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# Redox hydrogel based bienzyme electrode for L-glutamate monitoring

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**Abstract**

Amperometric bienzyme electrodes based on coupled L-glutamate oxidase ($GlO_x$) and horseradish peroxidase (HRP) were constructed for the direct monitoring of L-glutamate in a flow injection (FI)-system. The bienzyme electrodes were constructed by coating solid graphite rods with a premixed solution containing $GlO_x$ and HRP crosslinked with a redox polymer formed of poly(1-vinylimidazole) complexed with (osmium (4-4′-dimethylbpy)$_2$ Cl)$^{II/III}$. Poly(ethylene glycol) diglycidyl ether (PEGDGE) was used as the crosslinker and the modified electrodes were inserted as the working electrode in a conventional three electrode flow through amperometric cell operated at $-0.05$ V versus Ag|AgCl (0.1 M KCl). The bienzyme electrode was optimized with regard to wire composition, Os-loading of the wires, enzyme ratios, coating procedure, flow rate, effect of poly(ethyleneimine) addition, etc. The optimized electrodes were characterized by a sensitivity of $88.36 \pm 0.14$ μA mM$^{-1}$ cm$^{-2}$, a detection limit of 0.3 μM (calculated as three times the signal-to-noise ratio), a response time of less than 10 s and responded linearly between 0.3 and 250 μM (linear regression coefficient = 0.999) with an operational stability of only 3% sensitivity loss during 8 h of continuous FI operation at a sample throughput of 30 injections h$^{-1}$. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Glutamate; Redox hydrogel; Amperometric bienzyme electrode; Flow injection

## 1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain, being linked to synaptic plasticity and involved in the pathophysiology of various neurological disorders such as epilepsy, stroke and neurodegenerative diseases.

Development of new techniques for continuous monitoring of glutamate release at the synaptic level is highly warranted in order to clarify the role of this neurotransmitter both under physio-

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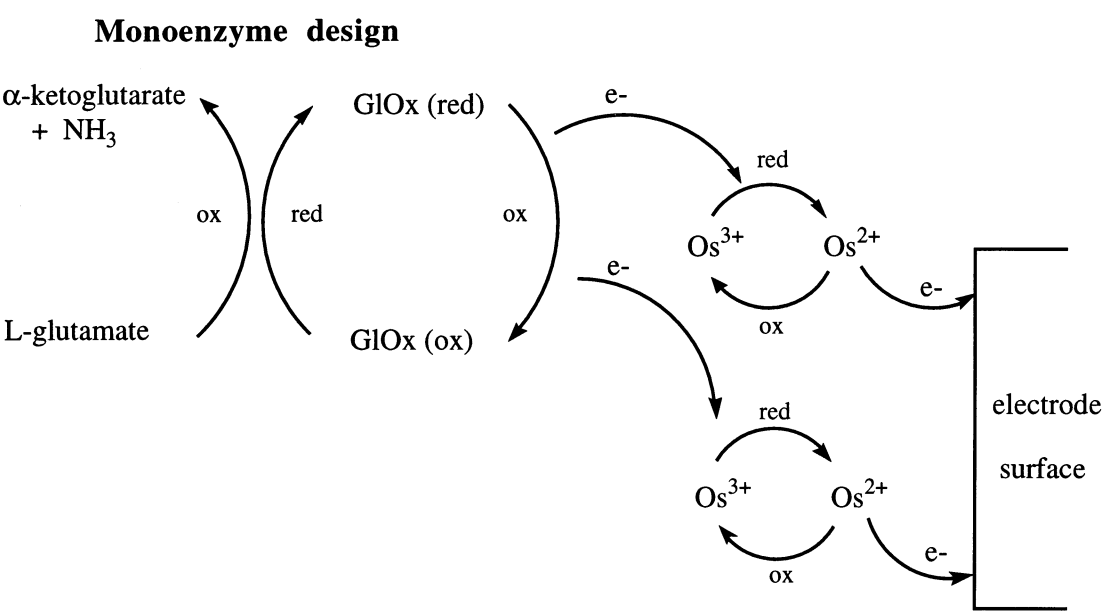
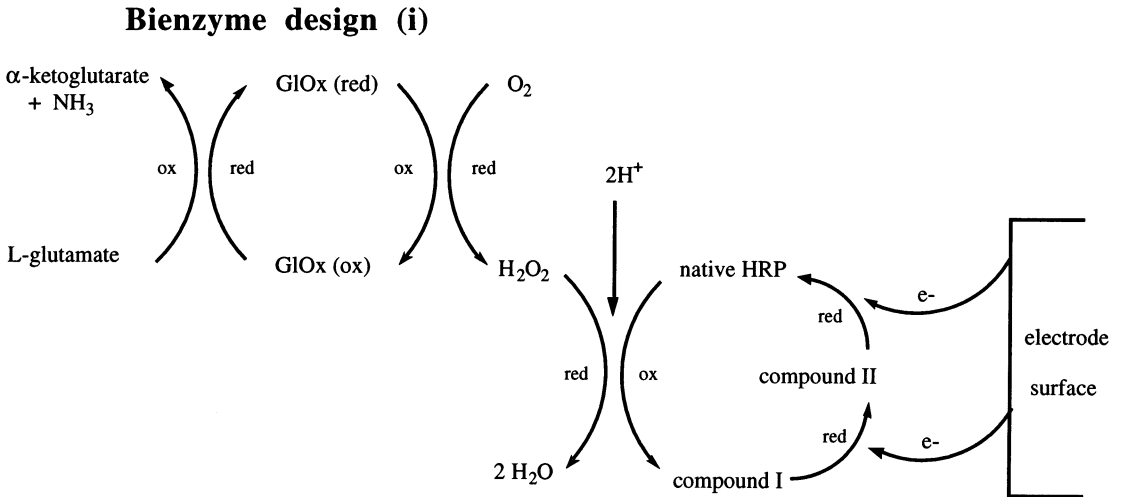
logical conditions, e.g. memory and learning and in certain pathological states such as epilepsy, stroke and neurodegenerative disorders. The existing classical chemical approach and instrumentation [1] do not allow for adequate measurements of transmitter release from individual synapses. The most advanced neurotransmitter release monitoring using microdialysis probes for sampling [2] often coupled to HPLC [3] and flow injection analysis [4,5], utilizes probes of several hundred μms in diameter. Biosensors applied as specific detectors [6–8] are often prone for biased signals and are not predisposed for miniaturization down to micrometer scale, which is required for monitoring localized events at synaptic level.

