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

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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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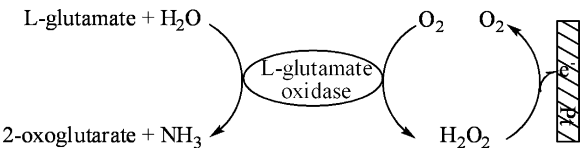

Scheme 1. Mechanism of L-glutamate microbiosensor.

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 7.85×10^{-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

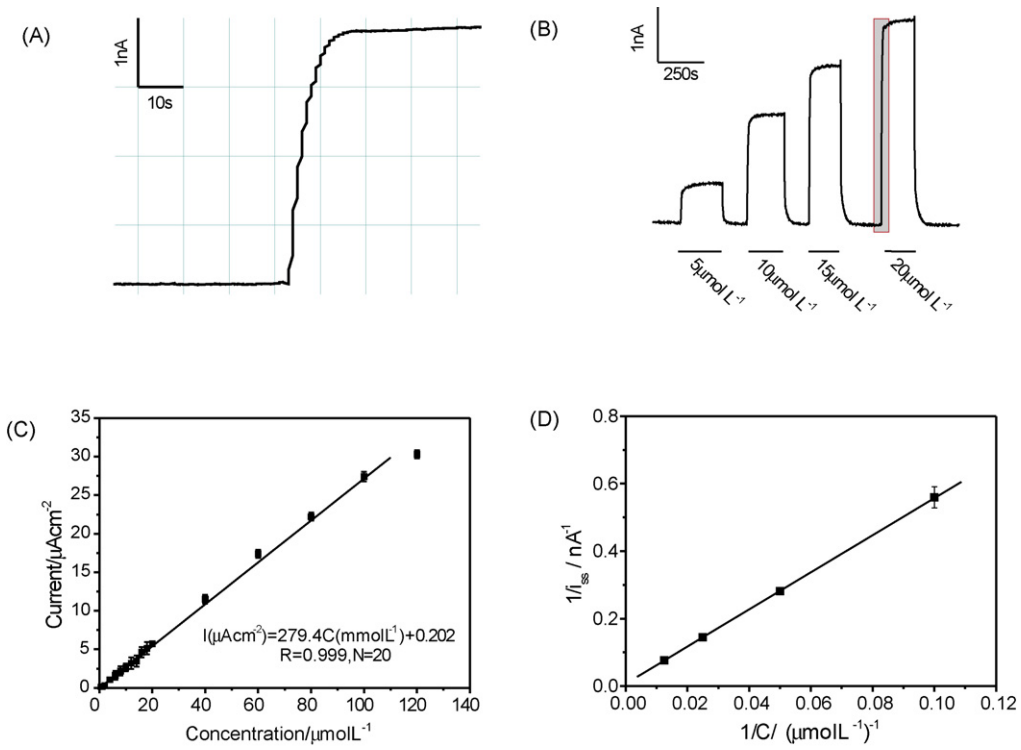

Fig. 1. Performance of the L-glutamate microbiosensor. (A) Enlargement of selected section in curve (B); (B) response of the microbiosensor to L-glutamate with different concentrations: 5, 10, 15 and 20 μmol L^{-1}. Applied potential is 600 mV *vs.* Ag/AgCl; (C) calibration of the microbiosensor and the linear regression equation; (D) linear fitting of a Lineweaver–Burk plot obtained from 3 individual L-glutamate microbiosensors.

sensors were aligned with the obex and placed into the nucleus tractus solitarus (NTS) regions (0.0 mm rostral, 0.5–0.7 mm lateral and 0.6–1.0 mm ventral of obex) where baroreceptor afferents terminate. The identical placement of the L-glutamate sensors and null sensors was achieved by aligning them to the obex and by means of the vernier scale of the manipulator. Once the sensors were placed, the exposed area of the brainstem and both sensors were covered with modified Bulmer's buffer and a period of 20–30 min was allowed for the biosensors to polarize to their operating potential (+500 mV with respect to Ag/AgCl). Sensors were calibrated *in vitro* immediately prior and after the recordings (some 1–2 h later) to test whether they retained sensitivity. During *in vivo* recordings L-glutamate microbiosensor lost on average ~50% of its initial sensitivity due to unspecific poisoning absorption on the microbiosensor surface. To convert changes in sensor current to changes in analyte concentrations, the mean of the initial and final calibrations was used. At the end of the experiment the animals were killed humanely by an overdose of pentobarbitone sodium (200 mg kg^{-1}, I.V.), the brains were removed and the locations of the recording sites were identified histologically. Histological analysis of the sensor placements confirmed that recording sites were within the targeted region of the NTS.

