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Prussian Blue and enzyme bulk-modified screen-printed electrodes for hydrogen peroxide and glucose determination with improved storage and operational stability

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Abstract

The addition to the carbon ink, a major component of a screen-printed electrode (SPE), of an aliquot (10%) of Prussian Blue (PB)-modified glassy carbon (PB-GC) particles, resulted in an interference free "ready to use" amperometric H_2O_2 sensor ($V_{app} = -50$ mV) with a LOD of 3×10^{-7} mol/l and a sensitivity of 135 mA (mol l^{-1} cm^2). A storage stability of up to 8 months and an operational stability of 3 days has been achieved making these sensors suitable for mass-production.

Glucose and lysine biosensors have been assembled immobilizing glucose oxidase (Gox) and lysine oxidase (LyOx) with glutaraldehyde and Nafion® onto the PB bulk-modified electrodes. A LOD of 4×10^{-6} mol/l for glucose and 5×10^{-6} mol/l for lysine with a linear range up to 0.5 and 0.7 mmol/l, respectively, have been observed.

A ready to use glucose biosensor was then developed mixing PB-modified glassy carbon (7.5%), carbon ink (87.5%) and glucose oxidase (GOx, 5%) and using the mixture for the printing step of a SPE working electrode. A LOD of 3×10^{-5} mol/l and a linearity range up to 6×10^{-3} mol/l of glucose have been achieved, together with a storage stability up to 20 weeks (at RT) and an operational stability of 1 day with 11 calibration curves performed.

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

1. Introduction

The characteristics of Prussian Blue (PB) or ferric exacyanoferrate have been thoroughly investigated by many research groups [1–4] involved in the development of new H_2O_2 sensors. In fact, Prussian Blue was found to have a catalytic effect on the reduction of H_2O_2 [1,5]. Moreover, the peculiar cubic geometry [6] of the PB molecules seems to be the cause for an effective electrochemical selectivity. In fact, molecules with a molecular weight higher than H_2O_2, such as ascorbic acid and uric acid, cannot penetrate the PB lattice, and give a catalytic redox reaction. These two promising advantages have been used to obtain a sensitive and interference free probe for H_2O_2 detection. This was firstly achieved by a modification of the

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electrode surface (i.e. Pt [7], graphite [8,9], glassy carbon [1,5,26], Au [10], etc.) with a Prussian Blue layer grown either with a chemical or electrochemical synthesis. The electrochemical detection of H_2O_2 at these electrodes can be obtained at low applied potential (about 0.0 V) as in the case of horseradish peroxidase (HRP)-modified electrodes [25]. In the case of PB, additional advantages such as low cost and easy of deposition are achieved. The successive immobilization of an oxidase enzyme (i.e. lactate, glucose, choline oxidase) on the PB-modified electrode surface makes possible the detection of the enzyme substrate [11–14,22]. The enzyme provides the selective oxidation of the substrate and the production of H_2O_2 which is proportional to the substrate amount and can be easily reduced by PB, giving a detectable electrical signal. One of the problems related to PB-modified electrodes was the instability of the PB layer at basic pH values [7,15,16]. This fact limited the choice of the oxidase enzymes to those having an optimum pH in the acid range. Recently, we reported a new procedure for PB modification on graphite particles based on a "in situ" chemical synthesis of PB, obtaining PB-modified electrodes with high stability even at basic pH values [14,17,18]. This procedure has been applied to the construction of PB-modified carbon paste [17], screen-printed (SPE) [14] and glassy carbon paste [18] electrodes. A recent paper by Guilbault's research group [19] has already showed the preparation of Prussian Blue bulk-modified screen-printed electrodes using activated PB microparticles ($<38\,\mu m$) mixed with the carbon ink. These electrodes exhibited a H_2O_2 LOD of 0.4 μmol/l with a sensitivity of 135 mA/(mol l^{-1} cm^2) [19]. A major drawback related to these probes was the low stability of the PB. A decrease of 50% of H_2O_2 signal was in fact observed after 4 h of continuous work, in addition their use was limited only to pH values not higher than 7.0 [19]. In the present paper, a new concept for PB bulk-modified screen-printed electrodes is proposed. In this case, an improved pH dependence behaviour, due to the optimized modification procedure, together with a high sensitivity and selectivity towards H_2O_2 is obtained. A detailed study of the operational and storage stability is presented. An application with lysine and glucose biosensors, obtained by the immsobilization of the enzymes lysine oxidase (LyOx) and glucose oxidase (GOx) on the PB-modified SPEs, is also showed. Moreover, by the simple addition of an aliquot of GOx to the PB–carbon ink mixture, before the printing process, a "ready to use" glucose biosensor has been obtained. Analytical parameters, such as detection limit, linearity range and sensitivity have been evaluated, together with operational and storage stability.