Excised pieces of cell membranes attached to the patch-clamp pipette tips as outside-out patches can serve as glutamate sensors [9], as long as ionic currents activated by incorporated glutamate receptors can be measured and can partially solve the problem of selectivity, spatial and temporal resolution but lack mechanical stability and reproducibility. Alterations in pre-synaptic release can be monitored indirectly by measuring currents at the post-synaptic site induced by activation of respective neurotransmitter receptors (whole-cell patch-clamp technique—PCT) but this information might often be misleading. The small size of the synapses, the very complex matrix to be analyzed and the extremely low concentration of many neurotransmitters in brain, together with their restricted lifetimes, require highly selective, sensitive, fast responding, stable and reliable detectors in μm range.

In order to meet these requirements, several biosensor designs were concepted and developed biosensors were tested either under laboratory or in vivo conditions (brain slices, microdialysates, cultured neurocells). They usually are enzyme based, using either $NAD^+$-dependent glutamate dehydrogenase [10–12], or $GlO_x$ [4,5,13–20] in mono- or bienzyme [15,18,19] design often immobilized into various polymeric materials [14,16–18,21,22], or carbon paste [10,11,19] also often containing mediating molecules. The detection systems could use enzymes immobilized in microreactors [5,23] or on the surface of a suitable electrode. However, site-specific monitoring of glutamate release at synapses, will require accurate positioning of a suitable microdetector, which is more likely to be solved using a microelectrode. Many of the mentioned enzyme electrodes are




Scheme 1. Monoenzyme design.




Scheme 2. Bienzyme design (i).

meeting the sensitivity and detection limits requirements (1 μM) but they often need the presence of additional interference eliminating membranes, increasing thus the response time of the sensors. Presently only two electrodes have an i. d. $<100$ μm [12,20]; the first [12] displays also an extremely fast response (450 ms), but requires further improvement of the detection limit. Moreover, its complicated design makes it more prone for other type of applications (integration with microdialysis (MD) or capillary zone electrophoresis (CZE)). The second one [20] is more promising for brain mapping, but it is still facing some interfering problems.

The purpose of this work was to design a glutamate electrode with a sensing chemistry which will allow a miniaturization of the electrode to μm size and will meet at the same time sensitivity, detection limit, response time, stability and interference elimination requirements. However, for initial neurological experiments the optimized electrodes are aimed to plausibly function in an integrated microdialysis-FIA detection system. Since osmium-based redox hydrogels were previously shown to be a very promising matrix for enzyme immobilization yielding high sensitivities and good stabilities [24–27], such a system was chosen in this work. Optimization steps and characteristics of the optimized electrode will be presented.

## 2. Experimental

### 2.1. Chemicals and reagents

L-Glutamate oxidase ($GlO_x$, EC 1.4.3.11) from *Streptomyces* sp. (Yamasa, Choshi, Chiba 288, Japan, Cat. no. 7804) was purchased as a lyophilized powder with an activity of 6.8 U $mg^{-1}$. Horseradish peroxidase (HRP, EC 1.11.1.7 from Boehringer-Mannheim, Mannheim, Germany (Cat. no. 814407) was purchased as a lyophilized powder with a minimum activity of 1000 U $mg^{-1}$. Poly(1-vinylimidazole) was complexed with (osmium (4-4′-dimethylbpy)$_2$Cl)$^{II/III}$ according to a previously published procedure [28]. Using this procedure two redox polymers with different Os loading, $PVI_7$-dmeOs and $PVI_{13}$-dmeOs, were synthesized and used in this work. The subscripts 7 and 13 indicate the number of vinylimidazole monomer units for every imidazol complexed to an osmium redox center. Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE, Polysciences, Warrington, PA, Cat. no. 08210) was used to crosslink HRP to the Os-polymer. L-Glutamic acid and poly (ethyleneimine) (PEI) purchased from Sigma Chemical, St. Louis, MO, Cat. no. G-6904 were used as received. Hydrogen peroxide (from Merck, Darmstadt, Germany) was standardized

by permanganate titration. Ringer's solution was prepared by dissolving the following salts in 1 l of $H_2O$: 8.6 g NaCl, 0.33 g $CaCl_2.2H_2O$ and 0.3 g KCl, pH 6.0. In all cases, if not otherwise mentioned, solutions were prepared using HPLC-grade water produced in a Milli-Q system from Millipore, Bedford, MA. All experiments were carried out at room temperature.

