Feature Article

Electroanalytical Study of Prussian Blue Modified Glassy Carbon Paste Electrodes

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Abstract
Two types of glassy carbon (GC) powder (i.e., Sigradur K and Sigradur G) have been mixed with mineral oil to obtain glassy carbon paste electrodes (GCPE's). The electrochemical behavior of such electrodes at different percentages of glassy carbon has been evaluated with respect to the electrochemistry of ferricyanide as revealed with cyclic voltammetry and the best paste composition was chosen. GC was then modified with Prussian Blue (PB), mixed at different percentages with unmodified GC and with a fixed amount of mineral oil in order to obtain PB modified glassy carbon paste electrodes (PB-GCPE's). PB-GCPE's with different percentages of GC modified with PB (PB-GC) were compared and the dependence on the amount of PB on their performances was evaluated by studying the parameters of cyclic voltammetry (i.e., current peak, ΔE_p, anodic and cathodic current ratio, charge density) and the amperometric response to H_2O_2. Data interpretation based on the GC surface area is presented. GCPE's with a selected amount of PB-GC were then tested as H_2O_2 probes and all the analytical parameters together with the dependence on pH were evaluated. Some preliminary experiments with these electrodes assembled as glucose, lysine and lactate biosensors are also reported.

Keywords: Glassy carbon paste electrodes, Prussian Blue, Amperometric sensor, Biosensor, Voltammetry

1. Introduction

In 1978 [1] the favorable electrocatalytic behavior of Prussian Blue (PB) was first revealed attracting the attention of a wide number of research groups. The deposition of PB on an electrodic surface, accomplished by a simple chemical or electrochemical synthesis, makes it possible to detect H_2O_2 amperometrically at a low applied potential (about 0.0 V vs. Ag/AgCl) [2, 3]. In fact PB acts as an "artificial peroxidase" [4–6] and its use in the preparation of oxidase based biosensors has been fully tested [5–8]. Review articles have been also published on this subject [9–11].

PB films have been deposited on a variety of electrodic surfaces such as Pt [2, 12, 23], graphite [8, 13–15], carbon fiber [16], gold [23], glassy carbon (GC) [16–20] and, recently, screen printed electrodes (SPE) [21, 22].

Mixing of PB with graphite powder to prepare carbon paste (CP) has been also reported [24–28]. CP's have been widely used for the construction of biosensors because of their easy preparation and modification [29–31]. The direct mixing technique represents a simple and effective way to add inorganic, organic or biological modifiers to the electrode material [28–32]. In addition CP's offer a very low background current [32, 33] and a renewable electrode surface.

Recently we reported on an efficient and simple way to prepare PB modified CP electrodes with improved characteristics in terms of life-time and operational stability [34]. Such electrodes have been also assembled with choline oxidase and glucose oxidase resulting in biosensors with promising analytical performances. The reason for the efficiency and for the improved stability of these electrodes has to be found in the new proposed method of modification of graphite powder with an in situ chemical synthesis of PB [34]. The same modification procedure for PB deposition has been used to prepare PB modified SPE's [22]. Such electrodes showed an effective sensitivity with respect to H_2O_2 detection, a very high selectivity and a long term stability.

In this work, we report on a new application of such PB modification procedure on a novel composite electrode material: glassy carbon paste (GCP). The use of GCP appeared recently in the literature [35–40]. These electrodes are based on the dispersion of glassy carbon (GC) spherical microparticles with a water immiscible nonconducting pasting liquid. Wang and coworkers demonstrated that GCP's showed high electrochemical activity (when tested with ferricyanide, catechol and hexaammineruthenium) and a wide potential window (between −2.0 and +1.7V vs. Ag/AgCl ca.) [38]. In this work glassy carbon paste electrodes (GCPE's), based on two kinds of glassy carbon (i.e., G and K) and mixed with different amounts of oil were prepared and electrochemically characterized.

Modification of GC microspheres with PB before mixing with mineral oil resulted in a PB modified glassy carbon paste (PB-GCP). These electrodes showed high H_2O_2 sensitivity at an applied potential of 0.0 V (vs. Ag/AgCl) and, when assembled also with the oxidase enzymes (glucose oxidase, lactate oxidase or lysine oxidase), a good substrate response was observed.

2. Experimental

2.1. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to a X-t recorder (L250E, Linseis, Selb, Germany). The electrochemical cell was assembled with a conventional three-electrode system: glassy carbon paste working electrode (3-mm diameter), an Ag/AgCl/KCl (3 M) reference electrode and a Pt counter electrode. Cyclic voltammetry (CV) and chronoamperometry experiments were performed using an Autolab electrochemical system (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands).

