Biosensor for Neurotransmitter L-Glutamate Acid
Designed for Efficient Use of L-Glutamate Oxidase and Effective Rejection of Interference

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An amperometric biosensor for L-glutamic acid (Glu) was constructed by the adsorption and dip coating of L-glutamate oxidase (GluOx, 200 U ml⁻¹ phosphate buffer, pH 7.4) onto 60-μm radius Teflon-coated Pt wire (1 mm exposed length). The enzyme was then trapped on the surface by electropolymerisation of o-phenylenediamine that also served to block electroactive interference. This procedure afforded electrodes with similar substrate sensitivity compared with the classical approach of immobilising enzyme from a solution of monomer, and represents an approximately 10 000-fold increase in the yield of biosensors from a batch of enzyme. A number of strategies were examined to enhance the sensitivity and selectivity of the Pt/PPD/GluOx sensors operating at 0.7 V versus SCE. Pre-coating the Pt with lipid and incorporation of the protein bovine serum albumin into the polymer matrix were found to improve the performance of the electrode. The sensors had a fast response time, high sensitivity to Glu, with an LOD of about 0.3 μmol l⁻¹, and possessed selectivity characteristics suggesting that monitoring Glu in biological tissues in vivo may be feasible.

Keywords: Enzyme-modified electrode; polymer-modified electrode; poly(o-phenylenediamine); ascorbic acid interference; amperometry; brain glutamate

L-Glutamate (Glu) is a ubiquitous excitatory amino acid neurotransmitter in the mammalian CNS, playing a major role in a wide variety of brain functions.¹⁻³ Glu concentrations in brain extracellular fluid (ECF) in vivo have been estimated in the 10 μmol l⁻¹ region using a variety of microdialysis techniques and detection systems.⁴⁻⁸ and a number of valuable studies on brain Glu have been reported using these methodologies.³⁻⁹⁻¹² The dialysis approach to monitoring brain chemistry has certain restrictions, however, such as limited time resolution and depletion of the ECF. As an alternative approach to detecting species in the ECF, implantable amperometric biosensors provide a continuous signal with significantly less interference by reducing agents.

An important problem in the use of enzyme-modified electrodes in biological media is interference by endogenous electroactive reducing agents, especially ascorbic acid (AA). This problem has been resolved, to a great extent, by the use of electro-synthesised polymers, such as poly(o-phenylenediamine) (PPD), that block access to the electrode surface of even relatively small organic molecules without affecting selectivity to hydrogen peroxide,⁴⁰ and a Pt/PPD/GluOx sensor has been reported.¹⁷₂⁴

The classical procedure for incorporating oxidase enzymes into a protecting polymer film has been to co-deposit the enzyme and polymer onto the electrode from a solution of the enzyme and monomer. This is convenient and effective but is very cost-inefficient for expensive enzymes such as GluOx. The aim of this work was to develop a more efficient procedure for producing Pt/PPD/GluOx sensors with high sensitivity to Glu. Since the intended application for these biosensors is monitoring Glu in brain ECF, where the concentration of AA is about 500 μmol l⁻¹,¹¹ twenty special attention was paid to blocking interference by reducing agents.

Materials and Methods
Reagents and Solutions

The enzyme L-glutamate oxidase (GluOx from Streptomyces sp. X-119-6, EC 1.4.3.11, 200 U ml⁻¹ in 20 mmol l⁻¹ potassium phosphate buffer, pH 7.4) was obtained as a generous gift from Yamasa Corporation, Chiba, Japan, and stored at −20 °C. The enzyme glucose oxidase (GOx) from Aspergillus niger (EC 1.1.3.4, Type VII-S) was from Sigma Chemical Co. (St. Louis, MO, USA). The lipid phosphatidylethanolamine (PEA, Type II-S) and bovine serum albumin (BSA, fraction V) were also obtained from Sigma. Stearic acid (STA), Nafion (NAF, 1100 EW, 5% solution in alcohol) and d-glucose were obtained from Aldrich (Milwaukee, WI, USA). All chemicals, including o-phenylenediamine (PD, Sigma), L-glutamic acid (Glu, Sigma), L-glutamate (Sigma) and L-ascorbic acid (AA, Aldrich), were used as supplied.

