Sensors and Actuators B 178 (2013) 606–614

Contents lists available at SciVerse ScienceDirect

Sensors and Actuators B: Chemical

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

Highly selective and stable microdisc biosensors for L-glutamate monitoring

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ARTICLE INFO

Article history:
Received 11 March 2012
Received in revised form 8 December 2012
Accepted 19 December 2012
Available online 16 January 2013

Keywords:
Glutamate
Biosensor
o-Phenylenediamine
Nafion
Selectivity
Stability

ABSTRACT

Glutamate mediates most of the excitatory synaptic transmission in the brain, and its abnormal regulation is considered a key factor underlying the appearance and progression of many neurodegenerative and psychiatric diseases. In this work, a microdisc-based amperometric biosensor for glutamate detection with highly enhanced selectivity and good stability is proposed. The biosensor utilizes the enzyme glutamate oxidase which was dip-coated onto 125 μm diameter platinum discs. To improve selectivity, phosphatidylethanolamine was pre-coated prior to enzyme deposition, and electropolymerization of *o*-phenylenediamine was performed to entrap the enzyme within a polymer matrix. A variety of coating configurations were tested in order to optimize biosensor performance. For stability measurements, biosensors were biased continuously and calibration curves calculated each day for a period of 5–6 days. The optimized biosensors exhibited very high sensitivity (71 ± 1 mA M^{-1} cm^{-2}), low detection limit of ~2.5 μM glutamate, selectivity (over 87% against ascorbic acid), very good temporal stability during continuous use, and a response time of <5 s. These biosensors are therefore good candidates for further development as devices for continuous monitoring during traumatic brain injury or neurosurgery.

1. Introduction

L-Glutamate biosensors have been in the forefront of neuroscience research due to the vital role played by L-glutamate in excitatory synaptic transmission in the brain [1,2]. Its effects are mediated through a large variety of ionotropic and metabotropic receptors abundantly expressed along the whole extent of the neuraxis [3,4]. Abnormal regulation of glutamatergic transmission has been found to be the key factor that underlies the appearance and progression of many neurodegenerative and psychiatric diseases [5–7]. In addition, glutamate monitoring in the food industry is also gaining interest [8]. At the site of brain injury, due to the sequence of mechanisms that occurs following injury, extracellular glutamate levels have been shown to increase prior to any change in intracranial pressure [9]. Therefore, in this paper, glutamate biosensors developed towards implantable applications are discussed.

In order to monitor glutamate, researchers have developed biosensors using various methods and materials to obtain suitable characteristics and performance for implantable applications [10–15]. In order to avoid issues relating to the performance of implanted biosensors, *i.e.*, biofouling, encapsulation and changes in pO_2, some authors have coupled the biosensors with microdialysis sampling for continuous *in vivo* monitoring [16,17]. Regardless of the placement of the biosensor, whether in-line using microdialysis or *in situ* in the brain, the transduction process is often performed by oxidoreductase enzymes, L-glutamate oxidase and glutamate dehydrogenase, or using glutamate receptors [18]. The principle of action of glutamate biosensors explored here has been elucidated by Ryan et al. [15], in which they utilized a Pt/Ir cylinder of 1 mm length and 125 μm diameter dip-coated with glutamate oxidase (GluOx) as the working electrode. However, McMahon et al. [19] found that miniaturizing the surface area had detrimental effects on the selectivity of these poly(*o*-phenylenediamine) (PPD)-based biosensors. Here, therefore, various configurations of sensors with different interference rejecting components were explored in order to improve the selectivity of microdisc-type glutamate biosensors. In addition, the temporal and pH stability of the best configurations of biosensors were investigated to examine whether they retained their sensitivity during continuous use and changes in the pH of the electrolyte solution, bearing in mind their intended ultimate application as implantable biosensors.

2. Materials and methods

2.1. Reagents and solution

The enzyme L-glutamate oxidase (GluOx, from *Streptomyces* sp. X-119-6, EC 1.4.3.11) was obtained as a generous gift from Yamasa

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http://dx.doi.org/10.1016/j.snb.2012.12.077

Corporation, Chiba, Japan, and stored at −20 °C. The lipid phosphatidylethanolamine (PEA, Type II-S), bovine serum albumin (BSA, fraction V), phosphate buffered saline tablets (PBS, pH 7.4) and polyethyleneimine (PEI) were obtained from Sigma. All chemicals, including *o*-phenylenediamine (*o*-PD, Sigma), L-glutamic acid (Glu, Sigma), L-ascorbic acid (AA, Aldrich) and Nafion (5% solution in a mixture of lower aliphatic alcohols and water, Sigma), were used as supplied. Double distilled water was used for all solutions.

The aqueous solution of the enzyme was made by dissolving 50 units of GluOx in 250 μL of 0.1 M phosphate buffer at pH 7.0 to make 200 units/mL of the enzyme. When not in use, the enzyme solution was stored at −20 °C. The monomer solution for electropolymerization was prepared by dissolving 300 mM *o*-PD with 0.125 g of bovine serum albumin (BSA) in 25 mL of PBS, with dissolution achieved by ultrasonication at 25 °C for 15 min. A 1% (w/v) solution of PEI in water was prepared and stored at room temperature. A solution of PEA was made by dissolving 25 mg of the lipid PEA in 250 μL of chloroform and stored at 4 °C. Stock solutions of 100 mM Glu were prepared in distilled water; 100 mM AA stock solutions were prepared by dissolving in 0.01 M HCl and stored at 4 °C. All experiments were carried out *in vitro* in the laboratory using 5 mL of PBS at pH 7.4 (0.01 M phosphate buffer and 0.137 M NaCl) in a 5 mL glass beaker at room temperature.

