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Oxygen tolerance of an implantable polymer/enzyme composite glutamate biosensor displaying polycation-enhanced substrate sensitivity

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Abstract

Biosensors were fabricated at neutral pH by sequentially depositing the polycation polyethyleneimine (PEI), the stereoselective enzyme L-glutamate oxidase (GluOx) and the permselective barrier poly-*ortho*-phenylenediamine (PPD) onto 125-μm diameter Pt wire electrodes (Pt/PEI/GluOx/PPD). These devices were calibrated amperometrically at 0.7 V versus SCE to determine the Michaelis–Menten parameters for enzyme substrate, L-glutamate (Glu) and co-substrate, dioxygen. The presence of PEI produced a 10-fold enhancement in the detection limit for Glu (∼20 nM) compared with the corresponding PEI-free configurations (Pt/GluOx/PPD), without undermining their fast response time (∼2 s). Most remarkable was the finding that, although some designs of PEI-containing biosensors showed a 10-fold increase in linear region sensitivity to Glu, their oxygen dependence remained low.

Keywords: Glutamate oxidase; Polyethyleneimine; PEI; Poly(*o*-phenylenediamine); PPD; Michaelis–Menten; Brain monitoring; Neurochemistry

1. Introduction

The importance of L-glutamate (Glu) in systems as diverse as food processing and brain monitoring (O'Neill et al., 1998; Wilson and Hu, 2000; Wilson and Gifford, 2005; Dale et al., 2005; Fillenz, 2005) has led to intense interest in the development of sensors for this amino acid. In a neurochemical context, Glu is the main excitatory neurotransmitter, and a range of biosensor designs, based mainly on glutamate oxidase (GluOx; MW_r, 140 kDa; solution K_M, 0.21 mM in neutral buffer; p*I*, 6.2) (Kusakabe et al., 1983), have been described for direct monitoring of Glu in brain extracellular fluid (ECF) (Hu et al., 1994; Cosnier et al., 1997; Kulagina et al., 1999; Matsushita et al., 2000; Burmeister et al., 2003; Nickell et al., 2005; Rahman et al., 2005) (see reactions (1) and (2)). Encouraged by success in the design and application of an implantable biosensor for brain glucose (Lowry et al., 1994; Dixon et al., 2002), based on the immobilization of glucose oxidase (GOx) in a permselective polymer (poly-*ortho*-phenylenediamine, PPD) electrosynthesized in situ on Pt wire (Pt/GOx/PPD) (Sasso et al., 1990; Malitesta et al., 1990; Lowry and O'Neill, 1992; Wang and Wu, 1993; Bartlett and Birkin, 1994), this design has been adopted in recent years for the detection of ECF Glu (Pt/GluOx/PPD) (Ryan et al., 1997; Lowry et al., 1998b; McMahon and O'Neill, 2005).

The task of detecting brain ECF Glu, however, is significantly more challenging than glucose monitoring, mainly because the baseline ECF concentration of Glu appears to be ≤5 μM (Miele et al., 1996; Lada and Kennedy, 1996; Baker et al., 2002; Chen, 2005; Fillenz, 2005), although values as high as 15 μM have been suggested (Kulagina et al., 1999), and compares to ∼500 μM for ECF glucose (Boutelle et al., 1992; Lowry et al., 1998a). Thus, optimization of Glu sensitivity is critical for physiological applications, and we reported recently a significant enhancement of the linear region slope (LRS) for Glu, by incorporating the polycation polyethyleneimine (PEI) in these PPD-based biosensors (Pt/PEI/GluOx/PPD) (McMahon et al., 2006b).

$$\text{l-Glutamate} + H_2O + \text{GluOx/FAD} \rightarrow \alpha\text{-ketoglutarate} + NH_3 + \text{GluOx/FADH}_2 \quad (1)$$

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$$\mathrm{GluOx/FADH_2 + O_2 \rightarrow\ GluOx/FAD\ +\ H_2O_2} \quad (2)$$

$$\mathrm{H_2O_2 \rightarrow\ O_2 + 2H^+ + 2e} \quad (3)$$

The protein immobilizing agent (Tang et al., 1998) and stabilizer (Bryjak, 1995; Andersson and Hatti-Kaul, 1999), PEI, has been used previously in biosensors for a number of analytes, including Glu (Belay et al., 1999; Rahman et al., 2005; Varma et al., 2006). Studies suggest that PEI can have beneficial effects on biosensor performance by augmenting enzyme stability through the formation of polyanionic/polycationic complexes (Andersson et al., 2000) and by decreasing the electrostatic repulsion between the enzyme substrate and biosensor components (Chi et al., 1997; Jezkova et al., 1997; McMahon et al., 2006b). However, the enhanced sensitivity of oxidase-based biosensors to enzyme substrate, achieved using PEI, could have the undesired effect of greater sensitivity to changes in the concentration of co-substrate (O_2; see reaction (2)).