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).

Cyclic voltammetry (CV) was 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. Electrodes

Screen-printed electrodes were produced in the Biosensor Laboratory of the University of Florence (Italy). Electrodes were printed with a 245 DEK (Weymouth, UK) screen printing machine using different inks obtained from Acheson Italiana (Milan, Italy). Graphite-based ink (Elettrodag 421), silver ink (Elettrodag 477 SS RFU) and insulating ink (Elettrodag 6018 SS) were used. To obtain a PB bulk-modified screen-printed electrode, the graphite ink was mixed at different percentages with PB-modified glassy carbon powder or PB-modified graphite powder.

The substrate was a polyester flexible film (Autostat HT5) obtained from Autotype Italia (Milan, Italy). The printing procedure is already described in previous papers [20,21]. The electrodes were produced in foils of 20 sensors. Each sensor consists of three printed electrodes, a carbon working and two silver electrodes, acting as counter and pseudoreference, respectively. Values of the potential measured with the Ag pseudoreference electrode and maintained constant using always a supporting electrolyte in the working buffer (i.e. KCl 0.1 mol/l), are shifted of 55 mV towards negative potentials in respect of a standard Ag/AgCl (3 M) reference electrode.

The diameter of the working electrode was 0.3 cm, resulting in an apparent geometric area of 0.07 cm^2. The resistance of these electrodes was in the range of 0.2–0.3 Ω.

2.3. Reagents

Spherical glassy carbon Sigradur G (GC-G) particles (diameter 0.4–12 μm) were purchased from Sigradur, HTM Hochtemperatur-Werkstoffe GmbH (Gemeindewald, Germany). Graphite particles (powder 1–2 μm) were obtained by Aldrich (Steinheim, Germany). All chemicals from commercial source were of analytical grade. All solutions were prepared with 0.05 mol/l phosphate buffer + 0.1 mol/l KCl. Standard solutions were daily prepared in the same buffer.

Glucose oxidase (EC 1.1.3.4, type VII, 185 U/mg) and lysine oxidase (LyOx) (EC 1.4.3.14, from *Trichoderma* sp., 6.27 U/mg) were obtained from Sigma (St. Louis, MO).

2.4. PB modification of glassy carbon or graphite

The Prussian Blue modification procedure has been already described in previous papers [14,17,18]. Briefly, the glassy carbon or graphite powder (1 g) was suspended in 10 ml of a solution of $K_3Fe(CN)_6$ 0.1 mol/l in 10 mmol/l HCl. Next, 10 ml of a 0.1 mol/l solution of $FeCl_3$ in 10 mmol/l HCl were added and the resulting mixture was stirred for 10 min. The powder (with adsorbed PB) was then collected by filtration and washed with 10 mmol/l HCl until the washing solution became colorless and then dried in a oven at 100 °C for 1.5 h. The powder modified with Prussian Blue (PB-modified glassy carbon (PB-GC) or PB-modified graphite (PB-graphite)) was stored dark in a desiccator at room temperature.

2.5. Preparation of PB-SPEs

The working electrode ink was mixed before the printing step to different weight aliquots (i.e. 2.5, 5 and 10%) of PB-GC or PB-graphite. The PB-modified screen-printed electrodes resulting from the printing of these inks will then be referred as PB-GC-SPEs (10%), PB-GC-SPEs (5%) and PB-GC-SPEs (2.5%) in the case of the use of PB-GC, and as PB-graphite-SPEs (10%), PB-graphite-SPEs (5%) and PB-graphite-SPEs (2.5%) in the case of the use of PB-graphite.

Resistance values for these electrodes were always in the same range of the unmodified SPE (i.e. 0.2–0.3 Ω).