2.2. Reagents

Spherical glassy carbon Sigradur K (GC-K) particles (diameter 0.4 – 12 μm), and spherical glassy carbon Sigradur G (GC-G) particles (diameter 0.4 – 12 μm) were purchased from Sigradur, HTM Hochtemperatur-Werkstoffe GmbH (Gemeindewald, Germany). The average values for the diameter were reported [41] to be 11.0 μm for the K-type and 4.1 μm for the G-type powder. Graphite particles (powder 1 – 2 μm) were obtained from Aldrich, (Steinheim, Germany). Mineral oil was obtained from Fluka (Buchs, Switzerland). All chemicals from commercial source were of analytical grade. All solutions were prepared with 0.1 M phosphate buffer + 0.1 M KCl, pH 6.0. Standard solutions were daily prepared in same buffer. For pH effect studies, citrate buffer (0.1 M + KCl 0.1 M, pH 3.0) and Tris buffer (0.1 M + KCl 0.1 M, pH 9.0) were also used.

Glucose oxidase (GOx) (EC 1.1.3.4, type VII from *Aspergillus Niger*, 185 units/mg), lactate oxidase (LOx) (EC 1.13.12.4, from *Pediococcus sp.*, 39 units/mg) and lysine oxidase (LyOx) (EC 1.4.3.14, from *Trichoderma sp.*, 6.27 units/mg) were obtained from Sigma (St. Louis, MO).

2.3. Preparation of Glassy Carbon Modified with PB

The PB was chemically synthesized in presence of GC particles starting from its precursors $K_3Fe(CN)_6$ and $FeCl_3$ as follows: the glassy carbon powder (1 g) was suspended in 10 mL of a solution of $K_3Fe(CN)_6$ 0.10 M in 10 mM HCl. Next 10 mL of a 0.10 M solution of $FeCl_3$ in 10 mM HCl

were added and the resulting mixture was stirred for 10 min. The glassy carbon (with adsorbed PB) was then collected by filtration and washed with 10 mM HCl until the washing solution became colorless and then dried in oven at 100 °C for 1.5 h. The powder modified with Prussian Blue (referred as PB-GC) was stored dark in a desiccator at room temperature.

2.4. GCP Electrodes Preparation

The glassy carbon paste mixture was prepared mixing glassy carbon and mineral oil at different ratios. The paste was carefully hand-mixed in a mortar and then packed into a cavity (3-mm diameter; 2-mm depth) at the end of a Teflon tube. Electrical contact was provided by a copper wire connected to the paste in the inner hole of the tube. The paste was kept at room temperature in a desiccator until used. Glassy carbon paste electrodes modified with Prussian Blue (PB-GCPE's) were prepared by hand-mixing different amounts of mineral oil and a mixture of PB-GC/GC at different percentages.

2.5. Glassy Carbon Pretreatment

The glassy carbon G powder (1 g) was cleaned with acetone, rinsed with distilled water, and then activated with aqua regia (HCl/HNO_3 3/1, 120 mL total) for 30 min. After this treatment, the particles were washed with distilled water until neutral pH was reached and then dried at 400 °C for 4 h.

2.6. Preparation of PB-GCP Biosensor

The PB-GC/GC mixture was first prepared at a fixed percentage then carefully hand-mixed in a mortar with oil and enzyme (glucose oxidase, lactate oxidase or lysine oxidase). Biosensors were prepared by hand mixing 80 mg of PB-GC/GC/enzyme mixture with 20 mg of mineral oil and then mixed continuously until a homogeneous paste was obtained. The PB-GC/GC/enzyme mixture had the following weight composition: 7.5% PB-GC, 87.5% GC and 5% oxidase enzyme. An aliquot of the final paste filled the tip of the electrode; the remaining was kept in an aluminium foil at 4 °C until reused. The sensors obtained with glucose, lactate or lysine oxidase will be referred to as glucose, lactate or lysine biosensor respectively.

2.7. H_2O_2 and Enzymatic Substrate Measurements

Measurements of H_2O_2 and glucose were performed using amperometric batch analysis in a stirred phosphate buffer solution (20 mL) with an applied potential of 0.0 V (vs. Ag/AgCl). When a stable baseline was reached, the analyte was added and the response was recorded.

3. Results and Discussion

3.1. Choice of the Paste Composition

Glassy carbon paste electrodes (GCPE's) were initially characterized before PB modification in order to evaluate the best paste composition and to study their electrochemical behavior. The first studied was glassy carbon G. The glassy carbon paste electrodes (GCPE's) were prepared mixing different percentages of GC-G (i.e., 66, 70, 75, 80 and 90%) and mineral oil. For all these electrodes, cyclic voltammetry was run in 10 mM $K_3Fe(CN)_6 + 1.0$ M KCl, between 0.5 V and -0.05 V and with a scan rate of 50 mV s^{-1}. The influence of the paste composition upon the separation of the cyclic voltammetric peak potentials (ΔE_p) and peak current (I_p) for $K_3Fe(CN)_6$ is represented in Figure 1.

