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Prussian Blue-modified microelectrodes for selective transduction in enzyme-based amperometric microbiosensors for *in vivo* neurochemical monitoring

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ABSTRACT

Prussian Blue-modified carbon fiber microelectrodes (CFE/PBs) are proposed as an alternative to the more conventional metal transducers used for H_2O_2-detecting biosensors in brain extracellular fluid (ECF). The main advantages of this approach are the very small dimensions (~10 μm diameter) and the low applied potentials needed (0.0 V versus SCE). Electrocatalytic and physiochemical properties of PB deposits were studied using cyclic voltammetric (CV), amperometric and spectroscopic methods (FTIR and VIS). Optimized CFE/PB microsensors displayed a H_2O_2 current density of 1.00 ± 0.04 A M^{-1} cm^{-2} with a detection limit of 10^{-8} M. Furthermore, to improve stability and selectivity properties, several polymeric films were investigated: Nafion, poly(o-phenylenediamine) (PoPD), and a hybrid configuration of these two polymers. Finally, the PoPD film was selected due to its excellent anti-interference properties. The use of this permselective film also enhanced the stability of PB against solubilization at high pH, albeit at the expense of slightly lower H_2O_2 sensitivity (0.48 ± 0.02 A M^{-1} cm^{-2}) and higher detection limit (~10^{-7} M). However, the use of the PoPD film significantly enhanced the selectivity against the main endogenous brain interference species (ascorbic acid, uric acid, dopamine and 3,4-dihydroxyphenylacetic acid), expressed as the ratio of the sensitivity slopes ($S_{H_2O_2}/S_{interference}$), which was close to 600 for all interference molecules studied. A prototype of a CFE/PB-based glucose microbiosensor design is presented, together with preliminary studies of its characteristics *in vitro* and its functionality in brain ECF *in vivo*.

1. Introduction

1.1. Prussian Blue

Prussian Blue (PB), $Fe_4[Fe(CN)_6]_3$, belongs to a transition metal hexacyanometallate family [1] and is the oldest coordination compound known and used [2]. Electrochemical properties of PB films are known since 1978 when Neff [3] reported the successful deposition of a thin layer on a platinum foil. A large number of studies followed, and different methods for the preparation of PB-modified electrodes have been described [4–7]. In 1984, Itaya et al. [8] showed that the reduced form of PB (Prussian White, PW) displayed catalytic activity for the reduction of O_2 and H_2O_2. The zeolite structure of PB, with a cubic unit cell of 1.02 nm and channel diameters of about 0.32 nm [9], allows the diffusion of low molecular weight molecules (such as O_2 and H_2O_2) through the crystal structure. Nowadays its electrochemical behavior is well-understood with cyclic voltammograms of PB-modified electrode showing two pairs of almost reversible and symmetrical peaks [10]. The first peak pair corresponds to the interconversion of PW and PB forms, and the second pair from PB to Prussian Green (PG) and its reversal. The electron transfer reactions in the presence of potassium chloride as supporting electrolyte may be formulated as follows:

$$(PB)\,Fe(III)_4[Fe(II)(CN)_6]_3 + 4e^- + 4K^+ \rightarrow (PW)\,K_4Fe(II)_4[Fe(II)(CN)_6]_3 \quad (1)$$

$$(PB)\,Fe(III)_4[Fe(II)(CN)_6]_3 + 3Cl^- \rightarrow (PG)\,Fe(III)_4[Fe(III)(CN)_6]_3Cl_3 + 3e^- \quad (2)$$

corresponding to peaks at 0.1 V and 0.9 V versus SCE, respectively [11–13].

Electrochemical properties such as formal electrode potential, sensitivity, stability and electron transfer rate constants of the PB/PW and PB/PG conversions depend on deposition method, pH, nature and concentration of the supporting electrolyte, etc. [14,15].

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As can be seen in Eqs. (1) and (2), these reduction and oxidation reactions are supported by free diffusion of cationic and anionic species, respectively. In the present work we focus on the first peak (conversion from PB to PW). Due to this mechanism, only cations with small hydrated radii, such as K^+, NH_4^+, Rb^+ and Cs^+, can support this electrochemical activity by diffusing across the PB structure during iron center oxidation/reduction. On the other hand, Na^+, Li^+, Mg^{2+}, Ca^{2+} can be described as blocking cations of the PB film, due to their larger hydrated radii inhibiting the free diffusion referred to above [1,16,17].

The principal handicap of PB is related to its high solubility at neutral and basic pH, although in acid conditions it shows good sensitivity and stability [18,19]. Different pH stability studies indicate that PB films can be made more stable in basic media by changing the deposition procedure, thus modifying slightly PB three-dimensional structure and avoiding OH^- diffusion across the crystal [20,21]. However, a more practical approach has been the use of protective polymer films deposited over PB to enhance both its operational stability and selectivity against electroactive species. Several polymers have been used, such as Nafion, polypyrrole, polyaniline and poly(o-phenylenediamine) [10,17,22]. These films partially block access of interference through different mechanisms (e.g., size and/or charge exclusion). Recently, Lukachova et al. have described a PoPD-coated, PB-modified macrodisk electrode with moderate hydrogen peroxide sensitivity of 0.3 A M^{-1} cm^{-2}, high selectivity ratio (~600) against ascorbic acid (main endogenous interference in physiological media), and high stability under continuous flow of 0.1 mM H_2O_2 (almost total retained sensitivity after 20 h) [22].