2.6. Preparation of membrane-based glucose and lysine biosensors

Lysine oxidase and glucose oxidase were immobilized onto PB bulk-modified SPEs using a procedure optimized in a previous work [18]. Seven microliters of a mixture of glutaraldehyde, Nafion® and a solution of enzyme + BSA were added onto the working electrode area. One hundred fifty microliters of the mixture have the following exact composition:

- 100 μl of enzymatic solution, prepared by dissolving 40 mg of BSA and 10 mg of enzyme into 1 ml of 0.05 mol/l phosphate buffer + 0.1 mol/l KCl, pH 7.4;
- 20 μl of glutaraldehyde (2.5 vol.% diluted in water);
- 30 μl of Nafion® (5 vol.% in ethanol).

After the addition of 7 μl of this mixture on the electrode surface, the solution was allowed to dry for 45 min at room temperature. The electrodes were then washed for 30 min with a solution of 0.1 mol/l glycine to saturate all the free aldehydic groups.

2.7. Preparation of bulk glucose biosensor

For the preparation of "ready to use" glucose biosensors, the working electrode ink was mixed, before the printing step, with 7.5 wt.% of PB-GC and with 5 wt.% of lyophilized glucose oxidase. The working electrode ink was then dried at various temperatures and times.

2.8. H_2O_2 and enzymatic substrate measurements

Amperometric batch measurements of H_2O_2 and glucose were performed in a stirred phosphate buffer solution 0.05 mol/l + KCl 0.1 mol/l, pH 6.0 (10 ml) with an applied potential of −50 mV versus internal reference electrode (int. ref.). When a stable baseline current was reached, the analyte was added and the current variation was recorded.

For real sample measurements once a stable baseline current was reached, an aliquot (25 μl) of the sample solution was added. The sample solution was obtained adding 1 ml of phosphate buffer to 9 ml of untreated sample. This procedure was used to compare the results with those obtained in the successive recovery experiments.

For recovery studies, the same aliquot (25 μl) of a spiked sample solution, obtained by adding 1 ml of glucose standard solution (1 mol/l) to 9 ml of untreated sample, was added. For each biosensor, sample and recovery measurements were made in triplicate.

Real samples of fruit juices from Yoga (Italy) and Coca-Cola (produced in Italy) were analyzed for glucose.

3. Results and discussion

3.1. Choice of the best ink composition

Previous studies on the preparation of PB-modified glassy carbon paste electrodes [18], showed the favorable characteristics of the glassy carbon particles material if compared with the more classic graphite powder (i.e. high electronic transfer rate and good conductivity). As the paste-based electrodes, the working electrode ink of a screen-printed electrode can be modified by simply adding biological or inorganic modifier.

Different amounts of PB-modified glassy carbon or PB-modified graphite were then added to the commercial ink used for the working electrode, in order to print PB bulk-modified screen-printed electrodes. Three different ink mixtures for each type of material (i.e. glassy carbon and graphite) were prepared adding a percentage of 2.5, 5 and 10 wt.% of PB-GC or PB-graphite to the working electrode ink. The first study consisted of the choice of the best PB-modified material (i.e. glassy carbon or graphite). This was made by the evaluation of the amperometric response to some H_2O_2 standards and by the study of the electrochemical behaviour in cyclic voltammetry experiments.

Results obtained in amperometric batch analysis (Table 1) clearly show that glassy carbon particles are better in terms of noise level than graphite powder. Moreover, the response to H_2O_2 is three to four times higher in the case of the SPEs obtained with PB-GC. This can be explained considering the different geometry of the GC and graphite particles. The spherical geometry of the GC particles makes possible a more efficient and homogenous coverage of the surface by the Prussian Blue as already explained in a previous paper [18], which results in a higher activity towards H_2O_2 reduction and a better electroactivity. Considering these results, PB-GC was then selected as the best material for the preparation of PB bulk-modified SPEs.

The dependence of the peak current towards the scan rate has been also studied for the PB-GC-SPEs. Fig. 1 shows the results obtained with the PB-GC-SPEs (10%). The direct relation between the peak current and the scan rate in a range between 2 and 100 mV/s demonstrates that the electrodic behaviour is not affected by diffusion problems. The same behaviour was shown by SPEs obtained with 2.5 and 5% of PB-GC. Moreover, with a scan rate of 5 mV/s a ΔE_p value as low as 14 mV and mirror image peaks were observed, demonstrating a very efficient transfer rate and a good reversibility of the PB deposited.