Solutions of PD (20–400 mmol l⁻¹) were made up in 10 ml of a phosphate-buffered saline solution (PBS) with dissolution achieved by sonication at 25 °C for 25 min. Stock solutions of 100 mmol l⁻¹ Glu and AA were prepared in doubly distilled water and 0.01 mol l⁻¹ HCl, respectively, and stored at 4 °C.

All experiments were carried out in vitro in PBS (pH 7.4) that consisted of 0.15 mol l⁻¹ NaCl (Merck, Poole, UK), 0.04 mol l⁻¹ NaH₂PO₄ (Merck) and 0.04 mol l⁻¹ NaOH (Merck). Solutions were kept refrigerated when not in use.

Instrumentation and Software

Experiments were microcomputer controlled with data acquisition achieved using a Biodata Microlink interface, a low-noise, low-damping potentiostat (Biostat II, Electrochemical and...
Medical Systems, Newbury, UK) and in-house software. The linear and non-linear regression analyses were performed using the graphical software package Prism (GraphPad Software, San Diego, CA, USA). All experiments were done in a 25 ml glass cell at 25 °C, using a standard three-electrode set-up with a saturated calomel electrode (SCE) as the reference and a silver wire in a glass sheath as the auxiliary electrode.

Preparation of the Working Electrodes

All the working electrodes were based on Pt cylinders (60 μm radius) prepared by cutting strips of Teflon-coated platinum wire, sliding the Teflon along the wire to expose about 1 mm of metal and sealing the Teflon rim with cyanoacrylate adhesive. The exposed metal was then dipped a number of times into 20 μl of a buffered solution of GluOx or GOx to deposit the enzyme. The number of dips varied (see below) but in all cases the first dip was left in the enzyme solution for 5 min to allow adsorption of the enzyme to occur.17,24,43,44 For all subsequent dips the electrode was immersed in the enzyme solution, removed immediately and allowed to dry for 5 min.

The enzyme-coated wire was introduced into PBS containing the monomer (20–400 mmol l\(^{-1}\)) PD and, in some cases, the non-enzyme protein BSA (5 mg ml\(^{-1}\)), and electropolymerisation carried out immediately at 0.7 V versus SCE. The polymerisation time for this self-sealing process was 30 min, unless stated otherwise. In some cases the lipid PEA45,46 or fatty acid STA was used to coat the Pt before enzyme deposition. This procedure involved dipping the bare Pt wire a number of times into PEA or STA dissolved in chloroform (100 mg ml\(^{-1}\)), allowing the solvent to evaporate each time (1 min).

Calibrations were performed amperometrically at 0.7 V versus SCE in quiescent air-saturated PBS for glucose in the range 0–10 mmol l\(^{-1}\) and for Glu in the ranges 0–100 mmol l\(^{-1}\) (for LOD and linearity studies) and 0–10 mmol l\(^{-1}\) (for \(K_m\) and \(V_{max}\) determinations). Calibrations for interfering substances were also carried out under the same conditions except Na\(^+\)-saturated buffer was used: ascorbate (AA, 0–1 mmol l\(^{-1}\)), dopamine (DA, 0–10 mmol l\(^{-1}\)), 3,4-dihydroxyphenylacetic acid (DOPAC, 0–100 μmol l\(^{-1}\)) and uric acid (UA, 0–100 μmol l\(^{-1}\)). Air-saturated solutions were used in the determination of l-glutamine interference (0–1 mmol l\(^{-1}\)).

