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Prussian Blue based screen printed biosensors with improved characteristics of long-term lifetime and pH stability

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Abstract

The promising advantages of Prussian Blue (PB) as catalyst and of the thick film screen printing technology have been combined to assemble sensors with improved characteristics for the amperometric determination of H_2O_2. PB-modified screen printed electrodes were applied to detect H_2O_2 at an applied potential of −0.05 V versus the internal screen printed Ag pseudoreference electrode, showing a detection limit of 10^{-7} mol l^{-1}, a linearity range from 10^{-7} to 5×10^{-5} mol l^{-1}, a sensitivity of 234 μA mmol l^{-1} cm^{-2}, and a high selectivity. Improved stability at alkaline pH values was also observed, which made possible their use with enzymes having an optimum basic pH. Then, the immobilisation of a single enzyme (glucose oxidase (GOD) or choline oxidase (ChOX)) or of two enzymes, acetylcholinesterase (AchE) coimmobilised with ChOX, has been performed on the surface of PB modified screen-printed electrodes (SPEs) using glutaraldehyde and Nafion®. ChOX has been selected as an example of enzyme working at alkaline pH. The choline biosensors showed a detection limit of 5×10^{-7} mol l^{-1}, a wide linearity range (5×10^{-7}–10^{-4} mol l^{-1}), a high selectivity and a remarkable long term stability of 9 months at 4 °C, and at least 4 weeks at room temperature. Similar analytical characteristics and stability were observed with the acetylcholine biosensors.

Keywords: Prussian Blue; Screen printed electrodes; H_2O_2 determination; Oxidase biosensors

1. Introduction

In the field of enzyme electrode probes, the most widely used enzymes belong to the family of oxidases. A product of the reaction catalysed by these enzymes is the hydrogen peroxide, and its electroactivity has been utilised to obtain a measurable current signal. Usually the electrochemical determination of H_2O_2 is accomplished by its oxidation at noble metal electrodes such as Pt and Au, although the required voltage is often sufficiently high to also oxidise other interfering compounds and/or organic molecules, which may be present in the sample.

Carbon electrodes can offer an inexpensive alternative to Pt, having in addition attractive features as a wide anodic potential range, low electrical resistance and low residual current. Moreover, they exist in a variety of forms as glassy carbon, carbon fibres, graphite pastes and carbon films (Gilmartin and Hart, 1995). Unfortunately, these electrodes require a high overpotential to detect H_2O_2 (Iannello and Iacynych, 1981), further reducing the selectivity of the sensors.

Many efforts have been made to improve the selectivity of carbon based electrochemical sensors, mainly through their modification with redox mediators (Cass et al., 1984; Frew and Hill, 1987).

Numerous other approaches have also been tested, such as the use of metallised (Rh, Ru) carbons to allow the non-mediated oxidation of H_2O_2 at a low applied potential (White et al., 1996; Newman et al., 1995) and electrode modification with peroxidase (HRP; Gorton, 1995).

In addition, the modification of electrodes with Prussian Blue (PB) has been particularly effective in enhancing the selectivity towards oxidase enzyme substrates.

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In the last years in fact, PB or ferric ferrocyanide ($Fe_4^{III}[Fe^{II}(CN)_6]_3$) has been the subject of studies by many research groups involved in the optimisation of amperometric biosensors. This growing interest was due to the peculiar characteristic of the reduced form of PB, Prussian White, to catalyse the reduction of H_2O_2 (Neff, 1978; Itaya et al., 1982) at low applied potential, around 0.0 V versus Ag/AgCl. In this way, the effect of the most common electrochemical interfering species is avoided or greatly reduced. The inorganic nature of PB and its low cost makes it more suitable than HRP in assembling modified biosensors. PB modified glassy carbon (Itaya et al., 1984; Karyakin et al., 1996, 1998), graphite (Chi and Dong, 1995; Jaffari and Turner, 1997; Deng et al., 1998), carbon paste (Garjonyte and Malinauskas, 1998) and platinum (Itaya et al., 1982) electrodes have been studied, leading to the construction of glucose (Karyakin et al., 1995; Garjonyte and Malinauskas, 2000), lactate (Garjonyte et al., 2001), glutamate (Karyakin et al., 2000), aminoacid (Chi and Dong, 1995) and alcohol (Karyakin et al., 1996) biosensors. Recently, a review on the analytical applications of PB has appeared in literature (Karyakin, 2001).

The use of PB as catalyst for H_2O_2 reduction is still a matter of concern because of its poor operational stability, limited to a range of hours, and to a very high decrease in sensitivity at neutral and alkaline pH.

During recent years the screen printing (thick film) technology applied to sensor and biosensor construction has been considerably improved and a large number of papers, and recently some reviews, have appeared in the literature (Hart and Wring, 1997; Albareda-Silvert et al., 2000). Screen-printed electrodes (SPEs) are in fact inexpensive, simple to prepare, rapid and versatile and this technology appears to be also the most economical mean for large-scale production of disposable electrodes.

Until now, just two papers have dealt with the modification of SPEs by hexacyanoferrates. The first is based on the dispersion of an analogue of PB, the cupric-hexacyanoferrate, and glucose oxidase (GOD) within the carbon ink (Wang and Zhang, 1999). The resulting glucose biosensor showed a good selectivity with regard to the common electrochemical interferences, but at physiological pH the signal decreased by 10% after 2 h.