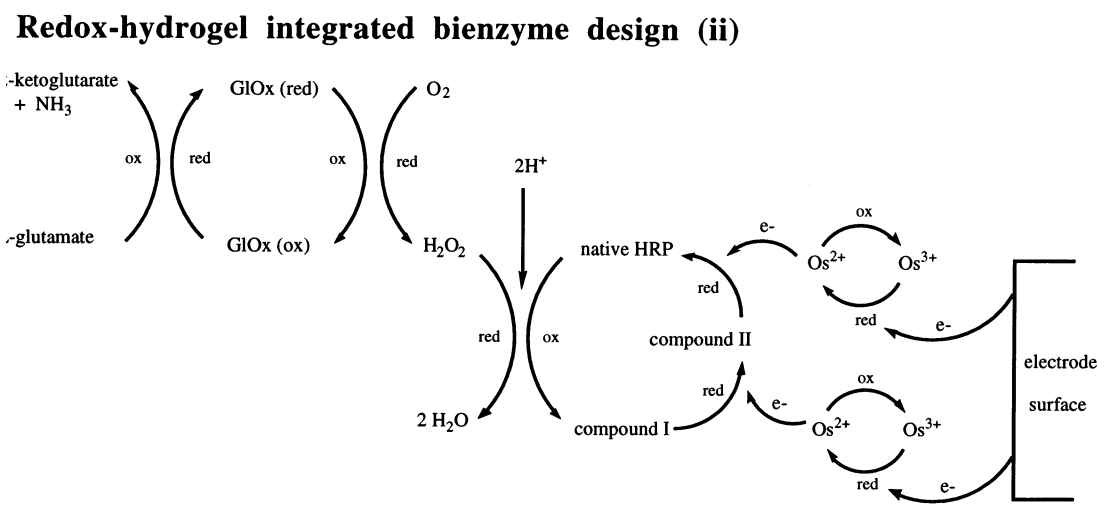
### 2.2. Instrumentation

Enzymatically modified graphite electrodes were inserted into a flow-through amperometric cell of the wall-jet type containing a platinum wire as the counter and a Ag|AgCl (0.1 M KCl) as the reference electrodes, respectively. The cell was connected to a potentiostat (Zäta-Elektronik, Lund, Sweden) and the output was recorded on an strip chart recorder (Kipp and Zonen, Delft, The Netherlands, Model BD 111). The construction of the flow-through cell was previously described [29]. A 0.1 M phosphate buffer (pH 7) or Ringer's solution was delivered into the electrochemical cell at a flow rate of 0.8 ml $min^{-1}$ (except during flow rate studies) using a peristaltic pump (Gilson, Villiers-le-Bel, France, Model Minipuls 2). Samples were pumped into the injection loop with another peristaltic pump (LKP, Bromma, Sweden, Model 2150). Samples of 50 μl were injected using a pneumatically operated injection valve (Cheminert, Cotati, CA, type SVA). Operational stability experiments were made using an Automated Sample Injection Analyzer (Ismatec, Glattburg-Zürich, Switzerland) by injecting samples of 0.05 mM L-glutamate, in Ringer's solution, every 2 min. All connections were made with Teflon tubings and commercially available screw couplings. Before use all solutions were filtered through a 0.45 μm filter (Millipore, Molshem, France, type HA) and degassed. 10 mM stock solution of glutamate was prepared daily. The dispersion coefficient of the FI system was determined as the ratio between the steady state and peak currents and was found to be 1.5.

### 2.3. Electrode preparation

First, graphite rods (I. D. 0.305 cm, type RW 001, Ringsdorff-Werke, GmbH, Bonn, Germany) were polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI) fixed on a home-made polishing machine and washed thoroughly with deionized water. After drying the electrodes at room temperature, their surfaces were coated in one or two steps:

1. In the one step procedure, the electrodes were prepared placing a defined amount of a premixed solution containing various amounts of




Scheme 3. Redox-hydrogel integrated bienzyme design (ii).

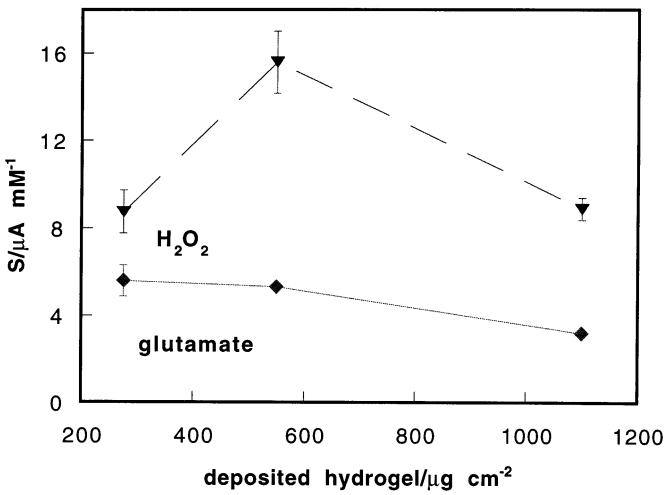



Fig. 1. Influence of the amount of deposited hydrogel on the current signals. Enzyme electrodes were operated in a FI-system at −50 mV versus Ag|AgCl using 0.1 M PB buffer at pH 7.0 as the carrier pumped at 0.8 ml $min^{-1}$ flow rate.

HRP (10 mg $ml^{-1}$ in 0.1 M phosphate buffer, pH 7), $PVI_7$-dmeOs (10 mg $ml^{-1}$), a freshly prepared PEGDGE solution (5 mg $ml^{-1}$) and $GlO_x$ (10 mg $ml^{-1}$ in 0.1 M phosphate buffer, pH 7). After curing the wired enzyme layer at 50°C for 10 min [30], the electrodes were immediately used.

