Application of a glutamate microsensor to brain tissue

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Chapter 2

Improving the reproducibility of hydrogel-coated glutamate microsensors by using an automated dipcoater.

This chapter is based on the following paper:

Oldenziel WH, Beukema W and Westerink BHC. Improving the reproducibility of hydrogel-coated glutamate microsensors by using an automated dipcoater. *J. Neurosci. Meth.* 2004; 140, 117-126.

Abstract

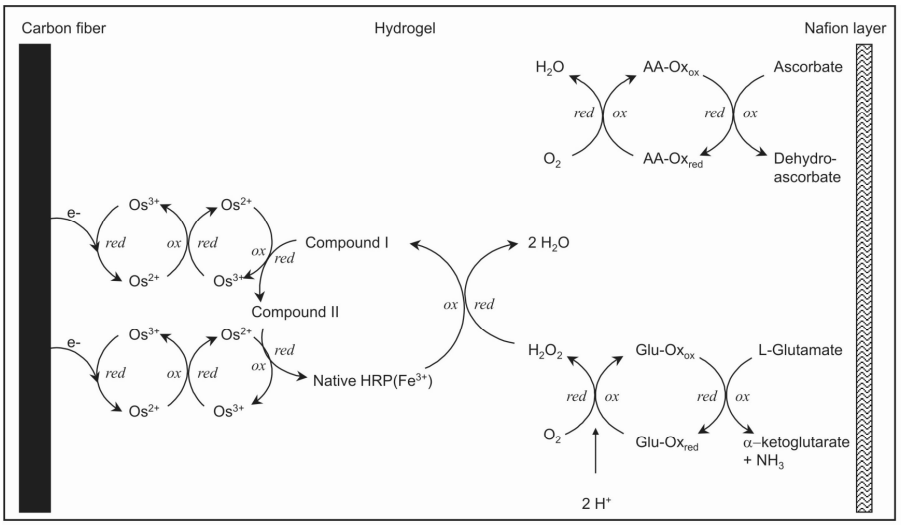
The hydrogel-coated glutamate microsensor is a promising tool for the analysis of extracellular glutamate both in vivo and in vitro. However, its construction depends on a manual fabrication procedure. This often results in a poor reproducibility of the analytical performance of the microsensor. The aim of the present study was to improve both the reproducibility and the performance of the glutamate microsensor. Various parameters that are likely to influence its performance were studied. It appeared that the most crucial step in determining the performance of the microsensor was the hydrogel-application procedure. To control this procedure we have developed an automated dipcoater. This dipcoater allowed mechanical application of the hydrogel on the carbon fiber electrode (CFE) under standardized conditions. A significant improvement in the performance of the microsensors was observed when the CFEs were dipcoated for 10 min at 37 °C. A further improvement was obtained when the automated hydrogel application procedure was combined with other cross-link methods, such as electrodeposition and electrostatic complexation. It appeared that a crucial factor in determining the performance of the microsensor was the thickness of the hydrogel layer. Microscopic observations revealed that, despite the use of an automated dipcoater, the layer-thickness was not constant. By combining the automated dipcoat technique with amperometry, the layer thickness could be monitored and controlled indirectly, which resulted in a significant improvement of the reproducibility of the microsensors.

2.1 Introduction

To monitor physiologically derived glutamate both *in vivo* and *in vitro,* an analysis technique with a high spatial- and temporal resolution seems required. Microsensors may provide a solution to these requirements (Hu et al., 1994; Cui et al., 2001; Burmeister at al., 2002). A promising microsensor concept was previously presented by Kulagina et al. (1999). The present work is based on this approach. Briefly, a 10 µm diameter CFE, with a length of 300-500 µm, is coated with a hydrogel containing the enzymes l-glutamate oxidase (Glu-ox), horseradish peroxidase (HRP) and ascorbate oxidase (AA-ox), which are coupled via poly(ethylene glycol) diglycidyl ether (PEDGE) to an $Os(bpy)_2Cl$ complexed redox-polymer (POs-EA). Casting of a thin Nafion film completes the fabrication. Scheme 1 shows the enzymatic detection of glutamate on the microsensor. Ascorbic acid (AA) is a strong reducing agent and is present in high concentrations (100-500µM) in the brain. (O'Neill et al., 1998). AA can interfere by reducing several intermediate steps in the reaction cascade, which are in an oxidized state (Garguilo and Michael, 1996). AA does not induce a signal itself, but diminishes the glutamate signal. Incorporation of AA-ox into the hydrogel is necessary to prevent this interference (Garguilo and Michael, 1996; Kulagina et al., 1999; Cui et al., 2001). In the next chapter (chapter 3) more attention is paid to this issue.

Although the microsensor concept has several specific advantages, its construction is complicated. Due to the small size of the CFEs, the various components have to be applied on the CFE by manual dipcoating. This is a difficult procedure to standardize and to control (Schuhmann, 2002). As a result, the reproducibility of the microsensors is poor.

This study investigates the possibility to improve the reproducible construction of the hydrogel-coated glutamate microsensor. To that end an automated dipcoater was developed. Various parameters, such as the conditions during dipcoating and combinations of different cross-link methods, were investigated with the automated dipcoater.



Scheme 1: Electrochemical detection of l-glutamate with the hydrogel-coated microsensor at -150mV vs. Ag/AgCl.

2.2 Materials 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 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). Poly(ethylene glycol 400 diglycidyl ether) (PEGDGE), silverchloride, 3-mercapto-1-propanesulfonic acid, H_2O_2 35 wt %, and Nafion (5 % Nafion solution,1100 equivalent weight) were obtained from Aldrich, (Milwaukee WI, USA). P-benzoquinone, acetone p.a., 2-propanol p.a. and sulfuric acid (96%) were obtained from Merck (Darmstadt, Germany). 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. 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. The redox-polymer, POs-EA, was made by complexing poly(vinylpyridine) with $Os(bpy)_2Cl$-groups and ethylamine-groups according an earlier described synthesis (Shankar et al., 1998).

