown above. The best operational stability is shown by the GC-G indicating a loss in the initial response of only 5.72% after 3 h. For the GC-R the decrease of the response signal was equal to 11.15% and for the GC-K to 11.05%.

Another important parameter to consider is the residual amount of Prussian Blue after 3 h of continuous injection of hydrogen peroxide in the flow system, measured with cyclic voltammetry. This is shown by the percentage of the remaining initial coverage as seen in Figure 4. The GC-R and GC-G show good stability of the electrodeposited Prussian Blue, 67.6% and 74.8% of the initial amount were retained. In contrast, the remaining total amount of Prussian Blue for the GC-K decreased rapidly to 26.15% of its initial value. The results obtained for the GC-G are in good accordance with previous work reported by de Mattos et al. [21] concerning a study of Prussian Blue electrodeposition on the same kind of GC.

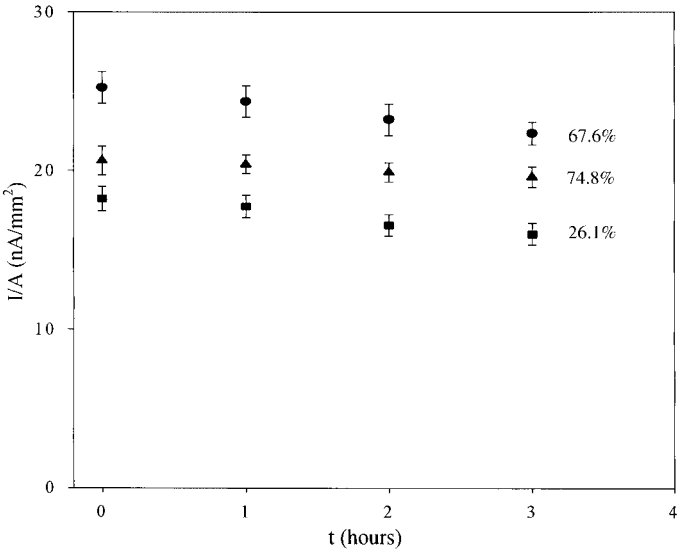

Fig. 4. Operational stability of the three Prussian Blue modified electrodes expressed as the current density for hydrogen peroxide (10 μM) detection during 3 h of injections. The percentage values represent the residual amount of Prussian Blue still deposited after the run. Applied potential − 0.05 V (vs. Ag | AgCl), flow rate 0.8 mL/min, phosphate buffer 0.05 M + 0.1 M KCl, pH 5.5. All values are the average obtained from five different electrodes for each kind of glassy carbon. (●) GC-R, (■) GC-G, (▲) GC-K.

3.3. Response to Glucose and Operational Stability of the Glucose Oxidase Prussian Blue Modified Electrodes

Since GC-R has shown the best results in terms of electrodeposition of Prussian Blue and since it is a new kind of glassy carbon, not yet used and tested widely, the GC-R was chosen to be investigated for constructing glucose biosensors. It is also possible to make a comparison with the results already obtained with the GC-G using the same procedure of electrodeposition and immobilization by de Mattos et al. [25].

After the immobilization of glucose oxidase, using a Nafion film, the electrochemistry of the PB film was initially investigated with CV (see Fig. 5) before the electrode was placed in the flow through cell of the FIA system. Then the response to hydrogen peroxide (10 μM), the linear range, and the detection limit for glucose were investigated together with the operational stability of the response for glucose (1 mM) during 3 h of continuous injections (once every 5 min). It is interesting to study the electrochemistry of the electrodes after the enzyme immobilization. As can be seen in Figure 5 the enzyme layer on the Prussian Blue modified electrode causes an increase in the ΔE_p value, since the total impedance of the electrode is higher. It seems that the enzyme layer additionally causes either a real loss of Prussian Blue or as an effect of shielding on the electrochemistry the Prussian Blue resulting in a registered decrease of the charge density and in the current peak values. Charge density values show an average decrease of 52.11% ($n=6$) and for the peak currents the observed decrease is 53.5% ($n=6$) as an effect of the enzyme immobilization. As a result of a less electrochemically active layer of Prussian Blue caused by the enzyme layer in combination with the mass transfer resistance caused by the same enzyme-Nafion layer, the response to H_2O_2 (10 μM) decreases by 63% ($n=6$) with respect of what was obtained with the same PB modified electrodes before enzyme immobilization. One possible reason for the decrease in electroactivity of PB and the increased ΔE_p could additionally be caused by the less accessible K^+ needed for the redox reaction of PB:

$$Fe_4^{III}[Fe^{II}(CN)_6]_3 + 4e^- + 4K^+ \rightarrow K_4Fe_4^{II}[Fe^{II}(CN)_6]_3 \quad (1)$$

The electroactivity of PB on glassy carbon is not only dependent on the electron transfer rate between the electrode and the electrodeposited layer of PB but also on the transfer, back and forth, of K^+-ions needed to set the electroneutrality at the electrode surface and also taking an active part of the electrodeposited layer (Reaction 1). Below pH 7.5 the stability and electrochemistry of PB seems to be unaffected by pH as revealed from previous investigations [2]. Possibly the Nafion-enzyme layer increases the overall resistance for charge transfer across the solution-electrode interface thereby increasing the ΔE_p of Prussian Blue.