2. In the two step coating procedure, the electrodes were coated first with a premixed solution composed of 1 μl HRP, 0.8 μl $PVI_7$-dmeOs and 0.4 μl of PEGDGE (50:40:10% w/w). The droplet was allowed to dry at room temperature for 10 min and coated thereafter with 2 μl of $GlO_x$ (HRP:$GlO_x$ = 73). Finally the electrodes were cured at 50°C for 10 min in a thermostat. All solutions had the same concentrations as mentioned for the single step preparation.

The electrodes were kept in a desiccator between measurements at room temperature. All results presented in this paper are means of three equally prepared electrodes if not otherwise mentioned. The geometrical surface area of these disk electrodes was 0.073 $cm^{-2}$. When PEI was integrated into the electrode configuration, 3 μl of a 0.1% (w/w) PEI solution were mixed with the other solutions.

## 3. Results and discussion

$GlO_x$ was considered to be used as the biological recognition molecule, since it does not require addition of a soluble cofactor (as glutamate dehydrogenase) and it was reported to be selective only towards glutamate and aspartate but only to an extent of 0.6% for the last [31]. It catalyzes the following reaction, yielding hydrogen peroxide and ammonia.

$$\text{l}-\text{glutamate} + O_2 + H_2O \xrightarrow{GlO_x} \alpha-\text{ketoglutarate} + NH_3 + H_2O_2$$

L-glutamate can be monitored via oxygen consumption or hydrogen peroxide formation, both reactions requiring high overpotentials resulting in bias signals from the interferents present in the biological matrix. There are, however, several possibilities to design a suitable glutamate sensor, mono- or bienzyme based ones, with and without the integration of various mediators.

The monoenzyme approach used in this work was based on the direct wiring of the $GlO_x$ using the above mentioned Os-redox hydrogel and PEGDGE crosslinker, operating at potentials $> +100$ mV versus SCE (the formal potential of the redox hydrogel), according to Scheme 1.

Unlike other FAD enzymes such as glucose oxidase, $GlO_x$ could be only poorly wired, signals being yielded only above 20 μM glutamate, with a very low sensitivity (8 μA $mM^{-1}$ $cm^{-2}$, $r = 0.995$) compared with that exhibited by glucose oxidase electrodes being of about 100 μA $mM^{-1}$ $cm^{-2}$ [32].

The bienzyme approaches concepted in this work were based on (i) the direct coupling of $GlO_x$ and HRP simply immobilized (adsorbed) on the electrode surface and (ii) entrapped into a redox hydrogel. The flow of the electrons is illustrated above (see Schemes 2 and 3) for both bienzyme approaches, both electrode configurations being operated at potentials $< 0$ mV.

The unwired electrode design resulted in 2–3 times lower sensitivities for glutamate than the wired one. Also the stability of these electrodes was not satisfactory. Their sensitivity decreased by 30–42% when continuously operated for 8 h in FIA mode. From our previous experience with hydrogel based electrodes it was expected that the wired bienzyme electrodes will exhibit improved stability [27]. Therefore, only the wired bienzyme approach was further considered for optimization.

### 3.1. Optimization steps

The bienzyme electrodes were optimized with regard to several parameters, namely, curing procedure, effect of wetting, Os loading of the wires, electrode coating procedure, amount of crosslinker used and the influence of various enzyme ratios. First the curing procedure was tested, comparing the sensitivities of electrodes cured at room temperature for various times, with that of electrodes cured at 50°C for 10 min. Increasing curing times at room temperature resulted in more stable peak signals. However, no difference in sensitivity could be observed between electrodes cured overnight at room temperature and those cured under thermostated conditions. Since curing at 50°C for 10 min did not deactivate the enzymes, yet, it shortened the time required for sensor preparation it was used throughout this work.

Next, the electrodes under potentiostatic control were flushed with the carrier buffer or Ringer's solution for at least 30 min before measurements were carried out. Signals recorded by the time stable base lines were attained (ap-

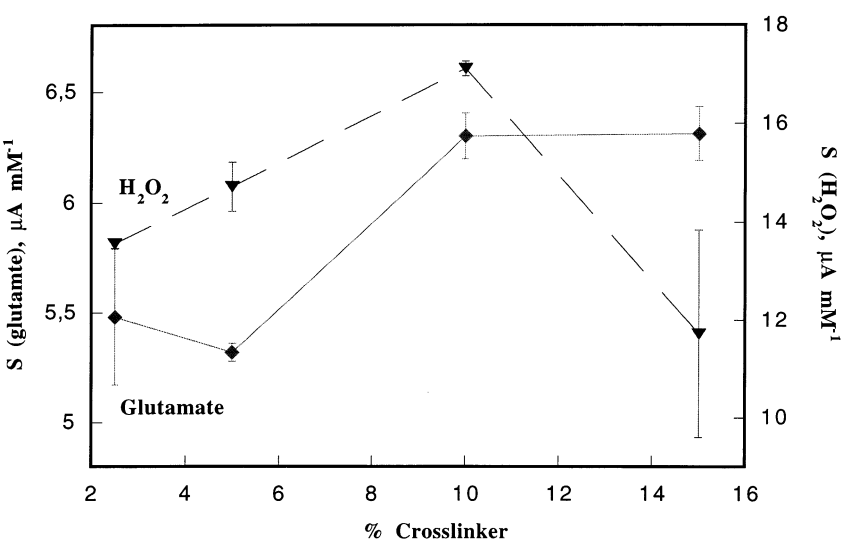



Fig. 2. Influence or wired HRP layer composition on the signals, effect of used crosslinker amounts. HRP and $PVI_7$-dmeOs ratio, HRP:$GlO_x$ ratio, and the amount of deposited hydrogel were kept constant.