The second paper (O'Halloran et al., 2001) is based on the bulk modification of the carbon ink by PB microparticles (<38 μm). The PB-SPEs show low detection limits (0.4 μmol l^{-1}) and a sensitivity of 137 μA mmol l^{-1} cm^{-2} for H_2O_2 and, when modified as glucose biosensor, a linear range up to 3 mmol l^{-1} of glucose, with no interferences. However, pH stability experiments, performed with the PB-modified SPEs, revealed again a decrease of the H_2O_2 amperometric signal (50% of the initial activity after 4 h of continuous use) at pH values above 7.

Recently, we reported a new method of graphite powder modification with 'in situ' chemically synthesised PB, which resulted in a very active and very stable matrix suitable for the construction of PB modified carbon paste and solid carbon paste electrodes (where solid paraffin replaces mineral oil in the paste; Moscone et al., 2001).

In this work, we report the results obtained by applying this new method of PB deposition to SPEs in order to assemble PB based biosensors. Our modified SPEs exhibit high sensitivity, good reproducibility and pH stability, yielding selective measurements of H_2O_2 in a synthetic mixture of electroactive substances.

Moreover, we report a new procedure of enzyme immobilisation on the PB modified SPEs, which shows both improved storage lifetime and operational stability. This procedure has been tested with immobilised GOD first, then with choline oxidase (ChOX), selected as an example of enzyme working at alkaline pH (Keesey, 1987) and finally with the coimmobilisation of two enzymes, ChOX and acetylcholinesterase (AchE).

The biosensors assembled with the SPEs have been designed to be reused, rather than to be disposable. Several examples of screen-printed biosensors principally inserted as detectors in flow injection systems can be found in literature (White et al., 1996; Rippeth et al., 1997; Tothill et al., 1997; Collier et al., 1998).

2. Experimental

2.1. Reagents

All chemicals from commercial source were of analytical grade.

Glutaraldehyde 25% (v/v), Nafion® 5% (v/v), bovine serum albumin (BSA), acetonitrile (99.5%) and Triton X-100 (*t*-octylphenoxypoly-ethoxyethanol), GOD from *Aspergillus niger* VII S (195 U mg^{-1}) (E.C. 1.1.3.4) and AchE from *Electric Eel*. Type VI S (200–400 U mg^{-1}) (E.C. 3.1.1.7) were from Sigma.

ChOX from *Alcaligenes* spp. (11.7 U mg^{-1}) (E.C. 1.1.3.17) was by Fluka.

Unless otherwise stated, all solutions were prepared with 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4. Standard solutions were daily prepared in the same buffer.

2.2. Apparatus

Amperometric measurements were carried out in batch using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected to a X-t recorder (L250E, Linseis, Selb, Germany).

Cyclic voltammetry experiments were performed with an Autolab electrochemical system (Eco-Chemie, Utrecht, Netherlands) equipped with PGSTAT 12 and GPES software.

The orbital shaker was from MPM Instruments (Bernareggio, Mi, Italy).

2.3. Electrodes

SPEs were obtained from the Biosensors laboratory of the University of Florence (Italy).

Electrodes were printed with a 245 DEK (Weymouth, UK) screen printing machine, and using different inks obtained from Acheson Italiana (Milan, Italy). Graphite-based ink (Electrodag 421) silver ink (Electrodag 477 SS RFU) and insulating ink (Electrodag 6018 SS) were used. The substrate was a polyester flexible film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The printing procedure utilised is already described in previous papers (Cagnini et al., 1995; Hernandez et al., 2000). They were produced in foils of 20 strips, each containing three printed electrodes, a carbon working and two silver electrodes, acting as pseudoreference and counter, respectively. The diameter of the working electrode was 0.3 cm, which resulted in an apparent geometric area of 0.07 cm^2.

2.4. Electrode pre-treatment

Prior PB modification, SPEs were pre-treated in a 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4, by applying an anodic potential of 1.7 V for different periods (from 20 s to 4 min).

All the applied potentials mentioned in the paper are referred to the internal Ag pseudoreference electrode of the SPEs.

2.5. Prussian Blue deposition

PB modification of SPEs was accomplished by placing a drop (40 μl of total volume) of precursor solutions onto the working electrode area. This solution is a mixture obtained 'in loco' adding 20 μl of 0.1 mol l^{-1} potassium ferricyanide ($K_3Fe(CN)_6$) in 10 mmol l^{-1} HCl to 20 μl of 0.1 mol l^{-1} ferric chloride in 10 mmol l^{-1} HCl. The drop was carefully placed exclusively on the working electrode area, in order to avoid the formation of PB on the reference and counter electrodes that could notably increase the internal resistance of the system. The electrodes were gently shaken on an orbital shaker for 10 min and then rinsed with few millilitres of 10 mmol l^{-1} HCl. The probes were then left 1 h in the oven at 100 °C to obtain a more stable and active layer of PB.

The PB modified electrodes were stored dry at room temperature in dark.

2.6. Enzyme immobilisation

Choline and GOD were immobilised onto the PB-modified electrode surface with the cross-linking method. A mixture of glutaraldehyde, Nafion®, enzyme and BSA was used.

