

Immobilization Method to Preserve Enzyme Specificity in Biosensors: Consequences for Brain Glutamate Detection

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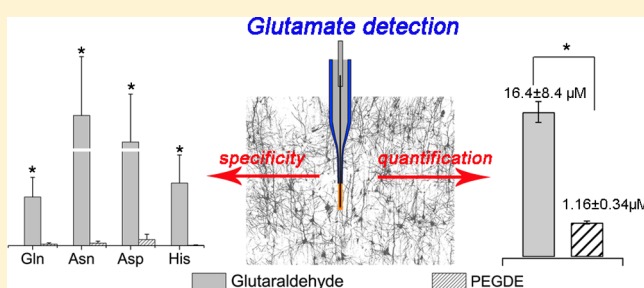
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S Supporting Information

ABSTRACT: Microelectrode biosensors are a promising technique to probe the brain interstitial fluid and estimate the extracellular concentration of neurotransmitters like glutamate. Their selectivity is largely based on maintaining high substrate specificity for the enzymes immobilized on microelectrodes. However, the effect of enzyme immobilization on substrate specificity is poorly understood. Furthermore, the accuracy of biosensor measurements for brain biological extracts has not been reliably established in comparison with conventional analytical techniques. In this study, microelectrode biosensors were prepared using different enzyme immobilization methods, including glutaraldehyde, a conventional cross-linker, and poly(ethylene glycol) diglycidyl ether (PEGDE), a milder immobilization reagent. Glutaraldehyde, but not PEGDE, significantly decreased the apparent substrate specificity of glutamate and glucose oxidase. For glutaraldehyde prepared biosensors, detection of secondary substrates by glutamate oxidase increased, resulting in a significant overestimate of glutamate levels. This effect was not observed with PEGDE-based biosensors, and when brain microdialysates were analyzed, the levels of glutamate detected by biosensors were consistent with those detected by capillary electrophoresis. In addition, basal concentrations of glutamate detected *in vivo* were approximately 10-fold lower than the levels detected with glutaraldehyde-based biosensors (e.g., 1.2 μM vs 16 μM , respectively). Overall, enzyme immobilization can significantly impact substrate specificity, and PEGDE is well-suited for the preparation of stable and selective biosensors. This development questions some of the previous biosensor studies aimed at detecting glutamate in the brain and opens new possibilities for specific neurotransmitter detection.



Glutamate is the primary excitatory neurotransmitter, and estimating its extracellular brain concentration is a key to understanding its physiological and pathological functions. The concentration of glutamate in the extracellular space is currently a matter of debate. Reported levels of glutamate largely depend on the analytical and sampling methods employed. *In vivo*, glutamate levels have been detected using microdialysis. For example, using a no-net-flux method, basal extracellular concentrations of glutamate in the rat brain have been estimated to range from 0.9 to 3.7 μM .¹ However, although microdialysis remains a reference technique for monitoring the composition of extracellular fluids in the field of neuroscience, this technique suffers from low temporal resolution and local brain injury that results from implantation of a probe into the brain parenchyma.² Alternatively, biosensors are minimally invasive and provide excellent temporal resolution.³ However, *in vivo* glutamate concentrations detected using biosensors have been extremely variable, ranging from 1.6 to 45 μM .⁴

Moreover, in addition to variability in detection, overestimates of glutamate concentrations by biosensors are problematic since excessive extracellular glutamate levels can cause excitotoxicity. Correspondingly, *in vitro*, it is difficult to maintain neurons for more than 24 h in the presence of 20 μM glutamate.⁵

To detect a single molecule in complex media, electrochemical biosensors rely on the specificity of the enzymes immobilized on the surface of an electrode. However, immobilization can impact enzyme substrate specificity. While these effects have been described in the field of enzymatic catalysis, they remain largely unexplored in the field of biosensor development. Therefore, we hypothesize that changes in enzyme substrate specificity during biosensor

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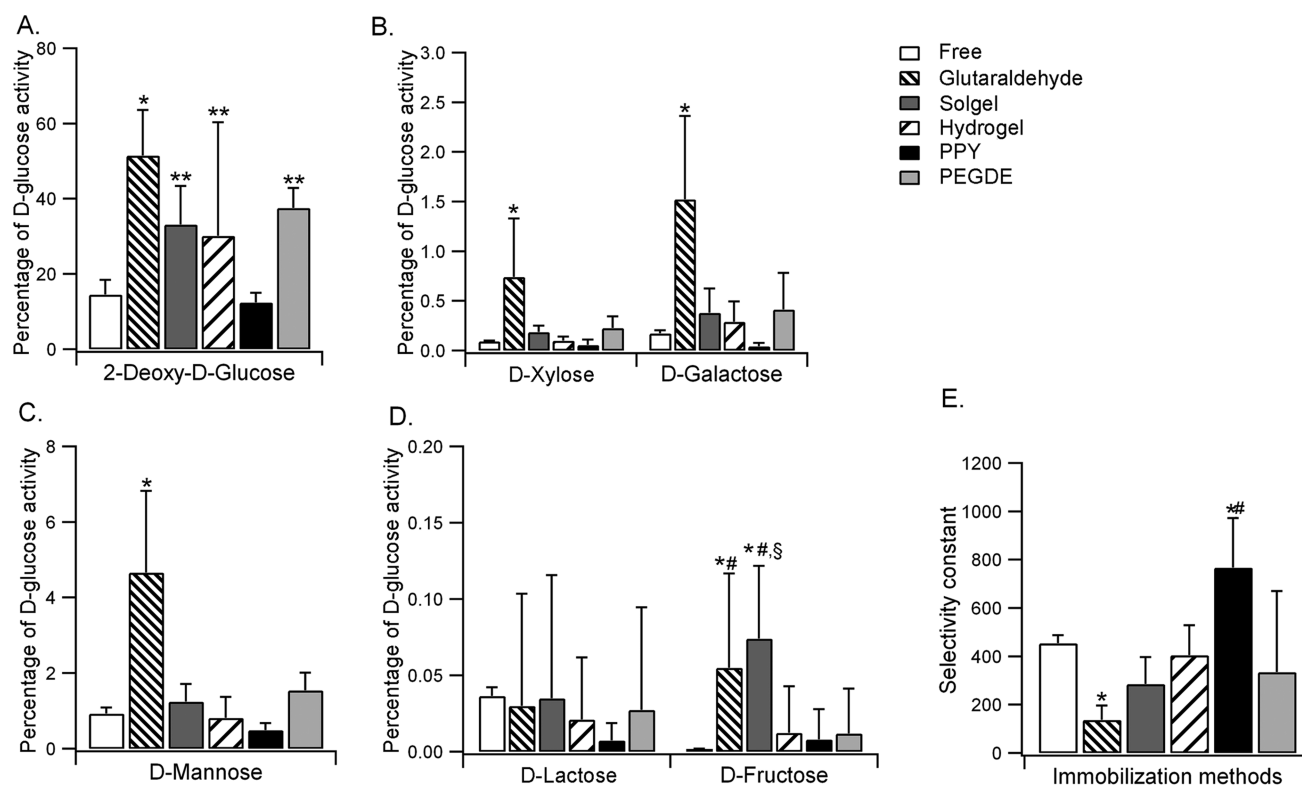