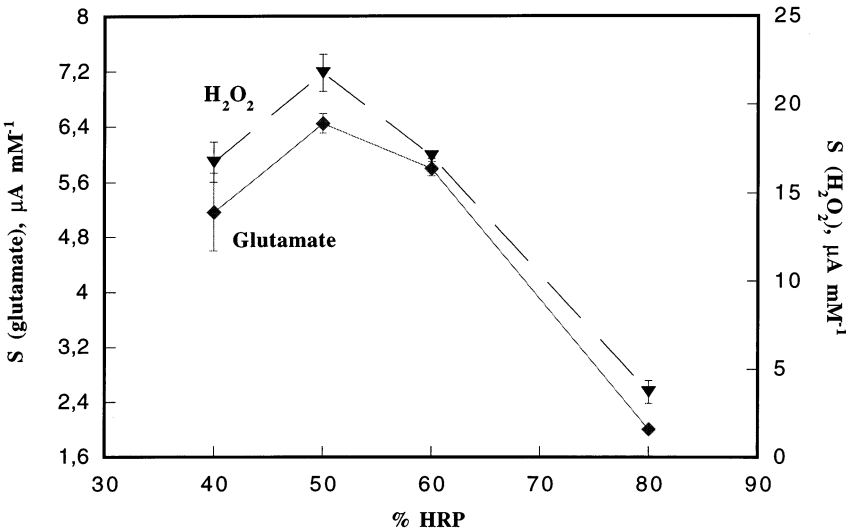



Fig. 3. Influence of wired HRP layer composition, effect of used HRP amounts. 10% PEGDGE and HRP:$GlO_x$ ratio were kept constant.

proximately after 10–15 min) were 15–25% less than the signals obtained after 30 min of flushing of the electrode. The redox hydrogel was reported to swell upon exposure to solvents, a highly swollen redox structure promoting permeation of the films by substrate and product molecules [33]. All electrodes were therefore flushed with carrier throughout this work.

Finally, the optimization of the electrode configuration was considered. The effect of the Os loading was tested by crosslinking HRP to redox wires with different Os content, namely $PVI_7$-dmeOs and $PVI_{13}$-dmeOs. Electrodes were prepared in the same way but containing either of the two polymers. For 50 μM glutamate, a 20% increase in current density was observed for the wire with higher osmium content. This observation is in agreement with previously reported results [25] showing that an increase in the number of the osmium redox centers resulted in increased sensitivities. Thus, only $PVI_7$-dmeOs was used for further optimization.

In a second step, the effect of the coating procedure was studied. Two coating procedures, i.e. coating with premixed solutions and sequential coating were tested. When sequential coating was used to prepare the bienzyme electrode, the various components were added as follows by gently mixing the solution on the top of the rod after each addition: HRP was added first followed by $PVI_7$-dmeOs, PEGDGE and finally $GlO_x$. When premixing was used to prepare the enzyme electrode, the same amounts of each of the components as used in sequential coating were first mixed in an Eppendorf tube and electrodes were coated with this premixed solution. Though similar sensitivities for glutamate (5.19 μA $mM^{-1}$ and 5.32 μA $mM^{-1}$, respectively) were obtained for both coating procedures, the reproducibility of the electrodes coated with the premixed solution was tremendously improved. Similarly prepared electrodes with sequential modification showed 20–30% differences in current output, whereas the difference was always less than 10% when electrodes were prepared by coating with premixed solutions.

Also the effect of one step or single-layer coating vs. two step or bilayer coating was considered. Coating in one step, might result in a partial wiring of the $GlO_x$ (short-circuiting), thus yielding small oxidation currents and resulting in somewhat biased signals. The sensitivity for glutamate was found to be about 15% higher for the two step coating procedure (bilayer electrodes), while

the hydrogen peroxide sensitivity remained practically unchanged, confirming the previously reported results for other bienzyme hydrogel electrodes [34,35]. However, since $GlO_x$ can be only poorly wired by osmium containing hydrogel and the difference in sensitivity for one step vs. two step coating approach was not relevant taking into consideration the reproducibility of electrode preparations, all further electrodes were coated in one step procedure using a premixed solution of all components.

The thickness of the redox film (amount of deposited enzyme/hydrogel mixture) was expected to play an important role on the stability and sensitivity of the electrodes. Good adhesion of the film to the electrode surface is highly desirable for better stability, while thicker films are expected to increase the current output, but adhering less efficient and thus displaying decreased stability. Therefore, in a next step the amount of deposited enzyme/redox polymer mixture was optimized. As shown in Fig. 1, an almost constant sensitivity was obtained for glutamate up to 550 μg $cm^{-2}$ of the deposited amount of hydrogel, thereafter a decrease in current output was noted with increasing amount of hydrogel on the electrode. Probably the mass transport of the substrate into the hydrogel is limited by a thicker layer. Interestingly, a maximum sensitivity was observed for $H_2O_2$ at a layer consisting of 550 μg $cm^{-2}$ of hydrogel clearly indicating an optimal layer thickness or amount of hydrogel on the electrode for electron transfer from the electrode surface. The reduced sensitivities at higher and lower layer thickness could be attributed to limitations of $H_2O_2$ and/or electron transport and the small amount of the hydrogel/enzyme (HRP) deposited on electrode, not being sufficient to convert the $H_2O_2$ flux reaching the electrode surface into current, respectively. Thus, a layer consisting of 550 μg $cm^{-2}$ of hydrogel was used for electrode preparation in all further experiments.

