Application of a glutamate microsensor to brain tissue

Oldenziel, Weite Hendrik

Chapter 3

Improving glutamate microsensors by optimizing the composition of the redox hydrogel.

This chapter is based on the following paper:

Oldenziel WH, Westerink BHC. Improving glutamate microsensors by optimizing the composition of the redox hydrogel. *Anal. Chem.* 2005; 77: 5520-5528.

Abstract

For an optimal use of the hydrogel-coated glutamate microsensor it is necessary that sensors with a high performance can be produced in a reliable and reproducible way. In the previous chapter we have observed that the balance between the five individual hydrogel components is critical in this respect. However, hydrogel application to a CFE needs to be performed by dipcoating. This is a difficult procedure to control and does not allow individual application of hydrogel constituents. To improve the construction of the microsensor and to better control the dipcoating procedure we have developed an automated device (previous chapter). Throughout this study, automatic dipcoating was performed with premixed solutions, in which the amount of a single component was varied. This allowed us to optimize the hydrogel composition, which resulted in a significant improvement of the properties of the microsensor in terms of sensitivity, current density, linearity, detection limit and interference by ascorbic acid.

3.1 Introduction

A prerequisite for successful application of the hydrogel-coated glutamate microsensor as an analytical tool is that sensors with a high performance in terms of sensitivity, selectivity, detection limit, linearity, etc can be constructed in a reproducible way. Moreover, the interference by AA should be minimized. In this respect the balance between the individual hydrogel constituents is critical. For example, when the amount of enzyme is in excess, the electron-current carrying capability of the matrix is insufficient, due to limitation in the collisional electron transfer between reduced and oxidized osmium redox centers. In addition, when the amount of POs-EA is in excess, there is insufficient enzyme to supply electrons or holes (electron vacancies) to the cascade. Other factors defining the optimization in hydrogel equilibration are the type of redox polymer, the type of enzyme(s), the activity of the enzyme(s), the accessibility of the prosthetic group of the enzyme, physical chemical characteristics of the different components, electron transfer properties, etc (Gregg and Heller, 1991b; Ohara et al., 1993a; Aoki et al., 1995; Rajagopalan et al., 1996; Kenausis et al., 1997; Belay et al., 1999). The fact that the total enzyme content within this particular microsensor architecture is divided over three different enzymes is an additional confounding aspect.

As a consequence, a reproducible construction of the microsensor with an optimized hydrogel constitution is required. However, this is a difficult task (Schuhmann, 2002; Mikeladze et al., 2002). Due to its small dimensions, the hydrogel application needs to be performed by dipcoating, which does not allow the exact control of the formation of the hydrogel on the CFE, or the individual application of the different hydrogel constituents. This contrasts with dropcoating on larger electrode surfaces, on which most work concerning optimization in hydrogel composition has been performed.

We have recently developed an automated device to improve the construction of the microsensor and to better control the dipcoating procedure (chapter 2). In the present study this automatic dipcoater was used to optimize the hydrogel constitution. To that end the quantity of each hydrogel constituent: POs-EA, PEDGE, HRP, Glu-ox and AA-ox was varied in the coating solution prior to dipcoating. This allowed us to determine the influence of each individual hydrogel constituent on the performance of

the microsensor. In addition the effect of the protective Nafion layer, which surrounds the hydrogel, was investigated.

3.2 Material and Methods

Reagents

Glutamate oxidase (Glu-ox; G-0400) from Streptomyces sp (E.C. 1.4.3.11) was purchased as a lyophilized powder with an activity of 12.1 units/mg. Horseradish Peroxidase type II (HRP; P-8250) from Amoracia Rusticana (E.C. 1.11.1.7) had an activity of 158 units/mg. Ascorbate Oxidase (AA-ox; A-0157) from cucurbita sp (E.C. 1.10.3.3) had an activity of 33.5 or 102.3 units/mg. [4-(2-hydroxyethyl)-1-piprazineethane-sulfonic acid] (HEPES), HEPES sodium salt, l-glutamate and l-ascorbic acid were all obtained form Sigma (St. Louise, MO, USA). AA-ox (type AO2) from cucurbita sp (E.C. 1.10.3.3) with an activity of 310 units/mg was obtained from Biozyme Laboratories (Blaenavon, UK). Poly(ethylene glycol 400 diglycidyl ether) (PEGDGE), silver chloride and Nafion (5 % Nafion solution,1100 equivalent weight) were obtained from Aldrich, (Milwaukee WI, USA). Acetone p.a. and 2-propanol p.a. were obtained from Merck (Darmstadt, Germany). 2-Bromoethylamine hydrobromide 98+ % was obtained from Janssen Chimica (Geel, Belgium). Artificial Cerebrospinal Fluid (aCSF), used for the calibration procedures, had the following composition: 145 mM Na^+, 1.2 mM Ca^{2+}, 2.7 mM K^+, 1.0 mM Mg^{2+}, 152 mM Cl^- and 2.0 mM phosphate; pH 7.4 with sodium hydroxide. Salts were obtained from Merck (Darmstadt, Germany). Solutions were made in ultra-pure (U.P.) water (Elgastat maxima, Salm en Kipp). Enzymes solutions were made in HEPES buffer; the salt form of HEPES was added to a 10 mM solution of the acid form, until pH 8.

Redox polymer

The osmium redox-polymer, abbreviated as POs-EA, was synthesized by complexing poly (4-vinylpyridine) with $Os(bpy)_2Cl_2$-groups and partially quaternizing it with 2-bromoethylamine, according an earlier described detailed procedure (Shankar et al., 1998). The synthesis is a three step procedure. Briefly, in the first two steps the $Os(bpy)_2Cl_2$ complex was synthesized. The UV spectra (UV spectrometer UV-160, Shiadzu, Kyoto, Japan) obtained for the $Os(bpy)_2Cl_2$ complex corresponded well to those in literature (Lay et al., 1986). In the last step of synthesis the $Os(bpy)_2Cl_2$ groups and ethylamine chains were connected to the poly (4-vinyl pyridine) backbone. In the present study the influence of the structure of POs-EA on the

microsensor performance was investigated. For this purpose several POs-EAs were synthesized with modified complexing of $Os(bpy)_2Cl_2$ - and ethylamine groups. Variations were made on a POs-EA structure, developed and characterized by Gregg and Heller (1991a), which herein is referred to as $POs_1Ea_{1.2}$. POs-EAs were also synthesized with a double ethylamine-chain loading ($POs_1Ea_{2.4}$), a half $Os(bpy)_2Cl_2$ complex loading ($POs_{0.5}Ea_{1.2}$) and a combination of both ($POs_{0.5}Ea_{2.4}$). Modifications were performed at the last step of the synthesis by adding respectively a double amount of 2-bromoethylamine hydrobromide, half of the $Os(bpy)_2Cl_2$ amount, or a combination of both. The subscripts indicate the number of $Os(bpy)_2Cl_2$ complexes and ethylamine groups per polymer repeat unit. Characterization of the POs-EAs was performed by C, H, N elemental analysis (Euro EA 3000) and elemental analysis on Os (ICP-AES; Vista AX CCD).

