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Covalent enzyme immobilization by poly(ethylene glycol) diglycidyl ether (PEGDE) for microelectrode biosensor preparation

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ABSTRACT

Poly(ethylene glycol) diglycidyl ether (PEGDE) is widely used as an additive for cross-linking polymers bearing amine, hydroxyl, or carboxyl groups. However, the idea of using PEGDE alone for immobilizing proteins on biosensors has never been thoroughly explored. We report the successful fabrication of microelectrode biosensors based on glucose oxidase, D-amino acid oxidase, and glutamate oxidase immobilized using PEGDE. We found that biosensors made with PEGDE exhibited high sensitivity and a response time on the order of seconds, which is sufficient for observing biological processes *in vivo*. The enzymatic activity on these biosensors was highly stable over several months when they were stored at 4 °C, and over at least 3 d at 37 °C. Glucose microelectrode biosensors implanted in the central nervous system of anesthetized rats reliably monitored changes in brain glucose levels induced by sequential administration of insulin and glucose. PEGDE provides a simple, low cost, non-toxic alternative for the preparation of *in vivo* microelectrode biosensors.

1. Introduction

Implantable enzymatic biosensors are widely used for *in vivo* monitoring applications (Vaddiraju et al., 2010; Dale et al., 2005). For instance, they have been used in neuroscience research to detect neurotransmitters and metabolites with outstanding temporal resolution. Enzymatic biosensors are typically constructed according to a common basic design in which an enzymatic layer is immobilized on a platinum (Pt) electrode. A variety of immobilization techniques are currently available. Enzyme cross-linking using glutaraldehyde, which is probably the oldest method, remains a reference procedure for simple, stable, and reproducible covalent enzyme immobilization (Wilson and Turner, 1992; Burmeister et al., 2000; Netchiporouk et al., 1995). Enzyme entrapment in a poly-o-phenylenediamine (PPD) layer has been reported (McMahon et al., 2005), but the resultant material is generally less stable and less sensitive than that produced with glutaraldehyde fixation. Enzymes have also been immobilized in sol–gels (Tian et al., 2009) and polypyrrole matrices (Cache-Guerente et al., 1995). However, both of these methods involve toxic components that limit their potential use in clinical applications.

One elegant procedure involves binding enzyme molecules to a redox hydrogel composed of water soluble poly(1-vinylimidazole) (Ohara et al., 1993) or poly(4-vinylpyridine) (Gregg and Heller, 1991a,b) and $[Os(bpy)_2Cl]^+$. Several enzymes, including glucose oxidase and glutamate oxidase, have been immobilized successfully using this technique (Kulagina et al., 1999; Oldenziel et al., 2004). Additionally, glucose biosensors based on this or similar principles have been approved for glucose monitoring in diabetic patients (Heller and Feldman, 2008; McGarraugh, 2009). However, the components of such redox hydrogels are expensive and the process requires several synthetic steps. Thus, there is a need for the development of simple, clean, and low-cost enzyme immobilization methods that can be easily reproduced in non-expert laboratories.

In this study, we investigated the potentially interesting process of enzyme immobilization using poly(ethylene glycol) diglycidyl ether (PEGDE) for biosensor preparation. PEGDE is an essential component of redox hydrogels used for glucose biosensors and is widely used in commercial devices such as the implantable Freestyle Navigator system (Abbott laboratories, review in Heller and Feldman, 2008). However, its ability to immobilize enzymes on

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its own has never been thoroughly characterized. Recently, Zigah et al. (2009) successfully immobilized glucose oxidase on an amino-modified silicon substrate using PEGDE, suggesting that biosensor fabrication using PEGDE alone is indeed possible. Here, we examined the feasibility of immobilizing several enzymes, including glucose oxidase, D-amino acid oxidase, and glutamate oxidase, on Pt microelectrodes using PEGDE alone. The *in vitro* and *in vivo* stability, sensitivity, and response time of biosensors produced using this method were measured and compared to respective reference values obtained for biosensors prepared using glutaraldehyde fixation.

2. Materials and methods

2.1. Enzyme preparation

Glucose oxidase (EC 1.4.3.11) from *Aspergillus niger* was purchased in powder form from Sigma–Aldrich (100–250 U/mg, Saint Quentin Fallavier, France). The final enzyme solution used for biosensor preparation contained 60 mg/mL glucose oxidase, 30 mg/mL bovine serum albumin (BSA, Sigma), and 1% glycerol in phosphate buffered saline solution (PBS 0.01 M, pH 7.4).

Recombinant *Rhodotorula gracilis* D-amino acid oxidase (RgDAAO, EC 1.4.3.3) was overexpressed in *Escherichia coli* and purified as reported by Molla et al. (1998). The final solution contained 58 mg/mL of the protein, 25 mg/mL BSA, and 1% glycerol in PBS (0.01 M, pH 7.4).

L-Glutamate oxidase (EC 1.4.3.11) from *Streptomyces sp. X119-6* was purchased from Yamasa Corporation (Choshi, Chiba, Japan). The working solution contained 57 mg/mL of the protein, 81 mg/mL BSA, and 1% glycerol in PBS (0.01 M, pH 7.4). Poly(ethylene glycol) diglycidyl ether, average MW 526 (Sigma–Aldrich) was added to the enzyme aliquot prior to biosensor preparation.