Figure 1. Effect of enzyme immobilization method on glucose oxidase substrate specificity. Enzymatic activity was evaluated for (A) 2-deoxy-D-glucose (2-DG), (B) xylose and galactose, (C) mannose, and (D) lactose and fructose. Glucose oxidase was tested in its free form (free), and following immobilization by glutaraldehyde (GA), sol-gel, hydrogel, derived polypyrrole (PPy), and poly(ethylene glycol) diglycidyl ether (PEGDE). Glutaraldehyde-based biosensors were the least selective, and the most selective biosensors were prepared using derived polypyrroles. (* Significantly different from the free enzyme, ** from the free enzyme and glutaraldehyde, # from PPy, \$ from PEGDE, $n = 6$.) (E) Selectivity constant representing the impact of the immobilization technique on apparent selectivity. (* Significantly different from free enzyme, # from all other methods.)

production can significantly impact the detection of glutamate that is present at low micromolar concentrations in the brain interstitial fluid. Correspondingly, mild immobilization techniques are needed to preserve the native catalytic parameters of immobilized enzymes, and the accuracy of biosensor measurements in brain biological media need to be evaluated using conventional analytical techniques.

In a recent study, enzyme immobilization using poly(ethylene glycol) diglycidyl ether (PEGDE) was identified as an especially mild method.⁶ In the present study, five enzyme immobilization methods were used to prepare microelectrode biosensors for implantation into rat brains. The methods included: cross-linking with glutaraldehyde or PEGDE, attachment to a hydrogel matrix, or entrapment in sol-gel or derived polypyrrole matrices. All of the biosensors were first tested with glucose oxidase, and the selectivity of these biosensors was evaluated *in vitro*. Glutamate oxidase biosensors were then prepared using PEGDE and glutaraldehyde immobilization methods. These biosensors were used to analyze brain homogenates and dialysates, and the accuracy of the concentration estimates obtained were confirmed using high-performance liquid chromatography (HPLC) and capillary electrophoresis coupled to laser-induced fluorescence (CE-LIF). Finally, both types of glutamate biosensors were used to estimate basal ambient glutamate levels and to detect changes in the extracellular concentrations of glutamate in the rat cortex in response to electrical and pharmacological stimulations.

MATERIALS AND METHODS

Glucose and glutamate biosensors were prepared from *Aspergillus niger* glucose oxidase (EC 1.1.3.4) and *Streptomyces sp.* L-glutamate oxidase (EC 1.4.3.11) immobilized on platinum wire microelectrodes covered with a screening layer of poly(*m*-phenylenediamine).^{6,7} Details on enzyme immobilization procedures are given in SI text. The substrate specificity of free glucose and glutamate oxidase was assessed in phosphate buffered saline (PBS) at room temperature with 0.1 U of enzyme and 10 mM of substrate. Peroxide accumulation over time was detected using bare platinum wire microelectrodes. All recordings were obtained with constant potential amperometry with a two-electrode potentiostat. Biosensors were held at +500 mV vs an Ag/AgCl reference electrode.

All *in vivo* experiments were performed in accordance with European directive 86/609/CEE and approved by the animals research committee of the Université Claude Bernard Lyon I (no. BH2010-19). Male Wistar rats weighing 300–500 g were used throughout the study.

Brain and blood samples were prepared by homogenization in 5% trichloroacetic acid (TCA), centrifugation at 8000g for 10 min, and extraction with diethyl ether. Sugar HPLC analyses were performed using a Dionex HPLC system coupled to amperometric detection. Amino acid samples were derivatized with 20 μ L of 6-aminoquinolyl-6N-hydroxysuccinimidyl carbamate (10 μ L sample, 70 μ L borate buffer, 55 $^{\circ}$ C, 10 min) and analyzed by reverse phase chromatography with an HPLC Waters 2695 Alliance system equipped with a

fluorimetric detector Waters 474 and AccQ-Tag Waters column containing a C18 modified silica phase.

Data are presented as the mean $\pm$ standard deviation (SD) for in vitro measurements in standard solutions, and as the mean $\pm$ standard errors of the mean (SEM) for ex-vivo/in vivo measurements. Comparisons between two data groups were performed using a paired or unpaired Student's *t*-test for equal or unequal variance as indicated by the F-test for equal variance (significance level, $p < 0.05$). Comparisons between three or more data groups were performed using ANOVA followed by a Fisher LSD posthoc analysis.

Detailed procedures are provided in the Supporting Information section.