Microsensor construction

The carbon fiber electrodes (CFEs) were made by sealing a single cylindrical carbon fiber (P-55s, Thornel carbon fibers, Amoco; 10 µm diameter) into a glass capillary (TW100F-3, World Precision Instruments, Sarasota, FL, USA). The capillary was pulled to a tip using a capillary puller (PN-3, Narishige, Tokyo, Japan). Electrical contact inside the CFE was made with a 250 µm diameter Teflon coated silver wire (Advent Research Materials, Eynsham Oxon, England). This contact was mediated by epoxy-silver (World Precision Instruments, Sarasota, FL, USA), inserted in the tip of the electrode with a spinal needle (Spinocan, Braun, Melsongen, Malaysia). After drying the silver epoxy for 8 hours at 70°C, the silver wire was further sticked in the capillary using a four component Spurr epoxy glue (Polysciences, Warrington, PA, USA) and was dried overnight at 70°C. Before use, the CFEs were trimmed under a microscope (Pleuger XSZ-107) to a length of 300-500 µm. To induce a uniform carbon surface prior to hydrogel coating, the CFEs were pretreated according a previously described standard cleaning procedure (chapter 2).

The glutamate microsensors were constructed by dipcoating the CFE in an aqueous mixture containing 20 µl POs-EA (1 mg/ml), 4 µl PEDGE (3 mg/ml), 10 µl HRP (474 units/ml (≈3 mg/ml)), 10 µl AA-ox (1400 units/ml (≈10 mg/ml)) and 10 µl Glu-ox (24.2 units/ml (≈2 mg/ml)). A 10 minute dipcoating procedure was performed by the automatic dipcoater at a temperature of 37°C, according to an earlier described procedure (chapter 2). The constitution of this aqueous mixture is referred

to as reference method. Throughout this study, variations were made to this mixture and the influence on the final microsensor performance was examined.

After coating, the microsensors were cured for 1 hr at 37 ℃, followed by 10 min dipping in U.P. water and 2 hours drying in ambient air. A Nafion coating completed the fabrication. Nafion coating was performed by dipping the hydrogel-coated microsensors repetitively 5 or 10 times for 10 seconds, with 20 seconds drying intervals at ambient air, in a 0.5 % Nafion solution (1:10 dilution in 2-propanol p.a). Only in the last series of experiments the glutamate microsensors were casted with a Nafion coating. The microsensors were stored in a refrigerator overnight before calibration the next day.

Electrochemical procedures

The microsensors were calibrated at a constant potential of –150 mV versus an Ag/AgCl reference electrode. The Ag/AgCl reference electrode was constructed by coating a bare silver wire with AgCl. Before calibration its potential was checked against a commercially available saturated Ag/AgCl reference electrode (Antec Leyden, the Netherlands). Reference electrodes were made daily. The potential between the microsensor and the reference electrode was applied by a home-made potentiostat. The amplifier used was a 2-stage amplifier. To reduce electrical noise the electrodes were connected to a “head stage” inside a Faraday cage. The amplifier was connected to a computer (Intel, Pentium II MMX, 384 MB RAM). Home-written software handled the incoming data.

Calibration of the microsensors

All calibrations were performed in a flow-injection analysis system. During calibration the microsensors were placed in a flow of air-equilibrated aCSF (1 ml/min) that was generated by an HPLC-pump (Pharmacia-LKB, 2150). A 0.5 ml bolus of substrate was injected via a HPLC injection valve (loop volume: 0.5 ml; Rheodyne, Cotati, CA, USA). This resulted in a 30 seconds lasting bolus injection of substrate. All stock-solutions were freshly prepared before the start of an experiment and the injected solutions were freshly prepared from the stock solutions. The AA stock solution was stored under N_2. Every solution was injected at least twice and the effect was averaged.

Calibration of the microsensors was divided into a few steps. First, a cyclic voltammogram (CV) of the microsensor was recorded by cycling the potential once between –150 and 650 mV at a speed of 100 mV s^{-1}. The CV reflects the cycling of the osmium groups between the 2^+ and 3^+ state. In addition, it gives an indication of the amount of osmium groups participating in the redox cascade and of the electron transfer kinetics through the hydrogel. Secondly, the microsensors were calibrated amperometrically at –150 mV. Glutamate concentrations of 5, 10, 50 and 100 µM were injected in duplicate or triplicate to determine the sensitivity, linearity and peak shape. The calibration was finished by investigating the interference by AA, determined by coinjecting 200 µM AA and 100 µM glutamate. This reduces the original glutamate signal by a certain percentage and is referred to as "interference" (see also Fig. 1).

Extracellular levels of glutamate in the brain are between 1-10 µM. However, during stimulation glutamate levels might come close to 100 µM. In accordance with the literature, we have chosen to determine the interference by AA at 100 µM glutamate (Garguilo and Michael, 1996; Kulagina et al., 1999; Cui et al., 2001). Extracellular concentrations of AA are between 100 and 500 µM. The interference was determined with 200 µM AA, unless otherwise stated.

After calibration, the following properties were determined for each microsensor: shape and height of the CV (nA; determined as the absolute value of the anodic (I_{pa}) t minus the cathodic (I_{pc}) peak current), sensitivity (pA/µM), current density (mA M^{-1} cm^{-2}), interference (%), linearity (R^2), detection limit (µM; 3 times signal to noise ratio) and response time (sec; time required for the signal to increase from 10 to 90 %). Data for sensitivity and CV height were corrected for CFE length, as the length fluctuated between 300-500 µm. For reasons of clarity only a limited amount of these data are shown in the Results and Discussion section. Most data shown concern the sensitivity and interference, as these are the most critical properties for the practical use of the microsensor.

Expression of results and statistics

Experiments are presented as mean ± SEM. A statistical program, Sigmastat 3.0, was used to calculate statistics. Data were, dependent on the type of study, analyzed with a Mann-Whitney Rank Sum test (M-W Rank Sum test) or with One Way ANOVA followed by a Dunn's post-hoc test. Significance was set at $p < 0.05$.