2.2. Microelectrode biosensor preparation

Biosensors were constructed using a 25 μm diameter 90% Pt/10% Ir wire (Goodfellow, Huntington, U.K.) glued to a copper wire using conductive silver paint (Radiospares, Beauvais, France). The sensor wire was inserted into a pulled glass capillary (Harvard Apparatus, Edenbridge, U.K.), and the tip of the pipette was cut so the Pt wire extended from the glass approximately 100 μm. The junction between the Pt and the pipette tip was sealed by locally melting the glass. The junction between the copper wire and the glass was sealed with epoxy resin (Araldite, Bostik, Paris, France). The electrodes were washed for 30 min in ethanol. For *in vivo* experiments, a poly-m-phenylenediamine screening layer was deposited by electropolymerization for 20 min in 100 mM m-phenylenediamine (Sigma–Aldrich) solution in 0.01 M PBS at pH 7.4 under a constant potential of +700 mV versus a Ag/AgCl reference electrode. The enzyme layer was deposited by dipping the Pt tip of the electrode in the enzyme solution. Control sensors were treated using a bovine serum albumin solution consisting of 400 mg/mL BSA, 10 mg/mL PEGDE, and 1% glycerol in 0.01 M PBS, pH 7.4. The electrodes were placed in an oven at 55 °C for 2 h, then stored at 4 °C for short-term storage and at −20 °C for long-term storage. In a few experiments, the enzyme was immobilized by exposure to saturated glutaraldehyde vapors for 2.5–3 min, as reported by Pernot et al. (2008).

Before the experiments all sensors were tested for detection of serotonin (5-HT, 20 μM in PBS), enzyme substrate (glucose, D-serine, or glutamate, 1 μM in PBS), and H_2O_2 (1 μM in PBS). Only those electrodes with an effective PPD screening layer and exhibiting less than 1.2 $\mu A \cdot mM^{-1}\ cm^{-2}$ response for 5-HT were included in the study.

Glucose microelectrode biosensors for *in vivo* experiments were covered with an additional polyurethane membrane to increase their dynamic range and to protect the enzyme layer from biofouling in the brain. The tip of the electrode was dipped four times in a 5% solution of polyurethane in THF (Sigma) with 10 min intervals between dips at room temperature to dry the layer. After this treatment, the glucose biosensors displayed a linear relationship between oxidation current and glucose concentration up to 3 mM.

2.3. Recordings

All recordings were obtained using a VA-10 electrochemistry amplifier (NPI Electronics, Tamm, Germany) equipped with a two-electrode potentiostat. Data acquisition was performed using a 16-bit USB-6221 acquisition board (National Instruments, Nanterre, France) controlled by homemade software based on Igor Pro 6.4 procedures (Wavemetrics, Eugene, OR). The oxidation current was recorded at 1 kHz and averaged over 1000 data points, yielding a final sampling frequency of 1 Hz (except for response time analyses, in which the signal was averaged over 100 data points, or 10 Hz).

In vitro calibrations were performed in standard PBS (0.01 M, pH 7.4) solutions. The reference electrode was a chloride-treated silver wire (Ag/AgCl) placed directly in the recording chamber. Recordings were obtained using constant potential amperometry at +500 mV versus the Ag/AgCl reference electrode. Holding potentials between +500 and +700 mV are optimal for H_2O_2 detection (Pernot et al., 2008). For *in vivo* studies, the biosensors were calibrated before and after the experiments to ensure the sensitivity remained stable.

2.4. In vivo experiments

All experiments were performed under isoflurane anesthesia, and the animals were treated according to European regulations. The experimental procedure was validated by the local committee on animals in research of Université Claude Bernard Lyon I. Adult, male Wistar rats weighing 300–500 g were placed in a stereotaxic apparatus (Stoelting, Dublin, Ireland). Their body temperature was maintained at 37 °C using a homeothermic blanket (Letica, Barcelona, Spain). A glucose microbiosensor was implanted in the frontal cortex (1.5 mm ventral to the dura) adjacent to a control biosensor covered with BSA. Recordings were started at least 1 h after implantation to ensure stabilization of the baseline electrochemical currents. Insulin (25 U/kg of body weight) was injected after an additional 30 min period of control recording, and 1 h later an injection of glucose (3 g/kg of body weight) was administered.

To achieve selective detection *in vivo*, all microelectrodes were coated with a layer of PPD and polyurethane. The PPD polymer creates a screening layer that prevents oxidation of endogenous molecules present in the brain such as serotonin, dopamine, ascorbate, and cysteine (Pernot et al., 2008) (Fig. S-1). The sensors were also prepared using an additional layer of polyurethane (see above). Glucose biosensors were implanted in combination with a control biosensor (BSA–PEGDE) on the contralateral side of the brain to control for nonspecific variations in oxidation current. Quantitative assessments of brain glucose concentrations were obtained by subtracting the non-specific current of the control biosensor from the output of the glucose biosensor. The resulting glucose oxidation current was then divided by the sensitivity of the biosensor and corrected for temperature in order to estimate the *in vivo* glucose concentration.

2.5. Statistics

Data are presented as means ± standard deviations (SDs), except for data from *in vivo* measurements, where means ± standard errors

