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Simultaneous deposition of Prussian Blue and creation of an electrostatic surface for rapid biosensor construction

Nikolay A. Pchelintsev, Alexander Vakurov, Paul A. Millner*

Biosensors and Biocatalysis Group, Institute of Membrane and Systems Biology, University of Leeds, Leeds, LS2 9JT, UK

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ABSTRACT

Polyethyleneimine (PEI) was deposited onto the surface of the screen-printed carbon electrode (SPCE) through simple adsorption from aqueous solution. Anion-exchange properties of the deposited PEI were then employed to confine ferricyanide ions $[Fe(CN)_6]^{3-}$ within the polymeric layer. After that, the resulting electrode was washed and incubated in $FeSO_4$ solution so that Fe^{2+} cations could react with the adsorbed $[Fe(CN)_6]^{3-}$. The reaction led to the formation of surface-confined Prussian Blue (PB) crystals that were highly active in the electrochemical reduction of hydrogen peroxide. During the formation of PB, negatively charged ferricyanide ions complexed with charged iron(II) ions thereby regenerating positive charges on the PEI and enabling electrostatic adsorption of enzymes. After glucose oxidase (GOx) was immobilised in this manner on the surface of a PEI/PB modified electrode an amperometric biosensor for glucose was produced which could sense the analyte at voltages as low as 0 mV, thus eliminating the redox activity of many common interferents. Within this report, we use GOx as a model enzyme to demonstrate principle. However, the methodology reported is amenable to immobilising virtually any enzyme whilst simultaneously depositing PB mediator.

1. Introduction

The conventional approach to the amperometric detection of H_2O_2, which allows monitoring of the biorecognition event in many amperometric biosensors, is its oxidation under relatively high potentials. Unfortunately, such high potentials often cause non-specific oxidation of other compounds that usually occur in crude samples such as ascorbic acid in blood. This compromises high specificity of biorecognition material of the biosensor and decreases the accuracy of the measurement or even makes it impossible due to the contribution of non-specific oxidation to the overall readings. Several approaches were developed to overcome this problem. However, most of them are suitable only for specific analytes and interferents. A more elegant approach suggested by Karyakin et al. [1] is based on the reduction of H_2O_2 rather than on its oxidation. Using iron hexacyanoferrate (Prussian Blue – PB) as an electrocatalyst, H_2O_2 can be detected at potentials below 0 mV (vs. SCE) with high sensitivity and specificity thus eliminating problems with interfering compounds [1–3].

Traditional synthesis of Prussian Blue on the electrode surface is accomplished electrochemically [1,2,4–25]. Even though such an approach resulted in the construction of extremely sensitive devices [2,25] most them were unique, hand-made items while certain areas of medical, food and environmental analysis require express and inexpensive monitoring methods, e.g. for glucose in blood or pesticides in water. This challenge is often addressed by disposable amperometric biosensors which can be industrially produced on a large scale and at a relatively low cost. However, electrochemical fabrication is generally not very attractive for the mass-production of biosensors since it imposes limitations on their layout, e.g. all the biosensors to be modified simultaneously must be electrically connected. Although there are some industrial examples of electrochemically produced biosensors, their fabrication on the basis of deposited PB is additionally complicated by enzyme immobilisation which is particularly difficult to achieve in a gentle and reproducible manner on the top of a non-reactive inorganic PB layer. So far, the most common approach for enzyme immobilisation on the surface of PB-modified electrodes was drop deposition of a mixture of enzyme(s) with various additives, e.g. Nafion, accomplished by evaporation of solvent [2,7,12]. This procedure was often extended by intensive cross-linking of the enzyme with bovine serum albumin introduced as a bulk protein carrier [5,11]. This process involves harsh conditions, i.e. dehydration and covalent modification, which only few enzymes can tolerate; additionally, fairly high quantities of enzyme are required. Only very recently Yen et al. described the adsorption of chitosan on the surface of electrodeposited PB which then could electrostatically immobilise glucose oxidase under mild conditions [24].

* Corresponding author.
E-mail address: p.a.millner@leeds.ac.uk (P.A. Millner).

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Another approach for the PB deposition relies on its chemical synthesis without the need for an electrochemical step and often provides some sort of activated surface to facilitate enzyme immobilisation. The reported methods include doping carbon inks or paste with PB powder [26–30], adsorption of PB-polymer [31–34] or PB-gold [31] composite nanoparticles, dendrimer-supported PB synthesis [35] and chemical deposition of PB onto the surface of pretreated screen-printed carbon electrodes [3,36–41]. The latter technique however, requires electrochemical pretreatment of the electrodes and also leads to an inert PB film on the electrode surface which again causes problems with the immobilisation of enzymes. More recent publications report custom-synthesised materials and complex protocols [42,43].

This paper describes a new procedure for the non-electrochemical synthesis of PB on the surface of SPCE which can be followed by electrostatic enzyme immobilisation. The method proposed is based on the anion-exchange properties of the surface bound polycations which are well known [44–46] but have not been applied for simultaneous deposition of PB mediator and electrostatic enzyme immobilisation.

2. Materials and methods

2.1. Materials

$K_3[Fe(CN)_6]$ (99%), 30% H_2O_2, polyethyleneimines with Mw of 400, 2000 (50% solution) 25,000 and 750,000 (50% solution), dianisidine, horse radish peroxidase, glucose oxidase from Aspergillus niger Type X-S (GOx) were purchased from Sigma–Aldrich. $FeSO_4$ (7 H_2O, 98%), KH_2PO_4 (99%), KOH (99%), KCl (99%) were supplied by BDH. Ethanol was bought from Fisher Scientific. Water was deionised using a Milli-Q reagent water system.