3.3 Results and Discussion

The redox hydrogel coating on the CFEs consists of 5 different components: POs-EA, PEDGE, Glu-ox, HRP and AA-ox. The influence of each component on the performance of the microsensor was investigated by dipcoating the CFE in different coating solutions, in which the concentration of an individual components was varied. Variations were made based on published methods (referred to as the reference coating solution: Kulagina et al., 1999; Cui et al., 2001; Oldenziel et al., 2004: see chapter 2). In addition the influence of a protective Nafion layer was investigated. The performance of the microsensor was determined by calibration in a flow injection analysis system. In Fig. 1 a typical example of such a calibration is shown. Glutamate concentrations of 5, 10, 50 and 100 µM were injected in duplicate to determine the sensitivity, linearity and peak shape. The calibration was finished by investigating the interference by ascorbic acid (AA). This was determined by coinjecting 200 µM AA

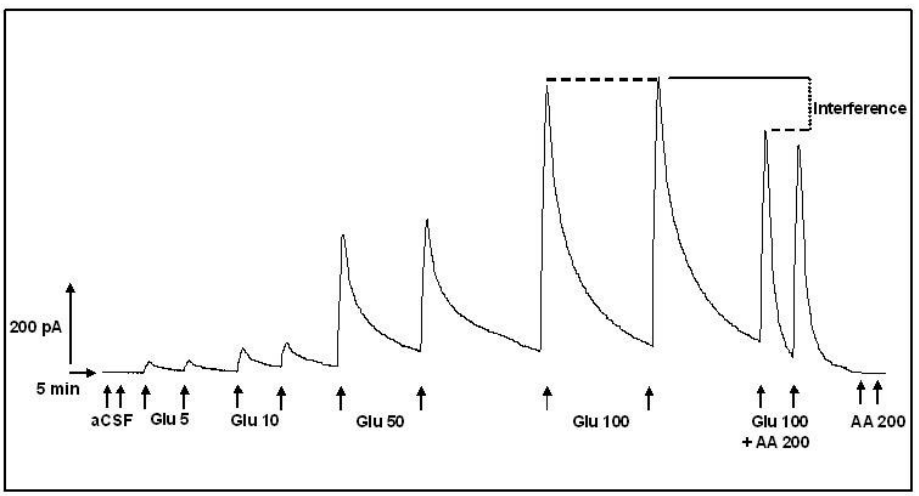


Figure 1: Schematic overview of a typical amperometric calibration of the glutamate microsensor. Calibration was performed in a flow injection analysis system at a flow of 1 ml/min. Different substrates were administered in duplicate as 30 second bolus injections: aCSF (blank), different concentrations of glutamate (numbers indicate the concentration in µM) and finally the coinjection of glutamate 100 µM with AA 200 µM to determine the interference by AA. This interference is defined as the percentage of decrease of the original glutamate signal. Note: a small baseline drift was noticed due to insufficient time interval between the different injections.

and 100 µM glutamate.

Note, AA is a strong reducing agent, which is present at high concentrations (100-500µM) in the brain (Grünewald, 1993; O'Neill et al., 1998). Although AA does not induce false positive signals itself, it decreases the original glutamate signal by reducing the intermediate steps in the reaction cascade that are in an oxidized state: Os^{3+}, HRP_{ox} and H_2O_2. This percentage of decrease of the initial glutamate signal is referred to as "interference".

Influence of the POs-EA concentration

First, the influence of different concentrations of the redox-polymer POs-EA on the performance of the microsensor was investigated. The concentration of POs-EA in the coating solution was varied between 0.5 and 10 mg/ml. In Fig. 2A is shown that with increasing POs-EA concentrations, an increase in both sensitivity and interference by AA was observed.

Cyclic voltammograms (CVs) were recorded to obtain an indication of the osmium content within the hydrogel and to examine the electron diffusion kinetics. An increase in POs-EA concentration resulted in an increase of current quantified by the CV, which is defined as [anodic (I_{pa}) - cathodic (I_{pc}) peak current] (Fig. 2B). A typical example of a CV of the microsensor, recorded at 100 mV sec^{-1} versus Ag/AgCl [0.15 M NaCl], is shown in the inset of Fig. 2B. The CV showed a formal potential (E^o) of approximately 310 mV and a peak splitting slightly more than 100 mV, which denoted some diffusion limitation within the hydrogel as a result of enzyme binding.

It appeared that a higher POs-EA concentration improved the sensitivity of the sensor for glutamate, its linear range (not shown) and its response characteristics. This was visualized in Fig. 2C, in which the glutamate 5 µM (± SEM) injections of the microsensors of Fig. 2A were depicted. With increasing POs-EA concentrations the initial response time decreased from 22.7 ± 0.2 to 15.6 ± 0.3 (mean ± SEM) seconds. Moreover, the time of decay of the glutamate peak back to baseline was faster.

Most likely a higher POs-EA concentration will result in an increased osmium-loading of the hydrogel. In turn a higher charge density of the hydrogel is expected, which may improve the hydrophilicity of the hydrogel and increase its hydratation when applied in an aqueous environment. This permits a faster diffusion of substrates and reaction products within the hydrogel and in particular the diffusion of negatively charged components (like glutamate), as these are attracted by the cationic osmium