2.2. Instrumentation and software

Experiments were conducted using two low-noise potentiostats. The custom built Neurochemical Recording System (NRS) (courtesy of Lance Thompson and the Medical Physics Department at the Newcastle General Hospital) provided a lowest ideal resolution of 0.15 pA at a 10 nA current range and the converted digital signals were fed to a computer for data storage and visualization. In addition to the NRS, a second potentiostat, Uniscan PG580 system from Uniscan Instruments Limited (Buxton, Derbyshire, United Kingdom) was also used.

2.3. Preparation of the working electrodes

All working electrodes were based on Teflon-coated platinum wires of 125 μm diameter. One end of the wire was connected to a gold pin for connection, and the other end of the wire with the Teflon coating intact served as the platinum disc electrode. Some electrode configurations were dipped in a 5% solution of Nafion and dried before being cured for 7–10 min at 170–180 °C [20,21]. The electrodes were dipped in a 1% solution of a polycationic polymer PEI and dried for 15 min immediately before immobilizing GluOx [22]. Deposition of the enzyme was carried out by immersing the electrodes in a buffered solution of the enzyme GluOx for 5 min to allow the adsorption of the enzyme on the discs and letting them dry for 5 min. Following the first adsorption, 2 subsequent dips were quickly performed with the drying times being the same to provide a total 3 dip–evaporation protocol for the enzyme. The electrodes containing the enzyme were introduced into the monomer solution and electropolymerization was performed by applying a potential between 0.40 V and 0.65 V *vs*. Ag/AgCl electrode depending on the sensor configuration, using a three-electrode electrochemical cell with a stainless steel needle serving as the counter electrode [23]. The polymerization time for the formation of the polymer was either 20 min or 15 min depending on the potential used and the sensor configuration. Following polymerization, the discs were left immersed in PBS overnight biased at 0.65 V to stabilize the biosensors. In the nomenclature used for the disc configurations, a forward slash separates layers coated one after the other, whereas a hyphen represents substances immobilized simultaneously, as suggested recently [24].

2.4. Experimental methods

Calibrations were performed in a two-electrode electrochemical cell with a solid Ag/AgCl pellet electrode (Harvard Apparatus, UK) as the reference/counter at a potential of 0.65 V in PBS (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH 7.4, at 25 °C). All potentials applied to the working electrode described in this work are relative to this Ag/AgCl reference. Calibrations for glutamate were performed in the range 0–4 mM, for AA between 0 and 1 mM and for hydrogen peroxide (H_2O_2) between 0 and 1 mM. Since it is established that the response of AA at PPD-modified electrodes is non-linear with the response displaying a plateau or decreasing at higher concentrations (see, *e.g.* Fig. 5, later), the approach to quantifying the interference was performed by doubling the nominal basal concentration of AA in brain striatal ECF (~500 μM) as suggested by Ryan et al. [15]. AA levels in the ECF are orders of magnitude higher than other interference species, such as dopamine, DOPAC, and uric acid, and AA was therefore the focus of this study. Although generic PPD–GluOx designs have performed well in a broader range of characterizations in the past [15,24], electroactive species, amino acids and proteins that could potentially affect the sensor performance *in vivo* will be considered in future studies for the designs developed here. Further details of specific fabrication protocols are provided in Section 3.

2.5. Data analysis

Calibration plots for glutamate were generated by plotting the steady-state current responses obtained *versus* the substrate concentration, and using a non-linear regression curve-fit to obtain the apparent Michaelis–Menten constants I_{max} (A), or J_{max} (A cm^{-2}), and K_M (μM). Linear regression analysis was used for the substrate concentration range of 0–50 μM to determine the sensitivity in the linear response region. The selectivity coefficient for each electrode configuration for glutamate with respect to AA (S_{AA}) was calculated for individual sensors using Eq. (1) [15], and then averaged.

$$S_{AA} = \frac{|I_{Glu}| - |I_{AA}|}{|I_{Glu}|} \times 100\% \quad (1)$$

$|I_{Glu}|$ and $|I_{AA}|$ are absolute values of the change in current for a 10 μM Glu addition to PBS and a 500 μM AA addition to a PBS solution containing 500 μM AA, respectively. A negative S_{AA} corresponds to the AA current response being higher than the response for Glu, a zero value would mean that both the current responses were equal, with a positive value indicating that the 10 μM Glu response was higher than the current response obtained for 500 μM AA.

A second selectivity coefficient, $S_{AA(H_2O_2)}$, was used with hydrogen peroxide and AA, when the electrodes were devoid of the enzyme. In this context, it is useful to consider the response for equimolar concentrations of AA and H_2O_2 (the oxidase signal transduction molecule) in calculating this selectivity coefficient [19]. Therefore, the equation for $S_{AA(H_2O_2)}$ can be formulated as:

$$S_{AA(H_2O_2)} = \frac{|I_{AA}|}{|I_{H_2O_2}|} \times 100\% \quad (2)$$

In the above equation, $S_{AA(H_2O_2)}$ indicates the selectivity coefficient expressed as a percentage interference by AA in hydrogen peroxide detection, $|I_{AA}|$ and $|I_{H_2O_2}|$ are the current responses obtained due to 1 mM concentrations of AA and hydrogen peroxide, respectively. As the current response due to AA should ideally be very small when compared with the current response due to hydrogen peroxide, the ideal $S_{AA(H_2O_2)}$ would be 0% and any electrode with a value closer to 0% is more selective. Equimolar concentrations of the analytes have been used in the definition of $S_{AA(H_2O_2)}$ so that it represents the ratio of effective polymer permeability for the two analytes with the

same number of electrons transferred per molecule, as suggested by McMahon et al. [19]. All the data reported here are Mean ± SEM values with n being the number of electrodes. The glutamate sensitivity values are represented using current densities (J) with units of the form A cm^{-2} M^{-1}.