To prepare 150 μl of this mixture, 20 μl of glutaraldehyde (2.5% v/v diluted in water) and 30 μl of Nafion® (5% v/v in ethanol) were mixed with 100 μl of a solution prepared dissolving 40 mg of BSA and 10 mg of enzyme in 1 ml of 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4.

The above mixture (7 μl) was placed onto the working electrode area and allowed to dry for 45 min at room temperature, then the electrodes were washed for 30 min with a solution of 0.1 mol l^{-1} glycine to saturate all the free aldehydic groups.

When the acetylcholine biosensor was prepared, the AchE enzyme was coimmobilised together with the choline oxidase enzyme. In this case, the whole procedure was the same, the only difference being in the enzymatic solution, which contained 30 mg BSA, 10 mg ChOX, and 10 mg of AchE dissolved in 1 ml of working buffer.

2.7. Electrode characterisation with cyclic voltammetry

The optimisation of the pre-treatment procedure was performed recording cyclic voltammograms in a ferricyanide buffer solution (0.05 mol l^{-1} phosphate +0.1 mol l^{-1} KCl, pH 7.4). Once deposited, the PB layer was studied cycling in a range of potential between −0.5 and +1.2 V, with a scan rate of 50 mV s^{-1} in above mentioned buffer solution.

2.8. H_2O_2 and enzymatic substrate measurements

H_2O_2 and enzymatic substrates (i.e. glucose, choline and acetylcholine) were measured dipping the electrode in 10 ml of a stirred phosphate buffer at an applied potential of −0.05 V versus screen printed internal silver pseudoreference electrode. When a stable current background was reached (30–60 s), the analyte was added and the response was measured after 30 s.

3. Results and discussion

To assemble oxidase-based biosensors, several optimisations were performed in order to obtain a reliable H_2O_2-PB based screen-printed sensor. To achieve this objective, the first step has been the pre-treatment and electrochemical characterisation of the electrode strips.

3.1. Optimisation of the pretreatment procedure

It is reported that electrochemical pretreatments of SPEs can improve their electrochemical behaviour (Wang et al., 1996). In order to obtain a more reproducible and active surface, the electrodes were polarised at 1.7 V for different periods (in a range between 20 s and 4 min) and the effect of this oxidation was evaluated by cyclic voltammetry in a ferricyanide solution. The increase of the total oxidation time up to 3 min resulted in a better electrochemical behaviour: the ΔE_p was lowered from 520 to 150 mV and the peak currents were doubled (Fig. 1). Then, a time of 3 min at 1.7 V has been chosen as the best compromise to achieve a consistent improvement of the SPEs electrochemical performances in the shortest time. In fact, no further improvements were observed with 4 min of oxidation.

The application of this anodic potential gave also a better interelectrode reproducibility. The R.S.D.% of the i_{pa} (anodic peak current) obtained with eight electrodes was in fact lowered from a value of 10% (before pretreatment) to 5% (after pretreatment). In the case of the i_{pc} (cathodic peak current), the R.S.D.% was 17% for the non-pretreated electrodes and 5% after the pretreatment.

3.2. PB modified electrode characterisation

The deposition of PB on different substrates is usually accomplished by the electrochemical reduction of an equimolar mixture of ferric and ferricyanide ions (Itaya et al., 1982, 1984; Chi and Dong, 1995; Karyakin et al., 1998; Zhang et al., 1999; Garjonyte and Malinauskas, 2000). In addition, non-electroplating methods can be applied (Neff, 1978; Weiβenbacher et al., 1992; Zakharchuk et al., 1995; Garjonyte and Malinauskas, 1998), but in this case they seem to be highly dependent from the nature of the substrate surface itself (Itaya et al., 1982).

In our previous work (Moscone et al., 2001), we successfully applied the latter procedure by chemical synthesis of PB onto graphite powder particles, obtaining a very effective modified matrix. The carbon ink used for the SPEs printing process is obtained by mixing graphite and other different compounds, such as polymeric binders and solvents in order to improve its viscosity. Due to this evident difference in the electrode composition, the achievement of good results as those obtained for the graphite powder was not obvious. For example, the same procedure applied to conventional solid glassy carbon electrodes, resulted in a very poor deposition of PB, which completely disappeared during cyclic voltammetry after only few scans (data not shown).

The effectiveness of the PB deposition procedure onto the SPEs was verified by cycling the modified electrodes from −0.5 to 1.2 V. Fig. 2 shows the voltammograms of the bare SPE and of the PB-modified SPE. In the latter case, the typical two pairs of redox waves showing the oxidation as well as the reduction of PB are present. Moreover, the increase of the cathodic wave at around 0.1 V in presence of H_2O_2 can also be noticed, proving the activity of the catalyst on the electrode.

