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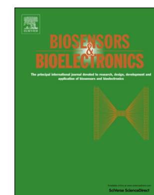

Glutamate oxidase biosensor based on mixed ceria and titania nanoparticles for the detection of glutamate in hypoxic environments

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ABSTRACT

We report on the design and development of a glutamate oxidase (GmOx) microelectrode for measuring L-glutamic acid (GluA) in oxygen-depleted conditions, which is based on the oxygen storage and release capacity of cerium oxides. To fabricate the biosensor, a nanocomposite of oxygen-rich ceria and titania nanoparticles dispersed within a semi-permeable chitosan membrane was co-immobilized with the enzyme GmOx on the surface of a Pt microelectrode. The oxygen delivery capacity of the ceria nanoparticles embedded in a biocompatible chitosan matrix facilitated enzyme stabilization and operation in oxygen free conditions. GluA was measured by amperometry at a working potential of 0.6 V *vs* Ag/AgCl. Detection limits of 0.594 μM and 0.493 μM and a sensitivity of 793 pA/μM (*RSD* 3.49%, $n=5$) and 395 pA/μM (*RSD* 2.48%, $n=5$) were recorded in oxygenated and deoxygenated conditions, with response times of 2 s and 5 s, respectively. The biosensor had good operational stability and selectivity against common interfering substances. Operation of the biosensor was tested in cerebrospinal fluid. Preliminary *in vivo* recording in Sprague-Dawley rats to monitor GluA in the cortex during cerebral ischemia and reperfusion demonstrate a potential application of the biosensor in hypoxic conditions. This method provides a solution to ensure functionality of oxidoreductase enzymes in oxygen-free environments.

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1. Introduction

L-Glutamic acid (GluA) or glutamate is one of the most important excitatory signaling molecules in the central nervous system and plays a critical role in a variety of brain functions, *e.g.* memory and learning (Holmes et al., 1993). During hypoxic injury, the normal functioning of the regulatory GluA receptors is compromised, the level of oxygen in the tissue decreases (Krajnc et al., 1996), and as a result, the level of extracellular GluA increases (Zemke et al., 2004). Imbalance in oxygen delivery to cells and tissues has been associated with neurodegenerative diseases such as ischemia, epilepsy, Parkinson's and Alzheimer's diseases (Ogunshola and Antoniou, 2009). Therefore, development of methods that permit evaluation of GluA levels during hypoxic insults is critical for studying the contribution of excitotoxicity to these disorders.

Analytical techniques commonly used to measure GluA include microdialysis (Baker et al., 2002), capillary electrophoresis (Vyas et al., 2011), fluorescent (Namiki et al., 2007) and luminescent probes (Kiba et al., 2002) and electrochemistry (Walker et al., 2007). Of these, electrochemical methods with microelectrodes allow direct real-time assessment of physiological GluA levels in both *in vitro* and *in vivo* systems with high spatial resolution (Lee et al., 2007). Several types of GluA biosensors have been developed (O'Neill et al., 2004) and some are commercially available (Pinnacle Technology, Inc.). The majority of GluA sensors designed for *in vivo* use are first generation biosensors that utilize glutamate oxidase (GmOx) to enzymatically convert GluA to electrochemically detectable hydrogen peroxide (H_2O_2). In this configuration, molecular oxygen is necessary for the formation of H_2O_2. Therefore, accurate measurements in low-oxygen conditions is a major challenge (McMahon et al., 2007).

Previous investigators have tried to reduce oxygen-dependent variability of oxidoreductase enzymes by including an oxygen chamber as an integral part of the biosensor so that oxygen levels in the region of the enzyme can be maintained at levels sufficient for an adequate sensor response (Simpson et al., 2006). This approach complicates miniaturization and fabrication of implantable devices. Alternatively, a mixture of graphite powder and

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perfluorocarbons with high oxygen solubility has been used (Wang et al., 2002; Zhao et al., 2003), but this sensor design has not been miniaturized, and there may be increased toxicity associated with these additional constituents. Few studies address oxygen variability of GmOx biosensors (McMahon et al., 2007). To our knowledge, there is no report of a GmOx biosensor operating in an oxygen-free environment. In this paper, we propose a new technology in which a biocompatible composite of ceria and titania nanoparticles in proximity to the GmOx enzyme dispersed in chitosan is used to provide oxygen to sustain the enzymatic reaction in conditions of oxygen depletion. The method makes use of the oxygen storage and release capacity of biocompatible ceria nanoparticles, which is enhanced in the presence of titania (Njagi et al., 2008).

Cerium oxide nanoparticles have unique redox and autocatalytic properties (Das et al., 2007). Moreover, ceria is an excellent co-immobilization material for a variety of enzymes such as cholesterol oxidase, glucose oxidase and horseradish peroxidase (Ansari et al., 2009). Its catalytic activity can be exploited to develop highly sensitive, enzymeless H_2O_2 sensors and for the fabrication of third generation biosensors (Ispas et al., 2008). Ceria has high oxygen mobility at its surface (Preda et al., 2011; Zhang et al., 2006b) and a large oxygen diffusion coefficient, which facilitates the conversion between valance states Ce^{4+}/Ce^{3+} (Dutta et al., 2006) that allow oxygen to be released or stored in its crystalline structure (Wang et al., 2011; Xu et al., 2010). The catalytic and oxygen storage and release capacity are used in this work to fabricate an 'oxygen rich' electrochemical biosensor for the detection of GluA in hypoxic conditions. We show that ceria nanoparticles, in conjunction with titania, embedded in the immobilization environment of GmOx can minimize problems associated with low oxygen levels and maintain the proper functioning of the enzyme, which requires oxygen as co-substrate, enabling this biosensor to work in a hypoxic environment. First, we have performed cyclic voltammetric (CV) characterization of the enzymatic sensor in the presence and absence of oxygen with and without metal oxides in the bioimmobilization matrix and established the optimum operational conditions. Next, the metal oxide GluA biosensor was studied using amperometry under aerobic and anaerobic conditions in a physiological buffer and further tested in cerebrospinal fluid. This strategy can be used as a general approach for the creation of other oxidoreductase-based biosensors that have requirements for operational functionality in anaerobic conditions.