The paste obtained with a GC-G/oil ratio of 66/34 (w/w) was rather inconsistent and particularly unstable, also a rapid leakage of the paste was observed when used in stirred solutions. This behavior was not observed with the graphite carbon paste prepared with the same paste composition [34] (graphite/oil 66/34, w/w) and the reason has probably to be searched on the different surface area of the two materials (i.e., graphite and glassy carbon). The average surface areas of the glassy carbon have been calculated considering the spherical geometry of the particles and using the values of the mass density (GC-K $\rho = 1.53$ g/cm^3; GC-G $\rho = 1.49$ g/cm^3) and of the average diameter of the GC spheres (GC-K 11.0 μm; GC-G 4.1 μm) [41]. The sphere-like shape of the particles has been certified by the manufacturer and was confirmed with optical microscope observations in our laboratory and in other previous articles [38, 41]. The average surface area (per mass) has been then calculated dividing the average area of a particle ($4 \pi r^2$) by the average mass, where r is the average radius of the particles and the average mass of a GC sphere has been evaluated multiplying the mass density (ρ) by the average volume ($4/3 \pi r^3$). For GC-G the average surface area was found to be ca. 0.96 m^2/g

Fig. 1. The effect of the paste composition on the (●) peak potential separation (ΔE_p) and (▲) peak current (I_{pa}) of 10 mM K_3Fe(CN)$_6$ + 1 M KCl. Only the anodic peak current is shown. Compositions: 66/34, 70/30, 75/25, 80/20, 90/10, GC-G/mineral oil (w/w).

while for GC-K it resulted 0.36 m^2/g. These calculated values are slightly lower than the experimental values (ca. 2 m^2/g for both GC) obtained by another group using BET method [41]. This difference between experimental and mathematical values is probably due to the roughness factor of the GC particles that was not considered in the mathematical approach. The value of surface area of graphite obtained from the manufacturer was between 6.5 and 10.5 m^2/g [42].

The higher surface area of graphite makes the construction of stable and resistant paste electrodes possible even with high amount of mineral oil. In contrast, the same relative amount of mineral oil when mixed with glassy carbon particles forms a less consistent paste that leaks in a stirred solution, causing the observed instability. This conclusion seems to be confirmed by the values of electric resistance of the two paste electrodes. GCPE's (GC/oil 66/34, w/w) show a high resistance (more than 400 Ω), whereas the graphite paste electrodes, at the same paste composition, were found highly conductive (less than 2 Ω). The reason of such a difference in conductivity is still due to the different surface areas between GC and graphite. GC particles, having a lower surface area, are probably completely covered by such amount of mineral oil (i.e., 34%). This hampers the electrical communications between different GC particles resulting in a higher resistance and thus a higher ΔE_p for $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox couple.

Decreasing the amount of mineral oil in the composition of the GCPE's, the electroactivity of the electrode is improved, resulting in sharper voltammograms (lower ΔE_p and higher I_p). GCPE's prepared by mixing glassy carbon (80 or 90%) and mineral oil (20 or 10%), gave the best results in terms of I_p and showed very low ΔE_p values (Fig. 1). Amperometric measurements at an applied potential of 0.0 V (vs. Ag/AgCl) performed in batch with GCPE's (GC/mineral oil 80/20, w/w) were characterized by the lowest current noise (0.1 nA). In the case of the electrodes obtained with 90% of glassy carbon, tested in the same conditions, a twice higher noise level was observed. According to these results, the best paste composition seemed to be the one prepared with 80% glassy carbon and 20% mineral oil.

The real area of GCPE's have been evaluated by using the chronoamperometric method in a 1 mM ferricyanide solution. The slope of the linear region of the $I - t^{-1/2}$ plot in the short time region provides the product $nFAC^0D^{1/2}\pi^{-1/2}$ by using the Cottrell equation [44]:

$$i_d = nFAC^0D^{1/2} (\pi t)^{-1/2}$$

where $C^0 = 1$ mM, $D = 7.6 \times 10^{-6}$ cm^2 s^{-1}, are the concentration and diffusion coefficient respectively of ferricyanide, and the other parameters have their usual meanings. For GCPE with 80% of GC-G and 20% of mineral oil, a real area of 0.083 ± 0.003 cm^2 was obtained, slightly different from the apparent geometric area of the electrode (0.070 cm^2). For GCPE obtained using GC-K (80/20) a real area of 0.086 ± 0.003 cm^2 was calculated. These values
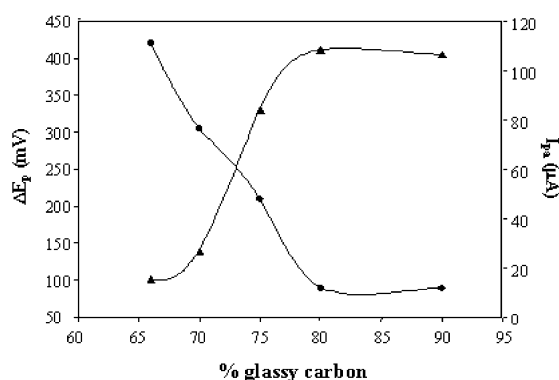
indicate no difference (within the experimental error) on the real area of GCPE's based on GC-G and GC-K.