3. Results and discussion

Sol–gel films are commonly applied onto surface of electrode by spin-coating or dip-coating. However, the uniformity of gel layer thickness is different from each other in a batch of prepared biosensors or even in different areas of a same gel layer fabricated using such a coating procedure. In our method, a sol–gel film can be rapidly electrodeposited onto microelectrode from a mixture of enzyme, sol and additives by altering regional pH on the electrode surface. The thickness of the deposition layers usually ranging from several to hundred micrometers can be well controlled by adopting different cathodic voltages and deposition times. Many aspects of biosensor performance such as reproducibility and sensitivity can be systematically optimized to retain high activity of L-glutamate oxidase within the gel layer.

3.1. L-Glutamate microbiosensor performance

The high porosity of gel layer deposited on the surface of microelectrode allows substances such as substrate and hydrogen peroxide produced by enzymatic reaction to quickly diffuse through it reaching the electrode surface. Thus, the quick mass transport within gel matrix promises the L-glutamate biosensor with fast response time. As shown in Fig. 1A, for a typical L-glutamate biosensor the response time was shorter than 10 s for the detection of 20 μmol L^{-1} L-glutamate.

Fig. 1B shows the amperometric *i–t* curve of L-glutamate with different concentrations recorded on a typical L-glutamate biosensor. The biosensor gives linearly increasing amperometric response with respect to the L-glutamate concentrations. From such an amperometric detection method, a calibration plot for the sol–gel modified L-glutamate microbiosensor was shown in Fig. 1C. A linear dependence of amperometric current on concentration of L-glutamate was obtained over the range 0.5–100 μmol L^{-1} with a detection limit of 5 nM, which was estimated as three times the noise. The sol–gel modified biosensor shows a high sensitivity to L-glutamate of $279.4 \pm 2.0\,\mu A\,(mmol\,L^{-1})^{-1}\,cm^{-2}$ ($n = 4$, R.S.D. = 2.8%).

The apparent Michaelis–Menten constant (K_M^{app}), which gives an indication of the enzyme–substrate kinetics, can be determined following the Lineweaver–Burk-type equation [35,36]:

$$\frac{1}{i_{ss}} = \frac{1}{i_{max}} + \frac{K_M^{app}}{i_{max}} \frac{1}{C}$$

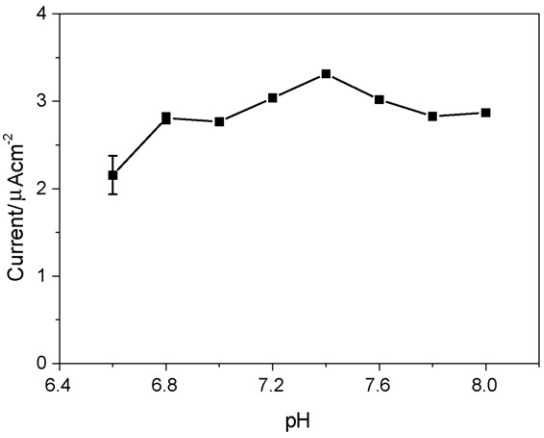

Fig. 2. Dependence of L-glutamate microbiosensor on pH. Responses to 15 μmol L^{-1} L-glutamate from pH 6.6 to 8.0. Each point is the mean obtained from 3 biosensors operated at 600 mV *vs.* Ag/AgCl.

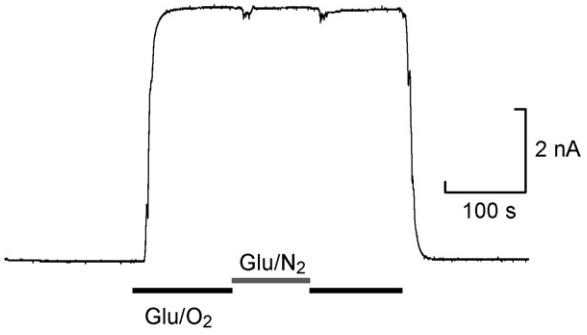

Fig. 3. The L-glutamate biosensor lacks appreciable O_2-sensitivity. Response to 10 μmol L^{-1} L-glutamate in aCSF separately saturated with 95% O_2 (Glu/O_2) and 95% N_2 (Glu/N_2) on L-glutamate microbiosensor with a length of 1 mm. Applied potential is 600 mV *vs.* Ag/AgCl.

