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Prussian blue modified glassy carbon electrodes—study on operational stability and its application as a sucrose biosensor

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Abstract

Stabilisation of electrochemically deposited Prussian blue (PB) films on glassy carbon (GC) electrodes has been investigated and an enhancement in the stability of the PB films is reported if the electrodes are treated with tetrabutylammonium toluene-4-sulfonate (TTS) in the electrochemical activation step following the electrodeposition. A multi-enzyme PB based biosensor for sucrose detection was made in order to demonstrate that PB films can be coupled with an oxidase system. A tri-enzyme system, comprising glucose oxidase, mutarotase and invertase, was crosslinked with glutaraldehyde and bovine albumin serum on the PB modified glassy carbon electrode. The deposited PB operated as an electrocatalyst for electrochemical reduction of hydrogen peroxide, the final product of the enzyme reaction sequence. The electrochemical response was studied using flow injection analysis for the determination of sucrose, glucose and H_2O_2. The optimal concentrations of the immobilisation mixture was standardised as 8 U of glucose oxidase, 8 U of mutarotase, 16 U of invertase, 0.5% glutaraldehyde (0.025 μl) and 0.5% BSA (0.025 mg) in a final volume of 5 μl applied at the electrode surface (0.066 cm^2). The biosensor exhibited a linear response for sucrose (4–800 μM), glucose (2–800 μM) and H_2O_2 (1–800 μM) and the detection limit was 4.5, 1.5 and 0.5 μM for sucrose, glucose and H_2O_2, respectively. The sample throughput was ca. 60 samples h^{-1}. An increase in the operational and storage stability of the sucrose biosensor was also noted when the PB modified electrodes were conditioned in phosphate buffer containing 0.05 M TTS during the preparation of the PB films.

Keywords: Biosensors; Flow injection; Amperometry; Sucrose; Prussian Blue; Tetrabutylammonium toluene-4-sulfonate

1. Introduction

Determination of hydrogen peroxide is important in several fields ranging from determination of very low concentrations in environmental and biological samples to high concentrations in cosmetics [1] and sterilisation solutions. Electrochemistry is a science encompassing several techniques, which can meet the demands for sensitive and linear detection of several electroactive species. It is possible to electrochemically monitor hydrogen peroxide from several orders of magnitude to concentrations lower than the μM level [2]. Electrochemical instrumentation is also reasonably cheap and can be used in field measurement. However, direct electrochemical detection of hydrogen peroxide, either oxidation or reduction, suffers from high overvoltage at conventional solid electrodes [3]. Several attempts have been made in order to produce chemically modified electrodes, at which the overvoltage for either the electrochemical oxidation or reduction reaction can be reduced so that measurements can be performed at ca. 0 mV versus Ag|AgCl and in neutral pH electrolytes. Such attempts include the use of finely dispersed noble metal(s) and heavy metal oxides [4–9], a variety of peroxidases [4,10]. However, all such modified electrodes either lack selectivity or long term stability. One possible approach to solve this problem would be the use of Prussian blue (PB) modified electrodes [11,12]. Electrodes modified with PB allow detection of hydrogen peroxide through electrocatalytic reduction at potentials below +100 mV versus Ag|AgCl and can be made selective for hydrogen peroxide even in the presence of oxygen [13–18].

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PB is a ferriferrocyanide complex, which can act as an electrocatalyst for hydrogen peroxide reduction. It is electrochemically reduced to form Prussian white (Everitt's salt), which is capable of catalysing the reduction of hydrogen peroxide at low potentials (−50 mV) like peroxidases hence it is also referred to as "artificial peroxidase" [12,16]. The PB complex can exist in two different states, *viz*. insoluble ($Fe^{(III)}{}_4(Fe^{(II)}(CN)_6)_3$) and soluble state ($KFe^{(II)}Fe^{(III)}(CN)_6$). The catalytic action of PB towards H_2O_2 reduction can be described as follows:

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

$$\underset{\text{Prussian white}}{K_4Fe_4{}^{(II)}[Fe^{(II)}(CN)_6]_3} + 2H_2O_2 \rightarrow \underset{\text{Prussian blue}}{Fe_4{}^{(III)}[Fe^{(II)}(CN)_6]_3} + 4OH^- + 4K^+ \quad (2)$$

Thus, the net reaction is the reduction of H_2O_2 to hydroxide ions:

$$H_2O_2 + 2e^- \rightarrow 2OH^- \quad (3)$$

However, there still exists some drawbacks associated with PB films that need to be solved before PB modified electrodes can be used as reliable sensors for the detection of hydrogen peroxide in real applications. The reduced form of PB, Prussian white, is dissolved by hydroxide ions at high pH values and also by those formed during the catalytic cycle (Eq. (2)), hence PB-modified electrodes have a restricted lifetime at pHs above 7 and it is only Prussian white, which has catalytic activity towards hydrogen peroxide reduction.

In 1999 Lin and Shih [19] proposed the use of tetrabutylammonium toluene-4-sulfonate (TTS) in the working solution to stabilise electrodes modified with chromium hexacyanoferrate. Later it was shown that also PB films deposited on screen printed Au electrodes were stabilised when TTS was added to the carrier stream in FIA experiments [20]. A rather high concentration of TTS was necessary (0.05–0.1 M) to obtain stabilisation and it would be too expensive to run buffers containing such high concentrations of TTS for practical applications. In this paper it is demonstrated that the addition of TTS during the electrodeposition of PB stabilises the PB films from both faradaic as well as nonfaradaic decay. In the present paper we have studied the effect of TTS in the activation step (*vide infra*) during the preparation of the PB films. The stability of the PB film was evaluated both with cyclic voltammetry (CV) and through the variation of the response to 0.1 mM hydrogen peroxide when the PB modified electrode was connected to a FIA system. It was observed that the addition of TTS in one of the steps during the preparation of the PB film results in an enhancement of its operational stability.