PB modified SPEs represent the support for enzyme immobilisation: their performances in respect of H_2O_2 response (detection limit, linearity range, and reprodu-

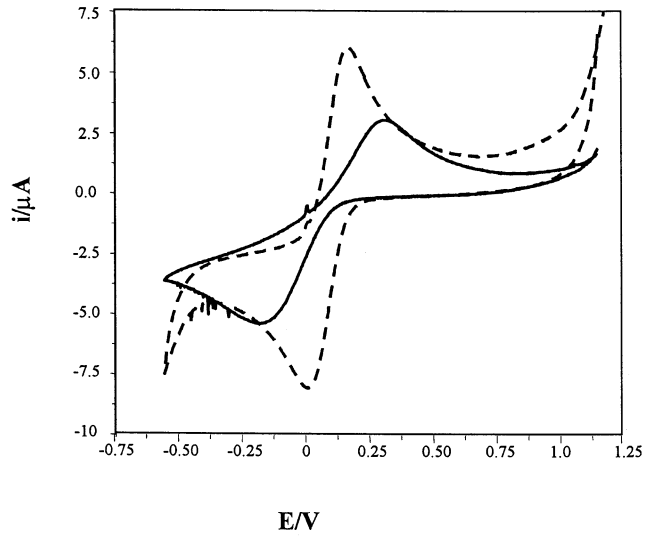


Fig. 1. Cyclic voltammograms of 1 mmol l^{-1} $K_3Fe(CN)_6$ in 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4 of a non-treated screen printed electrode (solid line) and a treated (3 min at 1.7 V vs. Ag/AgCl) screen printed electrode (dashed line). Potential range = −0.6 to 1.2 V. Scan rate = 50 mV s^{-1}.

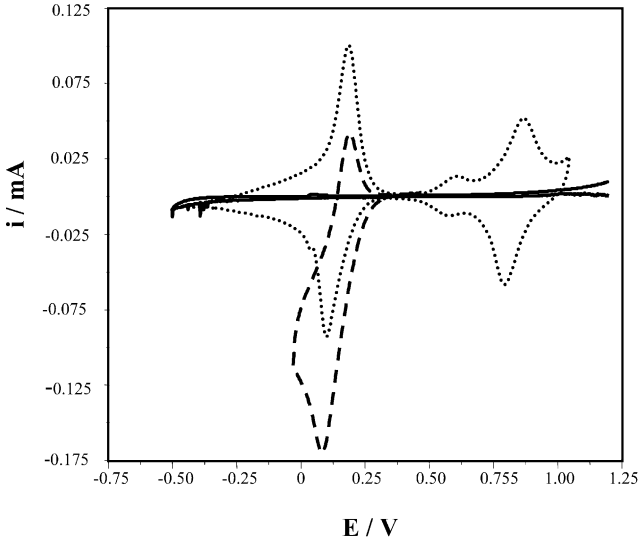


Fig. 2. Cyclic voltammograms (−0.5 to 1.2 V) in 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4 of bare SPE (continuous line); PB-modified SPE (dotted line); PB-modified SPE in presence of H_2O_2 10 mmol l^{-1} (dashed line; in this case the voltammogram was carried out between −0.05 and 0.35 V). Scan rate 50 mV s^{-1}.

cibility) were then carefully studied. Before this, the pH effect was evaluated and the best potential for H_2O_2 reduction was chosen, as illustrated below.

3.3. pH-effect

Cyclic voltammetry was also performed to evaluate the electrochemical behaviour and the stability of the PB layer to pH changes.

Fig. 3 shows cyclic voltammograms between −0.2 and +0.4 V (scan rate 50 mV s^{-1}) in buffer solutions at different pH values. Only the first cycle and the 250th one are depicted.

At pH 3 we obtained the best stability of PB in accordance of what reported in literature (Itaya et al., 1984; Garjonyte and Malinauskas, 1998, 1999; Zhang et al., 1999).

At pH 7, the PB layer is still well bound on the electrode surface and we can observe just a slight decrease of the peak current values after 250 cycles.

It has also to be stressed how the modified electrode behaves at pH 9. Although at this pH the PB layer deposited on the electrode surface shows a lower stability, after 250 cycles is still highly electroactive and sufficient to catalyse the H_2O_2 reduction. In fact, amperometric measurements of H_2O_2 (10 μmol l^{-1}) were carried out before and after the continuous cycling at pH 9, and the decrease of the signal was only 10% of the initial value.

The operational stability of all the PB modified sensors is a critical point, especially at neutral and alkaline pH. A possible explanation could be the presence of hydroxyl ions at the electrode surface as a product of the H_2O_2 reduction. The hydroxyl ions are known to be able to break the Fe–CN–Fe bond, hence solubilizing the PB (Karyakin, 2001). In our opinion, the good results here achieved, similar to those observed in a previous paper (Moscone et al., 2001), are due to the proposed method of deposition, which involves the chemical synthesis of PB in the presence of graphite, allowing its capillary adsorption. Moreover, the SPEs anodization pretreatment carried out at 1.7 V could produce carboxylic groups similar to those produced with aqua regia on graphite powder in our mentioned previous work, and the presence of these groups seems to contribute to the formation of PB films (Jaffari and Turner, 1997). In addition, recently has been demonstrated that electrochemical pretreatments induce some cracking of the surface of the carbon electrodes with the increase of its effective working area (Grennan et al., 2001). For all these reasons, a larger and deeper absorption of PB is probably obtained, and the carbon surface acts as a reservoir of well-bound PB, which minimises the leakage due to the hydrolysis of ferric ions, and increases the operational stability even at alkaline pH.

These PB modified electrodes could, therefore, be used also with enzymes having an optimum alkaline pH.