Microsensor construction

The carbon fiber electrodes (CFEs) were constructed 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). Inside the CFE the electrical contact between the CF and a 250 µm diameter Teflon coated silver wire (Advent Research Materials, Eynsham Oxon, England) was mediated by epoxy-silver (World Precision Instruments, Sarasota, FL, USA). This was 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 attached in the capillary using a four component Spurr epoxy glue (Polysciences, Warrington, PA, USA) and the glue was dried overnight at 70°C. Mediating the contact in the tip of the electrode with silver epoxy has a few advantages. It reduced the resistance of the CFE significantly to 200-600 Ohm, which in turn decreased the noise pick-up and made the CFEs better suitable for impedance spectroscopy. Before use, the fibers were trimmed under a microscope (Pleuger XSZ-107) to a length of 300-500 µm.

The glutamate sensors were constructed by coating the CFE with an aqueous mixture containing POs-EA (1 mg/ml; 20 µl), PEDGE (3 mg/ml; 4 µl), HRP (3 mg/ml (0.474 Units/µl); 10 µl), AA-ox (10 mg/ml (1.4 Units/µl); 10 µl) and Glu-ox (2 mg/ml (0.0242 Units/µL); 10 µl). Coating occurred by a 10 minute manually dipping procedure of a pair of CFEs under a microscope. HRP sensors were made in a similar way, only without Glu-ox and AA-ox incorporated into the hydrogel. The coating procedure was mechanized by using an automated dipcoater, which is explained in more detail in the Results & Discussion section (Fig. 3). After coating, the sensors were cured for 1 hr at 37 °C, followed by a 10 minute dip in U.P. water and 2 hours drying in ambient air. A final Nafion coating completed the fabrication. The Nafion coating was performed by dipping the hydrogel-coated microsensors repetitively 10 times for 10 seconds, with 20 seconds intervals, in a 0.5 % nafion solution (1:10 dilution in 2-propanol p.a). All the glutamate sensors were casted with a Nafion coating, unless otherwise stated. The sensors were stored in the refrigerator overnight before calibration at 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 dipping a bare silver wire in molten AgCl. Before each calibration the potential was checked against a commercial available saturated Ag/AgCl reference electrode (Antec Leyden, the Netherlands). New 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. In order to reduce the 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 microsensor

All calibrations were performed in a flow-injection analysis system (FIA). During calibration the microsensors were placed in a flow of air-equilibrated aCSF (1 ml/min), generated by an HPLC-pump (Pharmacia-LKB, 2150). Compounds were injected during 30 seconds via an HPLC injection valve (loop volume: 0.5 ml; Rheodyne, Cotati, CA, USA). All stock-solutions were made fresh before the start of an experiment. The AA stock solution was stored under N_2. Before each injection, the solutions were prepared fresh from the stock solutions. Each solution was injected at least two times and the results were averaged. Calibration of the microsensors was carried out as follows. First, a cyclic voltammogram (CV) of the sensor was recorded by cycling the potential once between –150 and 700 mV at a speed of 100 mV s^{-1}. The CV reflects the cycling of the osmium groups between their 2^+ and 3^+ state. Secondly, the sensors were calibrated amperometrically at –150 mV. Glutamate concentrations of 5, 10, 25, 50 and 100 µM were injected to determine the sensitivity, linearity and peak shape. The calibration was finished by investigating the interference by AA, determined by coinjecting 200 µM AA to 100 µM glutamate. This reduced the original glutamate signal by a certain percentage, which is expressed as the percentage of decrease of the initial glutamate signal and is referred to as "interference" (see chapter 3 for a detailed explanation). After calibration, the following properties were determined for each microsensor: shape and height of the CV (nA), sensitivity (pA/µM), current density (mA M^{-1} cm^{-2}), interference (%), linearity (R^2), detection limit (µM) and response time (sec.). For reasons of clarity only a limited amount of these data were shown in the Results and Discussion section. The detection limit is defined as three times the noise-level. The response time is defined as the time required for the signal to increase from 10 to 90 %.

Combination of cross-link methods

In a few studies additional cross-link methods were included. A pulse-generator (Wavetek, model 19) was used to apply the electrodeposition recipe: a 0.25 Hz square wave potential between –0.3 V and +0.4 V vs an Ag/AgCl reference electrode (Gao et al., 2002). Thiol-modified CFEs were constructed by dipping the CFE, which

was pretreated with the Standard Cleaning Procedure, for 0.5 hr in a 2.0 µM thiol solution. The latter was prepared by dissolving 3-mercapto-1-propanesulfonic acid in a 1.6 µM sulfuric acid solution (Forzani and Solis, 2000). Washing with U.P. water during 5 min completed the thiol-modification procedure. In the present study the automated dipcoat technique was also combined with amperometry. As such, a constant potential of 20 mV was applied between the thiol-pretreated microsensor and the Ag/AgCl reference electrode, which was placed in the coating solution. Both electrodes were connected to an amplifier and the generated current was amplified and recorded. The whole setup was placed in a specially constructed Faraday cage to reduce the noise pick-up.

Expression of results and statistics

All results were corrected to a standard CFE length of 400 µm. Experiments concerning the variability were presented as mean ± SD. Other experiments were presented as mean ± SEM. A statistical program (Sigmastat 3.0) was used to calculate statistics. Data were analyzed with a Mann-Whitney Rank Sum test (M-W Rank Sum test). Variability was analyzed with the equal-variance test of One Way ANOVA. Significance was set at $p < 0.05$. In Fig. 8 the best line of fit was drawn through a scatter diagram, respectively a linear and Lorentzian fit were used.

2.3 Results and Discussion

Effect of various CFE pretreatment procedures on the final performance of the microsensor

It is well-known that pretreatment of a CFE surface can changes its electrochemical properties dramatically (Kawagoe et al., 1993; Chen and McCreery, 1996). Therefore, the effect of pretreatment of the CFEs prior to hydrogel-coating was evaluated at three subsequent stages of the fabrication of the microsensor. The first stage represented uncoated CFEs and these sensors were evaluated by oxidizing AA directly at the CFE surface at +150 mV. The second stage represented HRP-sensors and these sensors were evaluated by detecting H_2O_2. Note, HRP-sensors have the same composition as glutamate sensors, only glu-ox and AA-ox are not incorporated into the hydrogel. The third stage represented the final glutamate sensors.