3. Results and discussion

3.1. Pt_D/GluOx/PPD-BSA

It is well known that AA is the main interfering substance which needs to be eliminated while monitoring chemicals *in vivo* in the human brain using electrochemistry [25]. When cylindrical configurations have been used previously, a PPD polymer has proved to be very efficient in blocking AA from oxidizing at the platinum electrode [15,25,26]. The Pt_D/GluOx/PPD-BSA disc sensors were fabricated as described earlier in Section 2, using a polymerization potential of 0.65 V for 15 min.

Glutamate and AA calibrations showed that the average sensitivity for glutamate in the linear region was 32.4 ± 0.2 nA μM^{-1} cm^{-2} ($r^2 = 0.99$; $n = 10$). The selectivity for glutamate over AA as calculated from Eq. (1) was found to be very poor at $-35 \pm 4\%$ ($n = 5$). The negative selectivity coefficient obtained arises from the higher response obtained for 500 μM AA compared to 10 μM glutamate. Therefore, it was essential to increase the sensitivity of the biosensor while decreasing its permeability to AA.

3.1.1. Scanning electron microscopy

The low magnification SEM of the PPD-coated disc electrode (Fig. 1, top) shows that the integrity of the Teflon–metal seal was not compromised significantly by the cutting procedure used to form the bare disc from the wire. Interesting aspects of the surface structure of PPD formed from the different isomers of phenylenediamine [27–30] and other electro-deposited polymers [31–33] have been revealed in the past by scanning electron microscopy. Crater-like features described previously for PPD formed on metal cylinders from the *ortho* monomer [27,30] were also evident at higher magnifications for PPD electro-deposited onto the Pt_D electrode (Fig. 1, bottom). The perforated appearance is, however, misleading because a comparison with the surface of bare Pt [27] shows that polymer also covers the floor of the craters. It should be noted that the electron-beam energy needed to reveal any significant further detail of these PPD-based layers tend to 'burn' the surface. Therefore, SEM images provided above are at relatively low magnification, sufficient to illustrate the characteristic cratering of PPD and the integrity of the Pt–Teflon interface. AFM studies are planned in the future which promises to provide more details of the structure of the multi-layered coatings.

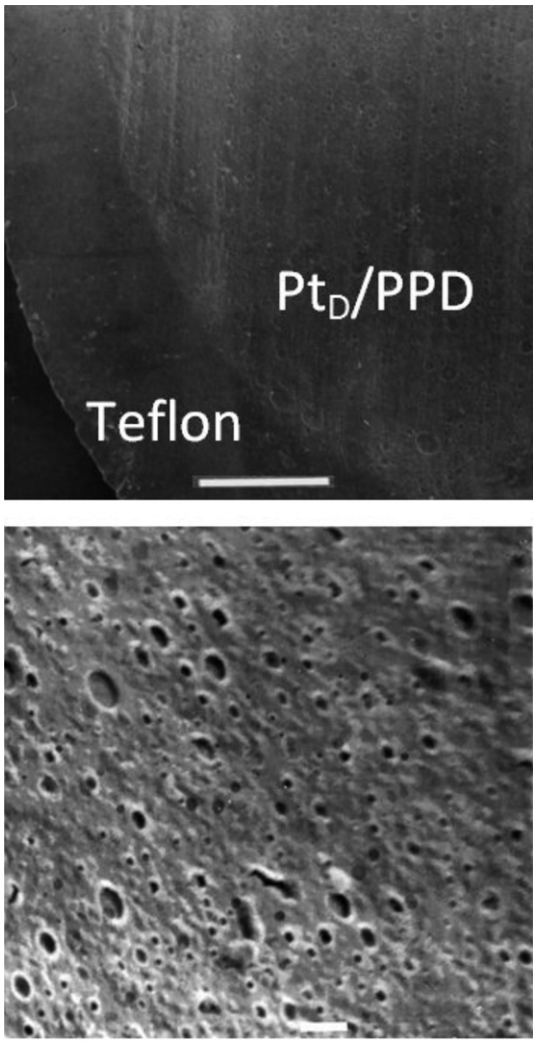

Fig. 1. Scanning electron micrographs of a quarter segment of a 125 μm diameter PPD-coated disc electrodes at low magnification showing the Teflon–metal interface (top, 20 μm bar), and at a higher magnification that reveals the cratered nature of the PPD layer (bottom, 2 μm bar).

3.2. Pt_D/PEI/GluOx/PPD-BSA

From Eq. (1), it is evident that the selectivity coefficient depends on the sensitivity of the biosensors to glutamate. To improve the Glu sensitivity of the biosensor, a cationic polymer PEI was incorporated into the sensor matrix [22]. Disc sensors were therefore formed by dip coating PEI onto platinum discs, followed by dip evaporation of the enzyme and electropolymerization of the monomer *o*-PD with BSA at 0.65 V for 15 min.

Upon calibration, the sensitivity to glutamate increased to 143 ± 2 nA μM^{-1} cm^{-2} ($r^2 = 0.99$; $n = 12$). Unfortunately, the AA response was also higher leading to a very low selectivity, S_{AA} of $-55 \pm 5\%$ ($n = 7$) calculated using Eq. (1). It could be expected that by introducing a polycationic polymer, a higher amount of anionic AA was being electrostatically attracted towards the surface, similar to the anionic glutamate molecules. Therefore, an increase in the sensitivity to glutamate alone was insufficient to improve the selectivity of these disc biosensors.

3.3. Pt_D/Nafion/PEI/GluOx/PPD-BSA

In an effort to improve the selectivity of the biosensor, a polyanionic Nafion layer was introduced before the polycationic PEI layer. The biosensors were made by dipping the platinum discs once in 5% Nafion solution and cured at 170–180 °C for 10 min [20,21]. The remaining steps were the same as described above. The Nafion-coated discs were dipped in the PEI solution and evaporated for 15 min before immobilizing the enzyme GluOx, also through dip–evaporation. The electrodes were then electropolymerized with the monomer solution at varying potentials from 0.45 V to 0.65 V for 15 min.