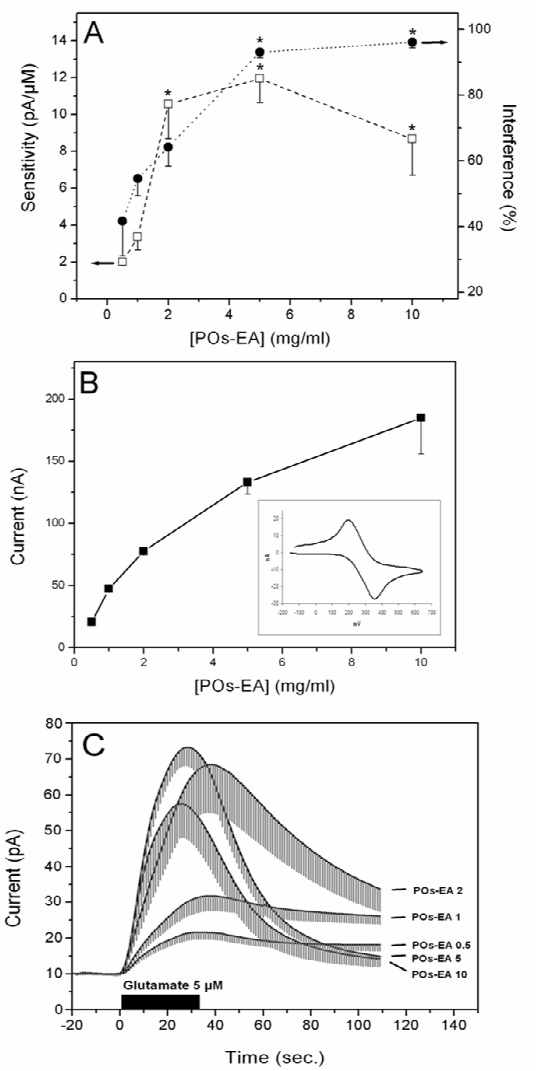


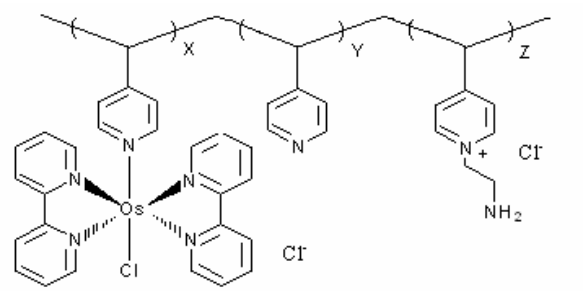
Figure 2: Influence of different concentrations of POs-EA on the performance of the microsensor. The following POs-EA concentrations were investigated: 0.5 (n = 8), 1 (n = 15), 2 (n = 9), 5 (n = 8) and 10 mg/ml (n = 8). ***A)*** *Sensitivity and interference at different POs-EA concentrations. * Denotes a statistically significant difference compared to the reference 1 mg/ml concentration (p < 0.05; M-W Rank Sum test).* ***B)*** *Correlation between the CV current ($[I_{pa} - I_{pc}]$) and the concentration of POs-EA. The inset shows a typical example of a cyclic voltammogram recorded at 100 mV sec^{-1}.* ***C)*** *Response of all microsensors depicted in (**A)** on a 30 second bolus injection of glutamate 5 µM, showing the variability in sensor response as a result of the used POs-EA concentration.*

complexes. In addition, improved electron diffusion kinetics within the hydrogel, and between the HRP redox center and osmium-complexes are expected, as the electrostatic complexation with negatively charged enzymes will be enhanced (Gregg and Heller, 1991a,b; Rajagopalan et al., 1996; Ni et al., 1999; Chen et al., 2000).

It is concluded that increasing the POs-EA concentration improved the glutamate detection properties. A concentration of 5 mg/ml appeared to be optimal, showing the highest sensitivity with optimal response characteristics. A further increase of the POs-EA concentration tended to decrease the sensitivity again, which may be explained by dilution of the enzyme content due to excess POs-EA.

Unfortunately, the interference by AA also increased dramatically with increasing POs-EA concentrations (Fig. 2A). It is likely that the increased cationic charge of the hydrogel not only improved the diffusion of glutamate into the hydrogel, but also that of AA, as glutamate and AA are both negatively charged and have about the same molecular size.

It is evident that with respect to the amount of POs-EA a compromise needs to be found between the sensitivity for glutamate and interference by AA. In the next studies a concentration of 1 mg/ml was maintained, as recommended in the reference method.



$POs_1Ea_{1.2}$: $X \approx 1$, $Y \approx 4$, $Z \approx 1.2$
$POs_1Ea_{2.4}$: $X \approx 1$, $Y \approx 2.8$, $Z \approx 2.4$
$POs_{0.5}Ea_{1.2}$: $X \approx 0.5$, $Y \approx 4.5$, $Z \approx 1.2$
$POs_{0.5}Ea_{2.4}$: $X \approx 0.5$, $Y \approx 3.3$, $Z \approx 2.4$

Figure 3: Chemical structure of the osmium redox-polymer POs-EA. Different POs-EAs with modified complexing of the $Os(bpy)_2Cl$ and ethylamine side groups were synthesized. The subscripts indicate the number of $Os(bpy)_2Cl$-complexes and ethylamine-chains per polymer repeat unit.

Influence of modifications of the POs-EA structure

Next, the influence of the chemical structure of POs-Ea on the performance of the microsensor was investigated. It is known that quaternization (Aoki et al., 1995; Rajagopalan et al., 1996) and osmium loading (Ohara et al., 1993a; Belay et al., 1999) changes the physical chemical properties of the POs-EA, which in turn may change the microsensor performance. Four types of POs-EA were synthesized and evaluated (Fig. 3).

In Table 1 the elemental analysis data of the different POs-EAs are shown. The determined values corresponded fairly well with the calculated values, confirming the presence of the expected quantity of side-groups on the poly (4-vinylpyridine) backbone. Our attempts to determine more accurately the exact loading of side-groups with ^{1}H-NMR,^{13}C-NMR and MALDI-MS were not successful.

POs-EA		**calculated**				**found**			
		C	H	N	Os	C	H	N	Os
$POs_1EA_{1.2}$	$C_{66}H_{67}N_{11}Os_1Cl_3$	59,83	5,08	12,09	14,40	59,40	5,27	12,23	14,74
$POs_1EA_{2.4}$	$C_{68}H_{77}N_{13}Os_1Cl_4$	57,83	5,25	12,46	13,43	56,57	5,04	12,32	13,96
$POs_{0.5}EA_{1.2}$	$C_{56}H_{59}N_9Os_{0.5}Cl_2$	64,81	5,71	12,73	9,20	65,66	5,71	12,27	8,80
$POs_{0.5}EA_{2.4}$	$C_{58}H_{66}N_{11}Os_{0.5}Cl_3$	61,88	5,87	13,14	8,42	58,89	5,74	13,92	8,63

Table 1: Elemental analysis data of the different POs-EAs presented in Fig. 3. The subscripts indicate the number of Os(bpy)$_2$Cl-complexes and ethylamine-chains per polymer repeat unit. Note: the determined values were corrected for trace amounts of water.

The properties of the microsensors prepared with the different POs-EAs are shown in Table 2A. No differences were observed between the microsensors, except for the CV current, which seemed dependent on the Os(bpy)$_2$Cl$_2$ complexing of the POs-EA. As will be shown in the next sections, the reference coating solution contains a rather high enzyme content, which limits the microsensor performance. For that reason, the different POs-EAs were also investigated with sensors lacking AA-ox, as the amount of AA-ox contributes largely to the total enzyme content. The results are shown in Table 2B. Again, no differences (beside the effect on the CV values), were observed between the microsensors. An increased sensitivity for glutamate and 100% interference by AA for all microsensors was observed when AA-ox was not included.

	POs-EA	N	Sensitivity (pA/µM)	Interference (%)	Response time (sec.)	Linearity (R^2)	CV (nA)
A	$POs_1Ea_{1.2}$	23	2.0 ± 0.3	31.6 ± 4.1	20.3 ± 0.5	0.991 ± 0.001	21.2 ± 2.5
	$POs_1Ea_{2.4}$	19	1.9 ± 0.3	23.1 ± 5.4	20.4 ± 0.5	0.993 ± 0.001	35.3 ± 3.1
	$POs_{0.5}Ea_{1.2}$	9	2.1 ± 0.2	29.1 ± 4.2	20.1 ± 0.2	0.992 ± 0.002	11.2 ± 0.9 *
	$POs_{0.5}Ea_{2.4}$	10	2.0 ± 0.5	40.3 ± 10.5	22.9 ± 2.6	0.989 ± 0.001	15.5 ± 2.8
B	$POs_1Ea_{1.2}$	10	12.5 ± 1.8	100.0 ± 0.0	21.0 ± 0.8	0.998 ± 0.001	67.2 ± 4.5
	$POs_1Ea_{2.4}$	10	10.1 ± 0.9	"	20.9 ± 0.7	0.994 ± 0.001	53.6 ± 3.4
	$POs_{0.5}Ea_{1.2}$	10	8.8 ± 1.0	"	19.5 ± 0.2	0.994 ± 0.001	21.6 ± 1.8 *
	$POs_{0.5}Ea_{2.4}$	12	12.3 ± 1.5	"	23.0 ± 1.0	0.987 ± 0.003	25.5 ± 1.9 *

Table 2: Influence of different POs-EAs on the performance of the microsensor. ***A)*** *Microsensors constructed according to the reference method.* ***B)*** *Microsensors constructed according to the reference method without AA-ox.*

These microsensors were also used to determine whether POs-EA modification changed the interference characteristics. The interference by different concentrations of AA (range from 0.01 to 500 µM) was investigated (Fig. 4). However, no differences in interference characteristics were observed as a result of POs-EA modification.