The next step was the choice of the best PB-GC percentage. As shown in Table 1, the PB-GC-SPEs

Table 1
Amperometric parameters of PB-GC and PB-graphite-based SPEs

	GC			Graphite		
	2.5%	5%	10%	2.5%	5%	10%
Noise (nA)	0.4	0.8	1.6	0.8	1.2	2.2
Background (nA)	26	52	103	24	25	105
Signal to 10^{-6} mol/l H_2O_2 (nA)	1.6	3.7	8.0	–	–	–
Signal to 10^{-5} mol/l H_2O_2 (nA)	18	44	91	4.9	9.5	37
Sensitivity (mA/(mol l^{-1} cm^2))	24	53	114	7	13	52

Batch amperometric analysis, applied potential = −50 mV vs. int. ref. Phosphate buffer 0.05 mol/l + KCl 0.1 mol/l, pH 6.0.

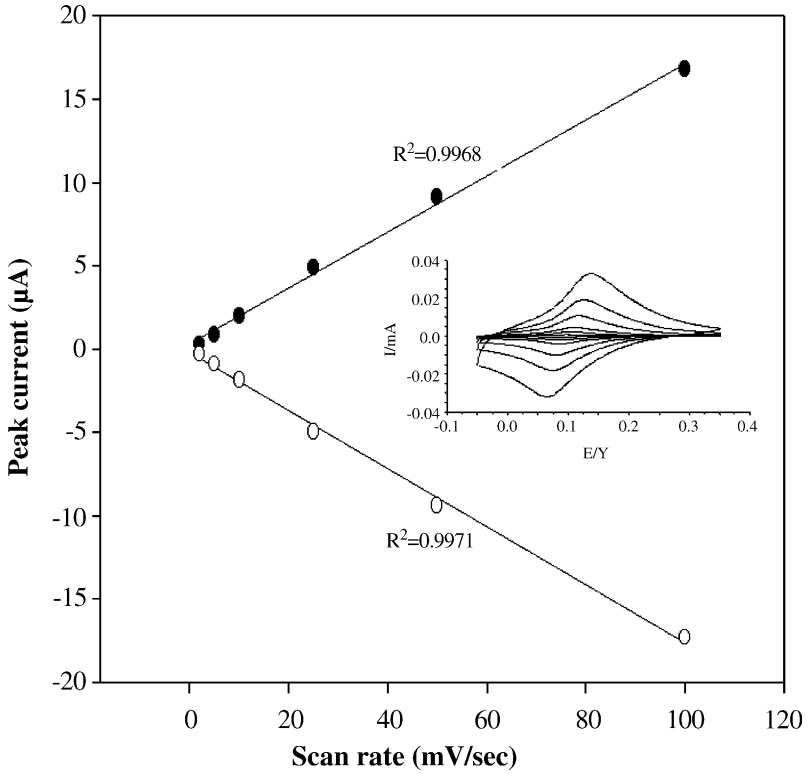

Fig. 1. Dependence of peak current (μA) towards cyclic voltammetry scan rate (mV/s). Cyclic voltammetry was performed between −50 and +350 mV in phosphate buffer 0.05 mol/l + KCl 0.1 mol/l, pH 6.0. Scan rate range between 2 and 100 mV/s.

(10%) gave the best sensitivity towards H_2O_2, so the percentage of 10% of PB-GC was selected for electrode preparation. This would also permit to have an amount of PB that should guarantee a longer life of the sensor.

3.2. H_2O_2 sensors

The response towards H_2O_2 of the PB-GC-SPEs (10%) was evaluated in a batch amperometric system. The applied potential used (−50 mV) was selected in our previous paper [14] dealing with conventional SPEs modified with PB. At this potential, a high H_2O_2/interference ratio was in fact observed.