Hydrogen peroxide detection is very important in conjunction with enzyme based amperometric biosensors as most of the commonly used enzymes in biosensor development are oxidases, which yield hydrogen peroxide as one of the products. A number of oxidases, such as glucose, oxidase, alcohol oxidase, L-glutamate oxidase, choline oxidase, lactate oxidase [12,21–23], have been immobilised on PB modified electrodes. Various techniques of enzyme immobilisation on the PB film have been investigated. Lack of a suitable functional group on the PB complex limits the enzyme system to be either coupled covalently or strongly adsorbed. Modification of PB films functionalised with isonicotinamide have been studied [24]. Several publications report on monoenzyme systems coupled to PB modified electrodes [12,21,22] and even a bienzyme system has been reported [25]. Hence, it was of interest to study a three enzyme system and we have co-immobilised three enzymes, invertase, mutarotase, and glucose oxidase (INV/MUT/GOX), together to make a sensitive sucrose sensor in order to emphasise the possibility of coupling PB films with an enzyme reaction sequence requiring more than one or two enzymes. PB was electrodeposited on glassy carbon electrode followed by cross-linking INV/MUT/GOX with bovine serum albumin and glutaraldehyde. The analytical characteristics of the biosensor with respect to sucrose, glucose and H_2O_2 detection were investigated using FIA. Additionally several parameters effecting the sensor characteristics of a sensitive sucrose biosensor were investigated.

2. Experimental

2.1. Materials, reagents and standards

Glucose oxidase (GOX, EC 1.1.3.4, from *Aspergillus niger*, 286 U mg^{-1}) was obtained as a lyophilised powder from Biozyme (South Wales, Great Britain). Invertase (INV, lyophilised powder, EC 3.2.1.26, from yeast, 252 U mg^{-1}), bovine serum albumin (BSA, fraction V, minimum 96%) and glutaraldehyde (GLU, grade 1, 25%) were purchased from Sigma (St. Louis, MO, USA). Mutarotase (MUT, EC 5.1.3.3, from hog kidney, 5000 U mg^{-1}) was obtained as a solution from Roche Applied Science (Stockholm, Sweden). Sucrose and glucose were obtained from BDH (Poole, UK) and Merck (Darmstadt, Germany), respectively. Nafion perfluorinated ion-exchange (5% solution in 90% light alcohol) was obtained from Aldrich (Steinheim, Germany). All inorganic chemicals including KH_2PO_4, KCl, $FeCl_3$, $K_3[Fe(CN)_6]$, HCl and H_2O_2 (30%) were of analytical grade and obtained from Merck. Tetrabutylammonium toluene-4-sulfonate was procured from Fluka (Buchs, Switzerland). All enzymes and buffer solutions were prepared using water purified from Milli-Q system (Millipore, Milford, CT, USA).

GOX and INV (1000 U) were dissolved in 125 μl of water. MUT (800 U) was diluted to 100 μl with water. Working solutions of BSA and GLU at the required concentrations

were prepared in water. 0.05 M KH_2PO_4 and 0.1 M KCl were used to prepare buffers, the pH adjustments were made using KOH. Stock solutions of glucose (1 M), sucrose (1 M) and H_2O_2 (0.1 M) were prepared in water and stored at 4 °C when not in use. Standard working solutions of glucose, sucrose and H_2O_2 were prepared freshly by diluting the stock solutions with buffer.

2.2. Apparatus

Electrochemical deposition of PB and cyclic voltammetric studies were performed using an EG&G potentiostat-galvanostat model PAR 273 (Princeton, NJ, USA). A conventional three-electrode set-up was used, a platinum sheet was used as the auxiliary electrode and a saturated calomel (SCE) electrode as the reference electrode. PB modified glassy carbon electrodes (2.9 mm diameter, type GC-2500, the Institute of Graphite Materials, Moscow, Russia) [26], kindly provided by Dr. A.A. Karyakin, Moscow State University were used as working electrodes.

FIA studies were done using a single line manifold with a three-electrode electrochemical cell of the wall-jet type [27]. A reference electrode (Ag|AgCl) was present in a circular chamber filled with 0.1 M KCl supplied with an external syringe. A separate chamber containing the working and a Pt counter electrode (encircling the chamber) was connected with the chamber containing the reference electrode through four holes concentrically surrounding the inlet. The working electrode used was PB modified glassy carbon. The distance between the inlet nozzle and working electrode was about 2 mm. A Gilson peristaltic pump (Minipulse 2, Villiers-le-Bel, France) equipped with Tygon tubing (0.89 mm i.d.) propelled the carrier solution into the flow line (Teflon tubing, 0.5 mm i.d.). The samples with substrates (50 μl) were injected via a LabPRO six-port injection valve (PR700-100-01, Rheodyne, CA, USA) into the carrier stream. A potentiostat (Zäta Electronics, Lund, Sweden) was connected to the electrochemical cell in the standard way. The output signals were recorded using a strip chart recorder (Kipp and Zonen, type BD111, Delft, The Netherlands).

2.3. Electrode preparation

2.3.1. Cleaning and polishing electrode material

Rods of glassy carbon electrodes were polished with alumina paste (Al_2O_3, 0.25 μm, Struers, Copenhagen, Denmark) to obtain a mirror-like surface (0.066 cm^2) followed by sonication for 2 min.