The glutamate response obtained in the linear region was 33.4 ± 0.3 nA μM^{-1} cm^{-2} ($r^2 = 0.99$; $n = 18$) and the average selectivity to AA (Eq. (1)) was determined as $40 \pm 25\%$ ($n = 9$). The results obtained clearly indicated that the introduction of the Nafion layer decreased the permeability to AA as it formed an electrostatic and physical barrier, but its effectiveness appeared to be decreased as this physical barrier seemed to have also lowered the amount of biocatalytically produced H_2O_2 from being oxidized at the

electrode surface, as reported by Brown and Lowry [20]. Upon further investigation, it was found that, for the electrodes electropolymerized in the lower range of applied potential between 0.45 and 0.50 V, the S_{AA} selectivity (Eq. (1)) was better ($65 \pm 3\%$ ($n = 6$)) than for electrodes polymerized at 0.65 V *vs.* Ag/AgCl ($15 \pm 3\%$ ($n = 3$)). Therefore, the insertion of a Nafion membrane into the matrix along with a lowered polymerization potential provided a significant increase in selectivity ($p < 0.001$; $n = 6$), albeit insufficient to be considered for implantable use.

3.4. Effect of PPD polymerization potential on the selectivity of Pt_D/PPD-BSA

The structure of PPD films and their mechanisms of formation are known to be complex, and have been studied in some detail by many authors [34–42]. The properties of PPD have been suggested to depend mainly on the conditions of the polymerization, such as pH, electrolyte composition, temperature and the applied potential. PPD films electrosynthesized at neutral pH at room temperature have been shown to form a non-conducting, self-sealing coating on the Pt surface [34,40,43]. Previous studies by McMahon et al. [19] suggested that miniaturization of the electrodes leads to more rapid formation of the PPD layer due to highly efficient hemispherical transport of the monomer molecules which results in a non-compact polymer structure thereby increasing the permeability towards AA [19]. Since the disc designs investigated were found to have a higher temporal stability than their cylindrical counterparts (comparative data not shown), a significant parameter to be considered for *in vivo* monitoring, it seemed necessary to improve the interference rejecting capability of disc electrodes. Therefore, an attempt was made to decrease the rate of polymer formation by decreasing the polymerization potential. By conducting cyclic voltammetric experiments, it was determined that the lowest possible potential at which successful polymerization can be achieved was around 0.37 V *vs.* Ag/AgCl (results not shown).

The effect of polymerization potential on discs has been evaluated and reported previously [44] in which the selectivity of electrodes polymerized at 0.40 V provided the highest selectivity against AA. Therefore, a range of disc biosensor designs were electropolymerized between 0.40 V and 0.45 V here in an attempt to further improve selectivity.

3.5. Experiment to observe the effect of Nafion coatings on the selectivity of the disc electrodes

It can be seen from earlier configurations that Nafion functions as a physical/electrostatic barrier, making the biosensor relatively impermeable to AA. However, an electrode with a single Nafion coating was insufficient to provide the selectivity required for implantable use. To investigate the relative selectivities of the electrodes with and without Nafion, an experiment was designed with three electrodes: (1) Pt_D/PPD-BSA, (2) Pt_D/Nafion/PPD-BSA and (3) Pt_D/Nafion(2)/PPD-BSA. The Pt_D/Nafion(2)/PPD-BSA electrode was prepared by dip–evaporating Nafion twice and curing it each time for 10 min at 180 °C before performing polymerization of PPD. The electropolymerization was performed at 0.45 V for 20 min for all three electrodes.

The responses of the electrodes towards 1 mM hydrogen peroxide and 1 mM AA were measured and their individual selectivity coefficients calculated using Eq. (2). Electrodes with selectivity coefficient values closer to 0% are less permeable to AA. The $S_{AA(H_2O_2)}$ values obtained for electrodes 1, 2 and 3 were 0.18%, 0.43% and 0.20%, respectively. To improve the sensitivity of the electrodes, a PEI coating was used prior to enzyme immobilization. Therefore, electrodes 1, 2 and 3 were dipped in PEI and calibrations performed once more for H_2O_2 and AA to determine the selectivity coefficients. After PEI coating, the $S_{AA(H_2O_2)}$ values obtained for electrodes 1, 2 and 3 were higher: 2.32%, 3.56% and 0.43%, respectively. Therefore, with the inclusion of PEI, electrode 3 with double Nafion dips provided the best effective selectivity. Therefore, two Nafion layers appear to protect the electrode from interference to a greater degree than a single Nafion layer, or an electrode without Nafion, when PEI is incorporated in the matrix.

3.6. Pt_D/Nafion(2)/PPD-BSA/PEI/GluOx/PPD-BSA

Disc biosensors of the above configuration were prepared after establishing the ameliorating effect of introducing two Nafion dips in the matrix. The discs were prepared by dip–evaporating Nafion and curing them at 180 °C for 7–8 min twice, as described above [20,21]. The double Nafion-coated discs underwent electropolymerization at 0.45 V for 20 min, followed by PEI and GluOx dip–evaporation. The enzyme-coated discs were electropolymerized for a second time between 0.50 V and 0.55 V for 15 min to entrap the enzyme on the electrode.