3.4. Selection of the applied potential

The choice of the applied potential at the working electrode is fundamental to achieve the lowest detection limit and to avoid the electrochemical interfering species. H_2O_2 and ascorbic acid, both at a concentration of 0.1 mmol l^{-1} were measured at five different values of potential near 0.0 V (Fig. 4). The ascorbic acid gave an oxidation signal, while the signal due to H_2O_2 resulted in a reduction. The optimum potential was −50 mV because of the highest signal for H_2O_2 and the low interference from ascorbic acid (only 2% of the ratio ascorbic acid/H_2O_2 × 100). In spite of the similar interference/signal ratio, a higher current background was obtained at −100 mV.

Moreover, at the selected potential of −50 mV, the time necessary to reach a stable background current was as low as 30–60 s.

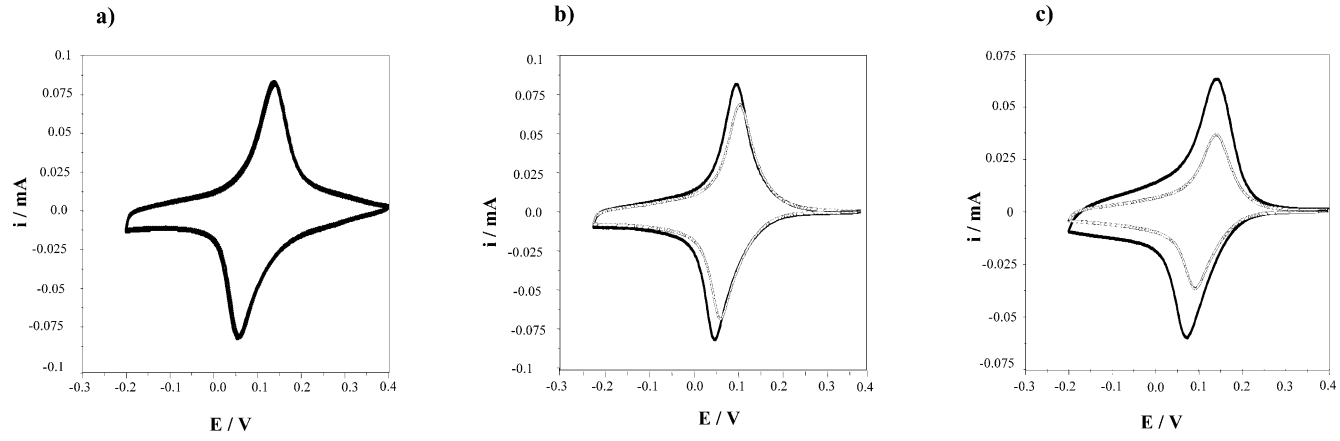


Fig. 3. Cyclic voltammograms in 0.05 mol l^{-1} phosphate buffer at (a) pH 3; (b) pH 7; (c) pH 9. Solid lines represent the first cycle of a series of 250 continuous cycling. The dashed lines are the 250th of the same series. Potential range = −0.2 to 0.4 V. Scan rate 50 mV s^{-1}.

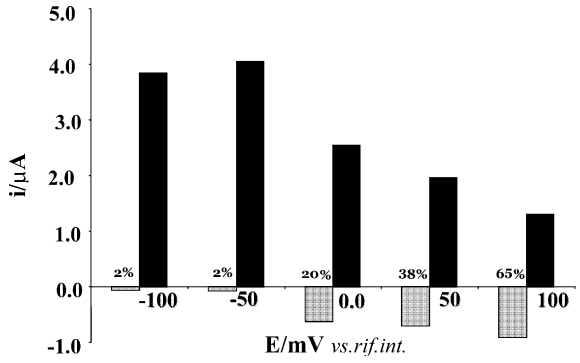


Fig. 4. Response of the PB-modified SPEs to H_2O_2 in 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4, at different applied potential; grey bars: 0.1 mmol l^{-1} ascorbic acid (oxidation current); black bars: 0.1 mmol l^{-1} H_2O_2 (reduction current).

3.5. PB modified electrode performances

PB-electrodes were then studied using cyclic voltammetry in a range between −0.2 and +0.4 V at different scan rates (in a range between 2 and 200 mV s^{-1}). A linear correlation between the scan rate and the current peaks (both anodic and cathodic) has been observed in a range between 2 and 50 mV s^{-1}, as expected in the case of an adsorbed electroactive substance on the electrode surface.

Then PB modified electrodes have been tested as H_2O_2 probes, and response time, detection limit, linearity range, sensitivity and electrode reproducibility were studied. All the measurements were performed in a 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4 and at an applied potential of −0.05 V.

The H_2O_2 calibration curves obtained with six different electrodes (using each electrode for all the concentration values tested) showed a good linearity in a range between 0.1 and 50 μmol l^{-1} with a detection limit of 0.1 μmol l^{-1}. Fig. 5 shows the calibration curve together with the representation of few authentic responses in the micromolar range. The regression equation of the linear part of the curve was $y = 22.90x - 0.013$, where y represents the current in μA and x the H_2O_2 concentration in mmol l^{-1}; the R^2 was 0.9980. The sensitivity was 324 μA mmol l^{-1} cm^{-2}, and the R.S.D.% was up to 5% (for all the concentrations tested by six different electrodes). The response time needed to reach 90% of the steady state response was 30 s.

