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Microbiosensors for glucose based on Prussian Blue modified carbon fiber electrodes for *in vivo* monitoring in the central nervous system

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ABSTRACT

Carbon fiber electrodes (CFEs) were used to develop microbiosensors for glucose as an alternative to the classical Pt and Pt–Ir transducers. Their low dimensions (~250 μm CFE length and ~10 μm diameter) are important factors for measurements in physiological environments. An electrocatalytic Prussian Blue (PB) film facilitated detection of enzyme-generated hydrogen peroxide at a low applied potential (~0.0 V against SCE), contrasting the high potential used in many previous designs (~0.7 V). The electrosynthesized polymer, poly-o-phenylenediamine (PoPD), was used to improve biosensor stability and selectivity against endogenous interference species, such as ascorbic and uric acids. Optimization of the fabrication procedure is described, including activation of CFE/PB, enzyme immobilization and stabilization, anti-interference films, optimizing applied potential, and pH effects. Analytical properties were also characterized such as sensitivity, LOD, linear range, and enzyme loading. Finally, an optimized biosensor displaying a linear sensitivity of $9.3 \pm 0.1\ \mu A\ mM^{-1}\ cm^{-2}$ ($n = 3$), a 2% RSD and free of interference, is proposed as a suitable candidate for *in vivo* glucose monitoring in the CNS.

1. Introduction

First generation biosensors are based on the detection of any electroactive metabolite that is generated (e.g., H_2O_2) or consumed (e.g., O_2) during the enzyme reaction. The principal problem of these approaches for oxidase-based biosensors is the high overpotential needed to detect either O_2 or H_2O_2 on Pt (Clark and Lyons, 1962; Clark, 1979) and other native electrode materials (Schuvailo et al., 2005). At high potentials, interference and polymerization reactions can occur at the biosensor surface. Biosensors in biological media are subject to interference by endogenous electroactive reducing agents, especially ascorbic acid (AA) and uric acid (UA) (D'Orazio, 2003; Osborne and Hashimoto, 2004; Garjonyte and Malinaus, 1999). These problems have been solved by several approaches: (a) the use of polymer films with anti-interference properties, such as poly-o-phenylenediamine (PoPD) (Lowry and O'Neill, 1994; Dai et al., 2006); (b) the use of artificial redox mediators (Castillo et al., 2003; Gregg and Heller, 1990; Habermüller et al., 2000; Mitala and Michael, 2006); (c) the use of electrocatalytic films to detect H_2O_2 at lower applied potentials (see Fig. 1) (Karyakin et al., 1995; Karyakin and Karyakina, 1999).

The neurophysiological motivation for this work is based on glucose being the principal source of energy in the brain. Fellows and Boutelle (1993) showed that physiological stimulation of neural activity is associated with an increase in glucose concentration in the extracellular compartment, which has a much longer duration than the associated change in regional cerebral blood flow (rCBF). On the other hand, recent studies suggest that increased energy requirements of activated neurons are met by neuronal utilization of lactate (Pellerin et al., 2007). Thanks to the development and use of microbiosensors to determine principal energy metabolites and neurotransmitters (Dale et al., 2005; Lowry et al., 1998), we can now further explore the relationship between neural activation, rCBF and brain metabolites.

Biosensors offer a variety of advantages over classical techniques for neurochemical monitoring: high spatial and temporal resolution; good selectivity; ease of implementation; amenable to miniaturization; rapid response time; etc. Recently, O'Neill et al. (2008) reviewed the development of wire-based amperometric polymer–enzyme composite biosensors, focusing on the relationship between key design features and analytical parameters. Using a carbon fiber substrate, we now propose a novel glucose biosensor, developed following this systematic approach, with significantly

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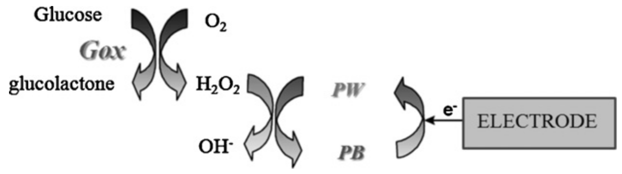

Fig. 1. Detection scheme for glucose biosensor based on a Prussian Blue (PB) modified electrode. Glucose is converted to gluconolactone, catalyzed by glucose oxidase (Gox) immobilized on the electrode surface. Secondary to this reaction is the production of hydrogen peroxide that can be reduced amperometrically at low applied overpotentials, electrocatalyzed by the PB.

smaller dimensions and a low working potential, which provide significant advantages for *in vivo* monitoring.