During first measurements with PB-bulk-modified SPEs, at a H_2O_2 concentration of 5×10^{-5} mol/l, the current began to increase without reaching a steady state. This drift was probably due to the diffusion of the unconsumed H_2O_2 through the external layer of the electrode, that results in a larger effective electrode surface area. The presence of a H_2O_2 concentration probably causes the activation of the PB that is not directly in contact with the solution. Indeed, a complete activation of the PB was obtained by applying a fixed voltage (−50 mV versus int. ref.) in 10^{-3} mol/l H_2O_2 solution for 5 min. It should be noted here that when PB was deposited on the surface of the working electrode after the printing step, no need of activation was necessary [14], because the redox reaction of PB occurs only at the surface of the electrode. The activated sensors showed, when tested in amperometric mode with H_2O_2 (10^{-4} mol/l), a steady-state signal after 15 s. Moreover, the noise level was quite lowered resulting in a higher probe sensitivity. The activation obtained just applying the potential (i.e. −50 mV) for 5 min to the electrode in a buffer solution was not so effective as in the case of the use of H_2O_2 solution. Similar results were also observed for the activation

Table 2
Analytical parameters of H_2O_2 sensor, glucose and lysine membrane-based biosensors

Sensor	Detection limit (mmol/l)	Noise (nA)	Linearity range (mmol/l)	Sensitivity (mA/(mol l^{-1} cm^2))	R.S.D. (%) ($n = 5$)	Time to stabilize baseline current (min)	Time to reach 90% of the signal (s)
PB-GC-SPEs (10%)	3.0×10^{-4}	1.5	5.0×10^{-4} to 1.0	135	5	3	5
Membrane-based glucose biosensor	4.0×10^{-3}	3.0	5.0×10^{-3} to 0.5	14	6	3	5
Membrane-based lysine biosensor	5.0×10^{-3}	6.5	6.0×10^{-3} to 0.7	52	6	3	5

Working electrode ink composition: 90% carbon ink, 10% PB-GC. Batch amperometric analysis, applied potential = −50 mV vs. int. ref. Phosphate buffer 0.05 mol/l + KCl 0.1 mol/l, pH 6.0 for H_2O_2 and glucose, pH 8.0 for lysine.

procedure consisting of the immersion of the sensors in a 10^{-3} mol/l H_2O_2 solution for 5 min. This means that the complete activation needs an applied potential (−50 mV) and a high concentration of H_2O_2 (i.e. 10^{-3} mol/l).

Table 2 summarizes all the analytical parameters evaluated in the batch amperometric analysis with the activated sensors and Fig. 2 shows some original responses (applied potential = −50 mV versus int. ref.). The H_2O_2 sensors exhibited a sensitivity of 135 mA/(mol l^{-1} cm^2) and a detection limit of 3×10^{-7} mol/l with a good linearity up to 1 mmol/l. It has to be stressed the good reproducibility obtained among different electrodes (R.S.D. = 5%, $n = 5$), the fast response time (ca. 5 s for 90% of the signal) and the fast stabilization of the current baseline (ca. 3 min). The interference effect was evaluated measuring the response to uric and ascorbic acid at a concentration of 10^{-4} mol/l. No detectable signals were recorded for both uric and ascorbic acid while for the same concentration of hydrogen peroxide a current of 0.95 μA was recorded.

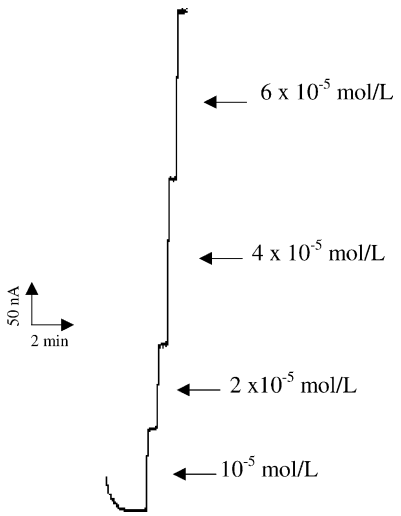

Fig. 2. Amperometric recordings for H_2O_2 using PB bulk-modified SPE. Working electrode ink composition: carbon ink 90%, PB-GC 10%. H_2O_2 final concentrations 10^{-5}, 2×10^{-5}, 4×10^{-5}, 6×10^{-5} mol/l. Phosphate buffer 0.05 mol/l + KCl 0.1 mol/l, pH 6.0. Applied potential −50 mV vs. int. ref.

3.3. Stability studies

For the storage stability studies, sensors were kept dry at RT and at 4 °C and then tested occasionally during a total period of 8 months. After 8 months, sensors stored both at 4 °C and at RT gave the same response to H_2O_2 as those sensors tested during the first day. Also their reproducibility and all the other analytical parameters, resulted unvaried. In order to evaluate the stability of the sensors after activation, a group of them was activated and then stored dry at 4 °C and at RT. In the case of the group of sensors activated and stored at RT, the loss of signal was about 50, 60 and 65% after 1, 4 and 8 weeks, respectively. In the case of the group of sensors activated and stored at 4 °C, we observed a loss of the original signal of 14, 35 and 70% after 1, 4 and 8 weeks, respectively.