2.3.2. Electrodeposition of PB films

PB modified GC electrodes were prepared as described elsewhere [28]. The CV of the polished GC was checked prior to deposition of PB to ensure cleanliness of the surface. PB modified electrodes were prepared in three steps as given below:

1. Deposition: A solution containing 2 mM $FeCl_3$, and 2 mM $K_3[Fe(CN)_6]$ in 0.1 M HCl and 0.1 M KCl was deareated by purging Argon for 10 min. PB was deposited on GC rods by applying a potential of +400 mV for 40 s.
2. Activation: The electrode was cycled 20 times from −50 to 350 mV at a scan rate of 50 mV s^{-1}. The electrolyte used was 0.1 M KCl and 0.1 M HCl. In order to check the effects of TTS on the stability, the electrode was cycled 10 times in 0.05 M TTS in the activation step at a scan rate of 10 mV s^{-1}. The electrode was then dried at 105 °C for 60 min and then allowed to cool down to room temperature.
3. Conditioning: A potential of −50 mV was applied for 10 min in 0.05 M KH_2PO_4 and 0.1 M KCl (pH 5.5). The CV was finally recorded in order to determine the amount of PB deposited.

2.3.3. Preparation of sucrose sensor

Various concentrations of enzymes (INV, MUT, GOX), BSA and GLU were standardised in a final volume of 5 μl. This mixture was placed on the surface of the PB-modified glassy carbon electrode and allowed to dry at room temperature for 1 h. Some of the PB modified electrodes were conditioned in 0.05 M TTS to obtain stable PB films. When the enzymes were immobilised in Nafion the same procedure was used as described previously [20].

3. Results and discussion

The CV of PB modified electrodes show an oxidation peak at ca. 170 mV and a reduction peak ca. 100 mV versus Ag|AgCl, see Fig. 1. If the electroactive material is immobilised on the electrode surface and the electron transfer across the immobilised species and electrode is facile then a separation between the anodic and cathodic peak potentials (ΔEp) of 0 mV should ideally be observed [29]. However, a ΔEp of ca. 70 mV is observed indicating that the electron transfer across the PB film and the electrode is rate limiting. Decreasing the thickness of PB layer causes a decrease in the ΔEp of the CV, hence this sluggishness can be attributed due to the thickness of the PB films, which results in high impedance.

3.1. Stability of PB films

It was observed that the PB films show decay due to both non-faradaic (electrolyte and buffer at high pH) as well as faradaic processes (due to substrate). The decay of the PB film is associated with the presence of OH^- at high pH buffers and its generation due to reduction of substrate as shown in reaction (3) (*vide supra*) [15]. The OH^- thus formed is responsible for the cleavage of the PB complex due to the formation of ferric hydroxide according to the

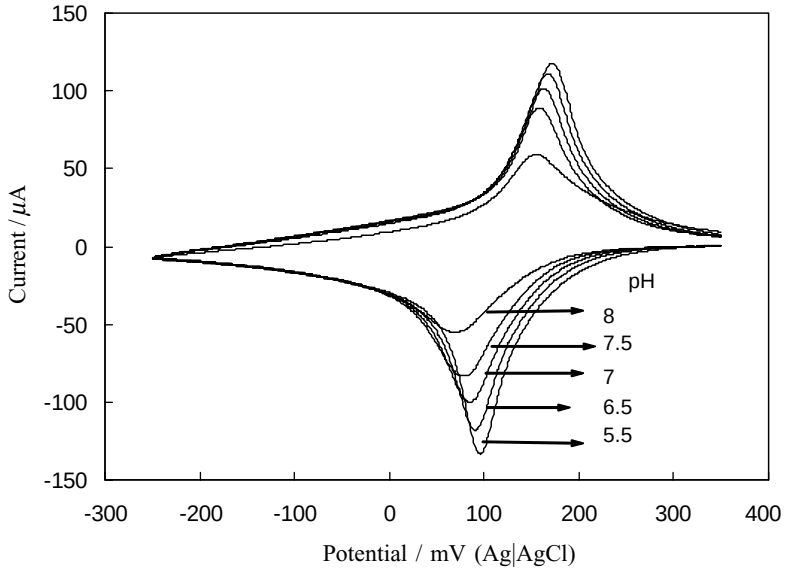

Fig. 1. Effect of pH of the carrier buffer (non-faradaic processes) on the CV of a PB modified GC electrode. The buffer was allowed to pass the flow through cell at a flow rate of 0.8 ml min^{-1} and the CVs were recorded every 5 min for a period of 30 min. Scan rate, 50 mV s^{-1}; carrier buffer, 0.05 M KH_2PO_4 and 0.1 M KCl adjusted to pHs of 5.5, 6.5, 7, 7.5 and 8. The CVs shown are for a single GC/PB modified electrode registered after 30 min at each pH.

reaction given below.

$$\underset{\text{Prussian blue}}{Fe_4^{(III)}[Fe^{(II)}(CN)_6]_3} + 12\,OH^- \rightarrow 4\,Fe(OH)_3 + 3\,Fe^{(II)}(CN)_6^{4-} \quad (4)$$

The decay of PB films due the above-mentioned processes was therefore studied in more detail. The effect of TTS was studied in two experimental set-ups (a) addition of TTS to the flow stream and (b) addition of TTS in the activation step.

3.1.1. Decay due to non-faradaic processes (CV studies)

The effect of the pH of the carrier stream of the flow system on the stability of the PB modified electrode was studied. A series of carrier buffer solutions with various pH values, 5.5, 6.5, 7, 7.5 and 8, were allowed to flow into the flow through cell (flow rate: 0.8 ml min^{-1}) and a CV was recorded every 5 min and measurements were repeated at least three times on a fresh new electrode. In between measurements open circuit potential was applied to the PB modified electrode. At pH 5.5 a slight initial increase in the peak height and area was observed due to an increase in the conductivity of the PB lattice cubes followed by a virtually stable electrode with time. A clear decrease in the peak current intensity of the cyclic voltammograms with an increase in the pH was observed (Figs. 1 and 2) especially at pHs higher than 7 indicating loss of the immobilised electroactive PB layer, which occurs due to dissociation of the PB complex into ferric hydroxide caused due to the presence of OH^- ions in the electrolyte (Fig. 2). It was, however, observed that the electrode when exposed to a buffer solution containing additionally 0.05 M TTS for 1 h showed an increased stability even when the carrier after 1 h was changed back to a buffer with no TTS seen in Fig. 2. The presence of TTS in the carrier stream, however, caused a decrease in the magnitude of the current (Fig. 2), however, when changing back to a plain buffer the current increased as well as the stability of PB. The reason for the decrease in current when TTS was added to the buffer remains unknown but could possibly be due to the high viscous nature of the TTS solution, which results in decreased mobility of electrolyte ions (K^+) participating in the redox reaction at the electrode surface.