where i_{max} and i_{ss} are the current measured under substrate saturation and the steady-state current for a given substrate concentration (C), respectively. Fig. 1D shows such a plot for sol–gel modified L-glutamate microbiosensor. From the linear fit, the apparent i_{max} and K_M^{app} of the sensor to L-glutamate were determined to be 141 nA and 776 μmol L^{-1}, respectively. The K_M^{app} of the microbiosensor is much smaller than that obtained on redox hydrogel modified L-glutamate biosensor (3 mmol L^{-1}) [26] and a Nafion® based L-glutamate biosensor (2.84 mmol L^{-1}) [19], which suggests the L-glutamate oxidase is well preserved and highly active within the sol–gel layer. As each molecule of L-glutamate can only result in the production of one molecule of hydrogen peroxide, and if it is assumed that all of hydrogen peroxide produced by enzymatic action can be sensed by the microbiosensor, i_{max} can be converted to V_{max} with units of moles per second. With such an assumption, we estimated the apparent V_{max} of the microbiosensor for L-glutamate to be 730 fmol s^{-1}.

The dependence of the L-glutamate microbiosensor response on pH was studied by determining the responses to 15 μmol L^{-1} L-glutamate solutions buffered to a range of different pH (Fig. 2). The amperometric response of the microbiosensor increased with pH from 6.6 to 7.4, and slightly decreased during pH range from 7.4 to 8.0. Thus, a highest amperometric response to L-glutamate was obtained at optimized pH 7.4.

The dependence of the biosensor response on dissolved oxygen was also studied in aCSF. This was saturated with 95% O_2 and 5% CO_2, 10 μmol L^{-1} L-glutamate was then applied in aCSF saturated with 95% O_2 before being switched to a solution of L-glutamate in aCSF saturated with 95% N_2 and 5% CO_2. Unlike the glass capillary-based L-glutamate electrode [37], the sensitivity of the microbiosensor was not affected by altering the O_2 level in the aCSF (Fig. 3). Although O_2 is required by L-glutamate oxidase to oxidize L-glutamate and produce H_2O_2, the O_2 will be regenerated at the surface of electrode. In the thin gel layer surrounding the electrode a sufficient amount of O_2 appears to be preserved to allow operation of the biosensor at low levels of oxygen pressure pO_2. This is a similar observation to other biosensors made with thin layers [32].

3.2. Selectivity of L-glutamate microbiosensor

The selectivity of the L-glutamate microbiosensor, which is an important issue for physiological measurements, has been assessed by measuring its response in the presence of several potential interfering substances that exist in physiological samples including L-glutamine (GLN), L-aspartic acid (ASP), L-ascorbic acid (AA), dopamine (DA), uric acid (UA), 5HT and catechol (CC). As shown in Fig. 4A, the microbiosensor shows its highly selective response against GLN and ASP as both of them with equivalent concentration (20 μmol L^{-1}) hardly give any response in contrast to the large responses produced by L-glutamate (GLU, 20 μmol L^{-1}) on the microbiosensor. This can be attributed to the unique specificity of L-glutamate oxidase with respect to its substance.

The poly(phenylene diamine) electrodeposited on the surface of Pt microelectrode efficiently eliminates the interfering response caused by co-oxidation of electrochemical active substances at high operating potential. Fig. 4B shows dependence of the microbiosensor amperometric response on GLU, AA, DA, UA, 5HT and CC detected in a flowing system. The relative interfering response of different substances on the L-gluatmate microbiosensor was shown in Table 1. The biosensor successfully screened interfering amperometric response from AA, DA, UA and 5HT. Whilst, an interfering signal about 19.8% for 10 μmol L^{-1} CC was obtained, this highly reactive material is unlikely exist in physiological media at such a concentration.

The interference rejecting performance of the microbiosensor was also evaluated in mixture of GLU without and with those interfering substances as shown in Fig. 4C. We found that the presence of these interferences made little difference to the size of the L-glutamate signal, suggesting that in our L-glutamate microbiosensor, the subsidiary reaction [23] between enzymatic produced hydrogen peroxide and oxidizable interferences does not happen.

3.3. Stability of the microbiosensor

The L-glutamate biosensors were highly reproducible showing a mean amperometric current of 2.79 nA with a SD of 0.51 nA (n = 12) for 15 μmol L^{-1} L-glutamate when they were fabricated on Pt microelectrodes with same dimensions (0.5 mm length and 50 μm diameter) using a jig.