It is emphasized that the three sensor stages represented different electrochemical signals. A direct comparison in this respect is not possible. For example, the electron transfer kinetics of AA and osmium-groups on the CFE surface are different (Chen and McCreery, 1996). In addition, with modified sensors the electron transfer kinetics of the osmium-groups, the binding of POs-EA to the CFE, the binding of enzymes to the POs-EA and the diffusion kinetics of substrates and intermediate products through the hydrogel also contribute to the final signal.

Three types of cleaning procedures were evaluated for the different microsensors. (1) CFEs were rinsed with acetone for 10 min. Acetone is often used to clean CFE-surfaces, as it removes a particular resin-coating (Stamford et al., 1995). (2) CFEs were electrochemically pretreated by applying a 7 sec 70 Hz triangle wave between –1.5 V and +1.5 V, followed by 0.5 hr 0V vs an Ag/AgCl-reference electrode. This method was derived from a pretreatment-procedure described by Cahill and Wightman (1995) and can be regarded as a relatively mild pretreatment. It cleans the CFE surface and induces uniformity in surface electronegativity, without generating a thick surface oxide layer. The latter resulted in bad sensor performance, i.e. it induced high basal currents and large noise levels (results not shown). (3) A combination of acetone rinsing, electrochemical pretreatment and rinsing with U.P. water, referred to as a standard cleaning procedure (SCP).

In Fig. 1A the sensitivity and reproducibility of uncoated CFEs is shown. Pretreatment with acetone or electrochemical cleaning only had a moderate effect on

the reproducibility, but application of the SCP improved the reproducibility significantly. It was observed that the sensitivity of the CFE to oxidation of AA was not affected by the different cleaning procedures. The results obtained with the HRP sensors (Fig. 1B) were comparable with the results of the uncoated CFEs, only the influence of the SCP on the variability of HRP-sensors was less compared to uncoated CFEs. In addition, the influence of the SCP on glutamate sensors was less in comparison to HRP sensors (Fig. 1C).

The results indicate that the influence of pretreating the CFE diminishes when the architecture of the sensor becomes more complex. Other factors apparently play a more important role in controlling the performance of the microsensor, which is investigated further in the following paragraphs. Despite, the SCP was applied on a routine base in the next experiments to prevent variation in CFEs.

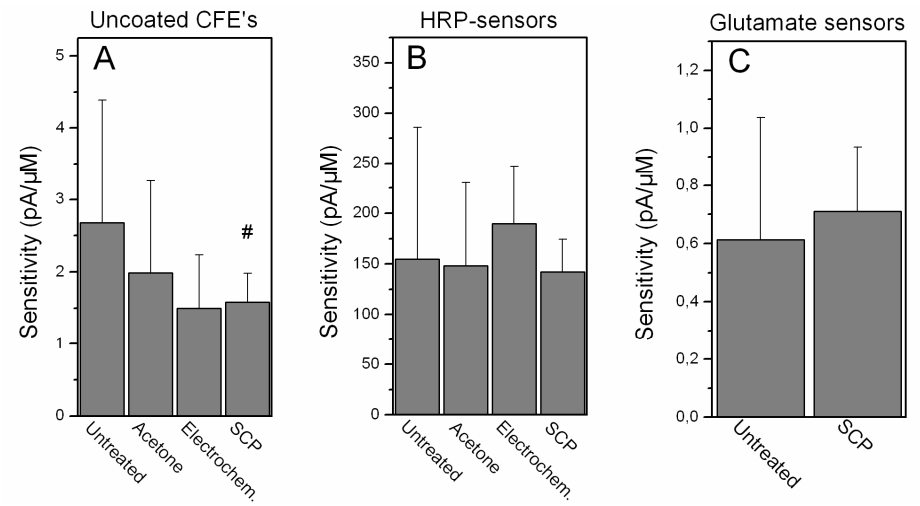


Fig. 1: Influence of different cleaning procedures on three types of microsensors. Cleaning procedures: no cleaning (untreated), 10 minute acetone rinsing (Acetone), electrochemical pretreatment (Electrochem.) and a standard cleaning procedure (SCP). The latter was a combination of acetone rinsing and electrochemical pretreatment. ***A****) Oxidation of AA at +150 mV at uncoated CFEs: untreated (n = 13), aceton (n = 13), electrochem (n = 7) and SCP (n = 12).* ***B****) HRP-sensors: untreated (n = 11), aceton (n = 15), electrochem (n = 4) and SCP (n = 6).* ***C****) Glutamate-sensors: untreated (n = 34) and SCP (n = 9). The sensitivity of the sensors was expressed as pA/µM ± SD. # Denotes a statistically significant reduction in variability (p < 0.05; equal-variance test).*

Correlation of the quantity of active CFE surface with the final sensitivity of the microsensor

Next, the relationship between the quantity of active CFE surface and the sensitivity of the microsensor after hydrogel-coating was investigated. The quantity of CFE surface was estimated by reducing p-benzoquinone at the CFE. The detected reduction current reflected the quantity of CFE surface according the Cotrell-equation (Gerhardt and Burmeister, 2000). After determination of the amount of surface, the CFEs were coated with a hydrogel in order to construct HRP sensors. Subsequently, the sensitivity of these sensors for H_2O_2 was determined. Note, p-benzoquinone was chosen for determination of the quantity of CFE surface, as it can be reduced at the same potential as the HRP sensors are being operated. In addition, before starting the hydrogel-coating procedure, the sensors were cleaned again in order to avoid remnant p-benzoquinone at the surface. In Fig. 2A the correlation between the sensitivity of the CFE for p-benzoquinone was correlated to the final sensitivity of the HRP sensors for H_2O_2. No correlation between both conditions was observed. Interesting to note is that the microsensors were hydrogel-coated in pairs, and a pronounced co-variance between the duplicates was observed when these sensors were marked (Fig. 2B).