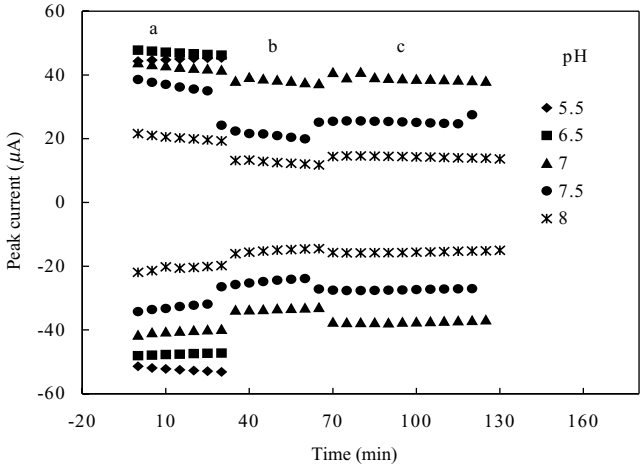

Fig. 2. Effect of pH on the intensity of the redox peak current (upper–anodic peak current, lower–cathodic peak current) as a function of time as seen in CVs. CVs were recorded every 5 min for a period of 30 min in a flow through system (0.8 ml min^{-1} flow rate): (a) with carrier buffer; (b) with carrier buffer containing 0.05 M TTS; and (c) when reverting back to the carrier buffer without TTS.

3.1.2. Decay of PB due to the Faradaic processes (FIA studies)

The operational stability of the PB electrode was studied using classical FIA experiments applying a potential of −50 mV [20,28]. A sample containing 100 μM H_2O_2 was injected every 1 min into the carrier buffer consisting of 0.05 M KH_2PO_4 and 0.1 M KCl adjusted to pH 7 and the reduction current caused by the electrocatalytic process (reactions (1) and (2)) was measured. The electrode not activated in TTS and investigated in plain buffer exhibited stable catalytic currents (peak height) in FIA for approximately 100 min, which was followed by a pronounced decrease in response to 100 μM H_2O_2 with time (Fig. 3). The stability of PB films has thus been substantially improved compared to our earlier results, where a H_2O_2 concentration of 100 μM H_2O_2 and at pH 7 resulted in a linear decay of the activity of immobilised PB [15], the reasons being mainly due to the deposition-activation-conditioning scheme developed [28,30] and also due to the choice of glassy carbon support shown previously in a recent publication [26]. At the concentration of substrate chosen (100 μM H_2O_2) any clear stabilising effect of adding 0.05 M TTS to the carrier buffer flow could not be seen (not shown) clearly, in contrast to when PB modified screen printed Au electrodes were investigated [20]. The stability effect of PB could, however, be clearly observed when the PB electrode had been activated in 0.05 M TTS during the preparation of the film. The electrodes activated in 0.05 M TTS retained more than 90% of maximum peak response height after approximately 220 min under identical conditions as seen in Fig. 3. Both electrodes showed an increase in response for the initial 60 min. The electrode activated in TTS clearly shows longer stability, ca. 220 min compared to the electrode not activated in TTS, which was stable for ca. 100 min. The addition of TTS thus helps in preventing the dissociation of the PB complex due to OH^- formed during the catalysis (faradaic processes). This prevention of dissociation of PB films could be due to formation of weak molecular forces of interaction (van der Waals type or hydrogen bonding) between PB and TTS.

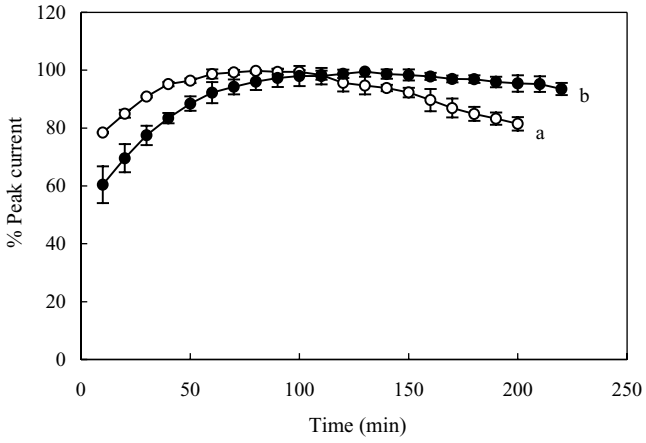

Fig. 3. Responses of a GC/PB modified electrode to repetitive injections of 100 μM H_2O_2 (Faradic processes). GC/PB electrodes ($n = 3$) not been activated in TTS (a) and activated in 0.05 M TTS (b). Each point is the average of 10 successive peaks obtained in FIA experiment. An applied potential of −50 mV, a carrier buffer of 0.05 M KH_2PO_4 0.1 M KCl adjusted to pH 7, a flow rate of 0.8 ml min^{-1} and an injection volume of 50 μl were used.

Various ways, which prevent the negative influence of hydroxide ions on PB modified electrodes, can play a significant role in the stabilisation of PB films. Stabilisation of PB films with non-conducting polymers like poly(*o*-diaminobenzene) [31]and conducting polymers such as poly(4,4′-bis(butylsulphanyl)-2,2′-bithiophene) [32] have been reported in the literature. The use of conducting polymers, e.g., polypyrrole and polyaniline [33], could help improving the stability of PB films on various electrode materials including gold, which is a poor electrode material for PB stability. TTS is a conjugate zwitterionic compound. It is expected to stabilise the PB film by weak molecular forces of interactions such as van der Waals type or hydrogen bonding. There is no change in the pH of the buffer upon the addition of TTS due to its conjugated zwitterionic nature, confirmed in our studies. Quaternary ammonium ions act as strong acids hence molecules like tetraalkylammonium and phosphonium ions may also stabilise the PB films [34,35]. Better stabilisation effects and immobilisation can be expected if alkylated PB derivatives complexes can be synthesised. This remains a possibility to be explored.