Table 1
Effect of interfering substances on L-glutamate microbiosensor response.

Interfering substances	Concentration (μmol L^{-1})	Relative interfering response[a] (%)
Ascorbic acid	100	9.2
Dopamine	10	4.0
Uric acid	100	3.5
5HT	100	6.3
Catechol	10	19.8

[a] The relative interfering response $I_{interference}/I_{GLU}$. $I_{interference}$: current of interfering substance with different concentrations; I_{GLU}: current of 10 μmol L^{-1} L-glutamate.

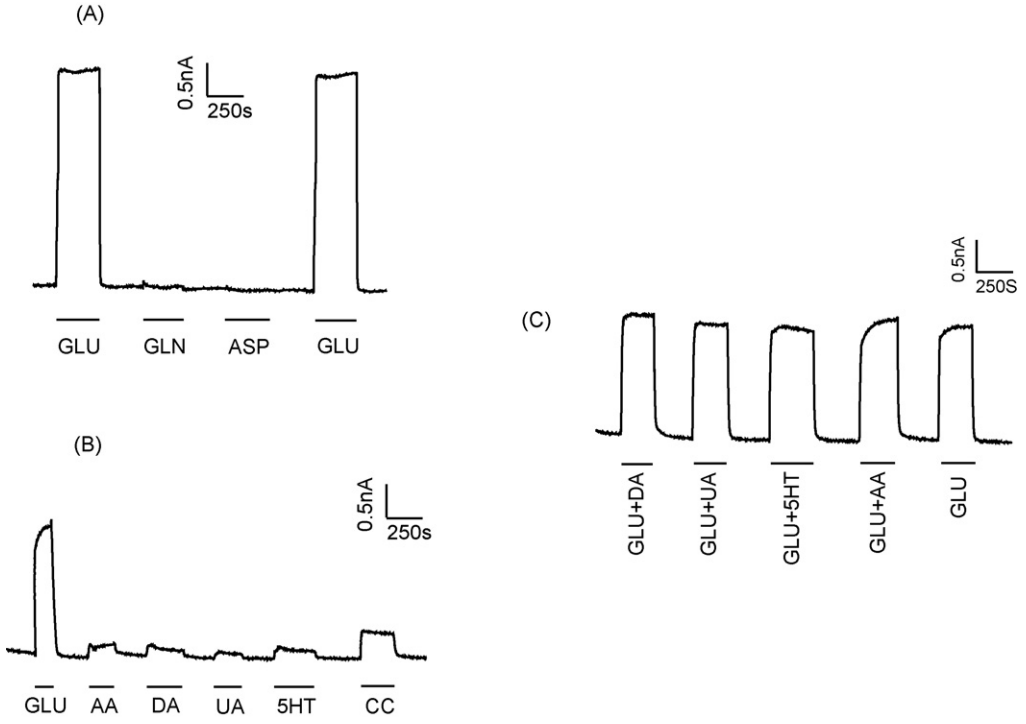

Fig. 4. Selectivity of the L-glutamate microbiosensor. (A) Selectivity of the microbiosensor against equivalent concentration (20 μmol L^{-1}) of L-glutamate (GLU), L-glutamine (GLN) and L-aspartic acid (ASP). (B) Recording of steady amperometric response on GLU (10 μmol L^{-1}), L-ascorbic acid (AA, 100 μmol L^{-1}), dopamine (DA, 10 μmol L^{-1}), uric acid (UA, 100 μmol L^{-1}), 5HT (100 μmol L^{-1}) and catechol (CC, 10 μmol L^{-1}). (C) Amperometric response of the microbiosensor on GLU (10 μmol L^{-1}) and its mixture with DA (10 μmol L^{-1}), UA (100 μmol L^{-1}), 5HT (100 μmol L^{-1}) and AA (100 μmol L^{-1}), respectively. Applied potential is 600 mV *vs.* Ag/AgCl in all measurements.

The long-term stability of the microbiosensor was evaluated by determining amperometric response of 15 μmol L^{-1} L-glutamate on a batch of simultaneously prepared microbiosensors ($n = 5$). As shown in Fig. 5, the microbiosensors exhibit favourable stability in five months dry storage. More than 95% sensitivity was retained for the microbiosensor during the testing period. This long-term stability may arise from the mild electrodeposition conditions for gel formation as well as biocompatible character of sol–gel matrixes.