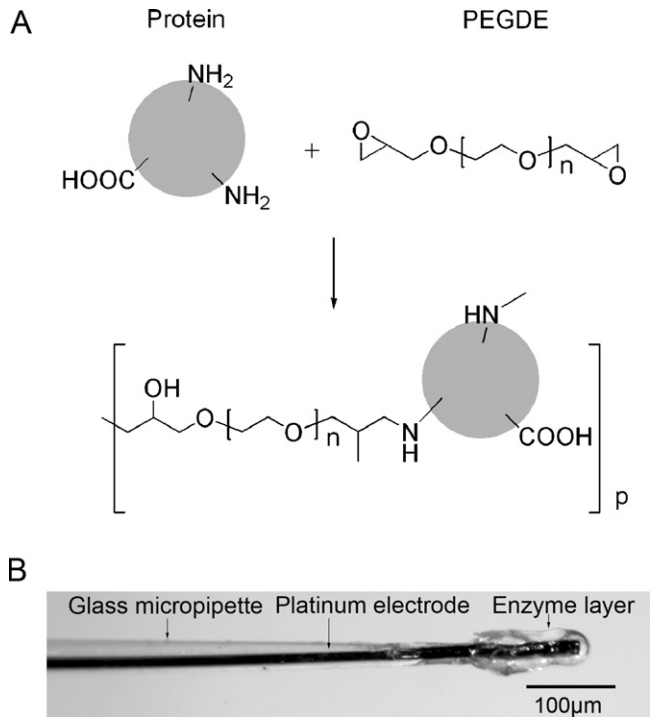

Fig. 1. (A) General reaction scheme of protein with poly(ethylene glycol) diglycidyl ether. All protein molecules (enzyme, bovine serum albumin, etc.) contain amino- and carboxyl groups that may react with the 2 epoxides of PEGDE, resulting in immobilization. (B) Photomicrograph of glucose biosensor tip.

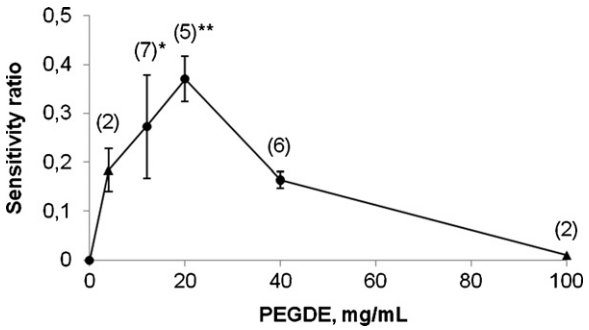

Fig. 2. Dependence of glucose biosensor sensitivity ratio (ratio of glucose sensitivity (pA/μM) to H_2O_2 sensitivity (pA/μM)) on PEGDE concentration in the enzyme solution. PEGDE concentration is expressed in mg/mL of 0.01 M PBS containing glucose oxidase (60 mg/mL), BSA (30 mg/mL), and glycerol (1%). The relationship between biosensor sensitivity and PEGDE concentration was a bell-shaped curve with a maximum near 20 mg/mL. Concentrations of 4 and 100 mg/mL resulted in unreliable biosensors with unstable enzyme immobilization (►). **Significant difference from all other concentrations, *significant difference from 40 mg/mL. The numbers of biosensors constructed using each protocol are presented above the data points.

of the mean are reported. Comparisons between two data groups were performed using the Student's *t*-test for equal, unequal, or paired variances as indicated by the F-test for equal variance (significance level, $p < 0.05$). The analyses were performed using the analysis tool-pack of Excel (Microsoft Office 2007) and SPSS 12.0 for Windows. The sample size (n) was equal to the number of biosensors constructed under the same protocol, or the number of animals used in the *in vivo* experiments (where a new biosensor was used in every experiment).

3. Results

3.1. Principles of enzyme immobilization on electrodes

PEGDE contains two epoxy groups that can react with amino, hydroxyl, and carboxyl groups. Reaction with the amine functional groups present in proteins creates a matrix for enzyme immobilization on the electrode surface (Fig. 1A). The reaction between the diepoxide and proteins is slow at room temperature (~48 h), but can be accelerated considerably by increasing the temperature (Gregg and Heller, 1991a). In the case of enzymatic biosensor preparation, the reaction is limited to conditions that preserve enzymatic activity. We therefore set the temperature at 55 °C, which is low enough to preserve the activity of all oxidase enzymes we tested. At this temperature, a 2-h incubation of the enzyme/PEGDE mixture was sufficient to achieve immobilization in a stable membrane. Therefore, this incubation time was used throughout the study.

3.2. Optimization of PEGDE concentration

We first tested the PEGDE enzyme immobilization process by preparing glucose oxidase-based biosensors. We studied the dependence of biosensor sensitivity on the amount of PEGDE added to the enzyme (Fig. 2), and normalized the response using the ratio of glucose sensitivity (pA/μM) to H_2O_2 sensitivity (pA/μM) to eliminate variability in the electrode surface and H_2O_2 sensitivity. The PEGDE concentration in the enzyme solution was varied from 4 to 100 mg/mL while the glucose oxidase concentration was fixed at 60 mg/mL, BSA at 30 mg/mL, and glycerol at 1% in 0.01 M PBS (pH = 7.4). This solution was viscous enough to be deposited on the microelectrode surface and then fixed for 2 h at 55 °C. The sensitivity of the biosensors followed a bell-shaped curve. At the minimum PEGDE concentration of 4 mg/mL, only 36.4% ($n = 11$) of the biosensors exhibited stable enzyme immobilization and the sensitivity ratio was low (0.18 ± 0.04). The biosensor sensitivity increased at higher concentrations, reaching a maximum of 0.37 ± 0.05 at 20 mg/mL. At concentrations of 100 mg/mL or greater, the biosensors displayed a very low sensitivity ratio (≈0.01). At these high PEGDE concentrations, the enzymatic membrane was stably immobilized on the electrode surface but the glucose activity was quite low, probably due to enzyme deactivation from excessive cross-linking. At the optimal PEGDE concentration of 20 mg/mL, enzyme immobilization was reproducible with a coefficient of variation of approximately 12% in the sensitivity ratio between microelectrodes.

Similar experiments were performed with other enzymes, including D-amino acid oxidase (DAAO) and glutamate oxidase, which also exhibited a bell-shaped dependence of biosensor sensitivity on PEGDE concentration (data not shown). The optimal PEGDE concentrations for DAAO and glutamate oxidase were 5.9 mg/mL and 78 mg/mL, respectively.