In order to evaluate any possible improvement of the electrochemical behavior of these GCPE's, glassy carbon G was also pre-treated with aqua regia. GCPE's were then prepared mixing pre-treated GC at a paste composition of 80/20 (GC/mineral oil, w/w) and cyclic voltammetry was performed in the same $K_3Fe(CN)_6$ solution used as above. A slight decrease of the ΔE_p values (from 89 to 83 mV) was observed when the glassy carbon powder pre-treated with aqua regia was used. However, it should be noted that the difference between the glassy carbon paste electrodes with and without pretreatment is in the range of experimental error, so a "time consuming" pretreatment procedure is not justified. For further experiments reported below, GCPE's have been prepared hand mixing 80% GC powder without any pretreatment and 20% mineral oil.

When the K type of glassy carbon was used instead of the G type, similar results were obtained. Results obtained with GCPE's based on K with the same composition (GC/mineral oil 80/20, w/w) are reported in Table 1 together with the results of GCPE-G of the same composition. In Table 1 the voltammetric parameters obtained with a graphite carbon paste electrode prepared mixing 80% graphite and 20% mineral oil are also shown. From this table it can be seen that the electron-transfer of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ at

GCPE's is faster ($\Delta E_p = 89$ and 97 mV) than at graphite based electrodes ($\Delta E_p = 121$ mV).

3.2. PB-Modified Glassy Carbon Paste Electrodes

Glassy carbon paste (G and K) were then modified with PB as reported in the experimental section. This procedure has already been optimized in a previous work using graphite paste [34].

Glassy carbon G and K modified with PB (PB-GC), were then used to prepare PB-glassy carbon paste electrodes (PB-GCPE's). The glassy carbon paste mixture was prepared mixing PB-GC (G or K) and mineral oil at the percentage previously chosen (80% and 20% respectively). Cyclic voltammograms for electrodes prepared by mixing only PB-GC and mineral oil revealed for both glassy carbon types G and K no waves when performed in phosphate buffer (Fig. 2h and Fig. 3h). PB-glassy carbon paste electrodes were therefore prepared using a mixture of PB-GC and unmodified glassy carbon (instead of only PB-GC) and mineral oil. The compositions of the PB-GC/GC mixtures were as follows: 0/100, 2/98, 5/95, 10/90, 25/75, 50/50, 75/25, 100/0 (PB-GC/GC, w/w). All the PB-GCPE's were tested with cyclic voltammetry in phosphate buffer. The voltammograms obtained, for both types of glassy carbon, G and K are shown in Figures 2 and 3 respectively. All the voltammetric parameters (ΔE_p, I_{pa}/I_{pc} ratio and the anodic charge density, Q_a, of the PB deposited on the electrode surface) are summarized in Table 2 for GC-G and for GC-K. In Table 3 are also shown the amperometric responses obtained for two representative concentrations of hydrogen peroxide (1 and 10 μM, respectively) for both types of GC. GCPE-G and GCPE-K with a PB-GC/GC composition of 100/0 and 75/25 (w/w), showed no voltammetric peaks and no amperometric response to H_2O_2. Furthermore, these electrodes showed very high background currents and a high noise. For both glassy carbon materials as the percentage of the PB-GC decreased to a value of 50% (Figs. 2f and 3f) of the total

Table 1. Peak potential separation (ΔE_p), peak currents (I_{pa}; I_{pc}) for glassy carbon paste electrodes (G and K) and graphite paste electrodes in 10 mM $K_3Fe(CN)_6 + 1$ M KCl. Scan rate; 50 mV s^{-1}, potential range: 0.5 V to -0.05 V (vs. Ag/AgCl).

	Graphite	Glassy carbon G	Glassy carbon K
ΔE_p (mV)	121	89	97
I_{pa} (μA)	82.4	108	81.4
I_{pc} (μA)	-94.9	-120	-96.1
I_{pa}/I_{pc}	0.87	0.90	0.88

Table 2. Voltammetric parameters for PB modified glassy carbon G and K paste electrodes (80% of PB-GC/GC mixture – 20% mineral oil). 0.1 M phosphate buffer + 0.1 M KCl, pH 6.0. Scan rate 50 mV/s.