3.2. Sucrose biosensor

Sucrose (commonly known as table sugar) is a major ingredient of most sweet drinks and is also an important analyte in clinical analysis. It is a disaccharide composed of one α-D-glucose unit and one β-D-fructose unit linked by a α-1,4-glycosidic bond. Hydrolysis of sucrose catalysed by invertase (INV) yields an equimolar ratio of α-1,4-glucose and β-D-fructose. Glucose oxidase (GOX) can then be used to oxidise the glucose formed. However, as GOX is only active for the β-form of glucose, mutarotase (MUT) is usually added to speed up the interconversion of α-1,4-glucose into β-1,4-glucose. Most of the sucrose biosensors made were either by random or asymmetric co-immobilisation of the three enzymes on an electrode or by integrating a glucose electrode to an enzyme reactor containing invertase and mutarotase [36] based on the sequence of the following enzymatic reactions:

$$\text{Sucrose} + H_2O \xrightarrow{\text{INV}} \text{D-Fructose} + \alpha\text{-D-Glucose} \quad (5)$$

$$\alpha\text{-D-Glucose} \overset{\text{MUT}}{\leftrightarrow} \beta\text{-D-Glucose} \quad (6)$$

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{GOX}} \text{D-Glucono-1, 5-lactone} + H_2O_2 \quad (7)$$

Either oxygen consumption or hydrogen peroxide formation can be measured amperometrically. Oxygen

consumption can be measured using a simple oxygen electrode at –600 mV versus Ag|AgCl [37–44] and hydrogen peroxide production can be monitored using a platinum electrode at ca. +600 mV versus Ag|AgCl [45–48]. Several methods have been reported to monitor the same enzymatic reaction. For instance the use of phosphate ions (to accelerate the interconversion of α-D-glucose to β-D-glucose) and yeast cells (source of invertase) has been reported [40]. Electrodes, which measure mediated electron transfer between GOX and graphite paste electrode using redox mediators such as tetracyanoquinodimethane have also been investigated [49]. Poly(vinylferrocinium) (a polymer which contains ferrocinium acting as mediator) was used to connect GOX with the electrode [50]. Kogure et al. [51] have described a new enzymatic cascade based on sucrose phosphorylase (sucrose receptor) coupled to phosphoglucomutase and glucose-6-phosphate-1-dehydrogenase. All the three proteins were immobilised in a reactor and conjugated with a flow injection analysis (FIA) set-up, in which spectroflourimetric detection of catalytically produced NADPH upon the addition of $NADP^+$ into the carrier stream and the response was linearly dependent on the concentration of sucrose. Maestre et al. [52] proposed a new sucrose biosensor based on the tri-enzyme configuration as described above and electrocatalytic detection and recycling of NADH by Os(4,4′-dimethyl-2,2′-bypyridine)$_2$(1,10-phenanthroline-5, 6-dione) redox couple was observed.

The use of INV/MUT/GOX as enzyme systems for the detection of sucrose limits the sensor from differentiation between sucrose and glucose. If glucose is also present in the sample interferences of the glucose signals with sucrose signals are seen. Hence glucose has to be either removed from the sample [47] or the corresponding glucose response must be subtracted from the total response [41,53–56].

3.2.1. Optimisation of sucrose biosensor

As the surface of PB does not contain any functionality that readily allows covalent binding of enzymes other strategies for enzyme immobilisation must be developed. Previously it was shown for several oxidases that they could be immobilised either together with a Nafion solution or could be covered with a layer of Nafion after being first adsorbed onto the PB surface [16,23,57]. As Nafion is a negatively charged polymer it not only acts as an entrapping material for enzyme immobilisation but also excludes to a large extent negatively charged interfering compounds such as ascorbate and urate common in real samples. Initial experiments were therefore performed trying to use Nafion as polymer for the three enzymes, GOX, MUT and INV. Two different immobilization procedures were investigated using 0.2% and 0.3% Nafion solutions that were first neutralised to pH 6: In the one step procedure the three enzymes and Nafion were mixed together and then a 5 μl solution of the mixture was spread onto the electrode surface [16,57]. In the other two step procedure a 5 μl aqueous solution containing the three enzymes was first applied onto the electrode surface and after drying at room temperature an additional 5 μl Nafion solution was added on top of the adsorbed enzyme layer [23]. However, in no case any response signal was observed for glucose or sucrose, revealing the incompatibility of using Nafion for construction of a sucrose sensor. Therefore another means to coimmobilise the three enzymes was used *viz*. the classic combination of glutaraldehyde (GLU) and bovine serum albumin (BSA) [58]. The developed biosensors were after enzyme immobilisation tested using FIA. The responses of the modified electrodes to sucrose, glucose and hydrogen peroxide were investigated. Parameters such as optimal concentrations of enzyme, BSA and GLU, pH, applied potential and carrier flow rate were standardised.

3.2.2. Optimisation of BSA concentration

Initial experiments were performed using a 5 μl mixture containing 4 U of each enzyme. Increasing the concentration of BSA from 0 to 2.0% (w/v) at a fixed concentration of GLU (1% (v/v)) resulted in a linear increase in the relative response of sucrose to glucose from 4 to 30% and an increase in the relative response of glucose to H_2O_2 from 26 to 30% when increasing the concentration of BSA from 0 to 0.5% (w/v). Further increases in BSA concentration (0.5 to 2%) resulted in a declining relative response of glucose to H_2O_2 from 30 to 10%. Increasing the BSA concentration to more than 0.5% (w/v) resulted in physical instability of the enzyme film. Physically stable films could be obtained if 0.5% BSA (w/v) was used in the reaction mixture and therefore further experiments with a BSA concentration of 0.5% were performed.