Since no literature is available on this topic, we investigate here the effects of incorporating PEI into Pt/GluOx/PPD biosensors on their oxygen dependence. The importance of oxygen interference in biosensor functionality is particularly relevant in applications involving in vivo monitoring, where pO_2 can fluctuate significantly (Clark et al., 1958; Bolger and Lowry, 2005). Thus, the suitability of a Glu biosensor design for a given application depends on the concentration of Glu being monitored, as well as the range of fluctuations in pO_2 relevant to that medium. For example, a combination of excessive Glu and low pO_2 could undermine the reliability of the Glu signal, and in extreme cases the biosensor becomes an oxygen sensor. The advantages and limitations of replacing O_2 in reaction (2) by various mediators has been discussed previously (O'Neill et al., 1998). Although these 'second generation' biosensors have the advantage of a low operating potential, they can suffer from a number of problems including leeching of untethered mediator from the enzyme layer, toxicity in biological tissues, and redox interference (e.g., oxidized ferrocenes can be reduced by ascorbic acid present in most biological media). Additionally, the complete insensitivity to oxygen tension sometimes claimed for mediated sensors has been questioned for certain mediators (Martens et al., 1995).

2. Materials and methods

2.1. Biosensor fabrication and calibration

Pt cylinders (Pt_C, 125 μm diameter, 1 mm length) were fabricated from Teflon®-coated Pt wire (Advent Research Materials, Suffolk, UK). GluOx (EC 1.4.3.11, 200 U mL^{-1}, Yamasa Corp., Japan) was deposited onto the metal surface by dip-evaporation (1–4 dips) (Ryan et al., 1997) and immobilized by amperometric electropolymerization (+700 mV versus SCE) in 300 mM *o*-phenylenediamine in phosphate buffered saline (PBS, pH 7.4) (Craig and O'Neill, 2003), as described previously to form Pt_C/GluOx/PPD biosensors (Ryan et al., 1997). Pt disks (Pt_D) were fabricated by cutting the Teflon®-coated wire transversely to produce 125 μm diameter disks, and Pt_D/GluOx/PPD biosensors were fabricated as for Pt_C. Additional sets of biosensors were prepared by pre-coating the Pt surface with the polycation polyethyleneimine (Aldrich, $MW_r \sim 750$ kDa, 1% aqueous solution), also by dip-evaporation, before enzyme deposition. The alternative polymer/enzyme configuration (enzyme deposited by dip-evaporation after the polymerization step) was also investigated: Pt_C/PPD/GluOx and Pt_D/PPD/GluOx, where the enzyme was immobilized by exposure to glutaraldehyde vapor (McMahon et al., 2005).

After rinsing and a settling period at 700 mV in fresh PBS, amperometric calibrations were carried out to determine the apparent Michaelis–Menten parameters (J_{max} and K_M(Glu); see below) and the linear region sensitivity (0–100 μM) of the biosensors to Glu and H_2O_2 in quiescent air-saturated buffer, unless stated otherwise. All electropolymerizations and calibrations were performed in a standard three-electrode glass electrochemical cell containing 20 mL quiescent PBS at room temperature. A saturated calomel electrode (SCE) was used as the reference electrode, and a large stainless steel needle served as the auxiliary electrode.

Experiments were computer controlled as described previously (Dixon et al., 2002; McMahon et al., 2005; McMahon et al., 2006b). Response times were recorded in constantly stirred solution, using a data acquisition rate of 100 Hz. A $t_{90\%}$ parameter was defined as the time taken for the analyte response to reach 90% of its maximum value from the start of the current upswing, and is similar to definitions used previously (Berners et al., 1994; Kulagina et al., 1999; Burmeister et al., 2003). The limit of detection (LOD) was determined using the widely applied criterion of three times the S.D. of the baseline.

2.2. Monitoring dissolved oxygen

A self-calibrating commercial membrane-covered amperometric oxygen sensor (CellOx 325 connected to an Oxi 340A meter, Carl Stuart Ltd., Dublin, Ireland) was used to quantify solution oxygen concentration as described previously (Dixon et al., 2002). This percentage was converted to an estimated concentration of O_2 by taking 200 μM to correspond to 100% (Bourdillon et al., 1982; Zhang and Wilson, 1993). To avoid contamination of the PBS by oxygen, the electrochemical cell was contained within an Atmosbag™ (Sigma) (Dixon et al., 2002). Oxygen sensor data and biosensor data were recorded simultaneously through the transition from N_2 saturation to air saturation. The biosensor response reached a plateau at oxygen levels which depended on the concentration of Glu in the cell, but typically by 30–50 μM O_2. Non-linear regression analysis of the current was performed up to this plateau region to determine the relevant $K_M(O_2)$ value (McMahon et al., 2006a; see Section 2.3).

2.3. Kinetic model and data analysis

A number of sophisticated mathematical models of the behavior of enzymes in membranes have been described (Albery and Bartlett, 1985; Bartlett and Pratt, 1993; Gooding et al., 1998; Phanthong and Somasundrum, 2003; Baronas et al., 2004). These complex analyses are often needed to understand and opti-