Calibrating the disc biosensors for glutamate and AA, it was found that the average sensitivity for glutamate in the linear region increased to 42.8 ± 0.1 nA μM^{-1} cm^{-2} ($r^2 = 1.00$, $n = 25$) which was an improvement over the Pt_D/Naf/PEI/GluOx/PPD-BSA configuration (33.4 ± 0.3 nA μM^{-1} cm^{-2}), possibly due to lowered curing times following the Nafion coating and/or the introduction of the electropolymerized layer prior to enzyme immobilization providing a higher surface area for adsorption of the enzyme on the surface [21]. With a lowered AA response for this configuration, an increase in S_{AA} value of $74 \pm 3\%$ ($n = 12$) was observed compared with the previous configurations. Although the average selectivity coefficient obtained was higher than the previous microdisc configurations discussed above, the sensitivity to glutamate was quite low, an important drawback for electrodes to be used *in vivo*. Therefore, it was imperative to increase the sensitivity to glutamate while maintaining a high selectivity.

3.7. Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA

This type of biosensor was developed to assess whether the introduction of the lipid PEA would improve the sensitivity of the biosensor to glutamate while maintaining a high selectivity. The lipid PEA has been used as an interference-blocking layer in cylindrical sensors for glutamate by O'Neill's group and shown to be effective in decreasing interference by AA [15]. These biosensors were prepared by electropolymerizing the discs from a solution containing the monomer *o*-PD and BSA at between 0.425 V and 0.45 V for 20 min followed by dip–evaporation of PEA. The discs were dipped five times into a solution of PEA dissolved in chloroform and evaporated for 15 min after each dip. The PEA-coated discs were then dip-coated with PEI and allowed to dry for 30 min before immobilizing the enzyme. Following enzyme adsorption, the discs were electropolymerized for the second time from the same monomer solution at 0.50 V for 15 min to bind the enzyme onto the electrode. During the second electropolymerization at a slightly higher potential, the initial current upswing obtained due to the oxidation of the monomer on PPD-BSA and enzyme-covered electrodes (Fig. 2 (inset)) was about 2 orders of magnitude lower than that obtained during electropolymerization on bare platinum electrodes (Fig. 2), representing near maximum coverage during the first polymerization at bare electrodes. However, Fig. 2 (inset) shows that the current drops and then increases again before stabilizing to a plateau demonstrating that a small number of new strands are formed which could have been due to the dislocation or rearrangement of existing strands during the adsorption of PEA, PEI and the enzyme layers and possible extension of the previously existing strands [42,45], thereby entrapping

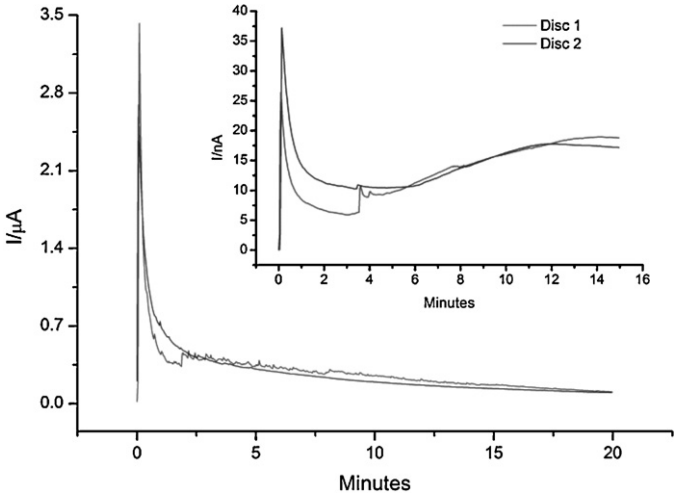

Fig. 2. Electropolymerization current response obtained at bare Pt discs when electropolymerized at 0.40 V (*vs.* Ag/AgCl). Inset: current obtained at Pt_D/PPD-BSA/PEA/PEI/GluOx electrodes when a second polymerization at 0.50 V (*vs.* Ag/AgCl) was performed (∼100× smaller initial current).

the enzyme between the PPD layers; the schematic is represented in Fig. 3.

These Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA electrodes were calibrated for glutamate and AA. The sensitivity of the biosensor in the linear region improved along with an improvement in the selectivity of the biosensor. The sensitivity was 71 ± 1 nA μM^{-1} cm^{-2} ($r^2 = 0.99$, $n = 21$) which was nearly 65% higher than that of the double Nafion-coated discs. The calibration results for glutamate and the average current response in the linear region are shown in Fig. 4. Despite having multiple layers, the response time of this configuration was found to be good at ∼5 s. The Michaelis–Menten constants of J_{max} and K_M for the configuration were found to be 77 μA cm^{-2} and 873 μM ($R^2 = 0.99$, $n = 21$), respectively. The limit of detection (based on 3× SD of the baseline current) of this configuration was calculated to be ∼2.5 μM glutamate, which is lower

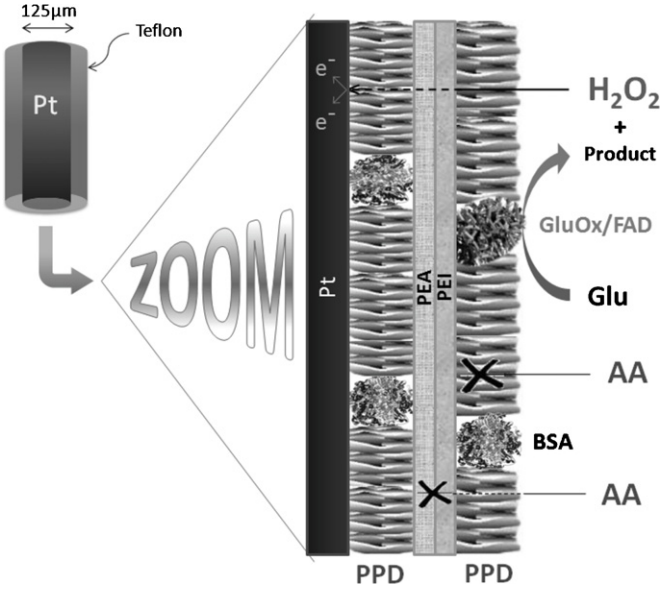

Fig. 3. Schematic representation of Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA configuration. The first polymerization was performed at 0.425–0.45 V *vs.* Ag/AgCl for 20 min and the polymerization following enzyme immobilization was conducted for 15 min at 0.50 V *vs.* Ag/AgCl electrode.
Source: Adapted from Ref. [15].