Since the resting level of AA in brain ECF has been estimated\(^{41,42}\) at 500 mmol l\(^{-1}\) and because the AA response at 0.7 V versus SCE for 1 Glu, recorded with sensors produced by GluOx and 300 mmol l\(^{-1}\) PD, was 1.8 ± 0.9 μA cm\(^{-2}\) (n = 13); this combination of enzyme and monomer has been shown previously to be optimal for these conditions.\(^{40}\) The currents for 500 mmol l\(^{-1}\) glucose recorded under the same conditions with sensors produced by different numbers of dip coatings using a 200 μl l\(^{-1}\) GluOx solution (to mimic the GluOx solution supplied) followed by polymerisation in 300 mmol l\(^{-1}\) PD were: 1 dip, 0.7 ± 0.1 nA (n = 2); 2 dips, 3.8 ± 2.3 nA (n = 3); 5 dips, 5.1 ± 2.0 nA (n = 3); 10 dips, 10.9 ± 4.8 nA (n = 3); and 20 dips, 3.1 ± 1.6 nA (n = 2). There was a maximum in the response for 10 dips that corresponds to a current density of 2.8 ± 1.3 μA cm\(^{-2}\) (n = 3).

Thus, the response of sensors produced by the dip coating method was at least as good as the classical co-deposition procedure. In addition, the reproducibility of the sensors’ sensitivity to Glu (as measured by the coefficient of variation) was the same for the two methods of production.

Pt/PPD/GOx Electrodes

Previous studies on the immobilisation of enzymes into the non-conducting form of the polymer PPD have involved either electropolymerisation from a solution of the enzyme plus monomer\(^{33,39}\) or from a monomer solution using electrodes with pre-adsorbed enzyme.17,24 The former approach uses large amounts of enzyme, typically 5 mg ml\(^{-1}\), whereas the latter leads to sensors with relatively poor sensitivity for substrate.\(^{17}\) To develop a sensor for Glu, using the expensive enzyme GluOx, we investigated here the use of ‘dip coating’ to deposit enzyme on the electrode surface prior to electropolymerisation in solution of monomer, PD.

To compare the effectiveness of the dip coating approach to the standard co-deposition of enzyme and polymer from solution, we used GOx as a model enzyme. Because our sensor development programme is motivated by applications in mammalian brain, we have used concentrations appropriate to this environment in many of the experiments.

The average current density for 500 mmol l\(^{-1}\) glucose, recorded amperometrically at 0.7 V versus SCE with Pt/PPD/GOx sensors produced by polymerisation in a 5-ml solution containing 5 mg ml\(^{-1}\) GOx and 300 mmol l\(^{-1}\) PD, was 1.8 ± 0.9 μA cm\(^{-2}\) (n = 13); this combination of enzyme and monomer has been shown previously to be optimal for these conditions.\(^{40}\) The currents for 500 mmol l\(^{-1}\) glucose recorded under the same conditions with sensors produced by different numbers of dip coatings using a 200 μl l\(^{-1}\) GOx solution (to mimic the GluOx solution supplied) followed by polymerisation in 300 mmol l\(^{-1}\) PD were: 1 dip, 0.7 ± 0.1 nA (n = 2); 2 dips, 3.8 ± 2.3 nA (n = 3); 5 dips, 5.1 ± 2.0 nA (n = 3); 10 dips, 10.9 ± 4.8 nA (n = 3); and 20 dips, 3.1 ± 1.6 nA (n = 2). There was a maximum in the response for 10 dips that corresponds to a current density of 2.8 ± 1.3 μA cm\(^{-2}\) (n = 3).

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Pt/PPD/GluOx Electrodes

Having established that GOx could be successfully immobilised by dip coating followed by polymerisation, the procedure was applied to GluOx. The amperometric responses at 0.7 V versus SCE for 10 μmol l\(^{-1}\) Glu, recorded with sensors produced by different numbers of dip coatings using a 200 μl l\(^{-1}\) GluOx solution followed by polymerisation in 300 mmol l\(^{-1}\) PD were: 1 dip, 0.62 ± 0.02 nA (n = 2); 2 dips, 0.59 ± 0.14 nA (n = 2); 5 dips, 0.78 ± 0.24 (n = 5); and 10 dips, 0.66 ± 0.35 (n = 5).
Surprisingly, and in contrast to GOx, there was no distinct peak in the current versus number of dips. Five dips was chosen as the standard for further experiments as this gave the largest (albeit not statistically different) response; this sensor is represented as Pt/PPD/GluOx. Approximately 60 sensors were made from each 20 μl aliquot of enzyme, representing an approximately 10 000-fold increase in the efficiency of GluOx immobilisation compared with the co-deposition method.