RESULTS

Effect of Enzyme Immobilization on Glucose Oxidase Substrate Specificity. Glucose oxidase is a key component of biosensors used to monitor glycemia or intracerebral glucose and is a model enzyme in many biosensor studies.⁸ In addition to glucose, glucose oxidase exhibits significant activity to other sugars like 2-deoxy-D-glucose (2-DG), D-galactose, D-mannose, and D-xylose. It is also weakly active on D-lactose, and is insensitive to D-fructose.⁹ To determine whether the substrate specificity of glucose oxidase is affected by immobilization, glucose oxidase was covalently immobilized using glutaraldehyde,¹⁰ PEGDE,⁶ or hydrogel,^{4d,e} or was entrapped in sol-gel¹¹ or a derived polypyrrole matrix.¹² The activity of the immobilized glucose oxidase was then measured and compared with the activity of glucose oxidase in its free form (e.g., in PBS solution). The substrates assayed included glucose, as well as the secondary substrates: 2-DG, galactose, mannose, xylose, lactose, and fructose (Figure 1).

When glucose oxidase was immobilized using glutaraldehyde, its activity for all secondary substrates increased significantly. In contrast, immobilization of glucose oxidase using PEGDE or hydrogel resulted in increased activity to 2-DG (Figure 1A) but not to other secondary substrates. For sol-gel immobilization, an increase in activity was observed for 2-DG and fructose (Figure 1A–D), whereas derived polypyrrole entrapment was associated with a slight decrease in glucose oxidase activity in response to all secondary substrates. To quantify these effects on enzyme specificity, a selectivity constant was calculated based on the ratio of activity to glucose to the geometric mean of activities to secondary substrates (the geometric mean is well-suited for averaging data in different numeric ranges, as is the case for 2-DG and fructose activities, Figure 1E). For glucose oxidase, the selectivity ratio was:

$$\text{selectivity ratio} = \frac{\text{activity to glucose}}{\sqrt[3]{\text{activity to 2-DG} \times \text{galactose} \times \text{mannose} \times \text{lactose} \times \text{fructose}}}$$

There was a significant effect of the immobilization method on the selectivity ratio ($F[5,29] = 12.8$, $p < 0.01$). The selectivity ratio for the free enzyme was 455 ± 33 , and similar constants were obtained for enzymes immobilized with PEGDE, hydrogel, and sol-gel (335 ± 21 , 405 ± 123 , and 286 ± 111 respectively, $n = 6$). However, the use of glutaraldehyde for immobilization resulted in a significant decrease in selectivity to 138 ± 60 ($p < 0.01$, $n = 6$). In contrast, biosensors prepared with derived polypyrrole were associated with a higher apparent selectivity (767 ± 205 , $p < 0.01$, $n = 6$, Figure 1E). These results demonstrate that the immobilization

method used for biosensor fabrication can significantly impact the apparent substrate specificity of the immobilized enzyme.

Effect of Enzyme Immobilization on Glutamate Oxidase Substrate Specificity. For studies of glutamate oxidase specificity, only data from glutaraldehyde and PEGDE immobilization methods were obtained, since hydrogel immobilization is chemically very similar to immobilization using PEGDE, and entrapment in derived polypyrrole or sol-gel membranes yielded biosensors that were stable for only a few hours at room temperature.

To test glutamate oxidase activity, four secondary substrates that are present in vivo were evaluated: glutamine, asparagine, aspartate, and histidine ($100\text{--}500 \mu\text{M}$). In its free form, glutamate oxidase was extremely specific to glutamate, and no activity was detected in the presence of the four secondary substrates (Figure 2A). Very little activity in response to the secondary substrates was detected when glutamate oxidase was immobilized with PEGDE (e.g., 0.03–0.3% of glutamate activity, Figure 2A). By contrast, a significant loss of specificity

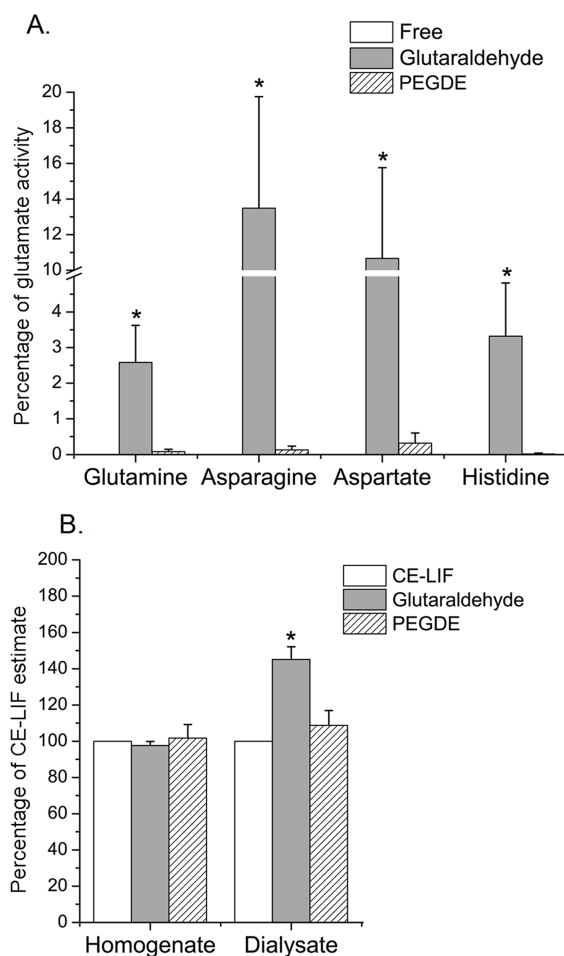


Figure 2. Effect of enzyme immobilization method on glutamate oxidase substrate specificity and glutamate detection in biological media. (A) Enzymatic activity to glutamine, asparagine, aspartate, and histidine. Free glutamate oxidase only detected glutamate. Glutamate oxidase detected glutamine, asparagine, aspartic acid, and histidine after immobilization by glutaraldehyde, but not by PEGDE. * Significantly different from free enzyme, $n \geq 12$. (B) Estimates of glutamate concentrations present in brain homogenates and microdialysates, using CE-LIF or biosensors prepared with glutaraldehyde and PEGDE. (* Significantly different from CE-LIF, $n \geq 24$.)