	Type of GC	%PB-GC/GC							
		100/0	75/25	50/50	25/75	10/90	5/95	2/98	0/100
ΔE_p (mV)	G	–	–	124	78	53	49	33	–
	K	–	–	52	34	26	20	19	–
E_{pc} (mV)	G	–	–	137	158	175	175	183	–
	K	–	–	158	169	181	173	176	–
I_{pa} (μA)	G	–	–	204	136	29	15	1.5	–
	K	–	–	56	27	13	3.6	2.6	–
I_{pc} (μA)	G	–	–	-165	-117	-26	-13	-1.1	–
	K	–	–	-51	-24	-12	-2.8	-2.2	–
I_{pa}/I_{pc}	G	–	–	1.24	1.16	1.11	1.15	1.36	–
	K	–	-	1.09	1.12	1.08	1.28	1.18	–
Q_a (μC)	G	–	–	550	320	60	28	2.4	–
	K	–	–	120	49	22	4.5	3.9	–

Table 3. H_2O_2 amperometric measurements for PB modified glassy carbon G and K paste electrodes (80% of PB-GC/GC mixture – 20% mineral oil). 0.1 M phosphate buffer   0.1 M KCl, pH 6.0. Applied potential 0.0 V vs Ag/AgCl.

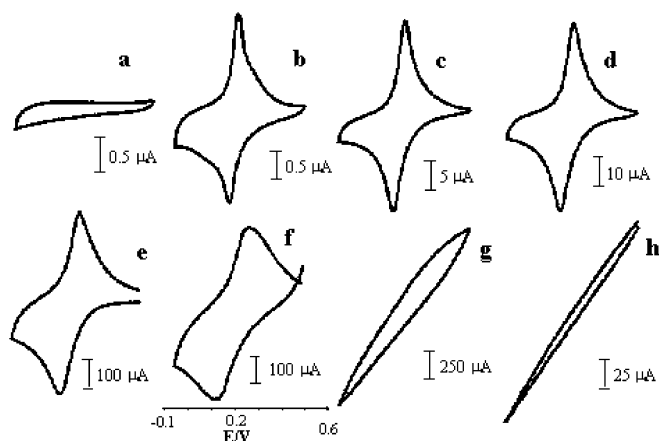| % PB-GC/ GC	Type of GC	100/0	75/25	50/50	25/75	10/90	5/95	2/98	0/100
Noise (nA)	G	–	–	10	5	2	1	0.2	0.1
	K	–	–	8	4	1	2	2	0.1
Time to stabilize the baseline current (min)	G	–	–	105	15	10	10	5	2
	K	–	–	30	10	5	5	5	2
I (H_2O_2-1 μM) (nA)	G	–	–	–	30	22	9.6	1.0	–
	K	–	–	38	34	11	6	4	–
I (H_2O_2-10 μM) (nA)	G	–	-	290	384	174	105	12.4	–
	K	–	–	328	304	85	68	20	

Fig. 2. Cyclic voltammograms of PB-modified glassy carbon G paste electrode (80% of PB-GC/GC mixture – 20% mineral oil) in 0.1 M phosphate buffer + 0.1 M KCl, pH 6.0. Scan rate; 50 mV s^{-1}, potential range: 0.5 V to -0.05 V (vs. Ag/AgCl). Compositions of PB-GC/GC mixture: a) 0/100, b) 2/98, c) 5/95, d) 10/90, e) 25/75, f) 50/50, g) 75/25, h) 100/0, PB-GC/GC (w/w).
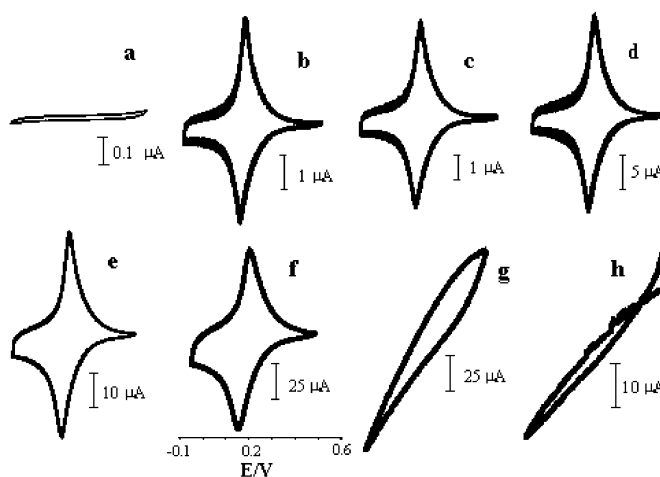
Fig. 3. Cyclic voltammograms of PB-modified glassy carbon K paste electrode (80% of PB-GC/GC mixture – 20% mineral oil) in 0.1 M phosphate buffer + 0.1 M KCl, pH 6.0. Scan rate; 50 mV s^{-1}, potential range: 0.5 V to -0.05 V (vs. Ag/AgCl). Compositions of PB-GC/GC mixture: a) 0/100, b) 2/98, c) 5/95, d) 10/90, e) 25/75, f) 50/50, g) 75/25, h) 100/0, PB-GC/GC (w/w).

glassy carbon mixture, the two typical redox peaks of PB appeared. Further decreasing the amount of PB-GC down to 2%, resulted in a sharper shape of the voltammograms with lower values of ΔE_p. Using a GC mixture of 50/50 PB-GC/GC (w/w), a long time was required to reach a stable baseline current, particularly for electrodes based on GC-G.