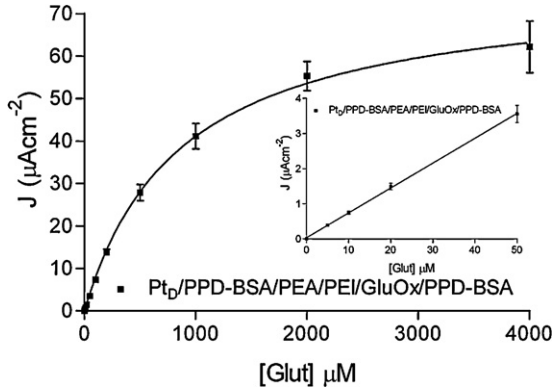

Fig. 4. The glutamate response at Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA biosensors, showing the calibration plot with non-linear Michaelis–Menten curve fitting equation applied to the data. $J_{max} = 77$ μA cm^{-2} and $K_M = 873$ μM ($R^2 = 0.99$, $n = 21$) were obtained. Inset: the sensitivity to glutamate in the linear response region was 71 ± 1 nA μM^{-1} cm^{-2} ($r^2 = 0.99$, $n = 21$).

than the baseline (∼10 μM [1,2]) and pathological (>200 μM [46]) concentrations of glutamate in the brain. The oxygen dependence of such biosensors has been established by McMahon et al. [47], who demonstrated that PPD-coated electrodes were highly efficient even at low oxygen concentrations, such as during ischaemia.

The most impressive result was the rejection of AA: S_{AA} was found to be $88 \pm 2\%$ ($n = 15$). The AA calibration at Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA electrodes is shown in Fig. 5, representing the Mean ± SEM of 15 electrodes. The response of the discs to a doubling of the AA concentration from 0.5 mM to 1 mM, resulted in a very small change in current indicating that the matrix formed by the lipid PEA and the electrosynthesized polymer at low potentials was sufficient to block the interference to the maximum extent without lowering the Glu sensitivity, unlike Nafion-coated discs. The more hydrophobic the polymer, the more difficult it is for hydrophilic solutes to partition into the film, thereby increasing its permselectivity [45]. As PPD is not very hydrophobic, ionic interference cannot be completely eliminated, but can only be decreased [45]. PEA immobilized on the electrode prior to enzyme adsorption aids in increasing the hydrophobicity of the biosensor. It has been proposed that PEA might improve the selectivity of the biosensor by posing as a physical barrier to interfering substances such as AA [15]. In addition, as PEA is a lipid molecule and therefore hydrophobic, it could also be presenting a phasic barrier to AA molecules, preventing them from reaching the metal

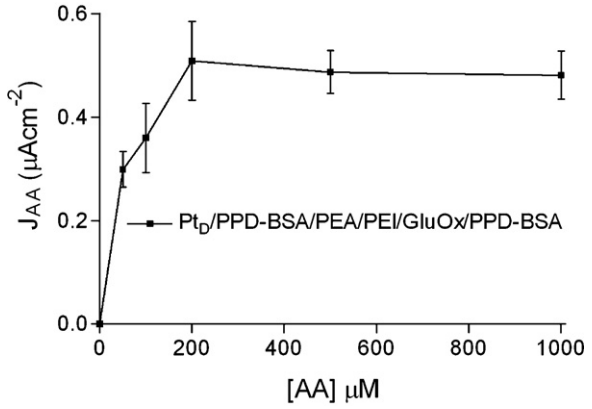

Fig. 5. Mean ± SEM values of AA calibration at Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA biosensors ($n = 15$). The average selectivity coefficient (Eq. (1)) of this configuration was found to be $88 \pm 2\%$ ($n = 15$).

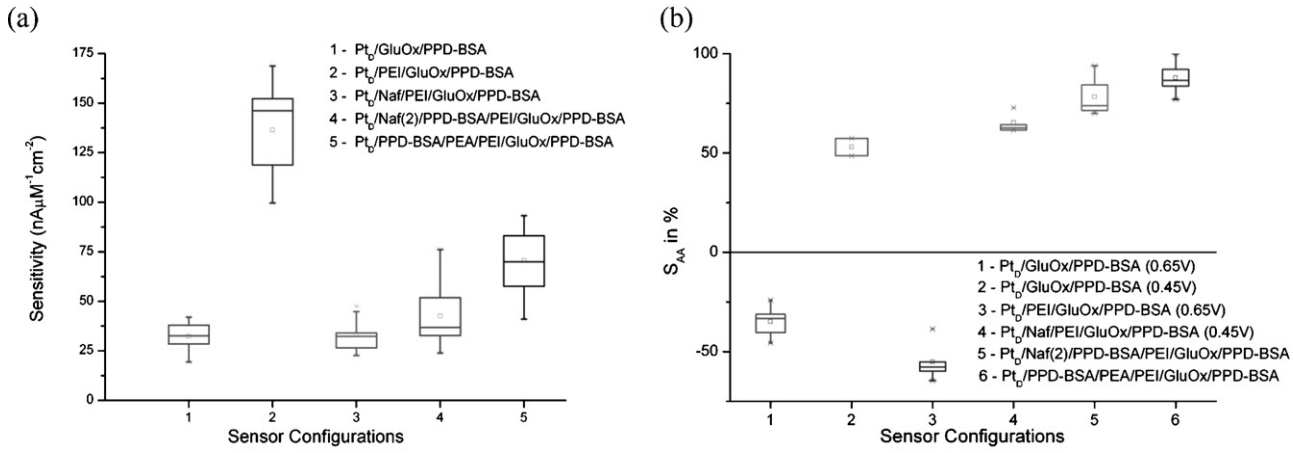

Fig. 6. Box and whisker plot: (a) sensitivity values at the different configurations of sensors explored. (b) S_{AA} values (according to Eq. (1)) plotted for the different configurations tested in the order in which they were developed. Although Pt_D/PEI/GluOx/PPD-BSA provided the highest sensitivity, its selectivity was very low. The best configuration in terms of both sensitivity and selectivity was found to be Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA.

