Analytica Chimica Acta 645 (2009) 86–91

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

# A microelectrode biosensor for real time monitoring of L-glutamate release

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ARTICLE INFO

*Article history:*
Received 21 November 2008
Received in revised form 19 February 2009
Accepted 29 April 2009
Available online 5 May 2009

*Keywords:*
L-Glutamate
Sol–gel
Microbiosensor
*In vivo*
*In vitro*

ABSTRACT

We have developed an amperometric microbiosensor for real time monitoring L-glutamate release in neural tissue, based on enzymatic oxidation catalyzed by the L-glutamate oxidase. By means of a sol–gel coating method, L-glutamate oxidase was entrapped in a biocompatible gel layer that provided a benign environment and retained enzyme activity on the surface of Pt microelectrode. Prior to gel layer formation, a modification on the surface of Pt microelectrode with poly(phenylene diamine) enabled the microbiosensor screen majority of common potential interfering substances existing in physiological samples. The miniaturized biosensor achieved a steady state response to L-glutamate within 10 s and exhibited a linear dependence on the concentration of L-glutamate from 0.5 to 100 μmol $L^{-1}$ with a high sensitivity of 279.4 ± 2.0 μA (mmol $L^{-1}$)$^{-1}$ $cm^{-2}$ ($n$ = 4, R.S.D. = 2.8%). The microbiosensor also exhibited excellent long-term stability in dry storage. We have successfully used the microbiosensor for real time measuring of L-glutamate *in vivo*.



## 1. Introduction

As the major excitatory neurotransmitter in the mammalian central nervous system, L-glutamate plays crucial role in a wide variety of brain activities [1–3] such as brain development, memory formation, various neurology disorders including stroke, epilepsy and several devastating neurodegenerative diseases. For better understanding of its potential roles, it is important to real time monitor the release of L-glutamate in the brain. Although several methods have been developed to detect L-glutamate, including capillary electrophoresis [4], chemiluminometric sensors [5] and fluorescent sensors [6,7], amperometric biosensors have been by far the most favoured method since miniaturized biosensors can provide fast, sensitive and selective detection both *in vitro* and *in vivo*. L-Glutamate amperometric biosensors are mainly fabricated based on two kinds of enzyme, L-glutamate dehydrogenase and L-glutamate oxidase. Dehydrogenase based biosensors [8,9] have some drawbacks as they involve amperometric detection of NADH. This can require a high detection overpotential and leads to biosensor fouling from by-products. Recent efforts have tried to circumvent these problems by adopting redox mediators [10] and platinum/carbon nanotube hybrid films [11] to decrease the NADH detection overpotential. However, L-glutamate oxidase based biosensors [12–15] have been widely developed as the hydrogen peroxide produced by oxidase based biosensor can be readily detected electrochemically [16].

The selectivity of peroxide-based amperometric biosensors can be dramatically affected by non-specific interference responses caused by oxidation of a range of compounds, which commonly exist in biological samples such as ascorbate, urate, *etc.*, at the working potential of the electrode. To obtain highly selective detection of hydrogen peroxide in the presence of different interfering compounds, electron mediators [17–21] have been widely adopted for fabrication of L-glutamate biosensor. These decrease the hydrogen peroxide detection potential and give greater selectivity. However, mediated L-glutamate biosensors suffer from long response times (20–50 s) [18,21], which limits their application in real time monitoring the release of L-glutamate both *in vivo* and *in vitro*. A popular method to improve specific selectivity of amperometric biosensors is to utilize a permselective polymer modified electrode. The permselective polymer (such as Nafion® [19], poly(phenylene diamine) [8,22–24] and overoxidized polypyrrole [25]) allows hydrogen peroxide passing through the electrode surface and being directly oxidized on electrode surface, whilst rejects various interfering compounds. The very thin permselective layer allows biosensors with rapid response times (about 1–2 s) suitable for physiological monitoring to be made, e.g. with the overoxidized polypyrrole modified L-glutamate biosensor [25].