Conducting carbon inks (type Electrodag PF-407A) and dielectric inks (type Electrodag PF-455) were obtained from Acheson. SPCE were produced as described previously [47]. Briefly, two carbon layers were sequentially printed onto ceramic support and cured for 1 h at 240 °C in air. Then, ceramic tiles were covered with an insulating layer of Electrodag PF-455 to restrict a round working area of 7 mm^2 (geometric area) per electrode. The insulating layer was dried in a vacuum oven for 1 h at 200 °C at less than 1 mbar pressure. After fabrication, the electrodes were stored at room temperature in a closed box. A DEK-248 screen printer was used for all printing procedures.

2.2. Adsorption of PEI

Surface modification of SPCE with PEI was carried at +50 °C in closed Eppendorf tubes and consisted of the following steps:

1. Surface cleaning: 3 min in ethanol, 3 min in water.
2. Adsorption of PEI: 30 min in 10 mg mL^{-1} PEI aqueous solution
3. Washing-off weakly bound polymer: 3 min in water.

No drying was allowed between steps. After step (iii) electrodes were thoroughly rinsed with water and incubated in water for another 5 min at room temperature before further modification with PB.

2.3. Synthesis of surface-confined PB

Synthesis of PB on the surface of PEI-modified electrode was accomplished in two steps at room temperature. Initially, surface bound PEI-film was saturated with $[Fe(CN)_6]^{3-}$ ions via 5 min incubation in 5 mM $K_3[Fe(CN)_6]$, pH 1, 100 mM KCl. Then electrodes were quickly rinsed with 100 mM KCl, pH 1, immersed into 5 mM $FeSO_4$, pH 1, 100 mM KCl and kept in the dark for 2 h. After that, electrodes were thoroughly washed with copious amounts of water, dried under a nitrogen flow and left in the dark for 1 h before incubation with GOx.

2.4. GOx immobilisation and glucose response

To immobilise GOx the PEI-PB modified electrodes were incubated for 10 min in 2 mg mL^{-1} enzyme solution in PBS, pH 7, 100 mM KCl. After careful rinsing with water an amperometric response for glucose was measured at 0 mV using sequential injections of 1 M glucose (2–20 μl) into 5 ml of PBS, pH 7, 100 mM KCl with intensive stirring.

Immobilisation efficiency was estimated from the comparison of enzymatic activity of GOx tethered on the electrode surface with that of the native enzyme in solution under low glucose concentration. In such conditions reaction rate is limited by glucose oxidation step and the decay of oxygen due to its consumption for FAD regeneration can be neglected. The activity of GOx was calculated as the rate of H_2O_2 production. For the immobilised enzyme the rate was calculated from the electrical current of H_2O_2 reduction considering that the current of 1 A corresponds to the reduction rate of 5.18×10^{-6} mol s^{-1} (two electron process). For the native GOx the rate was measured using chromogenic substrate *o*-dianisidine and peroxidase according to the standard procedure. Immediately before measurement a portion of *o*-dianisidine was dissolved in 25 mM KH_2PO_4, 100 mM KCl pH 7.0 containing 0.05 mg mL^{-1} GOx and 0.25 mg mL^{-1} peroxidase to get a final concentration of 250 μM. The reaction was initiated by the injection of 1 M glucose (2–50 μl) into 2 ml of this mixture and accumulation of the coloured product was followed at 500 nm.

2.5. Assay of ion-exchange capacity of the PEI film

The ion-exchange capacity of the PEI film was estimated as the amount of ferricyanide anions adsorbed on the modified surface after 5 min incubation in 5 mM $K_3[Fe(CN)_6]$, pH 1, 100 mM KCl followed by 3 min incubation in acidic pH 1 water with 100 mM KCl. The quantity of the ferricyanide ions was determined by CV in 100 mM KCl, pH 1 as the area under the oxidation peak. The potential during CV was changed from +700 mV to −100 mV and back at 100 mV s^{-1} scan rate.

3. Results and discussion

3.1. Assay of ion-exchange capacity of the PEI film.

Before comparing PEI with different molecular weights, a proper method for estimation of the PEI ion-exchange capacity had to be developed. $[Fe(CN)_6]^{3-}$ were considered as the most appropriate anions for this purpose because after adsorption on the PEI film they can be applied for synthesis of PB via condensation with Fe^{2+} cations according to Eq. (1):

$$4Fe^{2+} + 3[Fe(CN)_6]^{3-} \rightarrow Fe_4^{III}[Fe^{II}(CN)_6]_3 \quad (1)$$

Initially, the kinetics of ferricyanide adsorption were investigated where the electrode surface was modified by the PEI-750K, as described in Section 2.2, but the adsorption time was increased from 30 min up to 90 min. After washing-off weakly bound PEI the electrode was immersed into 5 mM $K_3[Fe(CN)_6]$, pH 1, 100 mM KCl for 3 min and the amount of adsorbed ferricyanide was measured by CV (Fig. 1).

Then, the electrode was incubated in the same $K_3[Fe(CN)_6]$ solution for another 10 min and the CV measurement was repeated. The areas under both oxidation and reduction peaks did not change.