The composition of the electrode was fulfilled by first optimizing the composition of the redox wire with regard to the HRP/$PVI_7$-dmeOs-PEGDGE ratio and next optimizing the ratio of the two enzymes (HRP:$GlO_x$). When optimizing the bienzyme electrode with respect to the amount of PEGDGE and the HRP:polycation ratio, the film thickness and the amount of $GlO_x$ were kept constant. One of the basic requirements to obtain 'wired' electrodes with high sensitivity is the flexibility of the hydrogel which allows high electron transfer rates within the polymer [24,36]. The

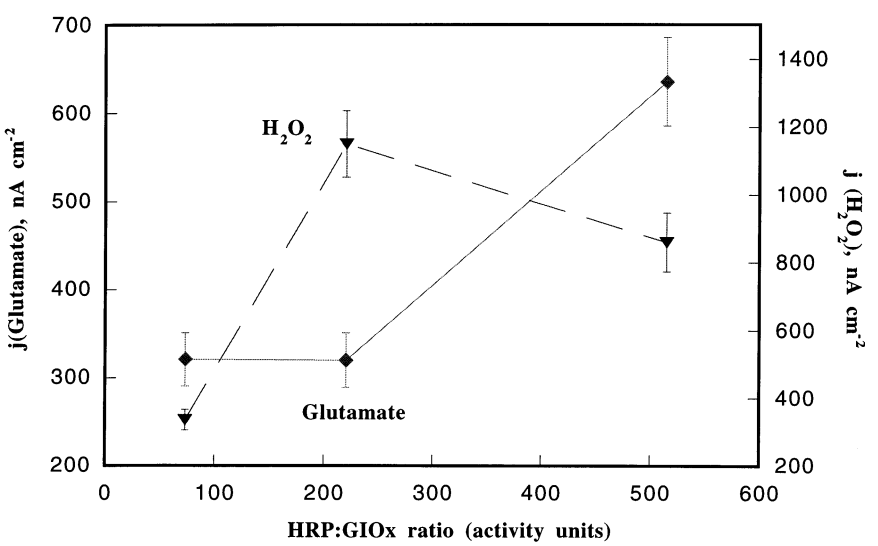



Fig. 4. Influence of HRP:$GlO_x$ ratio on the signal output. The composition of the wired HRP layer was HRP:$PVI_7$-dmeOs:PEGDGE of 50:40:10% w/w.

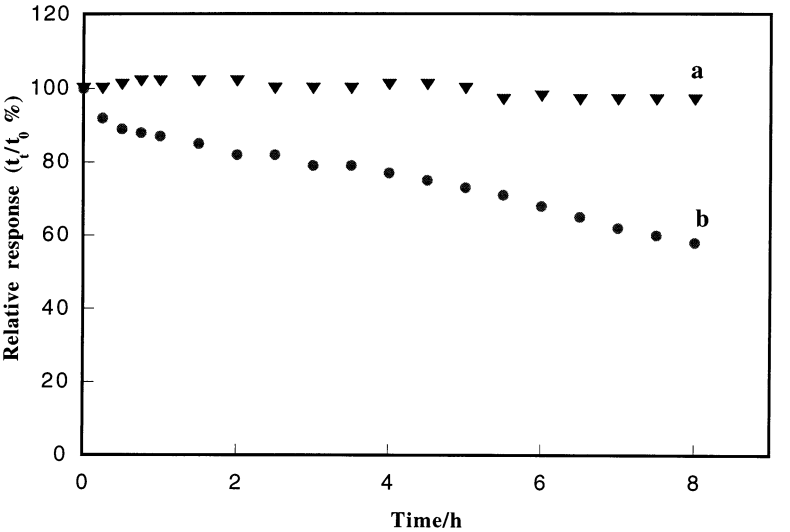



Fig. 5. Operational stability of different bienzyme electrodes: (a) HRP:$GlO_x$ ratio of 73.5; and (b) HRP:$GlO_x$ ratio of 515. Sample throughput was 30 injection $min^{-1}$. Glutamate concentration was 50 μM in Ringer's solution.

crosslinker reacts with the amino groups of the enzyme and the redox polymer to form a network in which the enzyme is immobilized. Excess amount of crosslinker in the hydrogel, however, will decrease its flexibility and thereby lower the segmental motion of the polymer chains thus also decreasing the rate of electron transfer within the hydrogel. Therefore, bienzyme electrodes were optimized with respect to the amount of crosslinker at a fixed HRP and redox polymer ratio. All compositions are referred to as w/w%. As depicted in Fig. 2, the sensitivity of the electrodes increased up to 10% crosslinker for both substrates. However, at a higher PEGDGE amount different trends were observed. The sensitivity for $H_2O_2$ decreased as the amount of PEGDGE was raised to 15% indicating that more rigid films resulted in decreased electron transfer rates within the hydrogel. For glutamate the sensitivity was practically the same at 10 and 15% PEGDGE. In this work, the optimum crosslinker amount (10%) was used for further studies of the bienzyme electrode as it resulted in maximum sensitivities for both substrates.