The enzyme membrane appeared as a transparent light-yellow film (Fig. 1B). The membrane was insoluble in water, but swelled slightly with increasing water content.

3.3. Characteristics of glucose biosensors

To further characterize the performance of the glucose biosensors, we determined their response time, linear range, and apparent Michaelis–Menten constant (K_m^{App}). For response time analysis, the biosensors were transferred rapidly between PBS and a 20 μM glucose solution, generating a step increase in the amperometric signal. The response time, defined as the time required to reach 90% of the plateau response, was equal to 3.1 ± 1.2 s ($n = 12$, Fig. 3A).

We then constructed a calibration plot for glucose concentrations in the range of 0–1 mM. A linear dependence of oxidation current on glucose concentration was observed up to 400 μM, with a sensitivity of 12.3 ± 3.8 pA/μM ($n = 35$). The electrical noise level was approximately 4 fA (RMS), yielding a theoretical *in vitro* detection limit of ~1 nM (3 × noise).

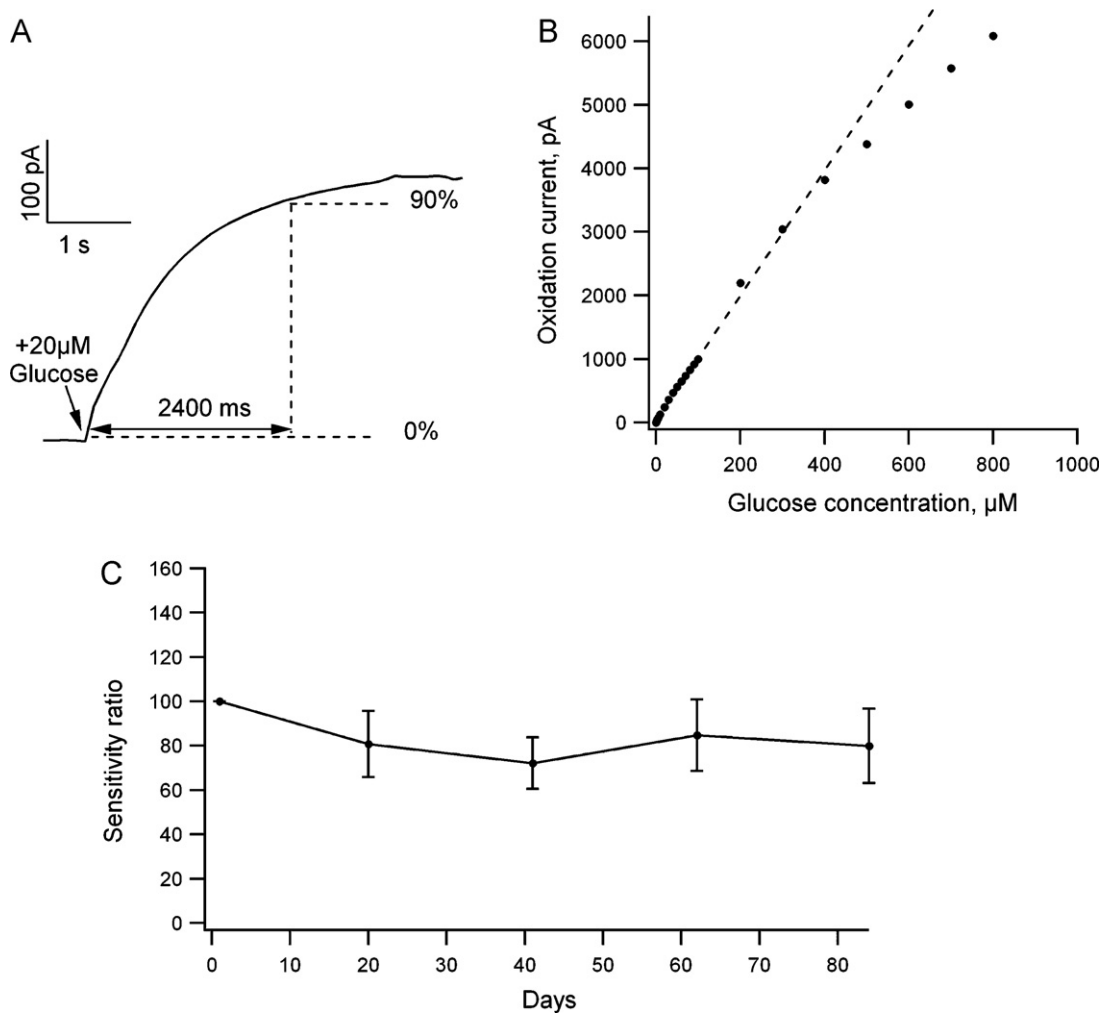

Fig. 3. Glucose biosensors. (A) Response of biosensor to changes in glucose concentration (0–20 μM). Biosensor response time was calculated as the time between 0 and 90% of the current step. (B) Calibration curve between 0 and 1 mM for computing Michaelis–Menten kinetic parameters. The linear range was 0–400 μM. $I(\text{pA}) = 9.80 \times C\,(\mu\text{M}) + 42.08$, $R = 0.998$. (C) Long-term storage stability of glucose microbiosensors at 4 °C ($n = 6$).

The apparent Michaelis–Menten constant K_m^{App} was estimated by fitting the calibration curve between 0 and 1 mM to the following equation:

$$I(C) = \frac{(I_{max}C}{(K_m^{App} + C)}$$

in which $I(C)$ is the oxidation current as a function of glucose concentration (C) and I_{max} is the maximum oxidation current recorded by the biosensor. I_{max} was equal to 12.8 ± 6.6 nA and K_m^{App} was 1.4 ± 0.3 mM ($n = 6$). The K_m^{App} of the biosensor was substantially lower than the K_m of the free enzyme, ranging between 26 mM (Hayashi and Nakamura, 1976; Nakamura et al., 1976) and 30 mM (Szajani et al., 1987; Kalisz et al., 1990). This effect, which has been described previously (Demers et al., 2001; Pernot et al., 2008), could be a result of substrate and product preconcentration in the membrane layer.