2. Materials and methods

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. Other chemicals, including o-phenylenediamine (oPD), glucose, ascorbic acid (AA), uric acid (UA), polyethyleneimine (PEI), KCl, $FeCl_3$, $K_3[Fe(CN)_6]$, HCl (35%, w/w), H_2O_2 (30%, w/v), Nafion (5%, w/w, in a mixture of lower aliphatic alcohols and water), bovine serum albumin (BSA, fraction V) and phosphate buffer saline solution containing 0.1 M NaCl (PBS), were obtained from Sigma and used as supplied. PBS stock solutions (pH 7.4) were prepared in doubly distilled water (18.2 MΩ cm, Millipore–Q), and stored at 4 °C. Enzyme solutions were prepared in PBS, which also served as the background electrolyte for all *in vitro* experiments. Stock 1 M and 250 mM solutions of glucose were prepared in water, left for 24 h at room temperature to allow equilibration of the anomers, and stored at 4 °C. The PEI solutions used were prepared by dissolving PEI at 1% (w/v) and 5% (w/v) ratios in H_2O. The cross-linking solution was prepared in PBS with 1% (w/v) BSA and 0.1% (w/v) glutaraldehyde. Monomer solution of 300 mM oPD was prepared using 48.6 mg oPD and 7.5 mg BSA in 1.5 mL of N_2-saturated PBS and sonicating for 15 min. A 300 U/mL solution of Gox was prepared by dissolving 3.7 mg in 2 mL of PBS. Carbon fibers (8 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.

2.2. Instrumentation and software

Experiments were computer controlled with data acquisition software EChem^{TM} for cyclic voltammetry and Chart^{TM} for constant potential amperometry (ADInstruments Pty Ltd., Australia). The data acquisition system used was e–Corder 401 and a low-noise and high-sensitivity potentiostat, Picostat (eDAQ Pty Ltd., Australia). The linear and non-linear regression analyses were performed using the graphical software package Prism (ver. 5.00 GraphPad Software, San Diego, CA, USA). To electro-deposit and activate the PB (see Section 2.5), an in-house Ag/AgCl/saturated KCl reference electrode and platinum wire auxiliary electrode were used.

2.3. Amperometric experiments

All experiments were done in a 25 mL glass cell at 21 °C, using a standard three-electrode set-up with a commercial saturated calomel electrode (SCE) (CRISON Instrument S.A. Barcelona, Spain) as the reference and platinum wire as the auxiliary electrode. The applied potential chosen for most amperometric studies was 0.0 V against SCE. Glucose calibrations were performed in quiescent air-saturated PBS (following stabilization of the background current for 30 min) by adding aliquots of glucose stock solution (1 or 0.25 M) to the electrochemical cell. After the addition of glucose aliquots, the solutions were stirred for 10 s and then left to reach the quiescent steady-state current.

2.4. Data analysis

Calibration plots for glucose were generated by plotting averaged steady-state currents against substrate concentration and fitting the data, using non-linear regression to obtain the apparent Michaelis–Menten constants, K' (mM), maximum biosensor response, V_m (pA), and the Hill parameter, h. Linear regression was used in the linear range to determine biosensor sensitivity, signal to noise ratio and coefficient of determination (r^2). In all cases, the currents recorded in background electrolyte were subtracted from the calibration responses. Data are reported as mean ± SEM, n being the number of biosensors or number of measurements (as specified).

2.5. Preparation of the working electrodes

2.5.1. Fabrication of carbon fiber electrodes

Carbon fiber electrodes (CFEs) were constructed using the following steps. A carbon fiber (diameter 8 μm, 20–50 mm in length) was attached to Teflon-coated copper wire (diameter 250 μm)

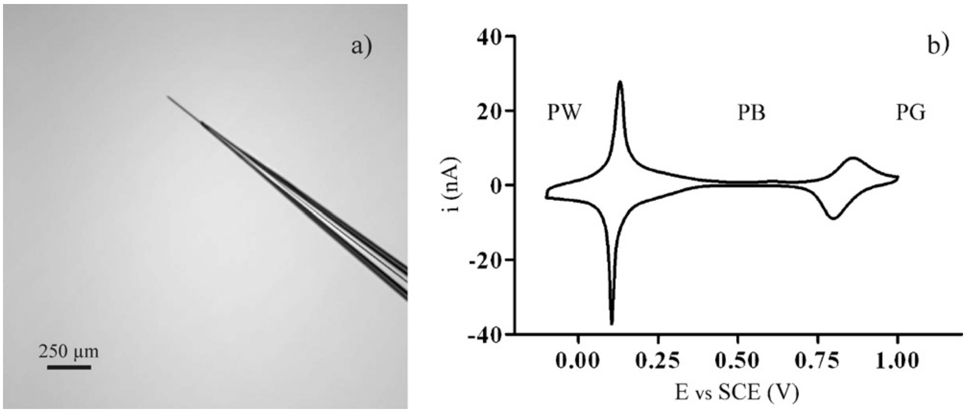

Fig. 2. a) Carbon fiber electrodes (CFEs) with a diameter of 8 μm and length of 250 μm. b) Cyclic voltammogram of the PB-modified CFE in de-aerated electrolyte solution (0.1 M KCl and 3 mM HCl) at a scan rate of 10 mV/s. PW is Prussian White, and PG is Prussian Green.