This experiment shows that the underlying CFE surface area is of minor importance for the final sensitivity of the microsensor. Apparently, the hydrogel-application procedure plays a more important role in determining the final microsensor properties.

It is emphasized that due to their small size the microsensors need to be constructed by dipcoating, as dropcoating of the aqueous mixture is not possible (Mikeladze et al., 2002). However, with manual dipcoating it is difficult to control the exact hydrogel coverage. To further study the hydrogel-application procedure we decided to mechanize the manual application procedure.

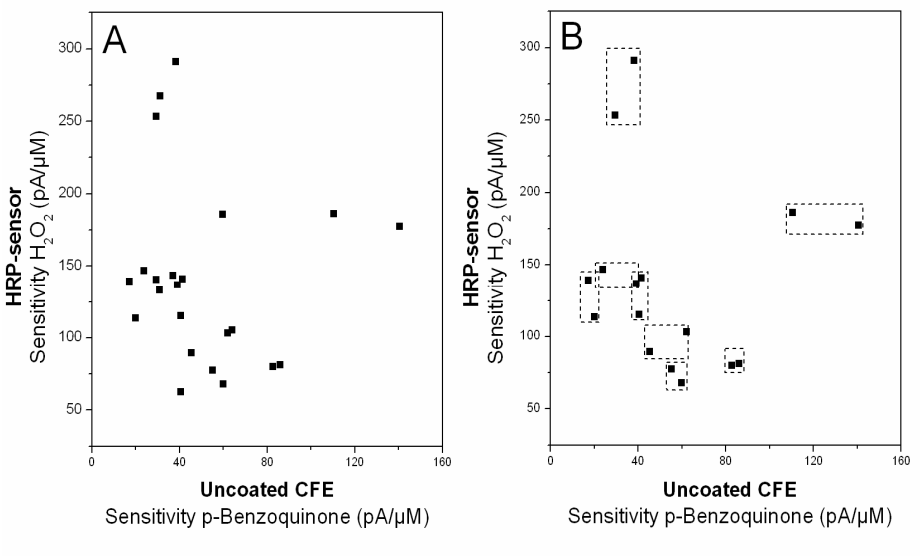


Fig. 2: Correlation between the sensitivity of uncoated CFEs for p-benzoquinone and the sensitivity for H_2O_2 after preparing HRP-sensors. ***A)*** *The reduction of p-benzoquinone is plotted against the H_2O_2-sensitivity after hydrogel-coating (n=23).* ***B)*** *Same graph, in which sensors that were constructed in pairs are marked. Note, the latter figure contains slightly less data, as the sensors from which one of a pair was missing were excluded.*

Mechanisation of the manual hydrogel-application procedure

In order to mechanize the manual application procedure, we have developed an automated dipcoater (Fig. 3). This dipcoater allowed us to prepare microsensors under well defined conditions. In brief, the microsensors were mounted in a holder. For each sensor a small cup was placed in another holder, which was situated on a speaker cone. These cups were filled with twenty microlitre of the aqueous mixture of redox-polymer, enzymes and cross-linker. The speaker cone was connected to a frequency modulator. By moving the cups, the four CFEs could be dipped simultaneously with a frequency and amplitude of choice. The sensors could be adjusted individually to a certain depth, which determined the time of exposure within the mixture. In addition, by placing the dipcoater in a perspex oven, the temperature and relative humidity could be monitored and controlled during the hydrogel-application procedure.

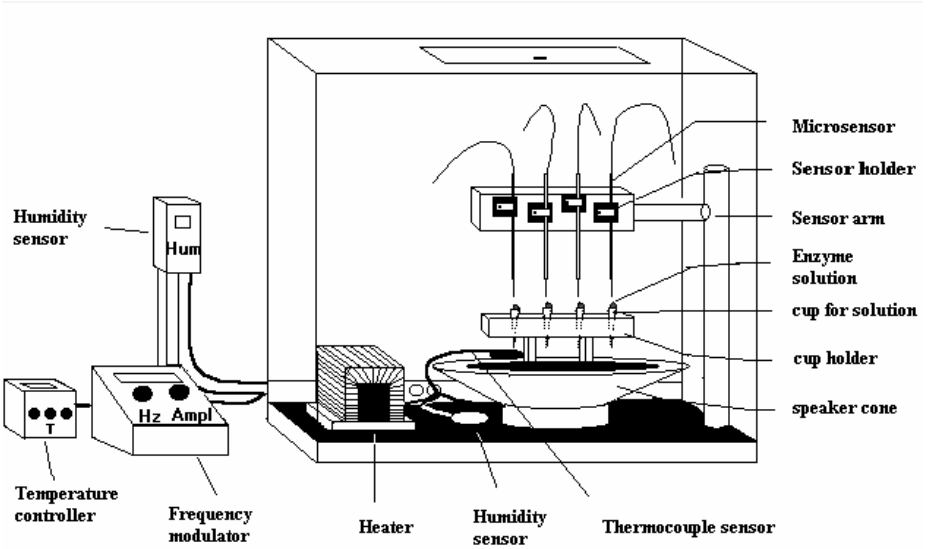


Fig. 3: Picture of the automated dipcoater.

The manual and dipcoater-fabricated glutamate microsensors were compared in Fig. 4. The manual fabricated group consisted of a large overall group (n = 104) of sensors on which the hydrogel was coated at temperatures varying from room temperature to 30°C. A representative group of sensors, that were all constructed at approximately 30°C (n = 22), was selected from this overall group. The dipcoater group (n = 18) was constructed with a frequency of 0.14 Hz, an amplitude of 1 mm and a temperature of 30°C, i.e. conditions mimicking the manual procedure of the selected group as close as possible. The sensors of the selected manual group and the sensors prepared with the dipcoater displayed a similar sensitivity and interference by AA (Fig. 4). They also displayed similar response characteristics (results not shown). On the contrary, the sensors of the overall-group displayed a significant lower sensitivity with larger variation and a higher interference.