2. Experimental

2.1. Reagents

Glutamate oxidase (GmOx) (EC 1.4.3.11, 25 U/vial; from *E. coli*) was purchased from Yamasa Corporation. Chitosan (practical grade) from shrimp shells, L-glutamic acid (99%), titania (nanopowder, 99.5% rutile, cat #637262), ceria (cat #544541), dopamine hydrochloride (DA), 3,4-dihydroxy-L-phenylalanine (L-DOPA), albumin (BSA; from bovine serum) and potassium phosphate (monobasic) were obtained from Sigma Aldrich. Sulfuric acid was purchased from Fisher Scientific. L-Ascorbic acid (AA) (99%), *o*-phenylenediamine (*o*-PD) (98%), serotonin hydrochloride (5HT) and sodium phosphate dibasic (anhydrous) were purchased from Acros Organics. Ascorbate oxidase (AsOx) (EC 1.10.3.3, 1 KU/vial; from *Cucurbita sp.*) was obtained from Alfa Aesar. Silver conductive epoxy was purchased from MG Chemicals, and non-conductive epoxy resin was obtained from Devcon. All solutions were prepared with distilled water collected from Millipore Direct-Q with a resistivity of 18.2 Ω cm.

2.2. Instrumentation

Amperometry and CV experiments were conducted using a CHI1030A electrochemical analyzer (CH Instruments Inc.). All electrochemical analyses were performed with a conventional cell with enzyme/nanoparticles/chitosan/*o*-PD/BSA modified platinum wire as working electrode, an Ag/AgCl/3 M NaCl (BAS MF-2052, RE-5B) as reference electrode and a platinum wire as a counter electrode. All potentials were referred to the Ag/AgCl reference electrode.

2.3. Biosensor fabrication

Platinum working electrodes with a diameter of 125 μm were fabricated from Pt-wires from A-M Systems. The Pt-wire was cut at 2 cm length, and the Teflon coating was removed. The biosensing active side was 2 mm in length and bent to a '*ν*' shape for increased loading of the active material. The inactive portion of the wire was ~5 mm in length and was glued to a copper wire with a conductive epoxy. The microelectrode was placed in a pulled glass capillary. The upper end of the capillary was sealed with non-conductive epoxy and cured for 10 min at 100 °C. The electrodes were treated electrochemically next in 0.5 M H_2SO_4 through CV in the potential range from −0.25 to 1.65 V at a scan rate of 0.1 V/s for 20 cycles. After the electrochemical treatment, the wire was rinsed thoroughly with distilled water. The clean electrode was electro-coated with *o*-PD by applying a potential of 0.9 V in a stirred solution consisting of 300 mM *o*-PD and 5 mg/ml BSA for 30 min. This method produces a thin, perm-selective and self-sealing film on the electrode surface (Killoran and O'Neill, 2008). The electrodes were rinsed with distilled water and immediately modified with the bioactive layer of nanoparticles and enzyme. This modification was made with a mixture containing 1% chitosan solution, CeO_2/TiO_2 dispersion (1:1 ratio of 1 mg/ml of each nanoparticle) and enzyme solution with a ratio of 2:1:1, respectively. The effect of CeO_2/TiO_2 loading on sensor response is shown in Fig. S1 (Supporting information). The enzyme stock solution was 0.1 U/μl GmOx in 0.1 M PBS (pH=7.4). Two aliquots of 2 μl of this mixture were casted onto *o*-PD modified platinum wire. In the final step, 1 μl of 200 U/ml AsOx stock solution was placed on microelectrode and dried. After each modification step, the electrode was dried under a N_2 environment. For the control electrodes, GmOx was replaced with the same amount of chitosan. Electrodes were denoted as GmOx/Chit/*o*-PD/Pt and CeO_2/TiO_2/GmOx/Chit/*o*-PD/Pt. All electrodes had AsOx incorporated, but it was omitted in the notations. SEM images of sensors can be found in Fig. S2.

2.4. Electrochemical measurements

Cyclic voltammetry was utilized to characterize the microelectrodes and establish the role of the metal oxides and the effect of oxygen deprivation. CVs were run in potential ranges from −0.25 to 1.65 V and from 0.2 to 1.2 V at a scan rate of 0.1 V/s in 0.1 M PBS (pH=7.4) (Fig. S3). Amperometry at a constant applied potential of 0.6 V was used to characterize the biosensor and determine the analytical performance characteristics for the detection of GluA in buffer and CSF. Buffer solutions were deoxygenated by purging nitrogen for 45 min to create anaerobic conditions. The nitrogen flow was maintained throughout the measurements. In control experiments, sensors were tested in the absence of metal oxides under anaerobic conditions. The effect of interferences was determined by co-injecting 5 μM GluA, 200 μM AA, 2 μM DA, 20 μM L-DOPA, and 10 μM 5HT.

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