The sensitivity of the sensor corresponds to a current density of 0.20 ± 0.07 μA cm⁻² (n = 5) for 10 μmol l⁻¹ Glu and compares very favourably with other biosensors for Glu.16,17,20 The latter value being the same as that observed for GluOx in solution.21 Considering the sensitivity of Pt to AA,40 bare cylinders of the dimensions used here would be expected to give about 400 nA for 500 μmol l⁻¹ AA. The actual response to this concentration of AA determined using Pt/PPD/GluOx sensors was only 0.8 ± 0.3 nA (n = 5), indicating that the PPD film is blocking access to the Pt surface quite efficiently. This barrier, however, is not sufficient for neurochemical applications since the selectivity coefficient for 10 μmol l⁻¹ Glu versus 500 μmol l⁻¹ AA, Sₐₐ (see eqn. 4), was −11 ± 40% (n = 5), i.e., the current for AA was similar to that for Glu at these concentrations.

Pt/PEA/PPD/GluOx Electrodes

The improved selectivity afforded by undercoating the enzyme layer with large molecules, such as PEA,45,46 is due to the fact that different sites are involved in the oxidation of interference molecules (Pt surface) and the substrate (enzyme) (see Fig. 1). Thus, PEA efficiently blocks access to the Pt surface for AA, say, but does not hinder access of Glu to the enzyme (reaction 1). Furthermore, the sensitivity of Pt to the small H₂O₂ molecule (reaction 3) is not affected by macromolecules on the Pt surface.46

Pre-coating Pt disks with the lipid PEA by drop coating from chloroform before electropolymerisation has been shown previously to enhance the interference blocking properties of biosensors.45,46 Since drop coating also led to a slower response time,45 the dip coating method was investigated here as an alternative means of depositing PEA onto bare Pt cylinders before immobilisation of the GluOx. The effect of 0–10 dips in a PEA solution (100 mg ml⁻¹ in chloroform) on the response of the sensor to 10 μmol l⁻¹ Glu and 500 μmol l⁻¹ AA was determined.

The presence of lipid had no significant effect on the Glu response. Increasing the amount of PEA on the sensor surface, however, decreased the AA response and had a maximum effect for 5 dips (0.33 ± 0.03 nA, n = 3, p < 0.05, compared with no PEA, 0.78 ± 0.29, n = 5). The observation that 10 dips was less effective than 5 dips may be caused by inhibition of PPD formation at this higher surface coverage of PEA. Therefore the characteristics of the Pt/PEA/PPD/GluOx sensor were improved by the PEA (Sₐₐ = 43 ± 10%, n = 3), the selectivity of the electrode was still not considered adequate for neurochemical analysis in vivo.

Pt/PPD/BSA/GluOx Electrodes

The ability of non-conducting PPD films formed at neutral pH (about 10 nm thick34,48) to block interference appears to be enhanced by the incorporation of protein (enzyme and non-enzyme) into the polymer matrix;30,45 both electrochemical and electron microscopy data suggest that PPD films are more compact when formed by electropolymerisation in monomer solution containing protein.40 Although enzyme is present on the Pt for the sensors described here so far, protein was not present in the polymerisation solution. Electropolymerisation in solutions of PD and BSA (5 mg ml⁻¹, a concentration that has been shown to be optimal for our conditions45) was therefore carried out in attempts to improve the selectivity further.