The electrodes were also tested in hydro-organic solvent (working buffer/acetonitrile ratio: 60/40 v/v) and in buffer containing Triton X-100 1% (m/v). No variation in the response to the addition of H_2O_2 (10 μmol l^{-1}) was found after dipping the modified SPE in buffer/acetonitrile solution for 48 h. The same behaviour was observed after 48 h in the Triton solution.

3.6. Enzymatic substrate measurements

The results obtained with the PB modified SPEs brought us to investigate their possible use in the assembling of oxidase enzyme based biosensors. Scheme 1 gives an overview of the reactions involved.

The PB-modified electrodes were then used as support for the oxidase enzyme immobilisation. The planar characteristics of sensors produced by screen-printed technology require a good and reproducible contact between the enzymatic layer and the working electrode as well as a good robustness of this layer onto the electrodic area.

Several experiments have been carried out to obtain a stable and active enzymatic layer. Nafion® and glutaraldehyde were used for this purpose. GOD was selected for the optimisation of the immobilisation procedure.

Preliminary experiments were carried out using enzymatic solutions and Nafion. Different enzyme amounts ranging from 0.75 to 150 U ml^{-1} were placed on the electrode surface, fixed with different Nafion® solutions (0.5–5% v/v) and tested. In all cases, no steady state signal was observed probably because of a not complete entrapment of the enzyme by the Nafion®. In fact, no response to the substrate was obtained when electrodes were reused.

A glutaraldehyde cross-linking method was then tested to obtain a more stable layer. Also in this case different enzymatic solutions (0.2–50 mg ml^{-1}) were mixed with different glutaraldehyde solutions (0.25–5% v/v). The resulting membranes were also unstable because a spontaneous detachment under stirring conditions was observed.

The combination of the crosslinking proprieties of glutaraldehyde with the ability of Nafion® to fix the enzymatic membrane on the carbon surface of the working electrode solved this problem. This novel optimised procedure, described in the Section 2 resulted in a strong improvement of the membrane stability together with a high enzymatic activity.

Under our experimental conditions, the PB-modified screen-printed glucose biosensors showed a linearity range between 2 and 100 μmol l^{-1}, and a detection limit of 1 μmol l^{-1}. The linear regression equation was: $y = 4.4x - 0.005$ where y represents the current in nA and x the glucose concentration in μmol l^{-1}; the R^2 was 0.9989. The calibration curves were repeated using five different biosensors and a R.S.D.% up to 5% was obtained. The sensitivity was 63 μA mmol l^{-1} cm^{-2}, and the enzymatic efficiency, i.e. the response of glucose in respect to the same concentration of hydrogen peroxide, measured with the same electrode, was about 32%.

The stability of the PB layer at basic pH brought us to investigate the use of PB modified electrodes with oxidase enzymes having an alkaline optimum pH. For