PB-GCPE's (G and K) based on 10/90 and 25/75 (w/w) mixtures of PB-GC/GC during amperometric H_2O_2 measurements showed good sensitivity, low background current and noise and a high signal-to-noise ratio. The amount of PB onto the PB-GCPE-G and PB-GCPE-K with a GC mixture of 5/95 and 2/98 (PB-GC/GC, w/w), seemed to be too low to give an effective H_2O_2 sensitivity. Electrodes based on GC-G and GC-K with a GC mixture of 0/100 (PB-GC/GC, w/w) gave no anodic and cathodic peaks and no response to H_2O_2.

The behavior of the PB-GCPE's with composition of PB-GC/GC 100/0 (w/w) seems to be surprising with respect to what was previously obtained with graphite paste electrodes reported from this laboratory [34]. The shape of the voltammograms of the PB-GCPE's (Figs. 3h and 4h) clearly follows the Ohm law demonstrating a very high resistance of the electrodes, furthermore, no response to hydrogen
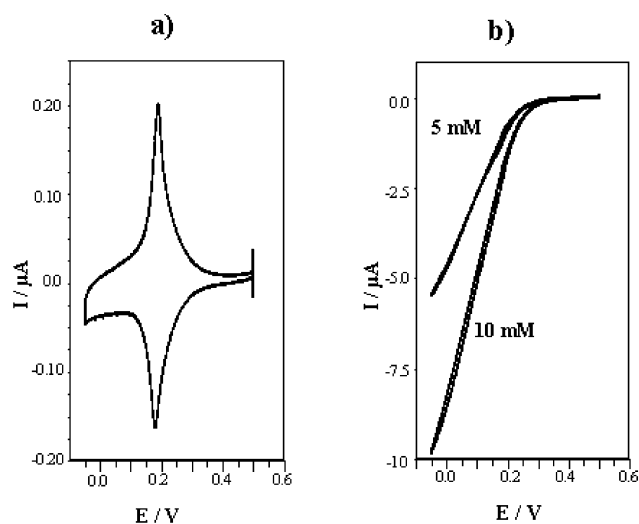

Fig. 4. Cyclic voltammograms of PB-modified glassy carbon G paste electrode in a) the absence and b) the presence of H_2O_2 (5 and 10 mM). 0.1 M phosphate buffer + 0.1 M KCl, pH 6.0. Scan rate 5 mV s^{-1}, potential range: 0.5 V to -0.05 V (vs. Ag/AgCl). Composition of PB-GC/GC mixture: 2/98 PB-GC/GC (w/w).

peroxide was observed during amperometric measurements. For PB-modified graphite paste electrodes [34], obtained by hand mixing the 100% PB-modified graphite and mineral oil the amperometric response to H_2O_2 was quite good and the voltammetric behavior showed the presence of redox peaks of PB, however, with large values of ΔE_p. In that case, mixing PB-modified graphite powder with unmodified graphite was not necessary. This different behavior could be again explained with the difference in the surface areas of GC and graphite. GC, due to its lower surface area, seems to be entirely covered by PB. The PB layer grown around the glassy carbon particles probably acts as a barrier to electrical contact between the PB-GC particles and also between them and the solution. The shapes of the voltammograms (Figs. 2h and 3h) are in accordance with this conclusion since they clearly follow Ohm's law showing a very high electrode resistance. In the case of graphite, since the surface area is higher, total coverage with PB is not obtained. This makes the presence of some unmodified graphite regions possible in the paste allowing electrical contact and resulting in a low resistance.

The results obtained with GC-G and GC-K, when compared, showed that GC-K performed much better in terms of ΔE_p, I_{pa}/I_{pc} ratio than GC-G at a fixed amount of PB-GC. In addition, the time to reach a stable baseline current during amperometric measurements was faster. However, GC-K showed a lower amount of deposited PB (low Q_a) and a lower signal for hydrogen peroxide in accordance with the lower surface area of GC-K when compared to that of GC-G. This probably confirms what has been previously observed: the electrochemical behavior of the electrode seems to be worse with too high amount of deposited PB. In this perspective a compromise has to be made between a mixture composition with an amount of PB that is enough to give a good response to H_2O_2 but not too high to affect the electrochemical performance of electrode in a negative way.