was observed when immobilization was performed using glutaraldehyde, with secondary activity in response to asparagine constituting up to 15% of the specific activity for glutamate (Figure 2A). A selectivity ratio could not be defined for the free form of glutamate oxidase since it would be infinite, however, the selectivity ratios for PEGDE immobilization and glutaraldehyde immobilization were 1494 ± 648 and 22 ± 14 , respectively ($p < 0.01$, $n = 12$). In this study, immobilization with glutaraldehyde was performed by exposing the biosensor to near-saturating glutaraldehyde vapors for 3.5 min (see the Materials and Methods section). This was the minimal duration below which the enzyme layer became unstable and dissolved into water. Alternatively, glutaraldehyde can be mixed in the enzyme solution at low concentration (1% BSA, 0.125% glutaraldehyde, and approximately 1% L-glutamate oxidase). In our hands, both methods for applying glutaraldehyde to produce immobilization yielded the same effects on apparent substrate specificity. When glutaraldehyde was mixed in the enzyme solution, glutamate oxidase activity to glutamine, asparagine, aspartate, and histidine was $1.8 \pm 1.7\%$, $8.6 \pm 6.7\%$, $5.5 \pm 1.4\%$, and $2.4 \pm 2.3\%$ of that to glutamate ($n = 4$). The apparent substrate specificity of glutamate oxidase biosensors was therefore clearly affected by immobilization, especially when glutaraldehyde was used as the cross-linking agent.

Detection of Glucose and Glutamate in Biological Media. Biological media such as serum, cerebrospinal fluid, brain homogenates, or microdialysates are complex mixtures containing several hundred different molecules. Accordingly, the loss of enzyme specificity following immobilization on a biosensor could profoundly impact the detection and quantification of a molecule of interest within these milieus. To evaluate this effect, concentrations of secondary substrates of glucose oxidase and glutamate oxidase were quantified in rat serum, brain homogenates, and microdialysates.

HPLC coupled to amperometric detection was used to detect concentrations of D-glucose, D-mannose, and D-galactose in serum and brain homogenates. 2-DG is a glycolysis blocker that is not present in biological media under physiological conditions,¹³ and therefore, was not detected. The concentration of D-glucose detected in both types of samples was the highest of the three substrates (11 ± 2 mM in serum and 97 ± 10 $\mu\text{g/g}$ wet tissue in brain homogenates; Table 1). Concentrations of D-mannose and D-galactose in serum and brain homogenates were much lower or undetectable (Table 1).

Table 1. HPLC Identification of Sugars Present in Serum and Brain Homogenates

sugar	serum	brain homogenate
	conc. (mM)	conc. ($\mu\text{g/g}$)
galactose	<0.00055	0.61 ± 0.11
glucose	10.86 ± 1.93	97.37 ± 10.37
mannose	0.0333 ± 0.0144	24.42 ± 6.19

Using HPLC coupled to fluorimetric detection, the concentrations of 20 proteinogenic amino acids were also detected in rat brain homogenates and microdialysates (Supporting Information, Figure S1). In brain homogenates, glutamate was the most concentrated amino acid detected (1980 ± 434 $\mu\text{g/g}$ wet tissue), followed by glutamine (1044 ± 244 $\mu\text{g/g}$ wet tissue) and aspartate (426 ± 100 $\mu\text{g/g}$ wet tissue;

Table 2). For brain microdialysate samples (a good representation of brain interstitial fluid), glutamate was found

Table 2. HPLC Identification of Proteinogenic Amino Acids Present in Brain Dialysates and Homogenates

amino acid	conc. in brain dialysate (μM)	conc. in brain homogenate ($\mu\text{g/g}$)
aspartic acid	1.58 ± 0.45	426.8 ± 100.1
glutamic acid	4.04 ± 0.90	1980 ± 434
serine	4.90 ± 1.84	104.4 ± 16.6
asparagine	1.53 ± 0.61	6.97 ± 0.70
glycine	2.96 ± 0.57	48.4 ± 2.48
glutamine	41.4 ± 14.6	1044 ± 240
histidine	2.89 ± 0.30	12.2 ± 1.84
threonine	5.01 ± 0.92	83.0 ± 18.4
arginine	3.89 ± 0.48	14.6 ± 1.33
alanine	6.04 ± 0.94	42.8 ± 1.26
proline	11.8 ± 7.86	57.3 ± 5.20
tyrosine	0.71 ± 0.20	10.0 ± 1.20
cysteine	0.83 ± 0.00	6.62 ± 5.55
valine	3.89 ± 0.44	6.87 ± 0.52
methionine	0.72 ± 0.23	4.49 ± 0.45
iso-leucine	3.07 ± 0.43	3.21 ± 0.00
leucine	2.54 ± 0.46	8.15 ± 0.38
lysine	8.71 ± 2.06	31.6 ± 4.77
phenylalanine	1.48 ± 0.24	6.93 ± 1.42
tryptophan	3.20 ± 0.75	97.9 ± 38.8

to be present at low concentrations (4 ± 1 μM) compared to glutamine (41 ± 15 μM), proline (12 ± 8 μM), and lysine (9 ± 2 μM ; Table 2). The comparatively low glutamate concentration compared to potential secondary substrates of glutamate oxidase suggests that unlike glucose, detection of glutamate by biosensors implanted in the CNS could be significantly impacted by the loss of specificity during enzyme immobilization.