The effect of HRP/redox wire ratio on the response of the wired electrode for glutamate and hydrogen peroxide was studied at a fixed amount of HRP:$GlO_x$ ratio and PEGDGE concentration (10%), by mixing HRP and the polycation in various ratios. Fig. 3 depicts the sensitivity of the electrode for glutamate and hydrogen peroxide as a function of the amount of HRP. For both substrates, increasing the amount of HRP caused an increase in the current output to a maximum followed by a drastic decrease. It is assumed that in the rising part of the curve, the current was controlled by the activity of HRP in the film, while in the declining part the electron transfer to or through the redox polymer became the limiting factor. Such a phenomenon has been observed and reported previously [27,28] and was found to be dependent of the redox polycation composition [33]. In the present study a ratio of HRP:$PVI_7$-dmeOs:PEGDGE of 50:40:10%, was found to represent the optimal wire composition, yielding highest glutamate sensitivity and it was used for further optimization and characterization of the electrode.

Keeping the total amount of hydrogel deposited on electrode and the relative ratio of HRP:Os:PEGDGE constant, the ratio (relative activities) of the two enzymes (HRP:$GlO_x$) was varied to assess its effect on the performance of the bienzyme electrode. The catalytic current output for 50 μM hydrogen peroxide and glutamate as a

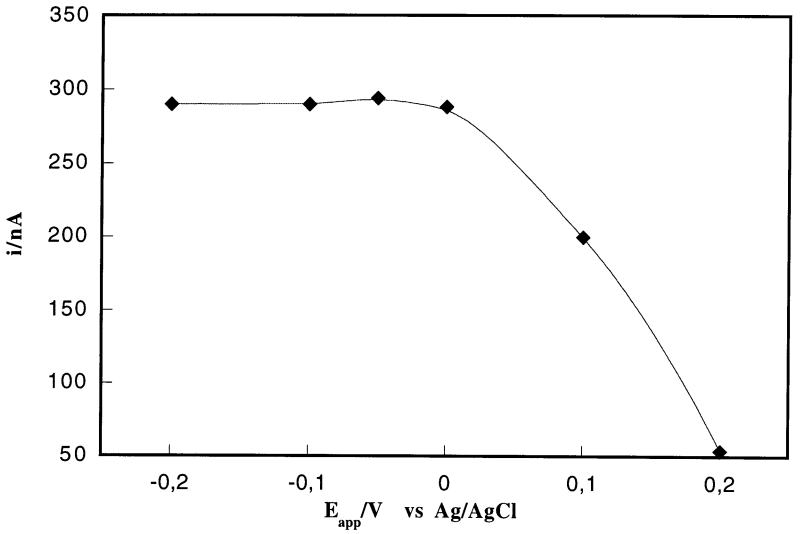



Fig. 6. Hydrodynamic voltammogram recorded for 50 μM glutamate in 0.1 M PB buffer pH 7.0

function of the HRP/$GlO_x$ activity ratio is shown in Fig. 4. A maximum in response is reached for $H_2O_2$ at the HRP:$GlO_x$ ratio of 220.The reduced current at lower HRP:$GlO_x$ ratio was attributed to the restricted amount of HRP and Os in the presence of high $GlO_x$ content. The electrode response to $H_2O_2$ is probably lowered by limited catalytic activity of HRP and slower electron hopping between more remote Os centers. Similarly, reduced currents could be observed at higher HRP:$GlO_x$ ratios too, however, keeping in mind a 10–15% standard deviation the decreasing trend is not conclusive enough. In contrast, the current for glutamate increased twice when the HRP:$GlO_x$ ratio was raised to 515. This probably indicate that the collection efficiency of $H_2O_2$ as the reaction product of the $GlO_x$ catalyzed reaction is better at higher HRP loading, resulting in higher response for glutamate at a relatively lower $GlO_x$ content in the sensing hydrogel layer. However, examining the operational stabilities (see Fig. 5), of the electrodes modified with the two extreme HRP:$GlO_x$ ratios (73.5 and 515, respectively) used in this work, the optimum hydrogel composition yielding high sensitivity and better operational stability was found to be given by the electrodes made from a premixed solution containing the two enzymes at the lower ratio (73.5). No explanation can be given to the observed phenomenon at this time. The optimized electrodes were coated in a single layer approach with 4.2 μl premixed solution formed of: 1 μl HRP, 0.8 μl $PVI_7$-dmeOs, 0.4 μl PEGDGE and 2 μl $GlO_x$, representing thus a composition of HRP: $PVI_7$-dmeOs:PEGDGE of 50:40:10 w/w% and HRP:$GlO_x$ ratio of 73.5.

### 3.2. Characteristics of the optimized electrodes

Hydrodynamic voltammogram of the optimized electrodes (see Fig. 6) showed that the reduction

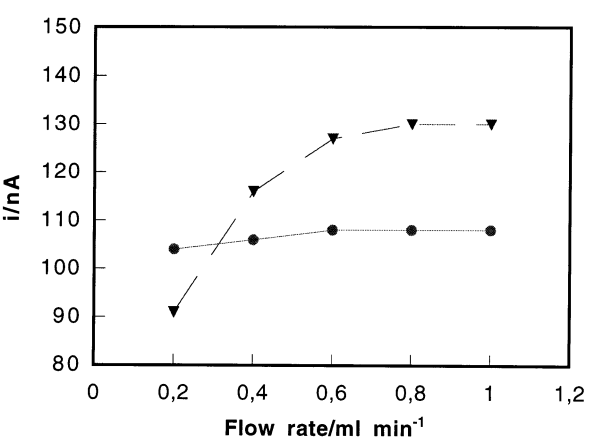



Fig. 7. Influence of flow rate on (●) glutamate and (▼) $H_2O_2$ signals, both substrates of 20 μM concentration.