It is concluded that the way the dipcoating procedure is performed is less important (i.e. manual vs. automatic), but that the conditions during the coating procedure seem to be crucial. Therefore, in the following experiments we have investigated the influence of different conditions during the hydrogel coating procedure on the final performance of the microsensor. The use of the automatic

dipcoater appeared to be of great benefit in these studies, as it allowed the control of experimental conditions such as temperature, humidity and air flow. Conditions that are difficult, or impossible, to control with manual dipcoating.

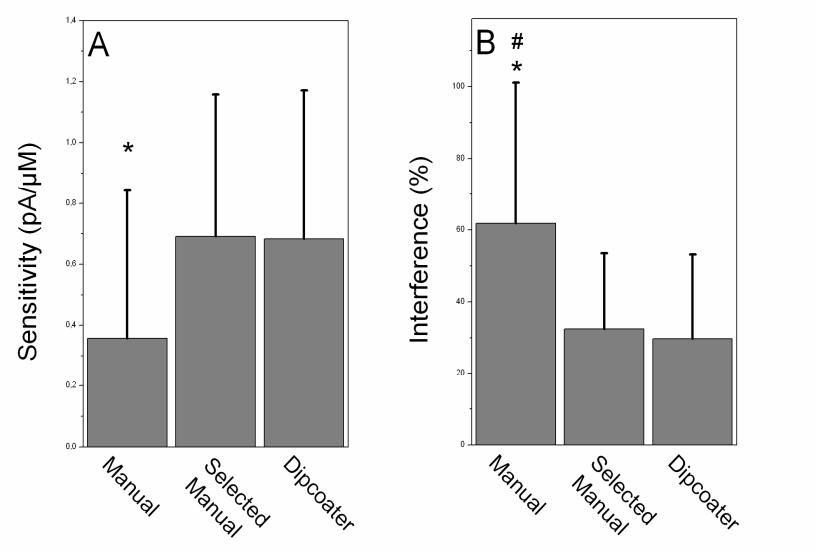


Fig. 4: Comparison of glutamate microsensors constructed by a manual or automated dipcoat procedure. The manual group (n = 104) is a large group of sensors constructed at non-standardized temperature conditions. The selected manual group is a selection (n = 22) of this group, which were constructed at a temperature of approximately 30°C. The dipcoater group (n = 18) consists of microsensors constructed with the automated dipcoater at 30°C. ***A****) Sensitivity for glutamate expressed in pA/µM.* ***B****) Interference by 200 µM AA on 100 µM glutamate, expressed in %. Data were presented as mean ± SD.* * *Denotes a statistically significant difference with both other groups (p < 0.05; M-W Rank Sum test). # Statistically significant higher variability compared with both other groups (p < 0.05; Equal-variance test).*

Effect of temperature during the hydrogel-application procedure on the performance of the microsensor

The dipcoater was used to study the effect of temperature during the hydrogel application procedure. Three different temperatures were compared: 25 °C (n = 4), 30 °C (n = 7) and 37 °C (n = 8). It was observed that a higher temperature during the construction of the microsensor improved the sensitivity of the sensor for glutamate and decreased its interference by AA significantly. In addition, a large improvement in

the performance of the microsensors that were constructed at 37 °C was observed (Fig. 5). The temperature was not further increased, to prevent damage to the enzymes. Due to increasing the temperature to 37 °C, the relative humidity in the dipcoater reached a stable value of about 20%. Varying the humidity during the dipcoating procedure at this temperature had no effect on the final properties of the glutamate microsensors (results not shown).

In Fig. 5 B a typical example of a microsensor response to a 30 second glutamate 100 µM bolus injection in a flow-injection analysis system is shown. In Fig. 5C the same sensors are shown, but now 200 µM AA was coinjected to 100 µM glutamate. The curves of Fig. 5B were included in Fig. 5C as dashed lines. Clearly visible were the higher sensitivity and lower interference of the sensors constructed at higher temperatures.

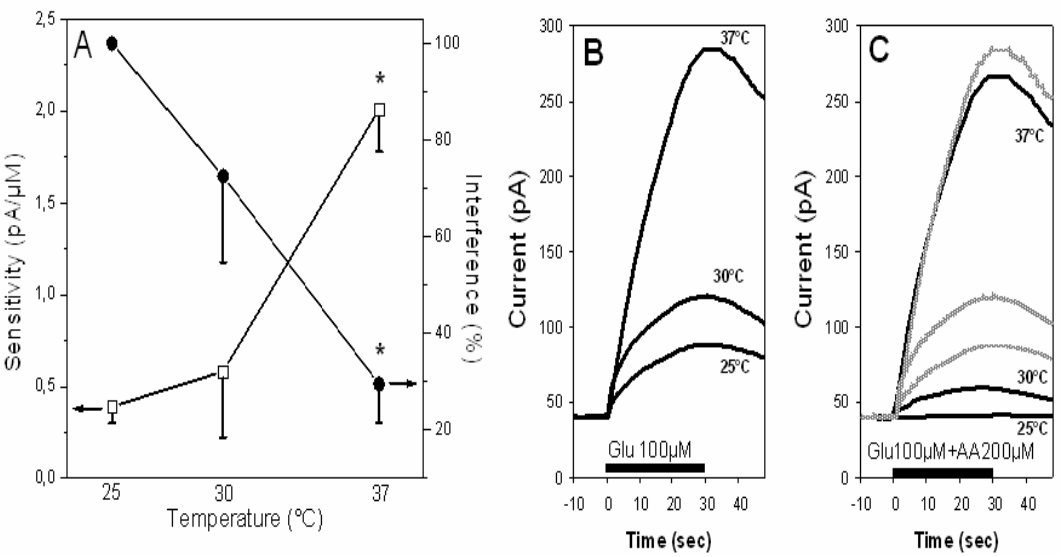


Fig. 5: Performance of glutamate microsensors prepared at different temperatures: 25°C (n = 4), 30°C (n = 7) and 37°C (n = 8). The sensors in this experiment were not coated with an additional nafion layer. ***A)*** *Sensitivity and interference (100 µM glutamate + 200 µM AA) of sensors constructed at different temperatures. Data were presented as mean ± SEM. (*) Statistically significant when compared with 25°C and 30°C (p < 0.05; M-W Rank Sum test).* ***B)*** *Typical response of sensors prepared at 25, 30 and 37°C to glutamate 100µM in the flow-injection system.* ***C)*** *Typical response of the same sensors as depicted in (**B**) to 100µM glutamate + 200µM AA. The dashed lines represent the initial 100µM glutamate signals.*