For the operational stability, the sensors were activated and then thoroughly tested for H_2O_2 response (Fig. 3). After the activation, each sensor was tested for 10 H_2O_2 successive calibration curves (30 min between each calibration, concentration range 10^{-6} to 10^{-4} mol/l H_2O_2). The same procedure was applied to the same sensor after 1 and 2 days, so that each sensor has been tested for a total of 30 calibration curves

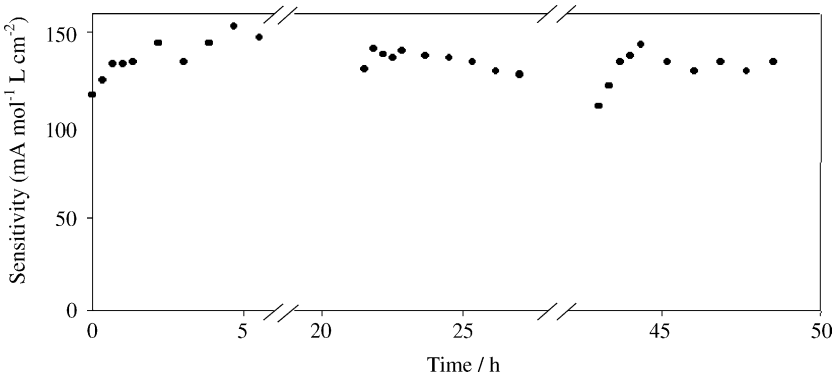

Fig. 3. Operational stability study for the PB bulk-modified SPE. Working electrode ink composition: carbon ink 90%, PB-GC 10%. Sensitivity obtained during 3 days for a total of 30 successive complete calibration curves. Phosphate buffer 0.05 mol/l + KCl 0.1 mol/l, pH 6.0. Applied potential = −50 mV vs. int. ref. Between each calibration curve and each day, the sensor was stored in buffer at 4 °C.

during three consecutive days. During the night and between each calibration curve, the sensors were kept in buffer at 4 °C. For all the calibration curves performed during the 3 days, the sensors ($n = 3$) retained 100 ± 5% of the original response. Probes were then tested for other 10 calibration curves 1 week after the first day and a loss of 10% of PB activity was observed.

A different study of the operational stability was performed in continuous mode. The activated sensors were kept in a 10 μmol/l H_2O_2 solution with the applied potential (−50 mV versus int. ref.). The signal to H_2O_2 was recorded every hour during the first 8 h and then, at the end of the experiment, after further 16 h of continuous work. This experiment was performed at pH values 6 and 9. For both pH values, the loss of signal after 24 h was about 10% demonstrating a good stability of the PB activity even at basic pH values. This peculiar stability is probably due to the modification procedure, consisting of an "in situ" chemical deposition of PB. This could provide a strong adsorption of PB microparticles onto glassy carbon powder which greatly reduces the leakage of PB at basic pH. On the other hand, using other published procedures, based on electrochemical deposition, the PB layer always resulted very unstable at alkaline pH [5,23].

3.4. Glucose and lysine biosensors

The PB bulk-modified SPEs have been then used to assemble glucose and lysine biosensors. Two different procedures are presented in this work, one involving a glucose or lysine oxidase immobilization onto the already modified electrodes and one the adding, before the printing process, of lyophilized glucose oxidase at the working electrode ink mixture.

3.4.1. Membrane-based glucose and lysine biosensors

Glucose or lysine oxidase were immobilized onto the activated PB-GC-SPEs (10%) using a procedure involving a cross linking method with glutaraldehyde, Nafion® and BSA. The analytical parameters of glucose and lysine biosensors are summarized in Table 2. A detection limit of 4 μmol/l with a linear range up to 0.5 mmol/l and a sensitivity of 14 mA/(mol l^{-1} cm^2) were observed in respect of glucose response. For lysine the detection limit was found to be 5 μmol/l with a linearity up to 0.7 mmol/l and a sensitivity of 52 mA/(mol l^{-1} cm^2).