surface. These properties do not have any effect on the oxidation of glutamate as it is oxidized at the enzyme centres close to the surface of the coatings and not at the platinum electrode. The PEA molecules do not seem to hinder significantly the small biocatalytically produced H_2O_2 molecules from being oxidized at the platinum surface.

It has been shown that simple PPD-coated disc-type platinum electrodes have low selectivity against AA [19,44]. McMahon et al. [19] initially suggested that faster hemispherical monomer diffusion at disc electrodes occurred during electropolymerization, forming non-compact polymer structure when compared with a slower linear diffusion at cylindrical electrodes, could have been the cause of decreased selectivity of disc electrodes. Recently, Rothwell et al. [48] reported that the higher permeability to AA at PPD-coated disc electrodes was primarily due to a novel insulation-related edge effect, which was more pronounced in disc-type electrodes than cylinders. In a previous study, it was shown that decreasing the polymerization potential for the formation of PPD at discs from 0.7 V to 0.4 V enhanced the selectivity of these electrodes [44]. In contrast to these results, Rothwell et al. [29] have recently shown that decreasing the electropolymerization potential decreases the interference-rejecting capability of PPD at platinum cylinder electrodes. Regardless, the results obtained from this study and those reported earlier [44] demonstrate that lowering the potential of electropolymerization of *o*-PD substantially enhances the selectivity of disc-type glutamate biosensors, despite the edge-effect. In addition, the effect of additional layers, such as the enzyme, PEA and PEI, on the formation and structure of the polymer is still unknown, although the previously mentioned study by Rothwell et al. [29] showed that PEI and GluOx did marginally decrease the outstanding AA blocking of PPD-coated platinum cylinders.

Fig. 6(a) and (b) summarizes the sensitivity and selectivity values at different sensor configurations tested, as a box and whisker plot. Although Pt_D/PEI/GluOx/PPD-BSA biosensors had the highest sensitivity to glutamate, they had the lowest selectivity amongst configurations containing a barrier to AA, as seen in Fig. 6(a) and (b). It is clear that, although Pt_D/Nafion(2)/PPD-BSA/PEI/GluOx/PPD-BSA blocked AA to the greatest extent (in terms of actual current response to AA), because of its lower permeability to H_2O_2 its effective selectivity decreased below that of the Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA sensor. The latter did not provide any significant hindrance to H_2O_2, thereby providing a greater sensitivity to glutamate and, as a result, a greater selectivity due to the presence of PEA.

3.8. pH and temporal stability of the disc biosensors

It is important for implantable sensors to provide a glutamate response which is not significantly dependent on changes in pH over the physiologically relevant range. Electrodes of the Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA configuration showed good insensitivity to changes in pH between 6.0 and 8.0 ($p > 0.5$), as seen from the linear region slope for this biosensor plotted in Fig. 7, normalized with respect to the response at pH 7.4.

The temporal stability of the biosensors is equally important for *in vivo* use as they cannot be removed and recalibrated once implanted. The main target for *in vivo* glutamate biosensors being patients with severe traumatic brain injury, it was learned from collaborating neurosurgeons (Prof. A.D. Mendelow and Mr. P. Mitchell (Department of Neurosurgery, Newcastle General Hospital, Newcastle, UK)) that once implanted the biosensors might have to remain within the patient's brain for a minimum period of 5–6 days post-operatively. Therefore, it was essential to determine whether the biosensors could provide similar sensitivities over many days of continuous operation.

Glutamate calibrations were carried out under conditions similar to continuous monitoring by biasing biosensors at 0.65 V in PBS (pH 7.4) for 5–6 days when not being calibrated. Physiological concentration of Glu was not added to the electrolyte solution when leaving the electrodes biased overnight because the presence or absence of ~10 μM of Glu would not have a

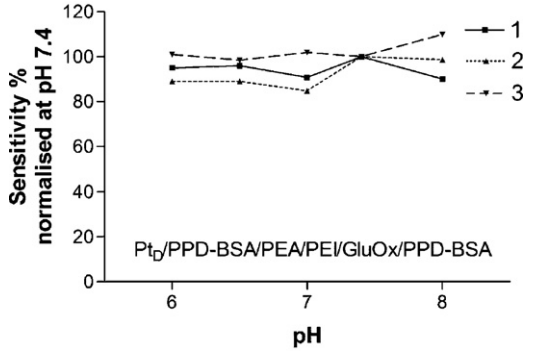

Fig. 7. Stability of Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA biosensors for changes in pH values is shown. The plot shows the linear region slope values of three electrodes normalized to pH 7.4 for glutamate calibrated in PBS at pH values ranging from 6.0 to 8.0.