The data in Table 1 show that there was indeed a significant (p < 0.01) improvement in Sₐₐ (83 ± 9%, n = 9) when BSA was incorporated into the PPD sensor. Surprisingly, this improvement was due to both an increase in the Glu current and a decrease in the AA response. Thus, the co-deposition of protein with the polymer may protect some GluOx molecules from inactivation by the polymerisation process in a similar way to that in which BSA protects enzymes during cross-linking with glutaraldehyde.49

A schematic illustration of a Pt/PEA/PPD/BSA/GluOx electrode, based on both structural and electrochemical data, is shown in Fig. 1. The thickness of the non-conducting PPD film formed under neutral conditions (about 10 nm thick44) is similar to the diameter of oxidases such as GOx39 (about 9 nm, 180 kD) and GluOx (about 140 kD21), whereas the size of the smaller BSA molecule (about 70 kD) is not critical. One might expect, therefore, that the binding site of a population of GluOx molecules would not be hindered by the polymer.53 This view is supported by the reported rapid response times (e.g., 1 s34 or < 10 s30,40,45) for glucose at PPD/GOx electrodes and for Glu at PPD/GluOx sensors (see Fig. 2, below). Although PPD efficiently blocks access to the metal for molecules the size of AA, Glu, glucose, etc., the small H₂O₂ molecules produced enzymatically (reactions 1 and 2) can diffuse to the Pt surface to be oxidised40 (reaction 3). The PEA underlying the enzyme layer provides additional blocking of the Pt surface for interferences, such as AA, but does not hinder access of Glu to the enzyme (reaction 1).

**Table 1** Average ± s Glu and AA currents calculated from individual calibrations in PBS (pH 7.4) at +0.7 V versus SCE, the corresponding selectivity coefficients (Sₐₐ, see eqn. 4) ± s and LOD ± s. Number of dip coatings of each modifier was: phosphatidyethanolamine (PEA, 100 mg ml⁻¹ in chloroform: 5), glutamate oxidase (GluOx, 200 U ml⁻¹ phosphate buffer: 5), stearic acid (STA, 100 mg ml⁻¹ in chloroform: 5) and Nafion (NAC, 1% alcohol solution: 1).

<table>
<thead>
<tr>
<th>Electrode design</th>
<th>10 μmol l⁻¹ Glu/nA</th>
<th>500 μmol l⁻¹ AA/nA</th>
<th>Sₐₐ (%)</th>
<th>LOD/μmol l⁻¹</th>
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deposition and subsequent polymerisation. The data in Table 1 show that the replacement of blocking agent had the opposite effect to that expected, decreasing the selectivity coefficient ($p < 0.05$) due to a larger AA response ($p < 0.02$). It appears therefore that the hydrophobic properties of these molecules are equally, if not more, important than electrostatic factors for blocking interference.

The perfluorinated polysulfonic acid Nafion has been used even more widely than STA to block AA interference in neurotransmitter detection.$^{52}$ However, the replacement of PEA in the sensor using 1 dip of a 1% solution of Nafion in alcohol had a catastrophic effect on $S_{AA}$, decreasing the Glu signal and increasing the AA current (see Table 1). Nafion may therefore inhibit both the deposition of GluOx on the electrode and the formation of PPD.

As final modifications of the sensor we investigated the effect of monomer concentration and polymerisation time. At least four sensors were made for each monomer concentration: 20, 100, 200, 300 and 400 mmol $l^{-1}$ PD. The value of $S_{AA}$ rose steadily from $-360 \pm 250\%$ ($n = 4$) at 20 mmol $l^{-1}$ PD, $-50 \pm 50\%$ ($n = 4$) at 100 mmol $l^{-1}$, $15 \pm 30\%$ ($n = 4$) at 200 mmol $l^{-1}$ to a peak value of $83 \pm 9\%$ ($n = 9$) at 300 mmol $l^{-1}$ PD, and declined again to $66 \pm 27\%$ ($n = 4$) at 400 mmol $l^{-1}$, a concentration close to saturation. The $S_{AA}$ value was also sensitive to polymerisation time in 300 mmol $l^{-1}$ PD solutions, mainly due to a decrease in AA response for longer times: 12 ± 16\% (1 min, $n = 3$); $10 \pm 100\%$ (5 min, $n = 3$); 86 ± 9\% (15 min, $n = 2$); and 83 ± 9\% (30 min, $n = 9$). Thus, although the polymerisation current associated with the formation of the self-sealing, non-conducting PPD (about 10 nm thick$^{34,48}$) falls rapidly to low steady-state values, it appears that 15 min are needed to minimise any small pores in the polymer matrix$^{39}$ (see Fig. 1).