Finally, we assessed the stability of our biosensors in both operating (i.e. exposed to glucose) and storage conditions. Glucose biosensors could be used repeatedly for *in vitro* and *in vivo* experiments lasting 4–5 h (see Section 3.6). We also tested their stability following a 3-d immersion in PBS at 37 °C. When glucose oxidase was immobilized directly on Pt microelectrodes, the sensitivity of the biosensors decreased by 40% after 2 d ($n = 5$), indicating poor stability of the enzyme layer. However, when the Pt surface was covered with an electropolymerized PPD layer, the enzyme was much more stable. This effect could have been the result of more efficient enzyme cross-linking as the PEGDE reacted with amino groups present on the PPD layer and improvements in enzyme grafting on the microelectrode surface. With this additional PPD layer, the sensitivity of the biosensors actually increased by 23 ± 17% after 3 d at 37 °C ($n = 3$). This slight increase could be the result of a reorganization of the PPD structure that increased H_2O_2 permeability over time.

Long term stability during dry storage at 4 °C over 3 mos. was also determined using biosensors containing an additional PPD layer. The sensitivity of the biosensors, assessed using a 4-point calibration, decreased slightly after the first 20 d, but remained stable for the next 2 mos. (Fig. 3C). Overall, the enzyme layer was stable over time when immobilization was performed on PPD.

3.4. D-Serine and glutamate biosensors

The same kinetic parameters reported for glucose biosensors above were also determined for D-serine (DAAO) and glutamate (glutamate oxidase) biosensors. The response times for serine biosensors ($n = 6$) and glutamate biosensors ($n = 4$) were found to be 3.7 ± 0.9 s and 3.6 ± 0.5 s, respectively (Fig. 4A and C). For the D-serine biosensors, the linear range was 0–200 μM, with a sensitivity of 15.3 ± 4.0 pA/μM ($n = 19$) and an *in vitro* detection limit