3.2.3. Optimisation of GLU concentration

The effect of varying the GLU concentration at a fixed amount of BSA (0.5% w/v) indicated that the relative response of sucrose to glucose was maximum (ca. 50%) at ca. 0.5% (v/v) GLU. Apart from this the relative response of glucose to H_2O_2 was maximum (ca. 35% conversion) at about 0.5% (v/v) GLU. Thus, the combination of 0.5% v/v glutaraldehyde and 0.5% w/v BSA was standardised for crosslinking all the three enzymes investigated and a maximum conversion of sucrose to glucose to H_2O_2 was observed. Additionally the membrane formed was quite stable and did not fall off the electrode surface.

3.2.4. Optimisation of enzyme concentrations

An increase in the enzyme concentration can enhance the electrode response but a very thick enzyme layer can also acts as a diffusion barrier [59]. In order to optimise the enzyme mixture for better film stability and response each enzyme concentration in the mixture (5 μl) was varied between 0 and 16 U keeping the other enzyme concentrations as 8 U. Fig. 4 shows the effect of the concentration of GOX on the electrode responses to sucrose, glucose and H_2O_2. It can be clearly seen that 8 U of GOX on the electrode surface produce the highest response to sucrose and glucose, but the electrode response to H_2O_2 decreases slightly

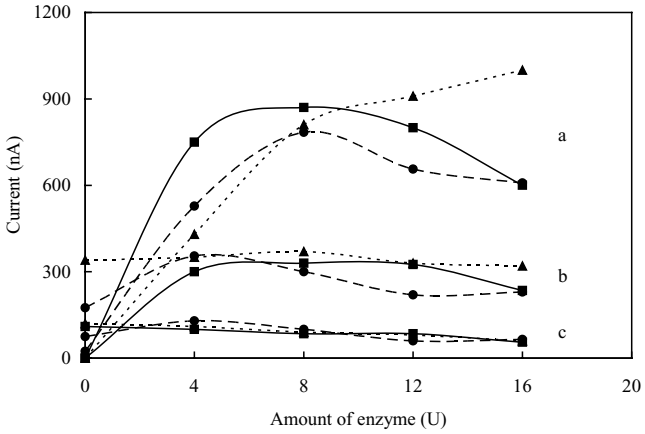

Fig. 4. Influence of amounts of GOX (■), MUT (●) and INV (▲) on the FIA responses of the GC/PB/enzyme modified electrodes to: (a) 500 μM sucrose; (b) 100 μM glucose; and (c) 10 μM H_2O_2. The amount of investigated enzyme was varied from 0 to 16 U while the amounts of the other two enzymes were kept constant at 8 U of each. Carrier: phosphate buffer at pH 6.0 (0.05 M KH_2PO_4/K_2HPO_4 + 0.1 M KCl); carrier flow rate: 0.8 ml min^{-1}; operating potential: −50 mV vs. Ag|AgCl; sample volume: 50 μl.

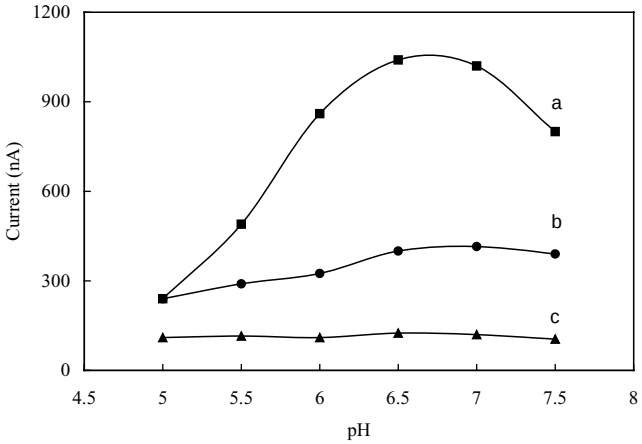

Fig. 5. pH profiles of the FIA responses of the GC/PG/enzyme modified electrode to: (a) 500 μM sucrose; (b) 100 μM glucose; and (c) 10 μM H_2O_2. The amounts of GOX, MUT and INV on the electrode surface were 8, 8, 16 U, respectively. Carrier stream: phosphate buffer (0.05 M KH_2PO_4/K_2HPO_4 + 0.1 M KCl) adjusted to various pHs; carrier flow rate: 0.8 ml min^{-1}; operating potential: −50 mV vs. Ag|AgCl; sample volume: 50 μl.

with increasing the GOX concentration. Varying the concentration of MUT (Fig. 4) shows that the highest electrode response to sucrose was observed when 8 U of MUT were used, but the optimum amount of MUT on the electrode surface for the determination of glucose and H_2O_2 is about 4 U. The results thus obtained indicate that the electrode response to sucrose increases with an increase in the amount of INV but the response to glucose and H_2O_2 decreases slightly when increasing the amount of INV on the electrode surface (Fig. 4). Thus the 5 μl mixture used for crosslinking the enzymes on the electrode surface (0.066 cm^2) was standardised to contain 8 U of GOX, 8 U of MUT and 16 U of INV, 0.5% BSA and 0.5% GLU and used for further studies.

3.2.5. Effect of pH

The effect of pH of the carrier solution 0.05 M phosphate buffer containing 0.1 M KCl adjusted to pHs ranging from 5 to 7.5 was investigated. The electrode responses to sucrose, glucose and H_2O_2 are shown in Fig. 5, the optimum pHs for determination of sucrose and glucose are 6.5 and 7.0, respectively, which is approximately close to the pH optima for GOX (5.5) and MUT (7.4) [46]. A change in pH of carrier solution has no significant effect on the response of the biosensor to H_2O_2. A pH of 6.5 was therefore selected as pH optima for the phosphate buffer based carrier for further study.