The results are most likely explained by the fact that a higher temperature stimulated several processes, such as the reaction between epoxide- and amine groups, evaporation of H_2O, absorption of the POs-EA to the CFE-surface and the convective flow in the mixture-droplet (Gregg and Heller, 1991; Dequaire and Heller, 2002). In general, a higher temperature during the dipcoating procedure most likely increases the coating of the hydrogel on the CFE. This resulted in an increased sensitivity, due to improved binding of HRP and Glu-ox, and a decreased interference, due to improved binding of AA-ox.

It is concluded that the conditions during the dipcoating procedure are of crucial importance for the final performance of the microsensor. These conditions seem to overrule other influences, such as the electrochemical state and quantity of the CFE surface (Fig. 1 and 2), and the way the dipcoating procedure is performed (manual vs. automatic, Fig. 4). Therefore, automatic dipcoating at 37 °C was used as a standard procedure in the next experiments.

Influence of the time of dipcoating on the performance of the microsensor

Next, the influence of the time of dipcoating on the final performance of the sensor was investigated. The time of dipcoating is an important factor in determining the thickness of the hydrogel layer. Three different time periods were compared: 5 min (n = 17), 10 min (n = 12) and 15 min (n = 7). By dipcoating at a constant frequency of 0.14 Hz, each dip lasted about 3.5 seconds and the total number of dips was respectively 42, 84 and 126. In Fig. 6 it is shown that the optimal time of dipcoating was approximately 10 min, which is in good accordance with earlier observations (Garguilo and Michael, 1996; Kulagina et al., 1999). Interesting to note is that the relation between the sensitivity of the sensor for glutamate and its interference by AA is biphasic. With an increasing thickness of the hydrogel the sensitivity of the sensor improved until an optimum. If the thickness of the layer increased any further, the sensitivity of the sensor decreased. Probably because of limitation in substrate diffusion (Ohara et al., 1993a; Belay et al., 1999). On the contrary, AA-ox is not dependent on communication with HRP, POs-EA and the CFE. The more AA-ox is incorporated into the hydrogel, the lower the interference by AA, as long as oxygen does not limit the activity of AA-ox. This implies that the interference by AA diminishes to a larger extent with an increasing thickness of the hydrogel.

Noteworthy, in this particular experiment the used AA-ox concentration was 0.7 Units/µl, instead of 1.4 Units/µl, which was used in the previous experiments. It was observed that all sensors displayed a higher sensitivity to glutamate, but also suffered from a higher interference by AA. This is most likely explained by the fact that at this lower AA-ox concentration more HRP and Glu-ox are able to bind to POs-EA, which results in a higher sensitivity. In addition, less AA-ox will bind to POs-EA, which results in a higher interference by AA. This observation stimulated us to investigate the influence of varying enzyme concentrations on the final performance of the microsensor (chapter 3).

The response of the sensors used in Fig. 6A to a 100 µM glutamate injection was visualized in Fig. 6B. Differences in sensitivity and response-characteristics were observed. It was examined that a longer time of dipcoating generated sensors with a slower response, i.e. both the initial response time, as the time required to return to baseline. The results were explained by the fact that a longer time of dipcoating resulted in a thicker hydrogel layer.

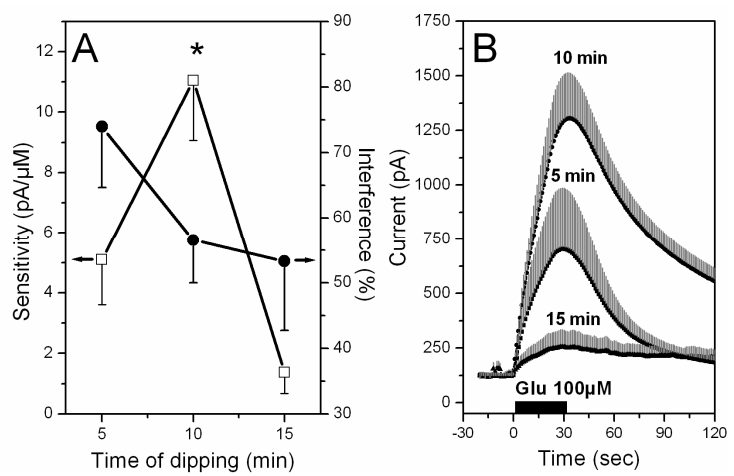


Fig. 6: Performance of glutamate microsensors prepared with different times of dipcoating: 5 min (n = 17), 10 min (n = 12) and 15 min (n = 7). The sensors in this experiment were not coated with an additional Nafion layer. ***A)*** *Sensitivity and interference (glutamate 100 µM + AA 200 µM) for sensors constructed with different dipcoat times. Data were presented as mean ± SEM. (*) Statistically significant when compared with 5 and 15 min ($p < 0.05$; M-W Rank Sum test).* ***B)*** *Response of all the sensors depicted in (**A**) to 100 µM glutamate. Data were presented as mean ± SD to show the variability in the response of the sensors.*