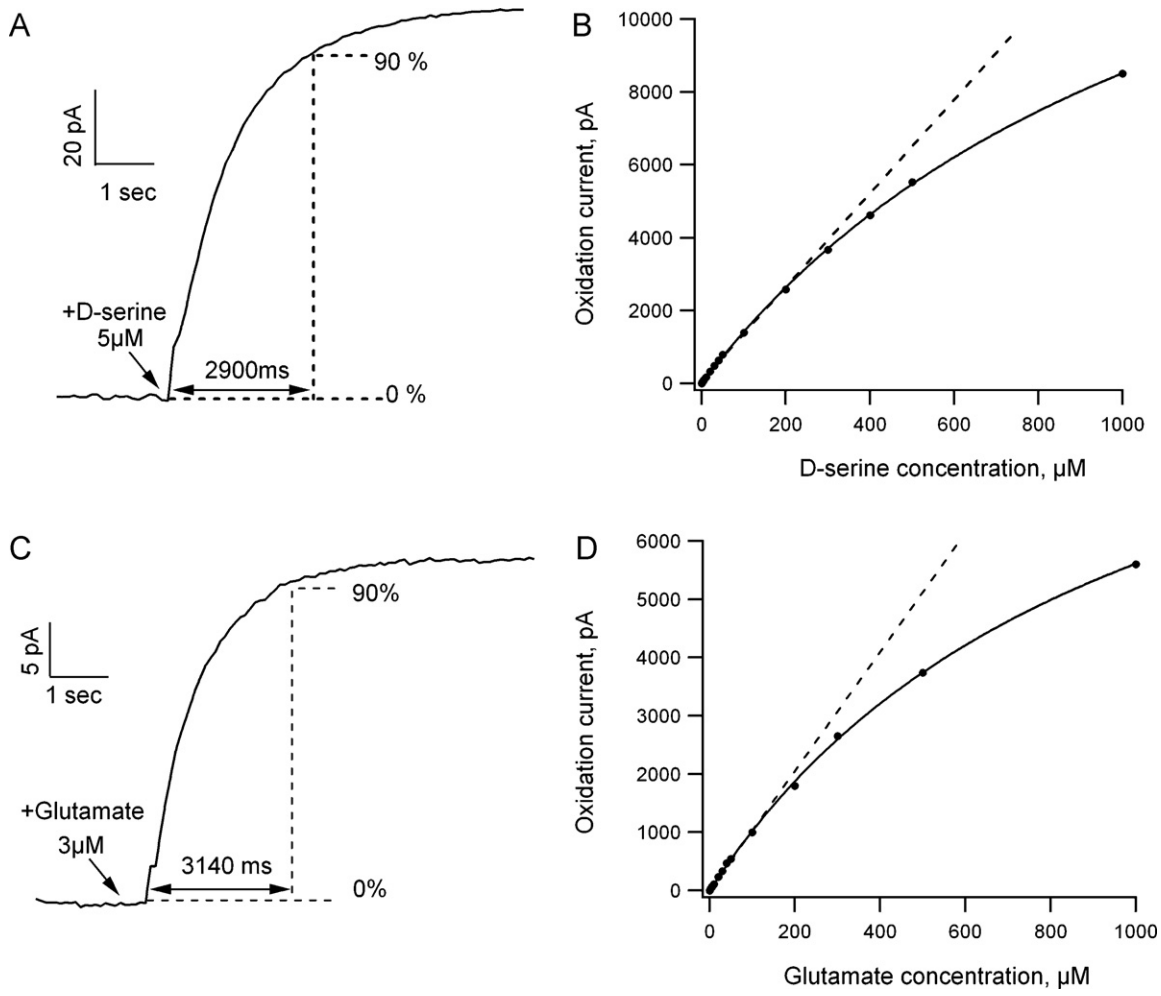

Fig. 4. D-Serine biosensors (top) and glutamate biosensors (bottom). (A), (C) Response of biosensor to changes in D-serine (0–5 μM) and glutamate (0–3 μM) concentration and response time estimation (see Fig. 2). (B), (D) Calibration curve for 0–1 mM D-serine and 0–1 mM glutamate for the computation of Michaelis-Menten kinetic parameters. Linear regression equation for D-serine biosensor: $I(\text{pA}) = 13.0 \times C\ (\mu\text{M}) + 48.7$, $R = 0.998$. Linear regression equation for glutamate biosensors: $I(\text{pA}) = 10.2 \times C\ (\mu\text{M}) + 16.2$, $R = 0.998$.

of approximately 0.8 nM. The Michaelis–Menten parameters I_{max} and K_m^{App} were 19.2 ± 5.5 nA and 1.3 ± 0.4 mM ($n = 4$, Fig. 4B). For the glutamate biosensors, the linear range was 0–100 μM, with a sensitivity of 14.4 ± 5.2 pA/μM ($n = 12$) and an *in vitro* detection limit of approximately 0.8 nM. Imax and K_m^{App} were 10.2 ± 1.3 nA and 0.79 ± 0.17 mM ($n = 4$, Fig. 4D). The apparent Michaelis-Menten constant of the D-serine biosensor was significantly lower than the K_m of the free enzyme in solution (13.75 mM, Pollegioni et al., 1992), an effect that was presumably related to the immobilization procedure itself as reported previously (Butò et al., 1994). On the contrary, the glutamate biosensors had a higher K_m^{App} than the free enzyme (0.2 mM Yamasa Corporation, Choshi, Chiba, Japan). It should be noted, however, that K_m^{App} values reported for glutamate biosensors are highly dependent on the immobilization method, ranging from 3 mM (redox hydrogel immobilization, Mikeladze et al., 2002) and 2.8 mM (immobilization in Nafion®, Maalouf et al., 2007), to 0.78 mM (immobilization in a sol–gel layer, Tian et al., 2009). Thus, the K_m^{App} of glutamate oxidase immobilized with PEGDE observed in our experiment is within the range of values obtained with other methods.

The DAAO biosensors were used repeatedly in *in vivo* experiments lasting 4–5 h without losing activity (data not shown). The glutamate biosensors were stored in dry conditions at 4 °C for at least 1 wk without losing enzymatic activity. Overall, these results indicate that enzyme immobilization with PEGDE yields stable, reproducible and sensitive biosensors for at least three different oxidase enzymes.

3.5. Comparison with glutaraldehyde fixation

We systematically compared the characteristics of our biosensors with those obtained after fixation with glutaraldehyde, one of the most widely used fixative reagents for enzymatic biosensor fabrication (Table 1). The response times of the PEGDE biosensors were similar to those obtained using glutaraldehyde (except for glutamate oxidase, for which PEGDE yielded a higher value). The K_m^{App} values were generally similar between the two methods (except for DAAO, for which PEGDE yielded a lower value). The PEGDE biosensor sensitivities were generally higher than those observed with glutaraldehyde fixation, except for the glutamate sensor, for which the sensitivity was similar. The kinetic behavior of the biosensors is summarized in Table 1A.

3.6. In vivo validation

We tested the utility of the PEGDE biosensors in applications requiring *in vivo* brain implantation. In initial experiments, we implanted glucose biosensors in the frontal cortex of anesthetized rats. Basal brain glucose concentrations were 0.68 ± 0.15 mM ($n = 8$), which is consistent with findings from other studies of

Table 1
Comparison of biosensor characteristics with proteins immobilized using glutaraldehyde versus PEGDE.

(A) Kinetic parameters

	Glucose oxidase		D-Aminoacid oxidase		Glutamate oxidase	
	PEGDE	Glutaraldehyde	PEGDE	Glutaraldehyde	PEGDE	Glutaraldehyde
Sensitivity ($\mu A \cdot mM^{-1}\ cm^{-2}$)	147 ± 45 ($n=35$)*	106 ± 29 ($n=10$)	212 ± 119 ($n=19$)*	87 ± 27 ($n=18$)	173 ± 63 ($n=12$)	178 ± 99 ($n=44$)
Response time (s)	3.1 ± 1.2 ($n=12$)	2.4 ± 0.8 ($n=6$)	3.7 ± 0.9 ($n=6$)	3.2 ± 0.5 ($n=4$)	3.6 ± 0.5 ($n=4$)	2.1 ± 1 ($n=6$)*
K_M^{App} (mM)	1.4 ± 0.3 ($n=6$)	1.1 ± 0.2 ($n=6$)	1.3 ± 0.4 ($n=4$)*	3.6 ± 0.4 ($n=4$)	0.79 ± 0.17 ($n=4$)	0.93 ± 0.48 ($n=4$)

(B) PPD layer stability

Sensitivity to serotonin ($\mu A \cdot mM^{-1}\ cm^{-2}$)	*In vivo* condition**		*In vitro* 37 °C	
	PEGDE	Glutaraldehyde	PEGDE	Glutaraldehyde
Before	0.3 ± 0.2 ($n=13$)	0.1 ± 0.1 ($n=9$)	0	0
After	0.9 ± 0.8 ($n=13$)	1.5 ± 2.0 ($n=9$)*	0.2 ± 0.3 ($n=3$)	1.8 ± 0.9 ($n=4$)*