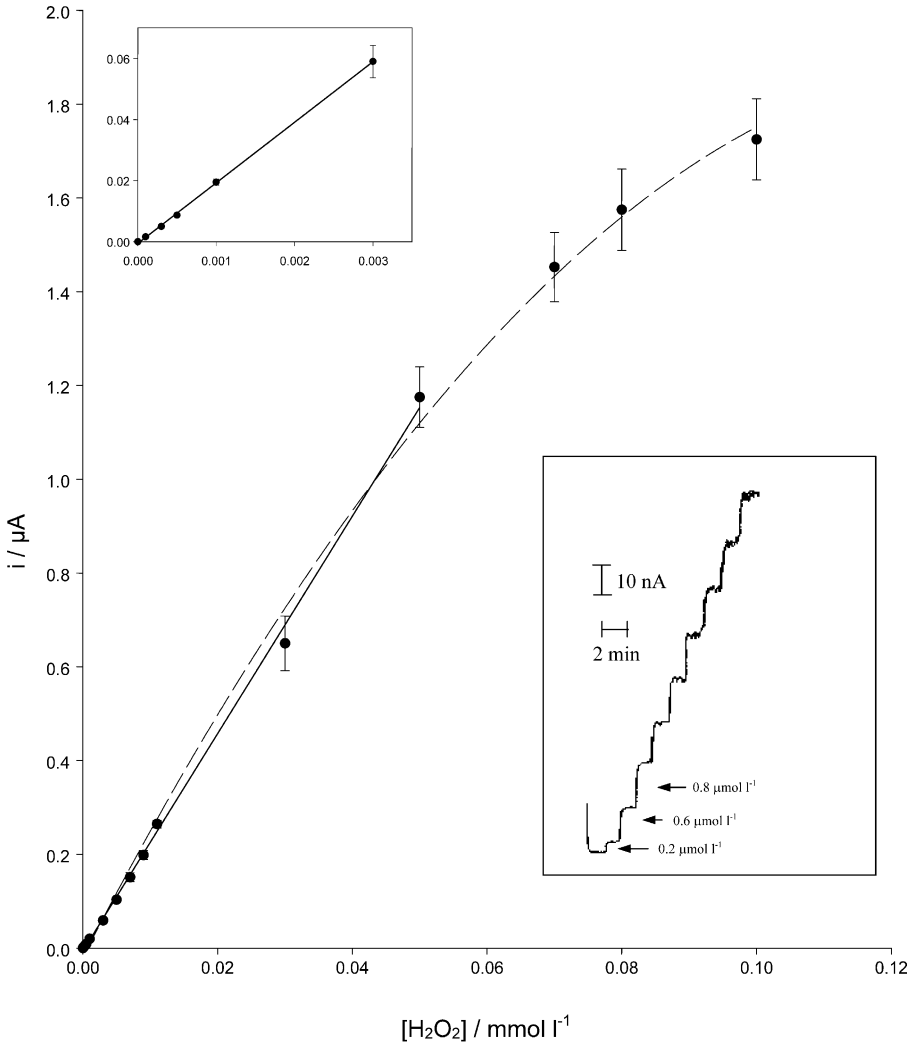
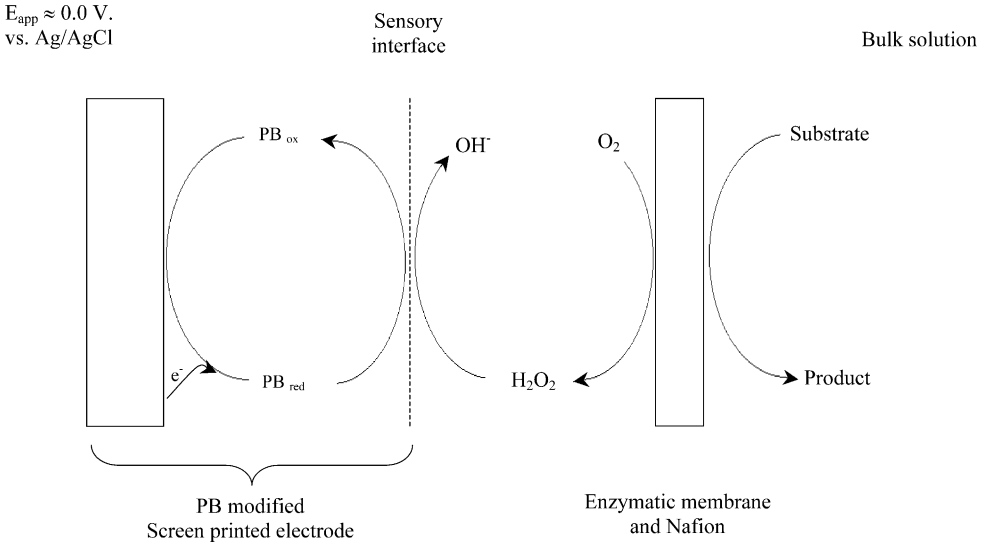


Fig. 5. H_2O_2 calibration curve obtained with PB modified SPE ($n = 6$). In the lower insert, the actual response curve for some H_2O_2 additions is also shown. All the additions successive to the second one are of the same entity (0.8 µmol l^{-1}). Applied potential −50 mV vs. the internal Ag pseudoreference electrode.



Scheme 1. General overview for an oxidase PB modified electrode.

this reason, choline oxidase was immobilised on the PB-electrode surface using the same procedure, and the analytical properties of these screen printed biosensors were studied.

3.6.1. Choline biosensor

Choline biosensors were then tested to define the detection limit, linearity range, sensitivity and reproducibility. Calibration curves were obtained with six biosensors, (for each biosensor all the concentration values were measured) and the analytical parameters were as follows: linear range between 0.5 and 100 μmol l^{-1}; detection limit 0.5 μmol l^{-1}, R.S.D.% up to 6%. The equation of the linear regression was: $y = 7.7x - 0.04$ where y represents the current in nA and x the choline concentration in μmol l^{-1}. The R^2 was 0.9949, and the sensitivity 110 μA mmol l^{-1} cm^{-2}. Since the enzymatic activity increased with the pH up to a maximum plateau of pH 7–8, all the measurements have been carried out at pH 7.4.

The enzymatic efficiency (i.e. percent of Choline/H_2O_2 response ratio) was around 80%. This percentage was higher than that obtained for the glucose biosensor (32%), also taking into account that the choline oxidase reaction produces two molecules of H_2O_2 for each molecule of choline. A possible explanation of this result could be the polyanionic structure of Nafion, able to attract the positive charged molecules of choline, increasing its concentration near the enzymatic layer.

3.6.2. Interferences study

The interference signal due to the most common electrochemical interfering species was also evaluated. The signal for a fixed concentration of choline was compared with the current value obtained in the presence of the same concentration (0.5 mmol l^{-1}) of the interfering species. The relative current signal gives a measure of the sensor selectivity. Results are summarised in Table 1 and show a high selectivity of the choline biosensor, probably due the low applied potential (i.e. −50 mV). The interference due to ascorbic acid remains the same of what obtained with the PB-modified electrode, and there was no signal due to the uric acid. Negligible responses were obtained by cathecol and by acetylcholine, probably because of the spontaneous hydrolysis of this latter to give small amounts of choline.