Effect of combining different cross-link methods

Next, the automated dipcoater was used to investigate the effect of combining different cross-link methods. The 10 min 37°C standard dipcoating procedure was combined with the following cross-link methods: (1) Electrodeposition. An electrodeposition procedure reported by Gao et al. (2002) was used. Both the microsensor and a reference electrode, which was placed in the aqueous mixture, were connected to a function generator. An electrodeposition potential was applied each time the sensor was dipped into the solution. This potential was a 0.25 Hz square wave potential between -0.3 and +0.4 V and it induces the deposition of the redox-polymer and enzymes on the CFE surface due to ligand-exchange. Note, the used deposition-procedure was the mildest variant reported by Gao et al (2002), in order to prevent oxidizing the CFE, as this may generate a thick surface oxide layer, which will deteriorate the performance of the sensor. (2) Electrostatic complexation. The CFE was negatively charged by thiol-modification prior to the hydrogel-coating procedure (Calvo et al., 2001; Forzani and Solis, 2000). This negatively charged CFE surface attracts the cationic POs-EA, which will improve the molecular orientation within the hydrogel. Note, direct electron transfer side reactions of the CFE with HRP are not to be expected (Schumann, 2002), as the thiol monolayer will not display self-assembling properties with the CFE surface. (3) A combination of (1) and (2). The results were presented in Table 1. Although, the different groups of sensors displayed similar sensitivities for glutamate, the sensors of the thiol-modified group and, especially, the combined group showed significant less interference by AA. Moreover, the sensors of the combined group showed improvements at all determined characteristics. The result are explained by the fact that the different cross-link methods are complementary, and very likely they will facilitate each other. For example, the electrochemical deposition may be improved when the absorption of the POs-EA on the CFE is stimulated (Dequaire and Heller, 2002). In turn, the molecular orientation within the hydrogel layer will be improved as well (Calvo et al., 2001). It is likely that the covalent cross-linking between the individual compounds is also more effective when the driving force of POs-EA and enzymes to the CFE is stimulated.

Combination of different cross-link methods							
Cross-link method	**N**	**Sensitivity (pA/µM)**	**Interference (%)**	**Response time (sec.)**	**Current Density ($mA/M*Cm^2$)**	**Linearity (R^2)**	**Detection Limit (µM)**
Standard-method	15	3.4 ± 1.9	54.6 ± 17.1	22.3 ± 1.7	27.2 ± 17.4	0.991 ± 0.005	0.149 ± 0.083
Electropolymerisation	13	3.6 ± 1.8	49.4 ± 29.3	22.6 ± 2.4	24.1 ± 12.6	0.995 ± 0.004	0.130 ± 0.077
CFE thiol pretreatment	11	3.8 ± 1.6	32.7 ± 17.4 *	21.9 ± 2.0	31.4 ± 16.3	0.987 ± 0.004	0.119 ± 0.069
Combination CFE thiol pretreatment and electropolymerisation	11	3.4 ± 1.0	17.0 ± 8.2 *	19.6 ± 0.9 *	28.8 ± 8.2	0.998 ± 0.001 * #	0.100 ± 0.024

*Table 1: Performance of glutamate microsensors prepared with different cross-link methods. Shown are the sensitivity to glutamate (pA/µM), the interference (glutamate 100 µM + 200 µM AA; expressed in %), the response time (sec), the current density ($mA\ M^{-1}\ cm^{-2}$), linearity (R^2) and the detection limit (µM). The sensors in this experiment were not coated with an additional Nafion layer. Data were presented as mean ± SD. * Denotes a statistically significant difference with the standard method ($p < 0.05$; M-W Rank Sum test). # Denotes a statistically significant reduction in variability ($p < 0.05$; equal-variance test).*

Monitoring the layer thickness with amperometry

Despite the improvements in performance and reproducibility, the variability was still a matter of concern. Most likely this was caused by variation in the thickness of the hydrogel-coating, as indicated by microscopic inspection. It is likely that growing of the hydrogel-layer does not occur at a constant speed. Therefore, a constant time of dipcoating does not result in a uniform layer thickness. Interestingly, we have noticed that a thicker hydrogel-layer correlated with a higher Ohmic resistance of the microsensor. This provided us an empirical tool for controlling the layer thickness. To that end, the automated dipcoat technique was combined with amperometry. A 20 mV potential was applied between the thiol-pretreated microsensor and a reference electrode (that was placed in the coating solution). The applied potential induced a small current that, by connecting both electrodes to an amplifier, could be amplified and recorded. The noise pick-up was reduced by placing the whole set-up in a Faraday cage. During the application of the hydrogel, the current gradually decreased as a result of an increasing resistance of the growing hydrogel layer. In Fig. 7 a typical registration of the current (y-axis) in time (x-axis) during the dipcoating procedure is shown. A current was produced each time the microsensor was dipped into the mixture. This resulted in a spike pattern, which was dependent on the frequency of dipping.

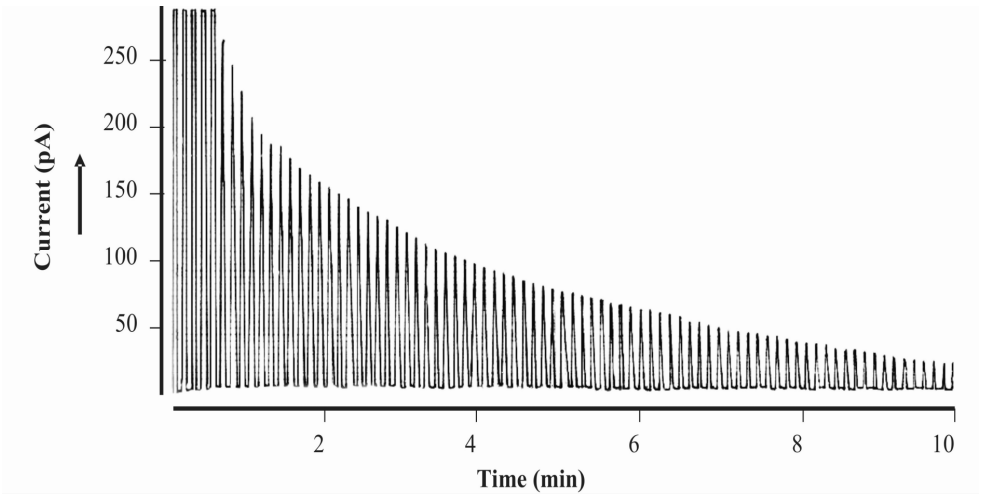


Fig. 7: Typical recording of the current (y-axis) during the dipcoating procedure, representing the growing hydrogel layer thickness in time.