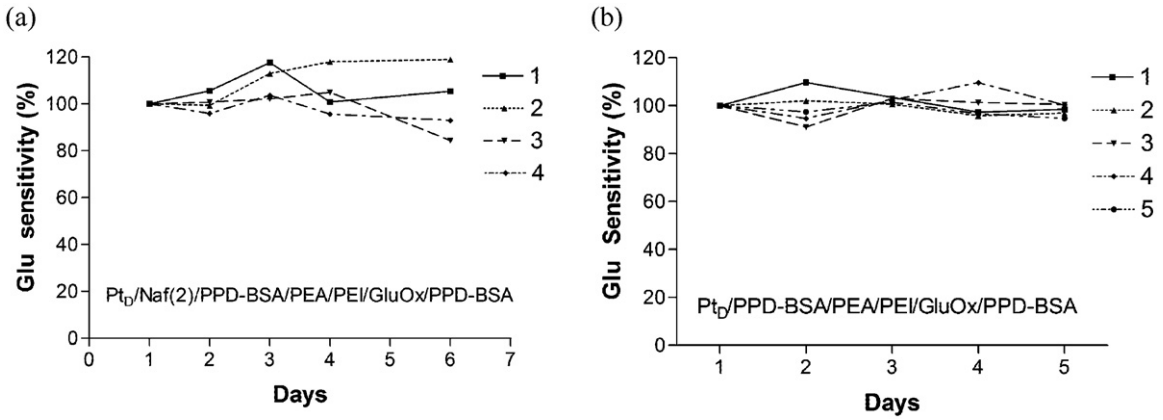

Fig. 8. (a) Stability of the individual Pt_D/Nafion(2)/PPD-BSA/PEI/GluOx/PPD-BSA biosensor responses to glutamate over 6 days of continuous functioning ($n = 4$). (b) Stability of the Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA response to glutamate over 5 days of continuous functioning ($n = 5$).

significant effect on the obtained results due to the high K_M of the optimized sensor (~880 μM Glu). The Glu linear-region slopes thus obtained for Pt_D/Nafion(2)/PPD-BSA/PEI/GluOx/PPD-BSA disc biosensors exhibited good stability over 6 days (Fig. 8(a)). However, after day 4, the difference between the sensor responses appears to vary to a greater degree, despite being statistically insignificant ($p > 0.5$). Fig. 8(b) shows that the Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA configuration also provided a high level of temporal stability. It also displayed lower intra-sensor variability, and is therefore well-suited for use as an implantable biosensor. The selectivity of the sensors over the same period was not performed because the sensitivity to glutamate, especially at the Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA discs, decreased by ~42% following AA calibration (results not shown). This is likely due to the homogenous interference from AA [49] observed during *in vitro* biosensor measurements. The effect of homogenous interference by AA during H_2O_2 detection has been reported to be more acute *in vitro*, apparently due to the presence of heavy metal ion impurities in the buffer, compared with *in vivo* monitoring conditions [49]. Therefore, despite being present at much higher concentrations *in vivo* compared with Glu, the homogeneous mechanism of AA interference might not be relevant *in vivo*, provided the direct AA blocking, biocompatibility and biofouling issues have been addressed [50]. Following specialized training, certification and licensing of the project, the next step in the development of this technique will involve implantation of the biosensor in specific brain regions. These electrodes can then be characterized *in vivo*, using microinfusion of compounds directly into the tissue, as well as pharmacological challenges known to influence AA and glutamate levels. These studies will be required to ascertain whether the sensitivity, selectivity and stability of the glutamate signal observed here will translate to reliable *in vivo* monitoring.

4. Conclusions

Glutamate, being an important marker for traumatic brain injury, needs continuous monitoring in the neuro-intensive care environment. Recent advances in sensor fabrication and design, improved biocompatible materials and nanoscale technologies are aiding in making implantable biosensors a reality. In this study, microdiscs were utilized in an attempt towards realizing an implantable glutamate biosensor for brain monitoring. This research started from a very basic design for which the sensitivity and selectivity of the biosensors were very low. By incorporating a polycationic polymer, PEI, the sensitivity of the glutamate biosensor was increased. Nafion, as an interference blocking polymer, was also investigated; however, it was found that adding PEA with dual polymerization steps with PPD-BSA at lower applied potentials provided the best results in terms of selectivity while maintaining high levels of sensitivity. Therefore, this work describes the research which led to the best configuration of disc biosensors for glutamate in terms of sensitivity, selectivity and stability.

It was found that introducing Nafion into the sensor matrix significantly decreased the glutamate and H_2O_2 responses at the surface of the sensor, apparently because Nafion layers formed a physical sieve with pore diameters small enough to block even H_2O_2. This was evident from the H_2O_2 responses obtained from electrodes without the Nafion layer and electrodes with one and two Nafion layers. The H_2O_2 response decreased to about ~45% for electrodes with a single Nafion layer. The response further decreased (~80% decrease from electrodes without Nafion) for electrodes with double Nafion layers. This shows that Nafion might be inefficient in providing a higher selectivity as it blocks both AA and H_2O_2 at the same time. Meanwhile, PPD films have been reported to block AA while having no effect on H_2O_2 [19]. Latest developments in highly sensitive detection of H_2O_2 using carbon nanotubes and gold nanoparticles have been described [51,52]. However, prior to their use in implantable biosensors, the *in vivo* toxicity concerns of nanotubes and nanoparticles leeching out into the brain tissue have to be investigated.

By performing calibration for 5–6 days and keeping the sensor continuously biased at 0.65 V in PBS when not being calibrated, it was determined that they retained their sensitivity over the measured period. Therefore, it is envisaged that the Pt_D/PPD-BSA/PEA/PEI/GluOx/PPD-BSA configuration, with its small size, high sensitivity, selectivity against AA and stability, could be the best candidate for implantation for short-term monitoring during surgery and post-operatively in patients with traumatic brain injury. However, further testing of these biosensors in brain extracellular fluid and animal models is necessary to verify their performance in *in vivo* environments.

Acknowledgements

The authors would like to thank Prof. A.D. Mendelow and Mr. Patrick Mitchell (Consultant Neurosurgeons, Newcastle General Hospital, Newcastle upon Tyne, UK) for their help and invaluable advice. The authors would also like to thank Northern Brainwave Appeal for funding a part of this research.

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