It is concluded that the different type of redox polymers did not modify the microsensor characteristics. Therefore, the use of the reference POs-EA type ($POs_1Ea_{1.2}$) was continued in the following studies. However, it needs to be emphasized that the different POs-EAs were only evaluated at a relatively low concentration of 1 mg/ml. At this condition the activity of POs-EA is limited, as mentioned before (see fig. 1). When higher concentrations of the modified POs-EAs were used, the differences in microsensor properties were more pronounced. However, the interference by AA increased in proportion with a higher sensitivity (data not shown).

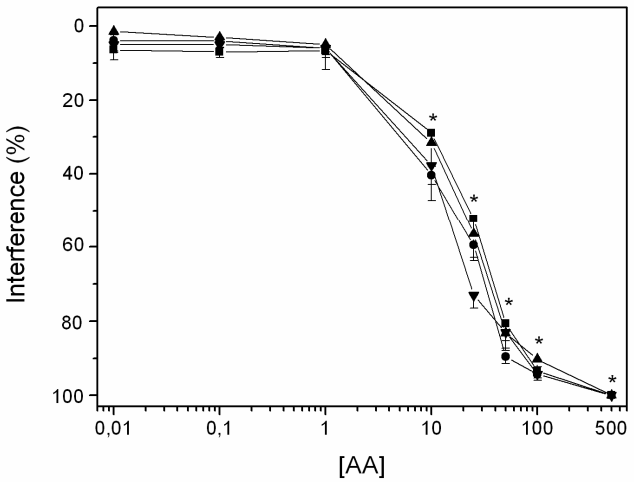


*Figure 4: Interference-characteristics of microsensors constructed with different POs-EAs. The concentration of AA coinjected to glutamate 100 µM was titrated from 0.01 to 500 µM. The sensitivity for 100 µM glutamate, without AA coinjection, was set at 100%. Similar microsensors were used as presented in Table 2B. -■- = POs_1-$EA_{1.2}$ (n = 10), -▲- = POs_1-$EA_{2.4}$ (n = 10), -●- = $POs_{0.5}$-$EA_{1.2}$ (n = 11), -▼- = POs_1-$EA_{2.4}$ (n = 12). * Statistically significant difference compared to the control values (p < 0.05; One Way Anova followed by a Dunn's post-hoc test).*

Influence of PEDGE

PEDGE is a commercial available diepoxide cross-linker. Together with the POs-EA and enzymes it forms a three-dimensional network which entraps the enzymes in the hydrogel. In addition, it also wires the enzymes to the pendant amine-groups of the POs-EA by covalent binding. The influence of different amounts of PEDGE on the sensitivity and interference of the microsensor was investigated. The amount of PEDGE was varied between 6.3 and 26.1 of weight % of the coating solution. No changes in microsensor performance were observed (Fig. 5). Likely, due to its chain-length (about 48 Å), PEDGE does not restrict the short-range polymer segmental motion. Consequently, the electron transfer process within the hydrogel is not affected (Gregg and Heller, 1991b, Mao et al., 2003). Therefore, the PEDGE quantity described in the reference method was maintained in the next experiments.

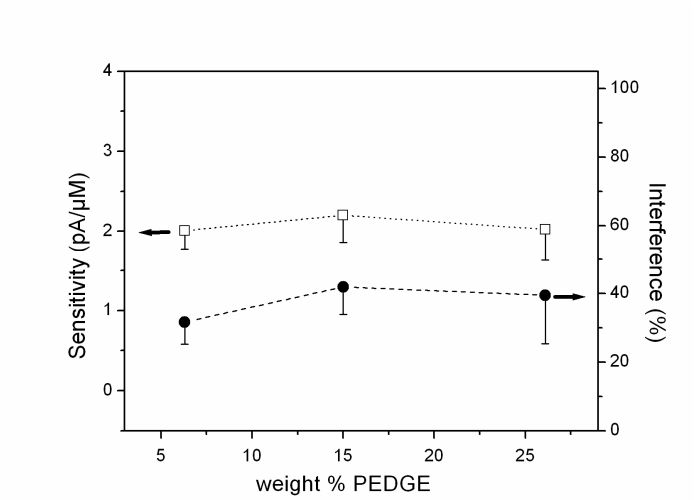


Figure 5: The influence of different concentrations PEDGE on the performance of the microsensor. The total weight % (w:w) of PEDGE in the coating solution was changed from 6.3 (n = 9) to 15 (n = 10) and 26.1 % (n = 6).

Influence of AA-ox

Next, the influence of AA-ox on the performance of the microsensor was investigated. The amount of AA-ox within the coating solution was varied between 0 and 2100 units/ml. In Fig. 6A the influence of AA-ox on the sensitivity and interference of the microsensor is shown. It is evident that increasing the AA-ox concentration resulted in a clear decrease of the interference by AA. However, the sensitivity for glutamate also decreased in proportion. The concentrations of AA-ox were relatively high compared to the concentrations of the other enzymes, therefore competition with Glu-ox and HRP for incorporation into the hydrogel might explain the large impact of AA-ox on the sensitivity of the microsensors.

It was also observed that the higher the AA-ox concentration, the slower the dynamics of the glutamate peaks (results not shown). Likely, a higher enzyme concentration decreased the relative POs-EA concentration within the hydrogel. This resulted in a decrease of the glutamate detection properties, such as response time, linearity, peak dynamics, sensitivity, etc. The observed effects were comparable to the effects observed previously in Fig. 2C and results can be explained similarly. With higher concentrations of HRP and Glu-ox similar type of observations were made (discussed below).