The surface coverage Γ of the PB is calculated from the voltammetric peak area at a low scan rate (10 mV s^{-1}) according to the equation [43] $Q = nFA\Gamma$, where Q is the anodic or cathodic charge, $n = 4$, F is Faraday's constant (96485 C mol^{-1}), A is the effective area of the electrode, which is assumed to be equivalent to the geometric area (0.07 cm^2) of the electrode. The Γ values obtained were in a range between 0.07 – 1.8 nmol cm^{-2} for 2 – 10% PB-GC/GC (w/w) for both types of glassy carbon (i.e., G and K). The ΔE_p is less than 15 mV for a Γ less than 1.8 nmol cm^{-2} at a scan rate of 10 mV s^{-1}. The ΔE_p value as low as 8 mV was obtained for a Γ of 0.07 nmol cm^{-2} at a scan rate of 5 mV s^{-1}. The anodic and cathodic waves are nearly mirror images (Fig. 4a). This indicates that the electron-transfer is fast [43]. The value of the formal potential, $E^{\circ\prime}$, calculated according to the equation $E^{\circ\prime} = (E_{pa} + E_{pc})/2$, remains almost constant and equal to 186 ± 5 mV at different scan rates (i.e., between 2 and 100 mV s^{-1}) and at different amounts of PB (i.e., between 2 and 10% PB-GC). The variation of i_p with scan rate (2 – 100 mV s^{-1}) was found linear as predicted for a diffusionless system [43]. All these voltammetric studies demonstrate that PB-GCPE's prepared with 2 – 10% of PB-GC fulfil almost all the criteria of an immobilized redox monolayer onto the electrode surface.

In the case of 25 – 50% of PB-GC (K and G) corresponding to a Γ of 1.8 – 17 nmol cm^{-2}, a large ΔE_p (> 25 mV) was obtained at a scan rate of 10 mV s^{-1}. An increasing separation between the anodic and the cathodic peaks with an increasing percentage of PB-GC is caused by the Ohmic drop effect. Indeed, PB modified GC particles are less conducting than uncoated (unmodified) GC particles and the resistance of the electrode increases by increasing the percentage of PB-GC (Figs. 2 and 3).

Figure 4b also shows cyclic voltammograms of PB-GCPE-G (2/98 PB-GC/GC, w/w) in the presence of two different concentrations of H_2O_2 (5 and 10 mM). In the presence of H_2O_2 (Fig. 4b) a considerable increase in the cathodic wave, demonstrating the high activity of the catalyst on the electrode surface for electroreduction of H_2O_2, was observed.

3.3. Calibration Plots

The responses to H_2O_2 of the PB-GCPE's obtained with both GC types (G and K) and a glassy carbon mixture composition of 10/90 (PB-GC/GC, w/w) were then studied. All the analytical parameters (obtained for 3 different electrodes) such as detection limit, linearity range, reproducibility and response time were evaluated and are summarized in Table 4. PB-GCPE's obtained with GC-G showed a lower detection limit and a higher sensitivity, probably due to the higher amount of PB deposited. For both electrodes a good reproducibility was observed resulting in a RDS% ($n = 3$ electrodes) of 6 – 7% for both kinds of glassy carbon tested.

Table 4. Analytical parameters for PB-GCPE's in respect of H_2O_2 response. Batch amperometric analysis in 0.1 M phosphate buffer + 0.1 M KCl, pH 6.0 (20 mL). Applied potential 0.0 V (vs. Ag/AgCl).

Electrode	Detection limit (M)	Linearity range (M)	Sensitivity ($\mu A\ mM^{-1}\ cm^2$)	RSD% ($n = 3$)	Response time (90% of steady state) (s)
PBGCPE (G)	2.5×10^{-7}	$2.5 \times 10^{-7} - 5 \times 10^{-4}$ (0.9906)	188	6	15
PBGCPE (K)	5×10^{-7}	$5 \times 10^{-7} - 5 \times 10^{-4}$ (0.9972)	99	7	15

Table 5. Amperometric measurement of H_2O_2 before and after 250 cycles at pH 3.0, pH 6.0, pH 9.0. Glassy carbon G paste electrode (GC-G/mineral oil 80/20, w/w). Composition: 10/90 (w/w) (PB-GC/GC).

	pH 3		*pH 6*		*pH 9*	
	before	*after*	*before*	*after*	*before*	*after*
	250 cycles		*250 cycles*		*250 cycles*	
Noise (nA)	4	2	4	4	6	4
I (H_2O_2-10^{-6} M) (nA)	24	22	23	21	18	10
I (H_2O_2-10^{-5} M) (nA)	196	186	180	160	186	140

Table 6. Analytical parameters for glassy carbon paste biosensors. All biosensors were made by hand mixing glassy carbon-enzyme mixture (80%) + paraffin oil (20%). Glassy carbon-enzyme mixture having the following composition: (PB-GC/GC/enzyme: 7.5/87.5/5, w/w/w).