Concerning the operational stability of the glucose oxidase layer, the method for the enzyme immobilization is very important. As Prussian Blue does not lend itself any sites for covalent immobilization of GOx, the use of the negatively charged polyelectrolyte-Nafion for this purpose has been widely tested in the last few years [19, 20, 23, 25, 28–30]. The resulting membranes possess high adhesion to the surface of the electrode and stabilize the ionic strength at the electrode surface. Moreover, by using this method, a decrease of the interference of ascorbate, urate and paracetamol has been observed [19, 20, 23, 25, 28, 29, 31–33]. After the immobilization of the enzyme using Nafion on the Prussian Blue modified electrode, the response was tested for hydrogen peroxide (10 μM) and for glucose (between 50 μM and 0.01 M) and the operational stability for 3 h of continuous injections of 1 mM glucose was evaluated.

GC-R Prussian Blue modified biosensor shows a good linearity ($r^2=0.9993$), in a range between 50 μM and 10 mM of glucose. In this range the regression equation is $y=2.32+40.35x$ where y is current signal in nA and x is glucose

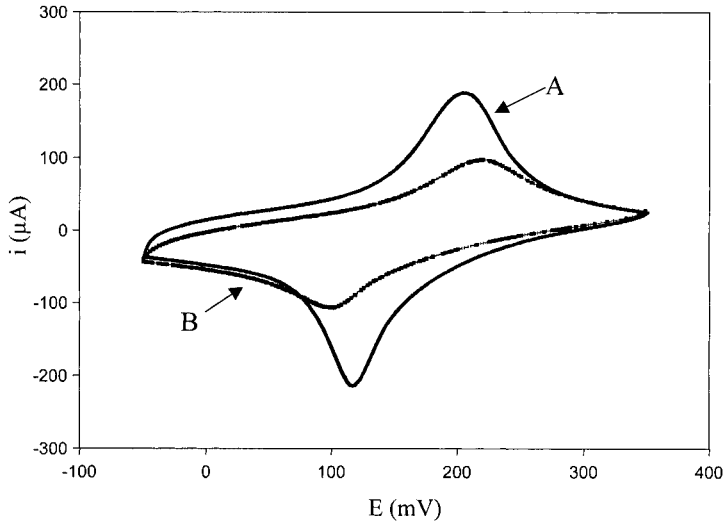

Fig. 5. Cyclic voltammograms of a Prussian Blue modified GC-R electrode before (A) and after (B) enzyme immobilization.

concentration in mM. The detection limit (S/N = 2) is 50 μM and the current density for the glucose response (1 mM) is 5.75 μA/cm^2. Compared with previous work [25] it can be stated that the current density for glucose is higher than for the GC-G Prussian Blue modified electrode, even though the detection limit is not improved (50 μM taken as three times the signal to noise ratio). The linear range does not show any major difference. The glucose sensitivity for 6 equivalently prepared glucose electrodes based on GC-R show slight variations (RSD% = 8%) but with a similar performance with respect to lower limit of detection and linear range. Comparing the responses for hydrogen peroxide and glucose the sensitivity for hydrogen peroxide was approximately 50 times higher than that for glucose. The operational stability of the GC-R Prussian Blue modified biosensor after 3 h of glucose injections was also evaluated. The decrease in the glucose response (i.e., glucose concentration 1 mM) was only 2.78% of its initial value, which is in accordance with the loss of Prussian Blue during the same time.

4. Conclusions

Of the three glassy carbon electrode varieties investigated the GC-R seems to be the best choice for developing Prussian Blue modified electrodes. It has shown the highest response for hydrogen peroxide with a slight decrease in the response signal for H_2O_2 after 3 h of continuous injection. The GC-R also allowed the electrodeposition of the largest amount of Prussian Blue on the electrode surface under otherwise equal conditions. The GC-K is surely the one that has shown the worst behavior concerning the electrodeposition of Prussian Blue including a low coverage of Prussian Blue, high ΔE_p and even the lowest signal for the hydrogen peroxide. Even if the performance of the GC-G is similar to that of GC-R it can be said that this comparative study has shown that the developed electrodeposition procedure is more suitable for the GC-R. The GC-R has also shown a good behavior after the immobilization of the enzyme for glucose sensing, proving that it can be used for further investigations for the construction of Prussian Blue based biosensors. Thus the choice of electrode material is obviously of great importance for the construction of PB modified electrodes. Even though the electrochemistry of PB and response to H_2O_2 were improved using the GC-R electrode compared with the other two, no improvement in operational stability of the PB-film could be noticed. As the object of this study was to investigate the influence of the electrode material other ways of improving the long term stability of the PB film were deliberately avoided, as already commented on above, as then the differences in behavior between the three glassy carbons would have been less obvious. However, to increase the long term stability it seems necessary as suggested elsewhere either to add a stabilizing chemical in the contacting solution, e.g., tetrabutylammonium toluene-4-sulfonate [34, 35] or to cover the PB-film by, e.g., electropolymerized *o*-phenylenediamine [36].

5. Acknowledgements

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