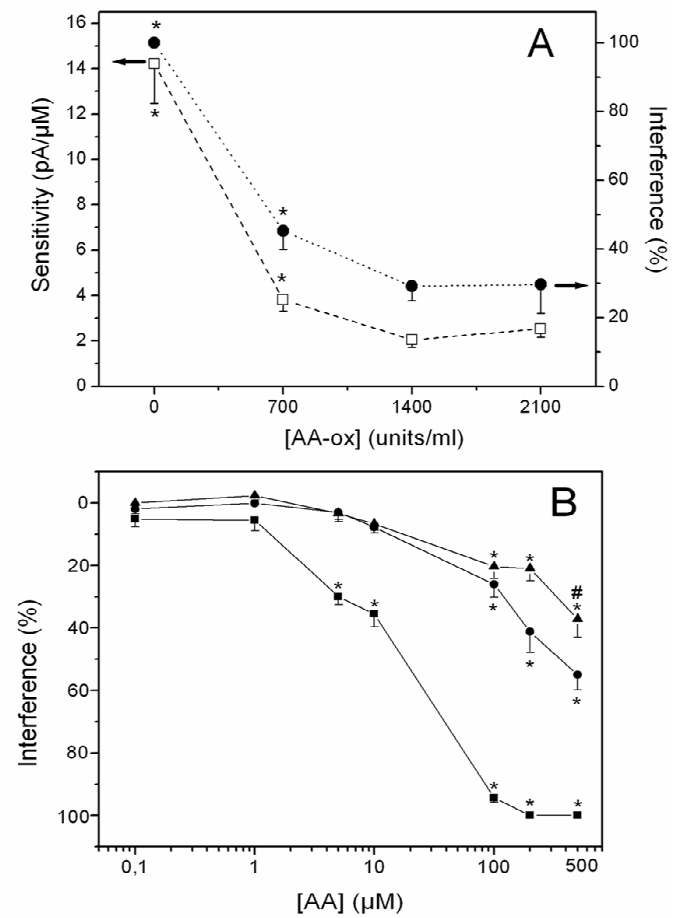


Figure 6: Influence of different concentrations of AA-ox on the performance of the microsensor. The following AA-ox concentrations were investigated: 0 (n = 16), 700 (n = 16), 1400 (n = 13) and 2100 units/ml (n = 6). ***A)*** *Sensitivity and interference of the microsensor at different AA-ox concentrations. * Denotes a statistically significant difference compared to the reference 1400 units/ml concentration (p < 0.05; M-W Rank Sum test).* ***B)*** *Interference characteristics of microsensors with different concentrations of AA-ox: -■- = 0 (n = 14), -●- = 700 (n = 7) and -▲- = 1400 units/ml (n = 8). The concentration of AA coinjected to glutamate 100 µM was titrated from 0.1 to 500 µM (x-axis). The sensitivity for 100 µM glutamate, without coinjection of AA, was set at 100%. * Denotes a statistically significant difference compared to the control value. (p < 0.05; One Way ANOVA followed by a Dunn's post-hoc test). # Denotes a statistical significant difference between the AA-ox 700 and 1400 units/ml curve (p < 0.05; M-W Rank Sum test).*

The interference by different concentrations of AA (range: 0.1 - 500 µM) on three type of sensors: no AA-ox, AA-ox 700 and 1400 units/ml is shown in Fig. 6B. It was observed that a concentration of 5 µM AA already interfered significantly when AA-ox was not incorporated. The interference behaviour of the sensors with AA-ox 700 and 1400 units/ml showed a similar pattern. A significant difference was only observed at AA 500 µM. It is observed that the interference increased only moderately when the concentration of AA was increased from 100 to 500 µM.

The extracellular AA concentration in the brain is between 100 and 500 µM (Grünewald, 1993; Yusa, 2001). This implies that changes in AA levels, that might occur *in vivo*, will only have a minor effect on the glutamate signal. The concentration of AA-ox used in the reference method was 1400 units/ml. Based on the slightly better sensitivity/ interference ratio, a concentration of 700 units/ml was used in the next experiments.

During the course of this study we experienced that different batches of AA-ox had different effects on the performance of the microsensor. Three batches of AA-ox were compared in Table 3. Each batch had different effects on the sensitivity and interference of the microsensor. This batch-to-batch variation was experienced as a serious handicap in the practical use of the microsensor. Therefore, this was further investigated in the next chapter (chapter 4). To prevent these enzyme batch variations, all microsensors used in this study were prepared with the same batch.

AA-ox activity (units/mg solid)	Supplier	Protein content (%)	Units/mg protein	n	Sensitivity (pA/µM)	Interference (%)
33,5	Sigma	3,3	1014	15	3.9 ± 0.4	54.6 ± 3.6 *
102,3	Sigma	9,9	1033	11	2.6 ± 0.8	33.7 ± 3.1 *
310	Biozyme	24,5	1265	8	2.7 ± 0.4	75.3 ± 7.2 *

Table 3: Influence of different batches AA-ox on the performance of the microsensor.

Influence of Glu-ox

The effect of different amounts of Glu-ox on the sensitivity and interference of the microsensors was shown in Fig. 7. The amount of Glu-ox within the coating solution was varied between 0.75 and 100 units/ml. The sensitivity for glutamate displayed a U-shaped effect with an optimum between 3.0 and 6.1 units/ml. It is likely that in the rising part of the curve the sensitivity was controlled by the activity of the enzyme within the hydrogel, while in the declining part the electron transfer through the hydrogel limited the sensitivity of the microsensor. The sensitivity may also have been limited by changes in substrate diffusion (Ohara et al., 1993a, 1994; Rajagopalan et al., 1996).

Interestingly, the same U-shaped effect was seen for the linearity in the response of the microsensor to glutamate: microsensors with the highest sensitivity also displayed the highest linearity, up to an R^2 value of 0.999 (data not shown).

The interference by AA remained unchanged at the different Glu-ox concentrations, except for the two lowest Glu-ox concentrations, which displayed a more pronounced interference. A possible explanation is that diffusion of AA through the hydrogel was improved due to the lower total enzyme content.

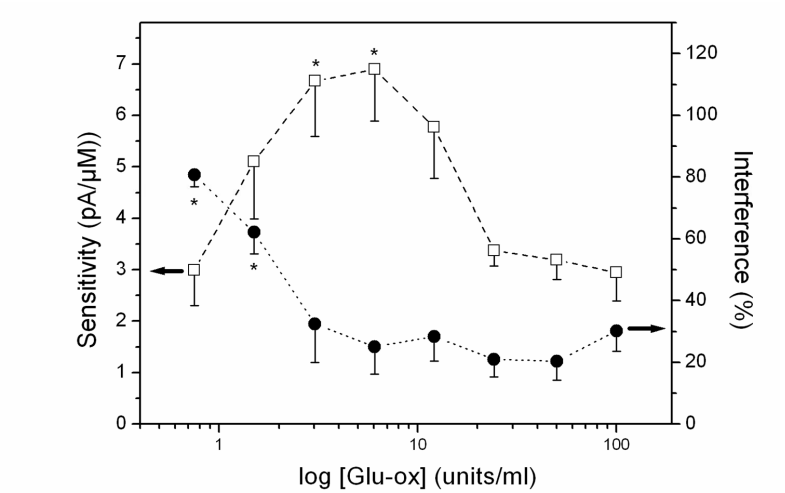


*Figure 7: Influence of different concentrations of Glu-ox on the performance of the microsensor. The following Glu-ox concentrations were investigated: 0.75 (n = 7), 1.5 (n = 8), 3.0 (n = 7), 6.1 (n = 7), 12.1 (n = 7), 24.2 (n = 8), 50 (n = 9) and 100 units/ml (n = 11). The sensitivity and interference of the microsensor were shown. * Denotes a statistical significant difference compared to the reference 24.2 units/ml concentration (p< 0.05; M-W Rank Sum test).*

In contrast to the observed batch-to-batch variation of AA-ox, variations due to different batches of Glu-ox were not observed. This may be explained by the fact that the used concentration of Glu-ox was much lower compared to the concentration of AA-ox. Based on these results, the optimum amount of 6.1 units/ml (compared to the reference 24.2 units/ml) was used in the next experiments.