Table 1
Electrode characteristics of the optimized electrodes fo L-glutamate

| Linear range (μM) | LDL (μM) | S (μA $mM^{-1}$ $cm^{-2}$) | $K_m^{app}$ (mM) | $j_{max}$ (μA $cm^{-2}$) | $i_{bg}$ (nA) | $t_{5-95\%}$ (s) |
|---|---|---|---|---|---|---|
| 0.3–250 | 0.3 | $88.36 \pm 0.14$ | $0.39 \pm 0.09$ | $30.4 \pm 0.55$ | $<10$ | $<10$ |

of the redox mediators incorporated into the hydrogel already starts at $+200$ mV vs. Ag|AgCl, reaching a maximum at 0 mV after which the reduction current is leveled off. All experiments were carried out at $-50$ mV versus Ag|AgCl, taking into account that the electrode is aimed to measure glutamate in complex biological matrices, thus also diminishing the interfering signals from electrochemically easily oxidizable interferents such as ascorbic acid ($E^{\circ\prime} = +14$ mV vs. Ag|AgCl [37] and acetaminophen $E^{\circ\prime} = 58$ mV versus Ag|AgCl [38], avoiding thus at the same time biases due to the reduction of molecular oxygen, which can start below $-100$ mV versus SCE at Os-hydrogel modified electrodes [35].

The dependence of peak current on the flow rate for the optimized bienzyme electrode is depicted in Fig. 7. The peak current for glutamate was practically independent of flow rate while a progressive rise in the output current was noted for $H_2O_2$ indicating that the response for glutamate is kinetically limited while being mass transfer limited for $H_2O_2$ at low flow rates. A stable plateau was reached starting from a flow rate of 0.8 ml $min^{-1}$ for both substrates.

Calibration curves were recorded for the optimized bienzyme electrodes both in Ringer's solution and 0.1 M phosphate buffer (pH 7). Typical electrode characteristics are given in Table 1. The operational stabilities of bienzyme electrodes prepared with the two extreme HRP:$GlO_x$ ratios were studied by continuously injecting 0.05 mM glutamate in Ringer's solution, at a sample throughput of 30 injections $h^{-1}$ at room temperature for 8 h. During this period the bienzyme electrode with the lower HRP activity lost only 3% of its initial sensitivity while the other one showed 40% loss in sensitivity. As seen from Table 1 the $K_m^{app}$ (calculated from a Linewear–Burk plot) of the optimized electrodes was found to be very closed to the one (0.21 mM) obtained in solution [31]. This together with data from Fig. 7 indicate that the response of the electrode is limited by the $GlO_x$ enzyme kinetics in the hydrogel.

### 3.3. Influence of poly(ethyleneimine) on electrode characteristics

It was previously shown that the formation of electrostatic complexes between a redox polymer and enzyme (glucose oxidase) in the gel enhanced the electrical communication, yielding thus higher signals [33]. This effect was studied by quaternizing a redox hydrogel, altering thus its charge. Since $GlO_x$ has a p*I* of 6.2, the enzyme is negatively charged at the working pH (7.4) [31]. The negative charges of the enzyme are assumed to electrostatically react with the positive charges of the redox polycation, resulting in a stable configuration. Enhancement of the positive charges was expected to increase the stability of the enzyme-redox polycation electrostatic complex and also to facilitate the electron transfer and thus yield higher signals. The effect of adding a positively charged polycation, poly(ethyleneimine) on the electrode characteristics was therefore considered. PEI was also reported to enhance the conversion efficiency of some other FAD enzymes in bienzyme configuration [39,40] and also to stabilize the immobilized enzymes, enhancing their operational stability [41].

Preliminary experiments showed that addition of PEI enhanced the glutamate sensitivity for both above mentioned bienzyme electrode configurations (simply adsorbed and crosslinked into redox hydrogel) the effect being much more accentuated for the non-mediated system (150 versus 50% increase, respectively). The hydrogen peroxide signal was on the other hand decreased by a factor of almost 2 for the non-mediated electrode configuration, while the signal for the

redox hydrogel-based one was practically unaffected by PEI addition. These results showed, that PEI addition caused an increase of the conversion efficiency for both electrode configurations, the effect being much more accentuated for the adsorbed (non-mediated) bienzyme electrodes. PEI is assumed to promote thus the electron transfer between the active site of the immobilized HRP and the electrode and/or increase the enzyme's turn over rate, besides its previously assumed stabilizing effect due to the positive charges it possess.

The apparent Michaelis–Menten coefficients calculated from Linewear-Burk plots, were also found to be influenced by PEI addition. Generally, an increase by a factor of three was observed for both bienzyme electrode configurations. A thorough examination of the mechanism of the influence of PEI on sensitivity, conversion efficiency, enzyme turn over rate and $K_m^{app}$ is in progress.

## 4. Conclusions

The optimized redox hydrogel incorporated bienzyme electrodes were characterized by high sensitivity, excellent operational stability, fast response time and low detection limit, making them very promising for glutamate monitoring in restricted brain areas. The sensing chemistry is such, that it allows miniaturization of the electrode in a shape which will allow its accurate positioning over targeted sites of brain. However, a study of the oxygen dependence and interference eliminating ability of the all, in this work mentioned electrode configurations both under laboratory and in vivo conditions is in progress and is the subject of a forthcoming publication. Similar electrodes were successfully used for the measurements of glutamate release at synapses of cultured rat cortex cells [42].

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