To verify the specificity of our biosensor measurements, glucose and glutamate concentrations were detected in the same biological samples using biosensors with glucose- or glutamate oxidase, respectively, immobilized with glutaraldehyde or PEGDE, and these values were compared with data from HPLC (for glucose) and CE-LIF (for glutamate) analyses. In serum samples, biosensors containing glucose oxidase immobilized by PEGDE or glutaraldehyde detected similar glucose concentrations of $100.1 \pm 6.0\%$ and $107.1 \pm 4.4\%$ of HPLC values. In addition, glucose concentrations detected in brain homogenates were $102.8 \pm 4.1\%$ and $92.1 \pm 5.2\%$ of HPLC values, respectively. These results confirm that detection of glucose by PEGDE- and glutaraldehyde-based biosensors was not impacted by potential secondary substrates of glucose oxidase.

Detection of glutamate in brain homogenates did not differ between biosensors prepared with PEGDE or glutaraldehyde and CE-LIF values (e.g., PEGDE $101.7 \pm 7.5\%$, glutaraldehyde $97.6 \pm 2.1\%$ of CE-LIF values). However, detection of glutamate in brain microdialysates using biosensors prepared with glutaraldehyde yielded significantly higher estimates ($145.1 \pm 43.0\%$ of CE-LIF estimates, $F[2,68] = 6.20$, $p < 0.01$) compared to PEGDE-prepared biosensors ($108.7 \pm 46.1\%$ of CE-LIF estimate, Figure 2B).

To estimate the impact of the interference by glutamine, asparagine, aspartate, and histidine, the electrochemical signal

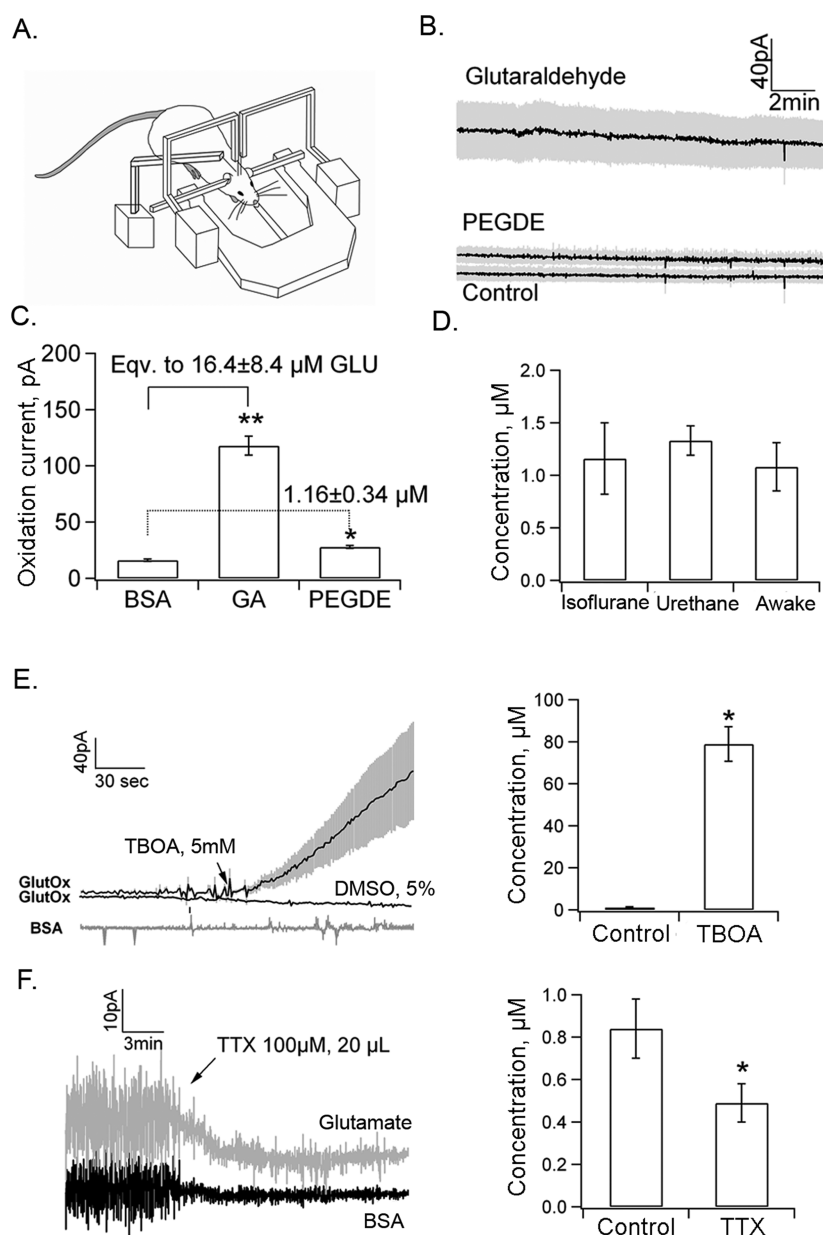


Figure 3. Detection of glutamate in the cortex. (A) Schematic representation of the experimental setup. (B) Oxidation currents recorded in the cortex of an anesthetized rat implanted with glutaraldehyde- or PEGDE-based biosensors and a control biosensor. (C) Quantitative estimation of basal glutamate concentrations in the cortex under isoflurane anesthesia. (* Significantly different from the control electrode, $n = 6$). (D) Glutamate concentrations under urethane or isoflurane anesthesia compared to those of an awake animal. (E) Effects of TBOA (20 μ L, 5 mM) on glutamate extracellular concentrations in the cortex. Mean glutamate concentrations increased from $1.16 \pm 0.33 \mu$ M to $79 \pm 8 \mu$ M (right). (F) Effects of a local TTX injection on the extracellular concentration of glutamate. (* Significantly different from the basal glutamate concentration, $n \geq 5$.)

corresponding to their presence in microdialysates was computed based on their concentrations measured by HPLC, and the enzymatic activity measured after glutaraldehyde immobilization. These interfering signals lead to an overestimation of glutamate concentration of 1μ M for glutamine (40 μ M, 2.5% enzymatic activity), 0.18 μ M for asparagine (1.5 μ M, 12% activity), 0.16 μ M for aspartate (1.6 μ M, 10% activity), and 0.09 μ M for histidine (3 μ M, 3% activity); therefore, a total glutamate overestimation of 1.43 μ M (+35.8%). Nonspecific detection of glutamine, asparagine, aspartate, and histidine by glutamate oxidase immobilized by glutaraldehyde thus accounted for most of the discrepancy between glutaraldehyde-based biosensors and PEGDE-based biosensors or CE-LIF. Therefore, it appears that enzyme

immobilization using PEGDE (but not glutaraldehyde) leads to specific glutamate detection in brain biological media that concurs with conventional analytical methods such as CE-LIF.