Influence of HRP

The influence of different concentrations HRP on the performance of the microsensor was investigated next. This was determined with microsensors using two concentrations of Glu-ox: the reference amount of 24.2 units/ml (Fig. 8A) and the optimized amount of 6.1 units/ml (Fig. 8B). The amount of HRP within the coating solution was varied between 47.4 and 711 units/ml (Fig. 8A), and between 23.7 and 1422 units/ml (Fig. 8B). Both curves showed a similar U-shaped pattern in sensitivity and linearity, and a similar interference pattern as observed in Fig. 7. These results can be explained similarly. Fig. 8A showed an optimum at 237 units/ml, Fig. 8B at 711 units/ml. This discrepancy can be explained by the different amounts of Glu-ox used. It is likely that if the amount of one enzyme is changed, the optimal amount of the other enzyme is also modified.

When Fig. 8A and B are compared, it is evident that for each investigated amount of HRP the sensitivity is significantly higher and the interference significantly lower in Fig. 8B. This finding is explained first by an increased efficiency in H_2O_2 collection, due to an increased HRP : Glu-ox ratio (Belay et al., 1999), and secondly by an improved electron diffusion in the hydrogel, due to a lower overall enzyme content.

Based on these data, the HRP concentration of 711 units/ml was used in combination with a Glu-ox concentration of 6.1 units/ml and an AA-ox concentration of 700 units/ml in the final experiments (referred to as optimized coating solution; Table 4).

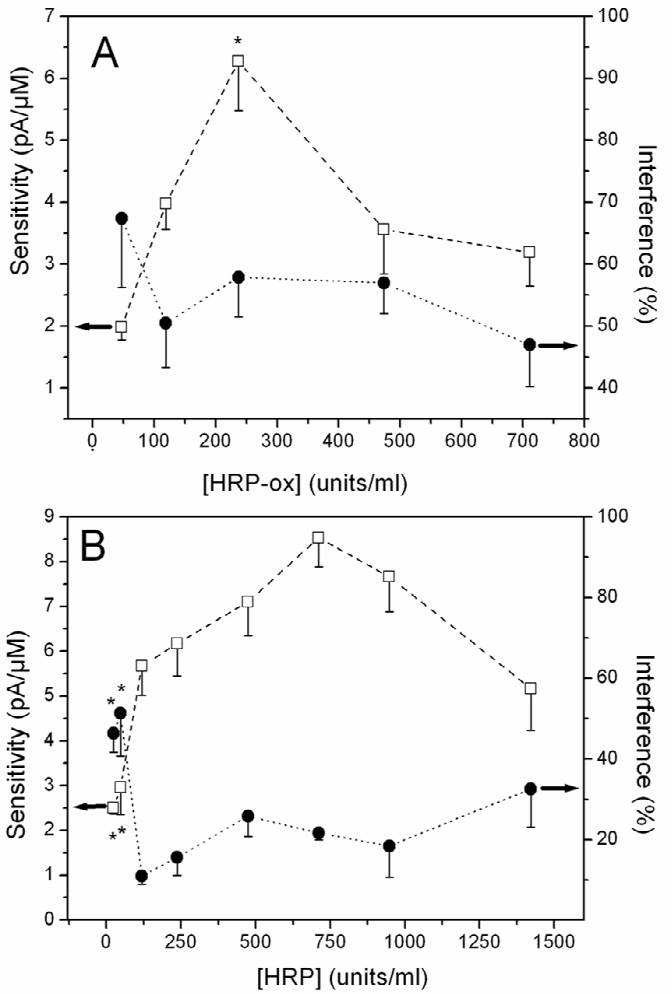


Figure 8: Influence of different concentrations of HRP on the performance of the microsensor. The sensitivity and interference were investigated for two type of microsensors: the reference amount (24.2 units/ml) ***(A)*** *and the optimized amount (6.1 units/ml) of Glu-ox* ***(B)****.* ***A)*** *The following HRP concentrations were investigated: 47.4 (n = 6), 119 (n = 6), 237 (n = 6), 474 (n = 14) and 711 units/ml (n = 10).* ***B)*** *The following HRP concentrations were investigated: 23.7 (n = 8), 47.4 (n = 7), 119 (n = 8), 237 (n = 8), 474 (n = 3), 711 (n = 10), 948 (n = 7) and 1422 units/ml (n = 6). * Denotes a statistically significant difference compared to the reference 411 Units/ml concentration (p< 0.05; M-W Rank Sum test).*

Influence of Nafion on the performance of the microsensor

Coating the microsensor with a Nafion layer completed the construction of the microsensor. Nafion is a commercially available perfluorosulfonic acid that rejects negatively charged components such as AA. For that reason, Nafion is often used as a discriminator within biosensors (Hu et al., 1994; Karyakin et al., 2000; Burmeister et al., 2002). Nafion is also used to improve the biocompatibility of biosensors and to provide protection against biofouling (Garguilo and Michael, 1996; Wisniewski and Reichert, 2000).

The influence of a Nafion coating on the performance of the microsensor was investigated under three conditions: no coating, and a 5 or 10 times dipcoating with 0.5 % Nafion. The effect of the different Nafion coatings was investigated on microsensors constructed with two amounts of AA-ox: the reference amount of 1400 units/ml (Fig. 9A) and the optimized amount of 700 units/ml (Fig. 9B). Nafion coating resulted in a 2-3 fold reduction of the sensitivity for glutamate and had no effect on interference by AA (Fig. 9A and B).

In Fig. 9C the effect of a Nafion coating on the interference by AA was investigated in more detail. Different concentrations of AA (range: 0.1 - 500 µM) were coinjected with glutamate 100 µM and applied to microsensors prepared with different Nafion coatings (similar microsensors were used as in Fig. 9B). No differences in interference were observed between the different microsensors. Application of the Nafion film also had no effect on the response time and the cyclic voltammogram of the microsensor (results not shown).