3.4. Detection of L-glutamate release in vivo

We tested the biosensor performance *in vivo* by recording changes in L-glutamate concentration in the dorsal medullary nucleus of the solitary tract. This area of the brain is crucially involved in regulating the activities of the cardiovascular system. Activation of arterial baroreceptors when blood pressure increases slows the heart and dilates the blood vessels—a key negative feedback reflex controlling the level of arterial blood pressure at rest. Arterial baroreceptors transmit information to the second order relay neurones in a discrete region of the NTS via release of L-glutamate and its actions at excitatory amino acid receptors [38–41].

We therefore determined whether the biosensors placed in the NTS region where arterial baroreceptor afferents terminate could detect release of L-glutamate evoked by increases in arterial pressure following an intravenous infusion of the α_1-adrenergic receptor agonist phenylephrine (40 μg mL^{-1}; 1–3 μg bolus). Fig. 6 demonstrates that the L-glutamate biosensors recorded rapid increases in extracellular L-glutamate concentration, which closely followed changes in arterial blood pressure (Fig. 6 inset). This increase in L-glutamate concentration in the NTS had mean ampli-

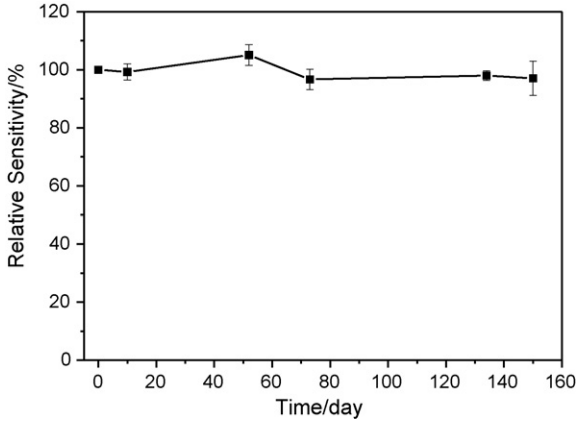

Fig. 5. Long-term storage stability of the L-glutamate microbiosensor. Each point represents the mean response from 5 biosensors to 15 μmol L^{-1} L-glutamate ($n = 5$) operated at 600 mV *vs.* Ag/AgCl.

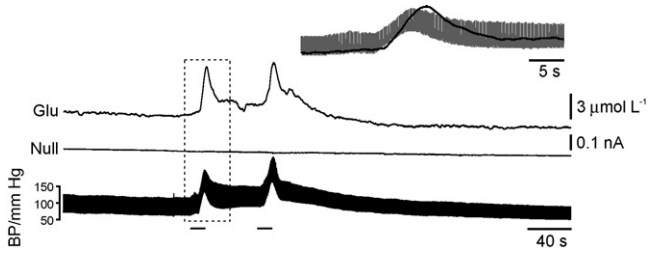

Fig. 6. *In vivo* performance of the L-glutamate microbiosensor. L-Glutamate and null biosensors were placed into the NTS of an anesthetized and artificially ventilated rat. All biosensors were operated at 500 mV *vs.* Ag/AgCl. Continual recordings of blood pressure (BP) were made. At the bars an intravenous infusion of the α_1-adrenergic receptor agonist phenylephrine (3 μg bolus) was given to increase arterial blood pressure. With a slight delay this caused transient release of L-glutamate in the NTS, no signal was seen on the null sensors. The inset shows an overlay of the blood pressure and L-glutamate biosensor traces from the dashed box to demonstrate the close correspondence between the two signals (same vertical scale as main figure).

tude of $5.4 \pm 0.4\,\mu mol\,L^{-1}$ ($n = 5$). No such signal was seen on the null sensor demonstrating that the biosensor specifically recorded release of L-glutamate in the NTS which was evoked by activation of the arterial baroreceptors.

4. Conclusions

We demonstrate the development of L-glutamate microbiosensor based on our sol–gel electrodeposition technique in this report. L-Glutamate oxidase along with additives is entrapped in sol–gel film formed under mild electrochemical deposition conditions. The abundant biocompatible function groups such as hydroxyl and amino groups within gel films provide benign environment suitable for retainment of activity of L-glutamate oxidase. After coated with a screening layer of poly(phenylene diamine), the microbiosensor displayed high selectivity against majority interfering substances existing in physiological samples. The attractive characteristics of high sensitivity, fast response time, favourable selectivity and excellent stability enable the microbiosensor to be ideally applicable for real time monitoring L-glutamate release both *in vitro* and *in vivo*.

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