Detection of Glutamate in Vivo. Finally, biosensors with glutamate oxidase immobilized using PEGDE or glutaraldehyde were implanted in vivo. To determine the basal concentration of glutamate in the cortex, three biosensors were implanted side-by-side in the cortex of rats anesthetized with isoflurane: two glutamate biosensors prepared using glutaraldehyde versus PEGDE, and one control biosensor with glutamate oxidase replaced by bovine serum albumin (BSA) (Figure 3A). After an initial 30 min stabilization period, the steady-state current reflecting the basal level of glutamate was detected at significantly higher levels by glutaraldehyde-based biosensors

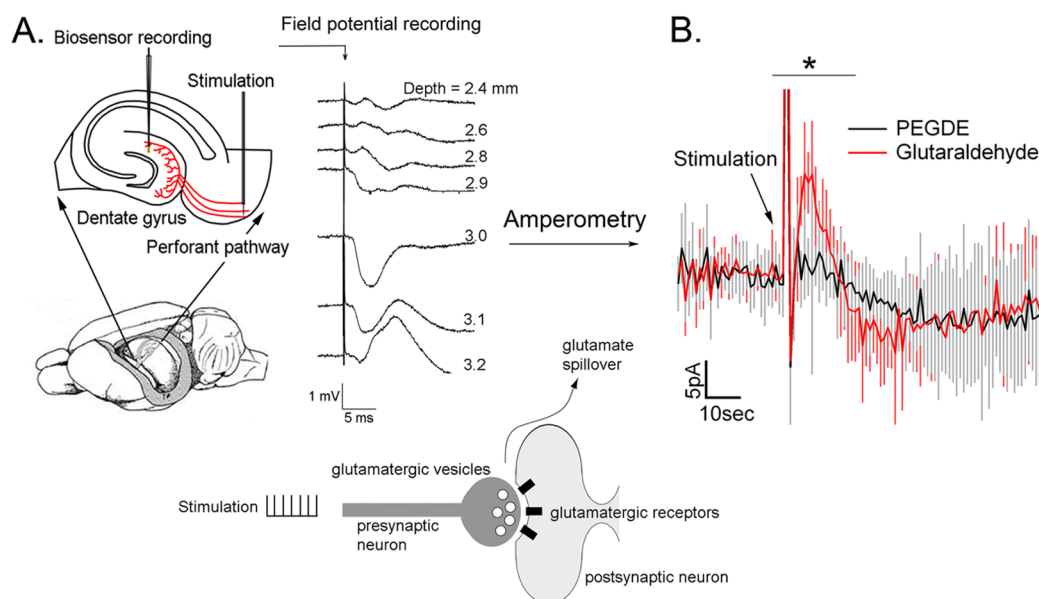


Figure 4. Detection of glutamate spillover in the dentate gyrus. A stimulating electrode and a microelectrode biosensor were inserted in the perforant path and the dentate gyrus, respectively, in an anesthetized rat (left). Field potentials evoked by perforant path stimulation were recorded from the biosensor in order to place it at the current sink of the stimulation (middle). 100 Hz perforant path stimulation evoked an increase in oxidation current at the biosensor prepared with glutaraldehyde (equivalent to $1.46 \pm 0.19 \mu\text{M}$ glutamate), which was not detected by biosensors prepared with PEGDE (right). (* Significant increase in current.)

($118 \pm 8.5 \text{ pA}$) versus PEGDE-based biosensors ($28.2 \pm 1.2 \text{ pA}$) and control biosensors ($16.2 \pm 1.0 \text{ pA}$, $F[2,15] = 20.69$, $p < 0.01$; Figure 3B). Extracellular glutamate concentrations were calculated based on these oxidation currents (see the Materials and Methods section). PEGDE-based biosensors estimated the resting level of glutamate present in the cortex to be $1.16 \pm 0.34 \mu\text{M}$ versus $16.4 \pm 8.4 \mu\text{M}$ ($n = 6$) detected by glutaraldehyde-based biosensors (Figure 3C).

To determine whether basal concentrations of glutamate varied with the level and type of anesthesia applied, extracellular concentrations of glutamate were detected using PEGDE-based biosensors in animals subjected to isoflurane or urethane anesthesia, compared to awake animals. However, there was no significant difference in the concentrations of glutamate detected in these three models (Figure 3D).

Extracellular levels of glutamate were then modulated pharmacologically using the glutamate reuptake blocker, (3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl] methoxy]-L-aspartic acid (TBOA), and the voltage-gated Na^+ channel blocker, tetrodotoxin (TTX). TBOA (5 mM) was applied to the pia and rapidly increased glutamate concentrations from $1.16 \pm 0.34 \mu\text{M}$ to $79 \pm 8 \mu\text{M}$ ($n = 5$, $p < 0.01$; Figure 3E). This increase in oxidation current was not detected by the control biosensor, or in response to vehicle ($5\% \text{ DMSO}$), thereby confirming that the electrochemical signal detected by PEGDE-based biosensors was dependent on levels of extracellular glutamate. In contrast, TTX produced a rapid silencing of neuronal firing as evidenced by the decrease in local field potentials detected by the biosensors (Figure 3F). The extracellular concentration of glutamate decreased by $34.8 \pm 9.3\%$ (21.47 to 14.14 pA , $n = 6$, $p < 0.01$, Figure 3F).