Biosensor	Glucose	Lactate	Lysine
Working pH	6	7	8
Detection limit ($s/n = 2$) (µM)	50	5	2.5
Linearity range (µM)	50 – 800	5 – 60	2.5 – 50
Reproducibility (RSD%) $N = 4$ biosensors	4	5	5
Response time (90% of steady state) (s)	30	30	30
Sensitivity (µA mM^{-1} cm^2)	5.2	33.0	68.9

3.4. pH Effect

The pH effect on PB-GCPE-G (10/90 PB-GC/GC, w/w) was also evaluated. The amperometric response towards some H_2O_2 standard concentrations was evaluated before and after 250 continuous cycles performed at pH 3, pH 6 and pH 9. Results are summarized in Table 5. The best stability of PB was obtained, as expected [10], at pH 3, where the signal for H_2O_2 (10^{-5} M) recorded after 250 cycles, was only 5% lower than that obtained before the series of cycles. At pH 6 a loss of signal of 11% was observed after 250 cycles. At pH 9, the H_2O_2 amperometric signal showed a loss of 25% of the initial signal. Further studies showed that, when the PB-GC/GC (25/75, w/w) was used as glassy carbon mixture, a slightly better stability to pH effect was achieved. After 250 cycles at pH 9 in fact, a 19% decrease of the H_2O_2 signal was observed. This confirms that with a higher amount of deposited PB, a better stability at alkaline pH values could be achieved.

3.5. Glassy Carbon Paste Biosensors

Glucose, lysine and lactate biosensors were assembled by hand mixing glucose oxidase, lysine oxidase and lactate oxidase to the paste mixture. Table 6 summarizes the key analytical parameters of these biosensors as the working pH, the detection limit, the linear range, sensitivity, response time and reproducibility, showing that these probes are promising devices to be used in biosensor and immunosensor studies. In Figure 5 are also shown some amperometric recordings for lactate and lysine biosensors. Particular attention has to be due to the alkaline pH range explored. There is no evident decrease of the PB activity as the
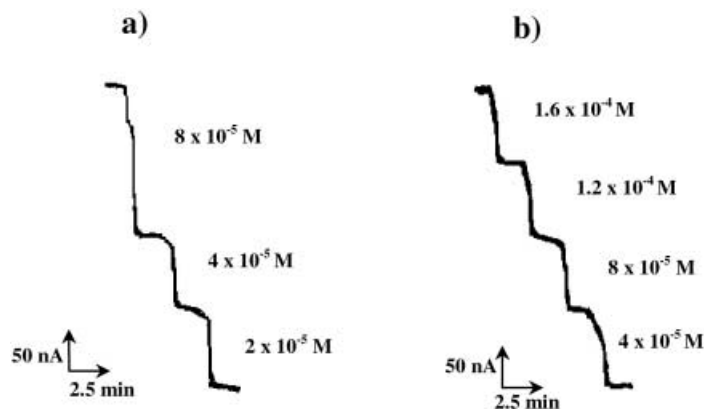

Fig. 5. Amperometric recordings for lysine (a) and lactate (b) biosensors. Phosphate buffer 0.1 M + KCl 0.1 M, pH 8.0 (lysine), pH 7.0 (lactate). Applied potential 0.0 V (vs. Ag/AgCl).

working pH increases demonstrating the versatility of such electrodes.

4. Conclusions

Studies on the composition of the glassy carbon paste demonstrated a different behavior with respect to graphite paste electrodes. Glassy carbon, due to the low surface area, requires a low amount of mineral oil (20%) to obtain a good composite electrode material. Glassy carbon paste electrodes showed an improved electroactivity of ferricyanide compared with that of graphite paste electrodes.

In the present study, a new kind of Prussian Blue modified electrodes have been presented. Glassy carbon paste electrodes modified with Prussian Blue resulted in an

effective probe for H_2O_2 detection also useful to assemble biosensors based on hydrogen peroxide producing oxidases. The high electroactivity of GC paste electrodes have been also confirmed after their modification with PB. Values of the peak potential separation (8 mV) from cyclic voltammetry of the optimized electrodes showed an almost theoretical behavior for a surface with immobilized electroactive species. Moreover, the method proposed here to assemble the PB-modified glassy carbon paste electrodes, based on the use of a mixture of PB-GC and GC, seems to be suitable for the control of the amount of PB on the composition of the electrode. The stability of the deposited PB at alkaline pH values obtained in our previous works [34] has been confirmed with these new electrodes. When the electrodes are further modified with hydrogen peroxide producing oxidases, the electrodes showed high sensitivity responses to their respective substrates and good reproducibility. The wide alkaline working pH range explored with the PB glassy carbon paste biosensors demonstrated also their versatility for biosensor applications.

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6. References

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