* Significant difference.
** Criterion for *in vivo* experiments is serotonin < $1.2\ \mu A \cdot mM^{-1}\ cm^{-2}$.

in vivo brain glucose monitoring with biosensors (Netchiporouk et al., 1996; Leybaert, 2005). Injection of insulin (25 U/kg body weight intraperitoneally) induced a steady decrease in oxidation current from the glucose microbiosensor, but not from the control electrode. Injection of glucose (3 g/kg) 1 h after insulin administration quickly raised the brain glucose signal above its original value (Fig. 5A). The extracellular brain glucose concentration 1 h after insulin injection was 0.19 ± 0.05 mM, and the concentration rose to 1.1 ± 0.2 mM after glucose injection (Fig. 5B). Calibration experiments performed after electrode removal from the brain revealed that our biosensors were still functional after *in vivo* implantation, with some even displaying a slight increase in sensitivity (Fig. 5C, Table 1B, probable explanation Section 3.3). The effects of insulin and subsequent glucose administration are well documented (Netchiporouk et al., 1996). The fact that we could detect them with our glucose oxidase/PEGDE biosensors therefore

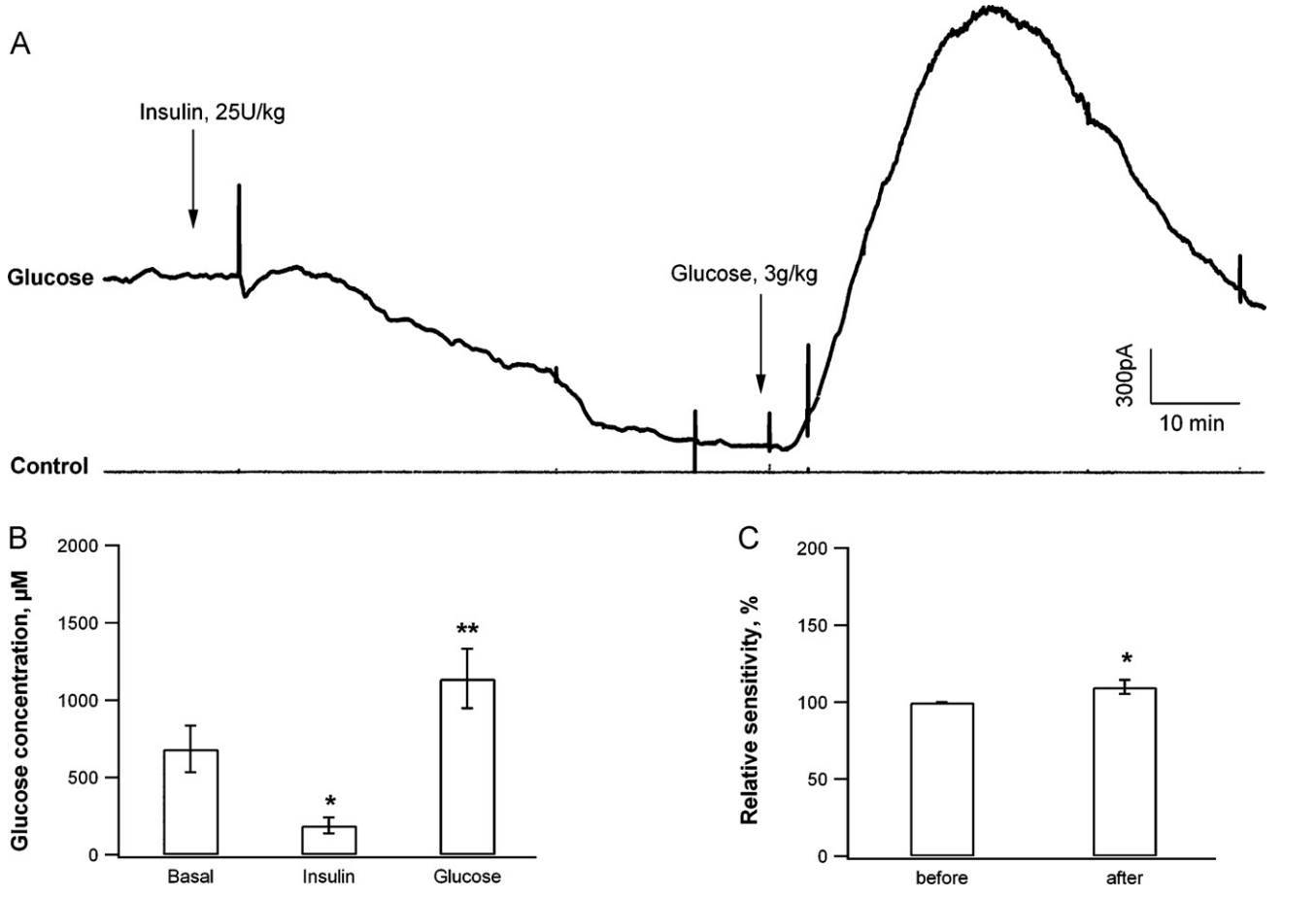

Fig. 5. Examples of biosensor recordings *in vivo*. (A) Glucose monitoring using a PEGDE/glucose oxidase biosensor implanted in the frontal cortex of an anesthetized rat. Brain glucose levels decreased following an intraperitoneal injection of insulin (25 U/kg body weight) and then increased following a subsequent glucose injection (3 g/kg body weight). For quantitative estimation of *in vivo* glucose concentrations, the signal detected by a glucose microbiosensor (bold line) was compared to that recorded by a control (BSA) biosensor implanted on the contralateral side of the brain (thin line). (B) Glucose concentrations estimated before and 1 h after insulin administration and at the peak of the rebound in glucose concentration. (C) Sensitivity to glucose before and after implantation revealing the stability of the biosensors *in vivo* ($n=6$).

indicates that our method is well suited for *in vivo* brain glucose monitoring.