If the data of Fig. 9A and B are observed it seems as if glutamate is more effectively blocked by Nafion than AA, as the sensitivity for glutamate is decreased about twofold, whereas the degree of interference remained stable. However, most likely this is not the case, as the degree of interference at high concentrations of AA is rather stable (for comparison see Fig. 6B and 9C). This implies that blockade of glutamate and AA to a certain extent, as induced by the Nafion outer layer, will decrease the sensitivity, whereas the interference will not be affected largely.

It was observed that the Nafion coating influenced the performance of the microsensor relatively to a minor extent. This is explained by the thin coating that was applied. When a thicker Nafion film was applied, a more efficient rejection of AA was observed, but this occurred at the expense of the sensitivity, detection limit and response time of the microsensor (results not shown).

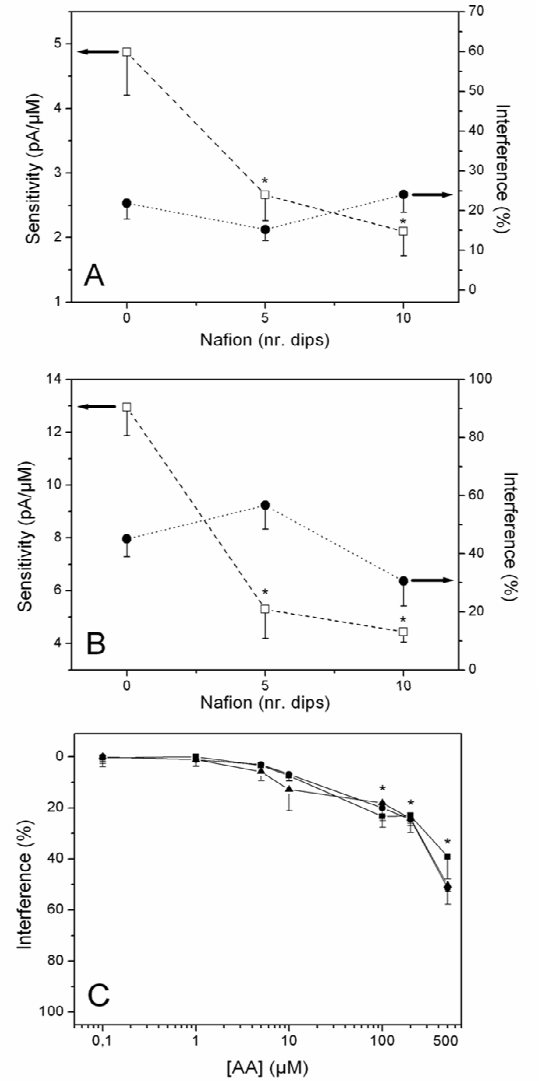


Figure 9: Influence of a Nafion coating on the performance of the microsensor. ***A)*** *Influence of different Nafion coatings: no coating (n = 6), 5 (n = 7) and a 10 times (n = 6) Nafion (0.5%) dip on the sensitivity and interference of sensors with AA-ox 1400 units/ml.* ***B)*** *No coating (n = 11), 5 (n = 8) and a 10 times Nafion dip (n = 6) on sensors with AA-ox 700 units/ml. * Denotes a statistically significant difference compared to microsensors without a Nafion coating (p < 0.05; M-W Rank Sum test).* ***C)*** *Represents the interference pattern of the microsensors depicted in (**B**). The concentration of AA coinjected to glutamate 100 µM was varied from 0.1 to 500 µM. Glutamate 100 µM values, without AA coinjection, were set at 100%. -■- = no coating (n=8), -●- = 5 times (n=5) and a -▲- = 10 times Nafion dipcoating (n=5). * Denotes a statistically significant difference compared to the control values (p< 0.05; One Way ANOVA followed by a Dunn's post-hoc test).*

It is concluded that application of a Nafion layer is not of advantage as a discriminating barrier. Both AA and glutamate are negatively charged and have about the same molecular size and it is likely that the Nafion coating similarly rejects both compounds. It is known that for an effective use of Nafion as a discriminating barrier, the Nafion coating should be annealed at temperatures of about 200 °C to the electrode surface (Gerhardt and Hoffman, 2001; Burmeister et al., 2002). However, these conditions are not suitable for this type of microsensor. The influence of Nafion on the biocompatibility and biofouling of the microsensor is investigated further in chapter 5.

3.4 Conclusion

In table 4 the properties of microsensors prepared with the optimized coating solution were compared with microsensors prepared with the reference coating solution. It is concluded that optimization in the composition of the hydrogel improved the properties of the microsensors in terms of sensitivity, current density, detection limit, interference by ascorbic acid and linearity. Finally, a more than 4-fold increase in sensitivity was obtained, while the interference by AA was reduced. Application of a Nafion film was not of advantage as a–discriminating barrier, as it reduced the sensitivity about 2-fold, without affecting other microsensor properties.

Coating solution	Nafion	N	Sensitivity (pA/µM)	Interference (%)	Response time (sec.)	Current Density (mA/ M*Cm2)	Linearity (R^2)	Detection Limit (µM)	CV (nA)
Reference	without	18	1.9 ± 1.0	34.0 ± 14.9	20.6 ± 1.7	17.6 ± 9.3	0.989 ± 0.007	0.6 ± 0.06	23.2 ± 9.71
Optimized	without	10	8.5 ± 1.7 *	21.7 ± 3.6 *	21.9 ± 2.1	82.4 ± 34.2 *	0.998 ± 0.001 *	0.09 ± 0.06 *	47.4 ± 17.5 *
Reference	10 times dip	63	0.9 ± 0.6	36.0 ± 21.2	20.6 ± 3.7	8.4 ± 5.7	0.990 ± 0.007	1.2 ± 0.2	21.2 ± 5.0
Optimized	10 times dip	12	4.9 ± 1.6 *	14.7 ± 12.8 *	21.5 ± 2.0	39.1 ± 13.2 *	0.997 ± 0.003 *	0.2 ± 0.02 *	51.6 ± 15.6 *

[a] Reference = 20 µl (Pos-EA 1 mg/ml), 4 µl PEDGE (3 mg/ml), 10 µl HRP (411 Units/ml), 10 µl Glu-ox (24.2 Units/ml), 10 µl AA-ox (1400 Units/ml)
[b] Optimized = 20 µl (Pos-EA 1 mg/ml), 4 µl PEDGE (3 mg/ml), 10 µl HRP (711 Units/ml), 10 µl Glu-ox (6.1 Units/ml), 10 µl AA-ox (700 Units/ml)

Table 4: Comparison of the performance of microsensors constructed with the reference (a) and optimized (b) coating solution. Both conditions were investigated with and without a Nafion coating (10 times dip in Nafion 0.5%). Denotes a statistically significant difference compared to the reference coating solution ($p< 0.05$; M-W Rank Sum test).*