The reproducibility for both biosensors (R.S.D. = 6%, $n = 5$) resulted as good as the one obtained without the enzyme layer. The time necessary to obtain 90% of the total signal was about 15 s and to reach a stable baseline current about 3 min. The glucose biosensors were then stored in a buffer solution containing NaN_3 (0.02%) at RT and tested occasionally during a period of 8 weeks. After 2 weeks, no decrease of signal was observed, and between the third and the eighth week, a gradual decrease till a final loss of 22% of the initial signal was observed.

3.4.2. Bulk glucose biosensor

We then investigated the possibility to prepare a "ready to use" glucose biosensor that would be suitable for mass production and would avoid time consuming procedures (i.e. enzyme immobilization). A new type of glucose biosensor was then obtained mixing at fixed weight percentage the carbon ink (87.5%), the PB-GC (7.5%) and lyophilized glucose oxidase (5%). We decided to use only glucose oxidase because of the possible problems related to the high temperatures needed for the drying of the working electrode ink. Moreover, glucose oxidase has been already mixed to a working electrode ink of a SPE [24,27]. Glucose oxidase is also known to be one of the most resistant enzymes and for its low cost makes possible the assembling of a high number of probes. The usual procedure adopted to allow the drying of the carbon ink (i.e. non-modified) requires high temperatures (i.e. 120 °C) for 10 min. At this temperature, the enzyme could undergo a partial inactivation that could affect the biosensor performances. Different conditions (lower temperature and higher drying time) have been then tested to avoid an enzyme inactivation. Three sensors for each drying procedure have been then tested for glucose response and the most representative analytical parameters have been evaluated and are summarized in Table 3. From these results, it seems that the different drying procedures do not affect the analytical performances of the biosensors. The detection limit (ca. 3×10^{-5} mol/l) and the linear range (ca. 5×10^{-5} to 5×10^{-3} mol/l) are almost the same for all the sensors. The sensitivity is slightly higher (i.e. 3.9 mA/(mol l cm^2)) for the biosensors obtained drying the working electrode ink at RT (25 °C) during the night. These biosensors have been then chosen for further experiments of stability and real sample analysis. The temperature used for the drying step (25 °C), in fact, would not affect the enzyme activity and the procedure would be also suitable to produce "ready to use" biosensors based on the use of less stable enzymes.

Even in this case, an activation step is needed to provide a stable and less noisy signal. The procedure used to activate the biosensors was the same already optimized for H_2O_2 sensors (i.e. applied potential = −50 mV in 10^{-3} mol/l H_2O_2 solution for 5 min). As it should be expected from a bulk-modified biosensor, the detection limit was higher than the one obtained with a membrane-based biosensor. All the analytical parameters obtained in batch amperometric analysis were evaluated. A detection limit of 30 μmol/l together with a linearity up to 6 mmol/l were observed. The sensors ($n = 5$) showed an unvaried reproducibility (R.S.D. = 6%) in respect of what was obtained with membrane-based biosensors. A very short time was also needed to reach a stable baseline current (ca. 3 min) and to obtain a steady-state signal (15 s). Storage and operational stability studies were performed with these biosensors. For the storage stability study, the biosensors were kept dry at RT. After 1 month, the probes ($n = 3$) were activated and three glucose calibration curves were performed with each biosensor. The same procedure was adopted with other two groups of new sensors ($n = 3$) after 3 and 5 months. After 5 months, the sensors showed an average variation of the signal of 5% and no loss of sensitivity was observed. The same storage stability was also observed using sensors obtained with two different drying procedures (120 °C for 10 min and 60 °C for 30 min).

Table 3
Amperometric parameters for bulk glucose biosensors

	Overnight, 25 °C	10 min, 120 °C	30 min, 60 °C	60 min, 60 °C	120 min, 60 °C	180 min, 60 °C	Overnight, 60 °C
Detection limit (mol/l)	3×10^{-5}	3×10^{-5}	3×10^{-5}	5×10^{-5}	3×10^{-5}	3×10^{-5}	5×10^{-5}
Linearity range (mol/l)	5×10^{-5} to 6×10^{-3}	5×10^{-5} to 5×10^{-3}	5×10^{-5} to 5×10^{-3}	7×10^{-5} to 5×10^{-3}	5×10^{-5} to 5×10^{-3}	5×10^{-5} to 5×10^{-3}	5×10^{-5} to 5×10^{-3}
Sensitivity (mA/(mol l^{-1} cm^2))	3.9	3.7	3.7	3.1	3.6	3.6	3.2

Comparison between biosensors obtained using different procedures for the drying of the working electrode ink containing glucose oxidase. For all biosensors working electrode ink composition was: carbon ink, 87.5%, PB-GC, 7.5% and lyophilized glucose oxidase 5%. Batch analysis, applied potential = −50 mV vs. int. ref. Phosphate buffer 0.05 mol/l + KCl 0.1 mol/l, pH 6.0.