The performance of an amperometric biosensor including its sensitivity and stability requires efficient immobilization of enzyme on the electrode surface. To prevent the leaching of entrapped enzyme from sensing layer, L-glutamate oxidase is commonly attached onto polymer using covalent bonding between enzyme

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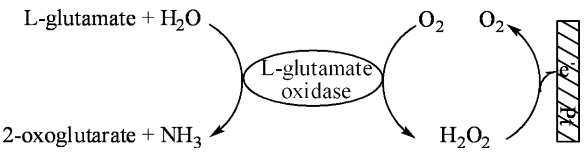
doi:10.1016/j.aca.2009.04.048




**Scheme 1.** Mechanism of L-glutamate microbiosensor.

and polymer [16,19,23,26–28]. However these methods run the risk of denaturing a significant proportion of the L-glutamate oxidase within sensing layer and can result in biosensors with low sensitivity and short shelf life.

The sol–gel techniques are of particular interest for enzyme immobilization [29,30]. And sol–gel film coated biosensors have attracted tremendous attention. Recently, a new methodology for forming sol–gel films on conductive substrates has been developed by our group [31,32]. Briefly, at a sufficient cathodic potential, $OH^-$ ions can be generated at the surface of an electrode and act as a catalyst to enhance the condensation and polymerization of hydrolyzed sol solution. The mild sol–gel formation condition makes it compatible with proteins such as enzymes and hence suitable for biosensor formation. Beside low temperature encapsulation and benign biocompatibility, this methodology also has advantages upon fast fabrication, high gel layer uniformity, ease of controlling enzyme-loading in the gel film and tunable gel properties including thickness and hydrophilicity. For instance, ATP biosensor and adenosine biosensor were successfully prepared on the Pt microelectrode using this method [32,33]. And the sol–gel film can even be deposited onto mediator (ruthenium purple) modified microelectrode to fabricate hypoxanthine biosensor [34].

In this paper, we demonstrate the fabrication of oxidase based biosensor for L-glutamate detection both *in vivo* and *in vitro*. First, a permselective poly(phenylene diamine) membrane was deposited on the surface of Pt microelectrode to reject the majority of interfering substances. Then, we use our simple and fast sol–gel electrodeposition method to entrap L-glutamate oxidase in the robust silica gel layer around a microelectrode. The detection principle of the biosensor depended on the amperometric detection of hydrogen peroxide produced by L-glutamate oxidase in the presence of L-glutamate, as shown in Scheme 1.

The resulting L-glutamate microbiosensors were characterized by a fast response, high sensitivity, favourable selectivity and excellent long-term stability.

## 2. Experimental

### 2.1. Materials and instrumentation

All silanes including tetramethyl orthosilicate (TMOS), 3-glycidoxypropyl-dimethoxymethylsilane (GOPTMOS) and 3-aminopropyltrimethoxysilane (APTMOS) and chemicals were commercially obtained from Sigma–Aldrich. L-Glutamate oxidase (EC 1.4.3.11) from *Streptomyces* sp. *X119*-6 was purchased in powder form from Yamasa Corporation, Choshi, Chiba, Japan. This product contained 6.3 units of enzyme activity per mg of powder. Sodium phosphate buffer solution (10 mmol $L^{-1}$, pH 7.4) was prepared and used as common supporting electrolyte in amperometric detection experiments. Fresh stock solutions of potential interferences (such as serotonin hydrochloride (5HT), ascorbic acid, dopamine, catechol and urate) were prepared just before use. Physiological measurements were performed in a Krebs artificial cerebrospinal fluid (aCSF, pH 7.4) composed of 124 mmol $L^{-1}$ NaCl, 3 mmol $L^{-1}$ KCl, 2 mmol $L^{-1}$ $CaCl_2$, 26 mmol $L^{-1}$ $NaHCO_3$, 1.25 mmol $L^{-1}$ $NaH_2PO_4$, 10 mmol $L^{-1}$ D-glucose and 1 mmol $L^{-1}$ $MgSO_4$. The aCSF was saturated with 95% $O_2$/5% $CO_2$. All aqueous solutions were prepared with 18.2 MΩ deionized water.