3.6.3. Reproducibility and repeatability

Despite the additional deposition of the enzymatic layer to the PB modified SPEs, the inter-electrode reproducibility was comparable to that obtained for the electrodes modified with only PB. A total number of 42 choline biosensors (seven groups of six biosensors each) have been tested for their reproducibility. Each group was prepared the same day. The R.S.D.% of the response to the choline of six different biosensors belonging to the same group was 6–7%, while between biosensors of different groups, prepared and tested during a period of 9 months, was around 14%.

The repeatability was evaluated using two choline biosensors and performing six successive calibration curves for each electrode. Results showed a R.S.D.% of 2.5%.

3.6.4. Storage stability

The storage stability of the biosensors in solution was evaluated at 4 °C and at RT. The response to choline 20 μmol l^{-1} of 20 biosensors, stored at 4 °C when not in use and tested every 2 weeks for 30 weeks showed an average decrease of 10% of the initial current value.

Further four electrodes were stored at room temperature during 4 weeks and tested every 2 days. The average activity was found to be 95 ± 10% of the original one at the end of the period.

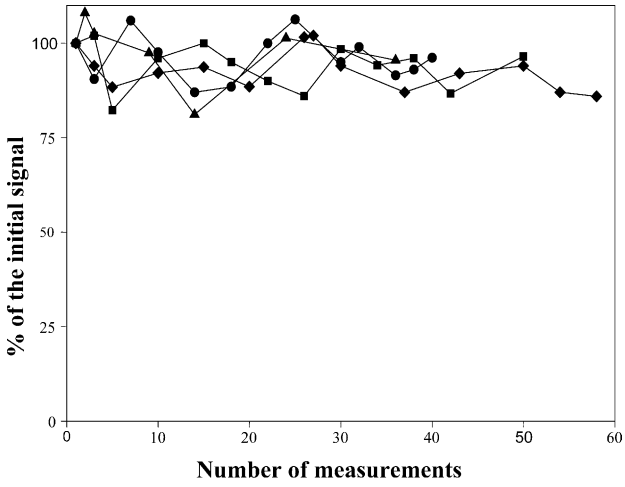


Fig. 6. Stability of choline biosensors ($n = 4$) during 6 months. Response to choline 20 μmol l^{-1} in 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4, applied potential −50 mV vs. the internal Ag pseudoreference electrode.

Table 1
Response of the choline biosensor towards interfering species (0.05 mmol l^{-1}) in 0.05 mol l^{-1} phosphate buffer +0.1 mol l^{-1} KCl, pH 7.4

Interfering specie (0.5 mmol l^{-1})	Choline	Ascorbic acid	Acetylcholine	Cathecol	Uric acid
Relative activity (%)	100	2	0.2	0.1	No int.

Applied potential −50 mV vs. the internal Ag pseudoreference electrode.

Fig. 6 shows the response occasionally obtained for choline (20 μmol l^{-1}) of four different biosensors, which, during a period of 6 months have been routinely tested for choline and occasionally for H_2O_2. After a large number of measurements (59, 50, 40 and 37, respectively) for each biosensor an average response decrease of 12% was observed.

3.6.5. Operational stability

The operational stability of the biosensors was evaluated keeping the biosensors continuously working in a solution of choline (20 μmol l^{-1}) for 50 h and renewing the solution every morning. Also in this case the signal, continuously recorded, showed an average variation of 10%.

3.6.6. Acetylcholine biosensors

This biosensor has been assembled to study the immobilisation of more than one enzyme. The enzyme AchE was coimmobilised with the choline oxidase enzyme as reported in the Section 2. The analytical characteristics can be summarised as follows: linear range between 5 and 100 μmol l^{-1}; limit of detection 1 μmol l^{-1}; the equation of the calibration curve was $y = 6.28x + 0.95$, and $R^2 = 0.9999$, where y was the current in nA and x the concentration of acetylcholine in μmol l^{-1}. R.S.D.% ($n = 5$ different biosensors) was found to be up to 7%. The sensitivity was 103 μA mmol l^{-1} cm^{-2}, and the enzymatic efficiency, i.e. the response of acetylcholine in respect to the same concentration of choline, was about 70%.

The storage stability of these biosensors was similar to that of choline biosensors: after 21 weeks of storage in buffer at 4 °C, the activity toward acetylcholine was still 100 ± 10%, and the same values were obtained after a 3 weeks storage at room temperature.

4. Conclusion

The new features of PB modified SPEs led to sensitive and reproducible probes for the detection of hydrogen peroxide. The use of PB resulted in a good selectivity towards electrochemical interferences. Moreover, the proposed novel PB modification procedure has brought to a stable layer even at basic pH values, overcoming the problems of operational stability and sensitivity in alkaline media, which still affect the use of this inorganic catalyst.

In addition, a new procedure for the immobilisation of enzymes on the surface of SPEs has been developed resulting in biosensors with improved characteristics of sensitivity and operational and storage stability. The choline biosensor obtained by cross-linking choline oxidase with glutaraldehyde and Nafion® onto the PB-modified SPE was found suitable for a stable and reproducible choline measurement in a range between 0.5 and 100 μmol l^{-1} within 9 months from its preparation. It has also shown the possibility to immobilise more than one enzyme on the surface of these modified sensors with similar performances in terms of storage stability, as in the case of the acetylcholine biosensor.

The results obtained with both the proposed methods of modification with PB and enzymes immobilisation, in our opinion, make these sensors an attractive alternative to the traditional biosensors.

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