1.2. Prussian Blue-modified biosensors

Although the birth of biosensors occurred in the early 1960s [23], when Clark and Lyons coupled an enzyme (glucose oxidase, Gox) to an amperometric electrode for detecting O_2, and PB electrochemical properties have been known since the late 1970s, the first work on biosensors involving the use of a PB-modified electrode was not reported until 1994 [24–26]. In these first reports, the authors proposed the use of PB-modified electrodes as an alternative to the traditional Pt transducer used to detect H_2O_2. During the last decade, a great number of studies involving PB have appeared using different biosensor configurations (carbon paste, screen-printing, glassy carbon, etc. substrates) and different oxidase enzymes (glucose oxidase, lactate oxidase, glutamate oxidase, etc.) [27–29].

On the other hand, in the neuroscience field traditional first-generation biosensors based on noble metal transducers [30], and second-generation biosensors based on artificial redox mediators, have been generally used [31]. Thus, the main purpose of the present work is to present CFE/PBs as an alternative for implantable microbiosensor designs.CFE/PBs were characterized, optimized, coated with permselective polymers, and a range of endogenous interferences in brain ECF (ascorbic acid, uric acid, dopamine, and 3,4-dihydroxyphenylacetic acid) tested. In addition, a prototype of a CFE/PB-based glucose microbiosensor is presented, together with preliminary studies of its characteristics *in vitro* and its responsiveness in brain ECF *in vivo*.

2. Experimental

2.1. Reagents and solutions

The enzyme glucose oxidase (Gox) from *Aspergillus niger* (EC 1.1.3.4, type VII–S, lyophilized powder), glutaraldehyde (Glut, 25% solution) were obtained from Sigma Chemical Co., and stored at −21 °C until used. All chemicals, including o-phenylenediamine (o-PD), polyethyleneimine (PEI), NaCl, KCl, $CaCl_2$, $MgCl_2$, $FeCl_3$, $K_3[Fe(CN)_6]$, HCl (35%, w/w), H_2O_2 (30%, w/v), Nafion (5 wt.% in a mixture of lower aliphatic alcohols and water), bovine serum albumin (BSA, fraction V), glucose and phosphate buffer saline (PBS, pH 7.4 containing 0.1 M NaCl) were obtained from Sigma and used as supplied. Endogenous interference species were obtained from Aldrich (ascorbic acid, AA, and 3,4-dihydroxyphenylacetic acid, DOPAC) and Sigma (dopamine, DA, and uric acid, UA). All solutions were prepared in doubly distilled water (18.2 MΩ cm, Millipore-Q). The stock calibration solution of H_2O_2 (10 mM) was prepared in water just before its use. Stock solutions of glucose (1 and 0.25 M) were prepared in water, left for 24 h at room temperature to allow equilibration of the anomers, and then stored at 4 °C. 300 mM of o-PD monomer solution was prepared using 48.6 mg of o-PD and 7.5 mg of BSA in 1.5 ml of N_2-saturated PBS, and sonicated for 15 min. Carbon fibers (7 μm diameter) were obtained from Goodfellow, glass capillaries from Word Precision Instruments Inc., 250 μm internal diameter Teflon-coated copper wire from RS, and silver epoxy paint was supplied by Sigma. An in-house microinjection cannula was constructed using fused silica capillary (75 μm inner diameter) supplied by Composite Metal Service, Hallow, UK.

2.2. Instrumentation and software

Experiments were computer controlled, using data acquisition software EChem™ for cyclic voltammetry and Chart™ for constant potential amperometry. The data acquisition system used was e-Corder 401 (EDAQ) and a low-noise and high-sensitivity potentiostat, Picostat (EDAQ). To electro-deposit and activate PB film, an in-house Ag/AgCl/saturated KCl reference and platinum wire auxiliary electrode were used. Transmittance spectra of PB films were recorded in the range 300–1000 nm with respect to air in a Transpec® photodiode array spectrophotometer. FTIR spectra were recorded with respect to air, using a Varian 670-IR spectrophotometer in the range 4000–400 cm^{-1}.

The electrochemical characterization experiments, H_2O_2 and glucose calibrations were done in a 25 mL glass cell at 21 °C, using a standard three-electrode setup with a commercial saturated calomel electrode, SCE (CRISON Instrument S.A.), as the reference and platinum wire as the auxiliary electrode. The applied potential for amperometric studies was 0.0 V versus SCE, unless stated otherwise. H_2O_2 and glucose calibrations were performed in quiescent air-saturated PBS (following stabilization of the background current for 20–30 min) by adding aliquots of H_2O_2 or glucose stock solution. After each addition, the solutions were stirred for 10 s and then left to reach the quiescent steady-state current.

2.3. Preparation of the working electrodes

2.3.1. Fabrication of carbon fiber electrodes

CFEs were constructed using the following steps. Carbon fibers (diameter 7 μm, 20–50 mm in length) were attached to Teflon-coated copper wire (diameter 250 μm), using high purity silver paint, and dried for 1 h at 80 °C. The borosilicate glass capillary was pulled to a tip using a vertical microelectrode puller (Needle/Pipette puller, Model 750, David Kopf Instruments). After drying, the carbon fiber was carefully inserted into the pulled glass capillary tube under a microscope, leaving 2–4 mm of the carbon fiber protruding at the pulled end. Subsequently, the carbon fiber was cut to the desired length (approximately 250 or 500 μm), using a microsurgical scalpel. At the stem end of the capillary tube, the copper wire was fixed by casting with non-conducting epoxy glue; the carbon fiber was also sealed into the capillary mouth, using the non-conductive epoxy glue. Finally, CFEs were dried again for 1 h, and were optically and electrochemically inspected before use.