Finally, we sought to detect glutamate spillover into the extrasynaptic space following neuronal stimulation. For these studies, a stimulating electrode was inserted in the perforant path and a microelectrode biosensor was implanted in the dentate gyrus to monitor glutamate levels in response to

stimulation (Figure 4A). Field potentials evoked by perforant path stimulation were recorded from the biosensor that was placed at different depths in relation to the stimulating electrode. Using these recordings, the biosensor was placed at the current sink, the site of maximal synaptic glutamate release.¹⁴ Stimulation of the perforant path evoked a transient increase in oxidation current in biosensors prepared with glutaraldehyde, equivalent to $1.46 \pm 0.19 \mu\text{M}$ glutamate for approximately 5 s. However, this apparent glutamate spillover was not detected by the biosensors prepared with PEGDE ($n = 6$, $p < 0.01$; Figure 4B).

DISCUSSION

Using microelectrode enzymatic biosensors, we found that enzyme immobilization can result in a significant loss of substrate specificity and that this effect can be avoided using the cross-linker PEGDE. The specificity of the microelectrodes used was confirmed using conventional analytical techniques such as HPLC and CE-LIF. Moreover, a new estimate of ambient glutamate concentrations present in the extracellular fluid of the rat brain was obtained.

Effects of Enzyme Immobilization Techniques on Apparent Substrate Specificity. An underlying principle of enzymatic biosensor technology is maintenance of substrate specificity for the enzymes used to recognize molecules in complex media. In the present study, different immobilization methods were used to prepare enzymatic biosensors. In particular, immobilization using glutaraldehyde was found to significantly decrease the selectivity of the glucose and glutamate oxidase sensors. In contrast, immobilization methods involving PEGDE, was found to preserve the substrate-specificity of the two enzymes.

Enzyme immobilization generates chemical bonds between an enzyme and the matrix used. Accordingly, these interactions have the potential to modify the three-dimensional conformation of an enzyme, and therefore, its substrate recognition.¹⁵

Currently, molecular mechanisms that underlie these effects remain largely empirical. Moreover, enzymes are immobilized within a macromolecular matrix through which substrates must diffuse before reaching the enzyme's active site. Changes in diffusion coefficients between the solution and the matrix, as well as in regard to substrate affinities toward the matrix, can cause substrates to be concentrated, or excluded, from the enzyme membrane. As a result, a partitioning effect can be generated.¹⁶

Modification of an enzyme's substrate specificity due to immobilization is frequently encountered in the field of enzymatic catalysis. Enzyme immobilization often results in a loss of substrate specificity due to enzyme rigidification, although it can also lead to improved apparent specificity.¹⁵ For example, α -D-galactosidase, lipases, and penicillin G-acylase exhibit improved substrate specificity following glutaraldehyde immobilization.¹⁷ For the preparation of biosensors, substrate specificity of an enzyme must be preserved during immobilization in order to ensure that selective measurements are made in complex media. Thus, to prevent unwanted distortion of the enzyme active site, or limitation in the enzyme mobility, mild immobilization methods are needed.¹⁵ In this regard, PEGDE immobilization is particularly mild. For example, the cross-linking reaction between PEGDE epoxy groups and amine groups on the enzyme surface is slow (48 h at room temperature, 2 h at 55 °C,^{6,18}). Moreover, the length of the PEGDE spacer arm is about 4 nm, which preserves enzyme conformation while accommodating conformational changes that occur during catalysis. For example, the overall dimensions of a deglycosylated glucose oxidase dimer are $7 \times 5.5 \times 8$ nm^{3,19} which is comparable to the length of the PEGDE spacer arm (4 nm). By comparison, glutaraldehyde immobilization is much faster (2–3 min at RT), and the spacer arm is more than five times shorter (~ 0.7 nm, Figure 5). As a result, protein rigidification can occur, thereby resulting in a loss of substrate specificity.

Consequences for Glutamate Detection in Vivo.

Changes in enzyme substrate specificity due to immobilization on a microelectrode can have crucial consequences for the in

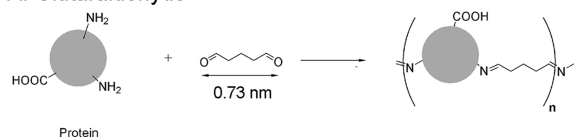
vivo detection of substrate(s). For glucose oxidase biosensors, detection of glucose in the serum or in the brain was unaffected by these effects. This is primarily because glucose is present at mM levels, while potentially interfering secondary substrates are present at much lower concentrations. However, levels of glutamate are in the low μ M range in the extracellular fluid of the brain.^{5,20} Therefore, glutamine, asparagine, and aspartate can potentially affect electrochemical currents of a biosensor if the specificity of immobilized glutamate oxidase enzymes is not preserved.

In particular, glutamine (1) is present at much higher concentrations than glutamate in the brain extracellular fluid, (2) can be detected by glutamate oxidase immobilized with glutaraldehyde, and (3) is actively shuttled between neurons and glia during neurotransmission.²¹ In the latter case, this could give rise to rapid transient changes in oxidation current, resembling those associated with neurotransmitter release and spillover. Our results therefore question the validity of at least some of the previous biosensor studies aimed at detecting glutamate in the CNS, especially those using glutaraldehyde as the cross-linker. Moreover, based on the results of the present study, PEGDE should be used as the immobilization agent for glutamate detection in the brain.

Based on the extracellular concentrations of glutamate that were estimated using PEGDE-based biosensors implanted in rats, many important observations were made: (1) the resting concentration of glutamate in the cortex was 1.16 ± 0.34 μ M, (2) levels of glutamate were not dependent on anesthesia (as evidenced in awake animals vs animals under isoflurane or urethane anesthesia), (3) glutamate concentrations were partly dependent (35%) on nerve impulse as revealed by administration of TTX, and (4) levels of glutamate were not significantly impacted by spillover from synaptic stimulation in the dentate gyrus.