A CHI 660B workstation was used in cyclic voltammetric and amperometric experiments. A PG580 potentiostat–galvanostat (Uniscan instruments) was used for sol–gel electrodeposition. A three electrode cell equipped with a platinum foil counter electrode and a Ag/AgCl (saturated KCl) reference electrode was used. Platinum microelectrodes (obtained from Sycopel International Ltd. with a diameter of 50 μm, a length of 0.5 mm and a surface area of $7.85 \times 10^{-4}$ $cm^2$ if unspecified) were employed as the working electrode in all experiments. The L-glutamate microbiosensor was operated at +600 mV (*vs.* Ag/AgCl) in a flow system for amperometric detection at room temperature.

### 2.2. Preparation of L-glutamate microbiosensors

The Pt microelectrode was coated with poly(phenylene diamine) by scanning cyclic voltammetry from 0.2 to 0.8 V for 6 cycles at a scan rate of 10 mV $s^{-1}$ in a 10 mmol $L^{-1}$ phenylene diamine solution in 100 mmol $L^{-1}$ phosphate buffer pH 7.4. Following this, the silica gel layer entrapped with L-glutamate oxidase was electrodeposited onto surface of Pt microelectrode under mild chemical conditions. This method has been well established and described elsewhere [31]. In brief, silane precursors such as TMOS, GOPTMOS and APTMOS were pre-hydrolyzed with diluted hydrochloric acid to desired concentration. Then they were mixed with 50 mmol $L^{-1}$ Tris buffer (pH 7) to neutralize their pH. Additives such as glycerol, and NaCl were added into the mixture to stabilize L-glutamate oxidase and to ensure production of a sufficient amount of $OH^-$ around microelectrode during gel film deposition. Next, 1 unit of L-glutamate oxidase was dissolved into 10 μL of the sol mix, and transferred into a small glass capillary. Finally, the Pt microelectrode together with a counter electrode and a reference electrode was carefully inserted into the capillary, and a reduction potential between −0.9 and −1.2 V was applied under potentiostatic conditions for 10–40 s. A transparent, smooth and robust gel layer was uniformly formed on the surface of Pt microelectrode.

The fabricated L-glutamate biosensor was stored in pH 7.4 phosphate buffer solution and ready for use. For long-term storage stability test the biosensor was dried and stored in refrigerator at 4 °C.

### 2.3. Measuring L-glutamate release in vivo

Experiments were performed on 5 male Sprague–Dawley rats (300–340 g) and carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. The rats were anaesthetized with pentobarbitone sodium (60 mg $kg^{-1}$, I.P.). Anesthesia was maintained with supplemental doses of pentobarbitone sodium injected intravenously as required (10 mg $kg^{-1}$ $h^{-1}$, I.V.). Adequate anesthesia was ensured by maintaining stable levels of arterial blood pressure (ABP), heart and central respiratory rate. The femoral artery and vein were cannulated for measurement of ABP and administration of anesthetic, respectively. The trachea was cannulated and the animal was ventilated with $O_2$-enriched air using a positive pressure ventilator with a tidal volume of 1.5–2.0 mL and a ventilator frequency similar to the normal respiratory frequency (~60 strokes $min^{-1}$). The animal was then injected with gallamine triethiodide (Flaxedil™, 10 mg $kg^{-1}$, I.V.; then 1–2 mg $kg^{-1}$ $h^{-1}$, I.V.) and was placed in a stereotaxic frame. An occipital craniotomy was performed and the cerebellum was partially removed to expose the dorsal surface of the brainstem. The body temperature was maintained with a servo-controlled heating pad at 37.0 ± 0.2 °C. The exposed area of the brain was protected by covering with modified Bulmer's buffer, which consisted of 100 mmol $L^{-1}$ NaCl, 1 mmol $L^{-1}$ $MgSO_4$ and 2 mmol $L^{-1}$ KPi buffer (pH 7.4).

The sensors were connected to a MicroC potentiostat (WPI, Sarasota, FL, USA) and held on a stereotaxic micromanipulator. The