**Other Characteristics of the Pt/PEA/PPD/BSA/GluOx Sensor**

The sensor type with the best average selectivity was the Pt/PEA/PPD/BSA/GluOx electrode produced by electropolymerisation in 300 mmol $l^{-1}$ PD for 15–30 min: $S_{AA} = 84 \pm 9\%$ ($n = 11$) for 10 mmol $l^{-1}$ Glu and 500 mmol $l^{-1}$ AA. For use in a given application, however, electrodes can be chosen that have the best characteristics. For example, half of the total sample of 11 electrodes gave $S_{AA} = 92 \pm 3\%$ ($n = 5$). This sensor design (see Fig. 1) may therefore be suitable for biochemical studies and therefore further characterisation was carried out in vitro.

The response time to Glu was of the order of the mixing time in the cell (about 10 s, see Fig. 2), i.e., similar to Pt/PPD/GluOx electrodes$^{33–39}$ but much faster than sensors incorporating PEA by the drop coating technique.$^{35}$ The sensitivity was high in the linear range (0–100 mmol $l^{-1}$ Glu, 3.8 ± 1.3 nA $c m^{-2} m l^{-1}$, $r^2 = 0.99 \pm 0.01$, $n = 4$) with a low LOD ($2 \times s$ of baseline, Table 1). Glutamine, a possible source of interference with an ECF level in the region of 100 mmol $l^{-1}$, but much faster than sensors incorporating PEA by the drop coating technique.$^{35}$ The sensitivity was high in the linear range (0–100 mmol $l^{-1}$ Glu, 3.8 ± 1.3 nA $c m^{-2} m l^{-1}$, $r^2 = 0.99 \pm 0.01$, $n = 4$) with a low LOD ($2 \times s$ of baseline, Table 1). Glutamine, a possible source of interference with an ECF level in the region of 100 mmol $l^{-1}$, but much faster than sensors incorporating PEA by the drop coating technique.$^{35}$ The sensitivity was high in the linear range (0–100 mmol $l^{-1}$ Glu, 3.8 ± 1.3 nA $c m^{-2} m l^{-1}$, $r^2 = 0.99 \pm 0.01$, $n = 4$) with a low LOD ($2 \times s$ of baseline, Table 1). Glutamine, a possible source of interference with an ECF level in the region of 100 mmol $l^{-1}$, but much faster than sensors incorporating PEA by the drop coating technique.$^{35}$
absent in brain tissue, presumably owing to the absence of suitable catalysts.39 Table 2 shows the response to a variety of other interfering substances found in brain ECF. Modification of the Pt with the PEA/PPD/BSA film (Fig. 1) reduced its sensitivity to DA, DOPAC and UA about 100 fold, and the responses were linear in the low concentration ranges tested. At the levels of these substances found in brain ECF, the corresponding current (see Table 2) would be negligible compared with the 10 μmol l−1 Glu signal (>1 nA, see Table 1). Interference by UA is complicated by the finding that its concentration in brain ECF depends on the size of the electrode39 but for sensors of the size used here, UA concentrations would be <5 μmol l−1. Thus, although the presence of AA (and not other endogenous species, such as DA17) is the most significant limitation for the application of biosensors in brain tissue, the selectivity of the Pt/PEA/PPD/BSA/GluOx electrode developed here suggests suitability for neurochemical applications in vivo. A more complicated combination of Nafion, ascorbate oxidase, cellulose acetate, glutaraldehyde, BSA and GluOx has been used in one study to produce a sensor with an equivalent SAA of about 99%;16 however, there has been a paucity of reports on applications using this sensor in the years since its first appearance.

Conclusions

The relatively simple fabrication of a sensor for Glu, based on the efficient use of GluOx, has been described. The sensor has high sensitivity for substrate and sufficient selectivity to provide a basis for neurochemical applications in vivo. Detailed characterisation remains to be carried out in brain tissue in vivo, however, before the sensor can be used to monitor Glu unambiguously in the ECF.

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References


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