3.2.6. Effect of flow rate

The effect of the flow rate (0.1 to 1.0 ml min^{-1}) of the carrier stream (0.05 M phosphate buffer, pH 6.5, containing 0.1 M KCl) on the response of the three analytes was studied (Fig. 6). Increasing the flow rate would increase the mass transfer to the electrode surface but at the same time the dispersion factor of the injected sample plug will increase. The slight decrease in response for H_2O_2 as the flow rate is increased should be a result of increased dispersion. For glucose the somewhat more pronounced decrease in response compared to that of H_2O_2 reflects the additional influence of enzyme kinetics on the response as well as loss of H_2O_2 to the carrier solution. For sucrose at low flow rates, the sensor response was high (due to longer residence time), in contrast at higher flow rates the response decreases significantly reflecting pronounced kinetic restrictions in the reaction sequence (reactions (5) and (6)). Considering two important characteristics of the biosensor i.e. sensitivity and sample through put, a flow rate of 0.35 ml min^{-1} was selected for further studies.

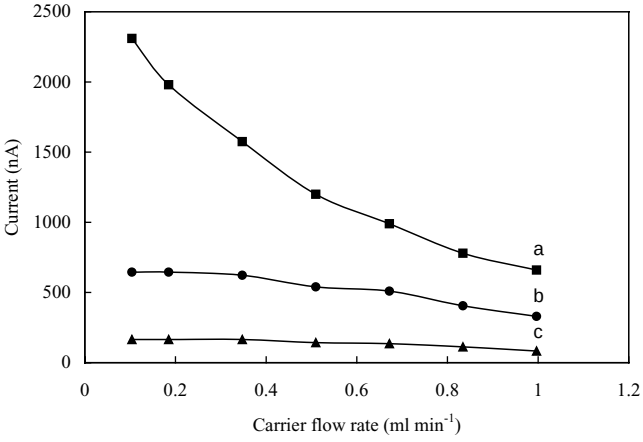

Fig. 6. Effect of flow rate on the FIA responses of the GC/PB/enzyme modified electrode to: (a) 500 μM sucrose; (b) 100 μM glucose; and (c) 10 μM H_2O_2. The amounts of GOX, MUT and INV on the electrode surface were 8, 8, 16 U, respectively. Carrier stream: phosphate buffer (0.05 M KH_2PO_4/K_2HPO_4 + 0.1 M KCl adjusted to pH 6.5); operating potential: −50 mV vs. Ag|AgCl; sample volume: 50 μl.

3.2.7. Effect of applied potential

The effect of the applied potential on the electrochemical response was studied and an optimum potential of −50 mV versus Ag|AgCl was [13–18] found optimal confirming previous studies on H_2O_2 detection at PB modified electrodes. Working at around 0 mV versus Ag|AgCl is desirable in practical applications as the immobilised protein molecules are less susceptible to potential induced degradation and it also decreases non-specific responses to easily oxidisable interfering compounds common in real samples [4].

3.3. Analytical characteristics of the sucrose biosensor

Amperometric FIA responses of the modified electrode to sucrose, glucose and H_2O_2 injections were studied in terms of sensitivities, detection limits and linear dynamic ranges and the results are shown in Table 1. The relative response of sucrose to glucose and glucose to H_2O_2 were ca. 80 and 35%, respectively, using freshly prepared enzyme solutions, but upon storage of the enzyme solutions (less than three weeks), the relative responses of sucrose to glucose and glucose to H_2O_2 reduced to 55 and 30%, respectively. It was observed that upon storing the activity of MUT declines at a faster rate than that of GOX and INV. The relative standard deviation of the optimised sucrose biosensor (100 μM, $n = 15$) was 1.2% and the reproducibility for five electrodes, prepared and used on different days was about 8%. The analysis rate for sucrose was about 60 samples h^{-1}.

3.4. Interferences

In order to examine the electrochemical responses of potent interfering compounds (in food samples) responses of various metabolites such as amino acids (glycine, alanine and valine), sugars and ascorbic acid were studied. The results are shown in Table 2, and as can be seen from the data the electrode did not respond to the amino acids investigated. Ascorbic acid contributed as 24% and 10% error at 100 and 50 μM, respectively. The only sugar that responded was glucose, no contributions from other sugars were observed. This can be attributed to the presence of GOX in the enzyme mixture at the electrode surface. The interference from glucose can be totally eliminated using different procedures [41,47,54,55,60,61].

3.5. Stability

The operational stability of the sucrose biosensor was studied by injecting 100 μM sucrose and the response as a function of time under optimised conditions was observed. After repeated use of the electrode for more than 40 min (30 assays) no decline in the response was observed. This was followed by a linear decay of the response signal with time (Fig. 7). The sensor lost 70% activity after 300 min (195 as-

Table 2
Effect of other compounds on the response of the sucrose biosensor

Substances	Relative response (%)
Sugars	
100 μM sucrose	100
+100 μM maltose	103
+100 μM fructose	101
+100 μM galactose	102
+100 μM glucose	286
Ascorbic acid	
100 μM sucrose	100
+100 μM ascorbic acid	124
+50 μM ascorbic acid	110
Amino acids	
100 μM sucrose	100
+100 μM glycine	101
+100 μM alanine	101
+100 μM valine	100