In a second set of experiments, we compared the *in vivo* performance of PEGDE-immobilized D-serine biosensors to that of glutaraldehyde-based electrodes. We implanted PEGDE or glutaraldehyde-immobilized D-serine biosensors in the frontal cortex of anesthetized rats, the D-serine baseline current was found to be $0.34 \pm 0.08\ \mu A\ cm^{-2}$ for PEGDE based sensors and $0.31 \pm 0.06\ \mu A\ cm^{-2}$ for glutaraldehyde-fixed sensors. The corresponding baseline D-serine concentration was approximately 2–2.5 μM based on the two methods.

A stable and selective PPD layer is essential to accurate electrochemical recordings throughout the *in vivo* experiments. We first tested the selectivity of our biosensors by measuring the oxidation current produced by the endogenous electroactive molecules serotonin, dihydroxyphenylacetic acid (DOPAC), uric acid and ascorbic acid. Interestingly, biosensors prepared using PPD and PEGDE immobilization were less sensitive to ascorbic acid that those prepared with glutaraldehyde (Fig. S1), indicating that the PPD layer was more selective with PEGDE. We then assessed the PPD stability in two independent sets of experiments, using serotonin. Following storage of the biosensors in PBS at 37 °C for 3 d, a 20-μM concentration of serotonin was undetectable using freshly prepared biosensors and was detected at only $0.2 \pm 0.3\ \mu A{\cdot}mM^{-1}\ cm^{-2}$ after 3 d ($n = 3$) when the sensors were fabricated using PEGDE, compared to $1.8 \pm 0.9\ \mu A{\cdot}mM^{-1}\ cm^{-2}$ when glutaraldehyde fixation was used ($n = 4$, Table 1B). The same comparison was performed after a 5-h implantation *in vivo*, revealing that PPD retained better performance as a screening layer with PEGDE fixation than with glutaraldehyde (Table 1B). For *in vivo* experiments, a sensitivity to serotonin of less than $1.2\ \mu A{\cdot}mM^{-1}\ cm^{-2}$ (i.e. <1% of the glucose, D-serine, or glutamate signal) is required both before and after implantation to ensure optimum selectivity. Therefore, more than half of the biosensors prepared with glutaraldehyde fixation did not fulfill this criterion at the end of a 5 h *in vivo* experiment. In this regard, the increased PPD selectivity and stability observed in PEGDE-immobilized sensors is a useful property for *in vivo* applications. Overall, these experiments demonstrate that biosensors prepared using PEGDE immobilized enzymes are fully functional *in vivo*, and display better performance for recording *in vivo* signals than those prepared with glutaraldehyde fixation.

4. Discussion

In the present study, a matrix based on covalent binding between enzymes, BSA, and PEGDE was employed for biosensor preparation. PEGDE preserved enzymatic activity and yielded biosensors with similar or better sensitivity and response time than biosensors produced with glutaraldehyde fixation, a classical method for enzyme immobilization on biosensors. When microelectrode biosensors fabricated using PEGDE were implanted in the brain of anesthetized rats, they produced reliable *in vivo* measurements for at least 4–5 h. The method was validated for three different enzymes: glucose oxidase, D-amino acid oxidase, and glutamate oxidase. These experimental results demonstrate that PEGDE may be used to fabricate stable, fast-responding, and sensitive biosensors designed for *in vivo* implantation.

PEGDE is an essential component of redox hydrogels used for biosensors to monitor blood glucose levels in diabetic patients (Gregg and Heller, 1991a,b; Ohara et al., 1993; Heller and Feldman, 2008). In those hydrogels, PEGDE is used as a cross-linking agent to bind enzymes to complexes of water soluble poly(1-vinylimidazole) and $[Os(bpy)_2Cl]^+$, thereby producing efficient electron diffusion between the enzymatic redox centers and the electrode (Ohara et al., 1993). This principle has also been applied successfully to microelectrode biosensors designed for brain implantation (Kulagina et al., 1999; Oldenziel et al., 2004). Our study demonstrates that reliable biosensors may be obtained using PEGDE alone. In this case, enzymatic activity is monitored through the oxidation of H_2O_2 on the Pt surface. For *in vivo* applications, an additional PPD screening layer designed to avoid the detection of endogenous oxidizable molecules such as ascorbic acid, uric acid, dopamine, and serotonin is deposited on the Pt electrode (Schuvailo et al., 2006; Malitesta et al., 1990). PEGDE therefore provides a simple, minimal setup for enzyme immobilization on electrode surfaces without the need for preliminary synthetic steps. Interestingly, omitting the redox mediator in the hydrogel yielded response times that were approximately 10 times faster than those obtained using $[Os(bpy)_2Cl]^+$ (Oldenziel and Westerink, 2005), which can be an important advantage for *in vivo* detection of neurotransmitters such as glutamate.

In conclusion, the present experiments showed PEGDE to be a feasible option for covalent enzyme immobilization in biosensor preparation. Several methods currently in use, such as glutaraldehyde fixation (Burmeister et al., 2000; Netchiporouk et al., 1995) and enzyme entrapment in a sol–gel (Tian et al., 2009) or functionalized polypyrrole matrix (Cache-Guerente et al., 1995), require the use of toxic components that exclude potentially important clinical applications. Thus it is noteworthy that PEGDE offers a clean, nontoxic alternative to these methods. Together with its low cost and ease of use, these advantageous properties should make PEGDE a promising method for future biosensor applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.03.012.

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