The 35% decrease in extracellular concentrations of glutamate detected by the PEGDE-prepared glutamate biosensors is consistent with the current model of extracellular glutamate regulation, where a cysteine-glutamate exchanger and a Na⁺-dependent antiporter preferentially located on glia, regulate concentrations of extrasynaptic glutamate.²² Moreover, the lack of detectable glutamate spillover following synaptic stimulation is also consistent with recent imaging studies where fluorescent detection of glutamate in brain slices and in superficial layers of the cortex have detected a glutamate spillover of ~ 2 μ M lasting less than 300 ms.²³ Such rapid changes in the concentration of glutamate would not be detected using enzymatic biosensors, which have a response time of ~ 2 s. The fact that glutamate spillover from the synapse is apparent after TTX administration but not after neuronal stimulation may appear as a contradiction. However, glutamate transporters have a K_m in the order of 10–100 μ M,²⁴ indicating that the speed of glutamate reuptake is much slower at resting concentrations (around 1 μ M) than after neuronal stimulation, when glutamate concentration in the synaptic cleft can exceed 1 mM. Therefore, it is reasonable to assume that glutamate transporters are more efficient at restricting the spillover of glutamate originating from quick bursts of neuronal activity, than that originating from regular spiking at rest. Overall, the use of glutamate biosensors prepared using PEGDE provided in vivo measurements that were consistent with predicted low levels of extrasynaptic glutamate predominantly regulated by glia.

A. Glutaraldehyde



B. PEGDE

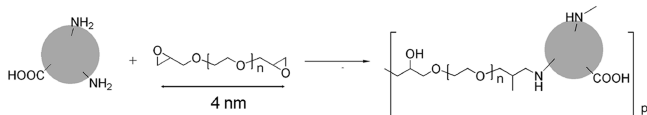


Figure 5. Covalent immobilization techniques. (A) Reaction scheme for protein cross-linking by glutaraldehyde. Aldehyde groups react preferentially with amino groups present on a protein to produce covalent bonds between proteins. (B) General reaction scheme of a protein with PEGDE. Protein molecules containing amino and carboxyl groups may react with the two epoxides of PEGDE, resulting in immobilization. One major difference between PEGDE and glutaraldehyde is the length of the spacer arm. For PEGDE it is 4 nm, and for glutaraldehyde it is 0.73 nm (computed using HyperChem Software).

The basal glutamate concentration detected in the present study, $\sim 1.2 \mu\text{M}$, is in the lower range of basal glutamate concentrations previously reported using a no-net flux microdialysis method (e.g., $0.9\text{--}3.7 \mu\text{M}$).¹ Moreover, it is a lower concentration than all biosensor estimates published so far. For example, using glutaraldehyde prepared microelectrode biosensors versus hydrogel-based biosensors, glutamate levels in anesthetized animals were estimated to range from 1.6 to $4 \mu\text{M}$ ^{4a–c} versus $19\text{--}29 \mu\text{M}$,^{4d,e} respectively. In awake animals, glutamate levels have been estimated to be even higher (e.g., $19\text{--}45 \mu\text{M}$) when glutaraldehyde-prepared biosensors were used.^{4f,g} Concentrations of extracellular neurotransmitters can vary for different brain regions, and potentially this applies to glutamate as well. However, it is unlikely that the discrepancies in glutamate concentrations published in the literature are only due to differences in brain regions. Focusing on biosensor studies performed, for example, in rat striatum, we found extracellular concentration estimates of 2.2 ,²⁵ 18 ,^{4e} and $29 \mu\text{M}$.^{4d} We hypothesize that some of the previous biosensor estimates, especially those using glutaraldehyde, were inaccurate due to compromised substrate specificity in relation to biosensor preparation methods. As a result, nonspecific detection of amino acids such as glutamine, asparagine, aspartate, and histidine may have occurred. In this study, glutaraldehyde immobilization was performed by exposition to chemical vapors or direct mixing into the enzyme solution and both methods produced a similar loss of apparent substrate-specificity. This result suggests that a similar effect probably applies to glutaraldehyde immobilization methods used in other laboratories. Therefore, based on the results of the present study, reevaluation of previous biosensor studies should be considered in order to resolve long-standing debates regarding estimates of resting ambient glutamate levels in the central nervous system, and to account for the effects of enzyme immobilization observed here.

Microelectrode biosensors are an invasive technique that inevitably produces some tissue injury upon implantation in the brain parenchyma. In this study, our microelectrode biosensors had a small external diameter of only $40\text{--}50 \mu\text{m}$ and probably minimized this effect. However, it is possible that the size of the biosensor and the corresponding tissue response is another important factor in determining ambient glutamate concentrations in the brain.

Overall, we have identified here an important effect of enzyme immobilization on biosensor selectivity that can significantly impact the detection of glutamate in the brain, and probably other neurotransmitters as well. PEGDE immobilization is mild enough to preserve enzyme substrate specificity, and represents an interesting solution to this selectivity problem. This method therefore provides a valuable tool for direct and accurate measurements of neurotransmitter concentrations for studying the chemistry of brain cell communication in vivo.

■ ASSOCIATED CONTENT

● Supporting Information

Supplementary figure S1: example chromatogram with the major amino acids present in the brain. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

N.V. developed the biosensor fabrication and enzyme immobilization protocols, performed in vitro and in vivo experiments, designed experiments, and wrote the manuscript. C.M. and A.M. tested enzyme immobilization and biosensor fabrication protocols and contributed to in vivo experiments. H.V. designed and performed HPLC experiments. T.L. designed and performed in vivo experiments. D.B. designed microelectrode fabrication methods. S.M. designed and supported the study, directed the work, analyzed data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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