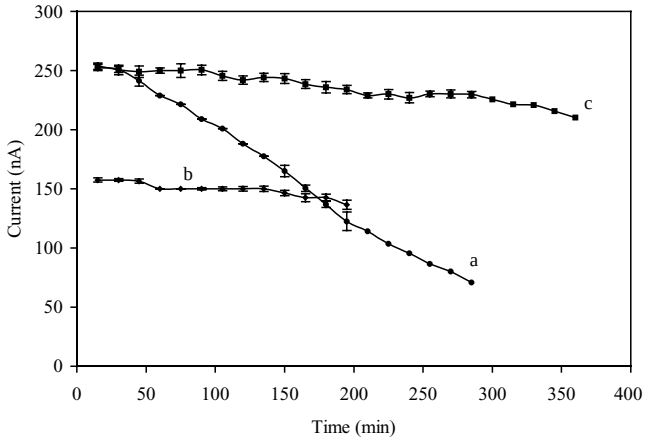

Fig. 7. FIA responses of the optimised sucrose biosensor to 100 mM sucrose as a function of time. Each point is the average of 10 successive peaks obtained in FIA experiment. The amounts of GOX, MUT and INV on the electrode surface were 8, 8, 16 U, respectively. Operating potential: −50 mV vs. Ag|AgCl; carrier flow rate: 0.35 ml min^{-1}; sample volume: 50 μl. Carrier stream: (a) phosphate buffer (0.05 M $KH_2PO_4/K_2HPO_4 + 0.1$ M KCl) adjusted to pH 6.5; (b) phosphate buffer (0.05 M $KH_2PO_4/K_2HPO_4 + 0.1$ M KCl + 0.05 M TTS) adjusted to pH 6.5; (c) phosphate buffer (0.05 M $KH_2PO_4/K_2HPO_4 + 0.1$ M KCl) pH 6.5 but the PB modified GC electrode was activated in 0.05 M TTS during the preparation of the PB modified electrode prior to enzyme immobilisation.

Table 1
Calibration data for sucrose, glucose and H_2O_2 detection using the optimised sucrose biosensor in flow injection amperometric measurements

Substrate	Intercept (nA)	Slope (nA μM^{-1})	r^2	Detection limit (μM)	Linear dynamic range (μM)
Sucrose	34.33	2.82	0.9992	4.5	4–800
Glucose	11.90	3.44	0.9999	1.5	2–800
H_2O_2	36.26	9.87	0.9992	0.5	1–800

says), compared to the initial response. The possible cause could be either the inactivation of immobilised enzymes and/or the disintegration of PB layer due to the high amount of hydroxyl ions produced [15]. It is known that immobilised GOX is highly stable [62,63]. Some researchers reported that H_2O_2 could deactivate MUT [64]. Additionally, Zhang et al. [48] reported that the stability of immobilised INV was the limiting factor for the stability of their sucrose sensor. From CV studies before and after sucrose and/or glucose and/or H_2O_2 injections a faster decrease of PB was revealed if high concentrations of substrates were used (not shown). Hence, it can be concluded that the disintegration of the PB layer is the one of the main factors responsible for the instability of the proposed sucrose biosensor. Approximately 70% loss in the PB films can be seen in Fig. 7(a) where the flow rate used was 0.35 ml min^{-1} compared to ca. 20% decrease as seen in Fig. 3(a) where the flow rate used was 0.8 ml min^{-1}. The reason to this behaviour is the use of a lower flow rate results in a longer exposure of the electroactive plug to the modified electrode hence the decay is more pronounced compared to higher flow rates. The effect of TTS was therefore also investigated. In order to stabilise the PB film 0.05 M TTS was added to the carrier buffer (0.05 M phosphate buffer, pH 6.5, containing 0.1 M KCl) and the long term stability of the biosensor was examined by injecting 100 μM sucrose. The results indicate that the use of TTS in the carrier buffer improved the operational stability of the sensor (Fig. 7). The electrodes were stable and lost less than 8% activity during repeated use for more than 200 min (130 assays), but the current intensity of the sensor decreased with 40%, compared to the initial response of the sensor in the absence of TTS. The decrease in sensitivity in the presence of 0.05 M TTS is in accordance with the results shown above for plain PB modified electrodes and is supposedly due to a decrease in of the mobility of electrolyte ions.

However, when the electrodes were conditioned in 0.05 M KH_2PO_4/K_2HPO_4, 0.1 M KCl (pH 6.5) also containing 0.05 M TTS, the operational stability of these electrodes studied under identical conditions indicates that the sensor was stable and lost less than 14% activity in repeated use over 370 min (240 assays) and the signal intensity of the sensor was the same as the initial response of the sensor in the absence of TTS, as seen in Fig. 7. In addition to operational stability, the storage stability was also improved by conditioning the PB films in the presence of 0.05 M TTS. The sensor was stable up to three days at 4 °C in a vessel seal with Parafilm.

4. Conclusion

In the present article an enhancement in the operational stability of PB films is reported when TTS was used in one of the steps in the electrodeposition developed in our laboratory (activation step) or when TTS was added to the carrier of the flow system. A novel sucrose biosensor was designed by integrating PB and a three enzyme mixture consisting of GOX, INV, MUT crosslinked with BSA and glutaraldehyde. The electrode was integrated with FIA for the determination of sucrose. The optimal amounts of enzymes on the top of the GC/PB electrode for analysis of sucrose were standardised as 16 U of invertase, 8 U of mutarotase and 8 U of glucose oxidase. The response time of the biosensor was about 1 min per assay with a relative standard deviation better than 1.2%. The sensor could operate at a low potential (−50 mV), which is a good feature for practical applications especially for sucrose determination in biological samples containing electrochemically easily oxidisable interferants. The detection limit of the sensor for sucrose determination at optimum conditions was 4.5 μM. The sensor was stable in repeated use up to 40 min (30 assays) but conditioning the PB film in phosphate buffer containing 0.05 M TTS improved not only the operational but also the storage stability of the sucrose biosensor up to 370 min (240 assays) and 3 days, respectively. The analytical characteristics of the proposed sucrose biosensor with respect to detection limit, linear range, analysis rate, operating potential and the amount of enzymes used were significantly better than most of previously reported sucrose sensors [38,43,44,47,52,53,65–69].

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