Table 4
Real sample measurements

Sample	Detected value[a] in diluted sample (mol/l)	Detected value after 2.5 $\times 10^{-4}$ spike (mol/l)	Recovery (%)	R.S.D. (%)	Real value[a] (mol/l)
Peer juice	$(3.5 \pm 0.2) \times 10^{-4}$	$(5.7 \pm 0.4) \times 10^{-4}$	92	6	$(1.4 \pm 0.1) \times 10^{-1}$
Peach juice	$(4.6 \pm 0.3) \times 10^{-4}$	$(7.0 \pm 0.5) \times 10^{-4}$	97	7	$(1.9 \pm 0.1) \times 10^{-1}$
Coca-Cola	$(4.7 \pm 0.3) \times 10^{-4}$	$(7.1 \pm 0.5) \times 10^{-4}$	97	6	$(1.9 \pm 0.1) \times 10^{-1}$
Ananas juice	$(4.1 \pm 0.3) \times 10^{-4}$	$(6.3 \pm 0.6) \times 10^{-4}$	92	7	$(1.6 \pm 0.1) \times 10^{-1}$
Multivitaminic juice	$(4.6 \pm 0.4) \times 10^{-4}$	$(6.9 \pm 0.6) \times 10^{-4}$	93	8	$(1.8 \pm 0.1) \times 10^{-1}$

Analytical recovery of glucose added to some beverage samples. Amperometric batch analysis (10 ml), applied potential $V = -50$ mV vs. int. ref.

[a] Real value is obtained by multiplying the detected value by the dilution factor 400 (i.e. 25 μl in 10 ml).

For operational stability studies, the biosensors were thoroughly tested during one working day. Each biosensor was used for 11 successive glucose calibration curves every 30 min. No loss of sensitivity was observed at the end of the day.

A group ($n = 6$) of biosensors were activated and stored dry at 4 °C for a total period of 5 weeks. After 3 weeks, the sensors ($n = 3$) tested showed a signal that was only 7% lower than the signal obtained with the probes used immediately after the activation. After 5 weeks, the other probes ($n = 3$) tested showed a total loss of signal of 20%.

3.5. Real sample measurements

Bulk glucose biosensors were then used to evaluate glucose content in some beverage industrial products. The recovery was investigated by standard addition of glucose (i.e. 10^{-1} mol/l) to the samples. Results are summarized in Table 4 and showed recovery values in the range of 92–97%.

4. Conclusions

Mixing the commercial carbon ink of a SPE with an aliquot of PB-modified glassy carbon particles, a sensitive, reproducible, interference free, low cost "ready to use" device for H_2O_2 detection has been obtained. Storage stability studies on these probes demonstrated also that the PB modification procedure can provide a long stability of the PB activity and probe robustness. The same stability has been also observed for 3 days of continuous work. Comparing to the SPEs published in [19], which showed a decrease of 50% of H_2O_2 signal after 4 h, our results represent a strong improvement in stability. For their promising features, the sensors were then used as supports for enzyme (i.e. glucose and lysine oxidase) immobilization resulting in a lysine and glucose biosensor. Next step was the assembling of bulk-modified glucose biosensor obtained mixing to the PB-GC–carbon ink an aliquot (5%) of lyophilized glucose oxidase. Good results in terms of sensitivity, LOD, linearity range and storage and operational stability have been achieved. The probes were also used to measure glucose concentration in beverage samples and recovery studies gave good results. The absence of any step of enzyme immobilization or mediator modification after the printing process and the low cost of the sensors make them an "easy to use", inexpensive and mass-producible probe for glucose detection. Moreover, the good stability showed during one entire day of work makes these probes suitable for "in situ" continuous analysis. Further, the absence during the printing procedure of any high temperature is a promising feature for the use of other oxidase enzymes.

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