In Fig. 8 the correlation between the finally measured current and the time of dipcoating (Fig. 8A), the sensitivity (Fig. 8B) and the interference by AA 200 µM (Fig. 8C) is shown for 31 sensors. The dashed lines in the graphs represented the best line of fit through the scatter-diagram. Interestingly, there was no correlation between the time of dipcoating and the finally detected current. This confirmed the idea that the speed of hydrogel-layer formation in time is not constant. An optimum in sensitivity, up to 7.5 pA/µM, was apparent between 400 and 280 pA. The interference by AA decreased gradually with an increasing layer-thickness and the lowest interference was seen between 250 and 150 pA.

As previously mentioned, the influence of the layer-thickness on an optimal performance of the microsensor is paradoxical. On one hand a high sensitivity with a low response time is required, which will reach an optimum at a thinner hydrogel layer. On the other hand a low interference by AA is required. The thicker the layer, the more AA-ox is included and the lower the interference by AA. It is concluded that controlling the layer thickness by recording the current during hydrogel application is a powerful tool to select sensitive sensors with a low interference for AA.

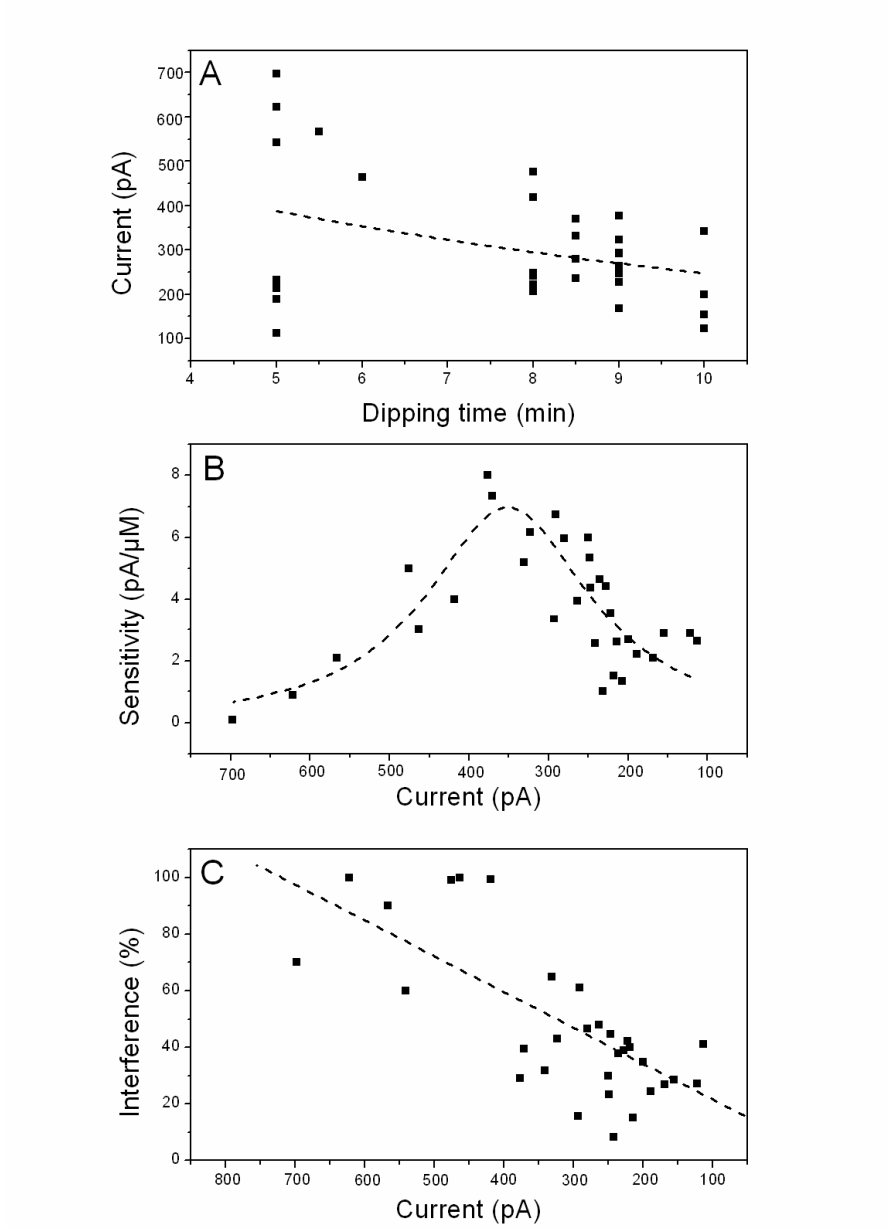


*Fig. 8: Correlation between the current measured at the end of the dipcoating procedure with (**A**) the time of dipcoating, (**B**) the sensitivity to glutamate and (**C**) the interference by AA (100 µM glutamate + 200 µM AA). The dashed line represents the best line of fit through the scatter diagram, respectively a linear fit (**A**,**C**) and Lorentzian fit (**B**).*

2.4 Conclusion

The aim of the present study was to improve the performance and reproducibility of the hydrogel-coated glutamate microsensor. It appeared that the final performance of the microsensor was determined to a large extent by its hydrogel layer. For a better control of the hydrogel coating procedure we have developed an automated dipcoater. The use of the dipcoater appeared to be of great benefit, as it allowed the control of the conditions during the coating procedure. Optimizing the conditions during the hydrogel application procedure and modifications of the cross-link procedure (combinations with electropolymerisation and electrostatic complexation) improved the performance and reproducibility of the microsensor significantly. In addition, a crucial factor in determining the microsensor properties appeared to be the thickness of the hydrogel layer. To monitor and control the thickness of this layer, the automated dipcoat technique was combined with amperometry. This approach resulted in the construction of reproducible microsensors with an optimal performance.