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Highly sensitive and selective glutamate microbiosensor based on cast polyurethane/AC-electrophoresis deposited multiwalled carbon nanotubes and then glutamate oxidase/electrosynthesized polypyrrole/Pt electrode

Malika Ammam*, Jan Fransaer

Department of Metallurgy and Materials Engineering (MTM), K.U Leuven, Kasteelpark Arenberg 44, B-3001 Heverlee, Belgium

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ABSTRACT

A highly sensitive and selective glutamate microbiosensor based on polypyrrole (PPy), multiwalled carbon nanotubes (MWCNT) and glutamate oxidase (GluOx) deposited on the transducer platinum electrode (Pt) is described. The sensor consists of a permselective membrane of polypyrrole for the rejection of interferences, followed by a layer of multiwalled carbon nanotubes and glutamate oxidase deposited by asymmetrical alternating current electrophoretic deposition (AC-EPD). The biosensor has a high sensitivity (3.84 nA/(μM mm^2)), low response to interferences such as ascorbic acid, uric acid and acetaminophen, a fast response time (7 s), low detection limit (~0.3 μM), a linear range of 140 μM and a satisfactory stability. In order to improve the linear range and the stability, a thin layer of polyurethane (PU) was applied to the Pt/PPy/MWCNT/GluOx sensor. The resulting sensor with the PU outer membrane showed an increase in the linear range up to ~500 μM glutamate and has a better stability at the expense of a decrease in sensitivity (2.5 nA/(μM mm^2)) and an increase in the response time (15 s).

1. Introduction

Glutamate is one of the major excitatory neurotransmitters in the brain and is believed to be responsible in learning, memory, neurodevelopment, biomaterial sensitivity, and synaptic plasticity (Martin et al., 2000; Umar et al., 2008). It is believed that some drugs, environmental pollutants, and food additives present, e.g. in soy sauce, chicken broth cubes, and soup base of instant noodles are responsible for excessive activation of glutamate receptors as well as glutamate release from neurons (Sitges et al., 2007; Szénási and Hársing, 2004). An increase in glutamate levels in the cerebrospinal fluids has been observed in neurological disorders, such as stroke, Alzheimer's, and Parkinson's disease (Bermeister et al., 2002; Hynd et al., 2004; Nedergaard et al., 2002; Belsham, 2001). Therefore, its determination is of interest to both the biological science and food industries. Various methods have been developed for glutamate determination based mainly on chromatography (Piepponen and Skujins, 2001; Kissinger, 1991) and capillary electrophoresis (Zhou et al., 1995; Lada and Kennedy, 1996; Lada et al., 1998). These methods are time-consuming and require expensive instruments.

In the last decade, the immobilization of glutamate oxidase or dehydrogenase on electrodes for the design of amperometric biosensors has been an area of intense research. Because glutamate dehydrogenase sensors require the addition of NAD^+ as a cofactor (Kuhr et al., 1993; Schubert et al., 1986; Nikoleis, 1987), the majority of glutamate sensors are usually based on glutamate oxidase (Hu et al., 1994; Tamiya et al., 1995; Cosnier et al., 1997; Berners et al., 1992; Zilkha et al., 1994; Niwa et al., 1996; Schuvailo et al., 2006), where the concentration of glutamate is measured by monitoring the formation of hydrogen peroxide from the glutamate oxidase-catalyzed oxidation of L-glutamic acid (glutamate) by dioxygen (Cooper et al., 1995):

$$\text{glutamate} + O_2 + H_2O \xrightarrow{\text{GluOx}} \text{2-oxoglutarate} + NH_3 + H_2O_2 \quad (1)$$

From the application point of view, the final goal of the biosensor technology lies in designing high-performance sensors with appropriate characteristics such as sensitivity, selectivity, response time, linear range, stability and reproducibility.

For the sensitivity, the key factor is the strategy employed for the immobilization of glutamate oxidase on the electrodes. Various strategies have been used including cross linking in glutaraldehyde (Udomsopagit et al., 1998) and entrapment in polymers or gels (Kwong et al., 2000; Oldenziel et al., 2004). One of the methods over-coming problems for depositing glutamate oxidase relies on electrochemical deposition of the enzyme,

* Corresponding author at: Department of Metallurgy and Materials Engineering (MTM), K.U Leuven, Kasteelpark Arenberg 44, Bus 2450, B-3001 Heverlee, Belgium. Tel.: +32 16 321260; fax: +32 16 321991.
E-mail address: Malika.Ammam@mtm.kuleuven.be (M. Ammam).

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because of the ease and control of the manufacturing process. The electrochemical fabrication of sensors usually involves a step of electropolymerization used to eliminate interferences such as ascorbate, urate and acetaminophen. The electrochemical fabrication of enzyme–polymer based biosensors can be accomplished in two different ways: (i) A polymer layer is formed directly on the electrode by electropolymerization of monomers such as terthiophene-3-carboxylic acid or diaminobenzene before the enzyme deposition (Kwon et al., 2005; Na Nakorn et al., 2003). (ii) The polymer is electropolymerized after the enzyme deposition (Cooper and Pritchard, 1993; Cooper et al., 1995). It is well known that the presence of polymers in the enzyme film is a very efficient means for the elimination of interferences, e.g. high selectivity. However, usually, this results in a sensor of moderate activity especially in the case of glutamate oxidase, where the employed enzyme activity is very low. The sensitivity of these sensors varied between 1.35 pA/(μM mm^2) and 1.46 nA/(μM mm^2).

Previously, we described a glucose sensor based on the asymmetrical alternating current electrophoretic deposition (AC-EPD) of glucose oxidase on the transducer platinum electrode from concentrated solutions (50 mg enzyme/0.5 mL solution), and have shown that the sensor is able to function with only the deposited enzyme layer (Ammam and Fransaer, 2009a,b). Because the deposited enzyme layer is thick and compact, the sensor yields a high sensitivity and reasonable selectivity. Compared to glucose oxidase, glutamate oxidase is a very expensive enzyme. The manufacturing of a glutamate sensor using the same enzyme concentration as used for the glucose sensor would cost about $30k (50 mg GluOx/0.5 mL solution). On the other hand, preliminary experiments showed that AC-EPD of glutamate oxidase from solutions with very low enzyme concentration (0.2 mg GluOx/0.5 mL solution) yields a high current response towards glutamate. Unfortunately, practically no exclusion of interferences was noticed in this case.

In this paper, we developed a strategy to manufacture a highly sensitive and selective glutamate sensor. Polypyrrole (PPy) was used as a permselective membrane for the rejection of the interferences. Films are grown under electrochemical control from aqueous buffered solution at physiological pH. The second step in the sensor construction involves deposition of a controlled layer of multiwalled carbon nanotubes (MWCNT) by AC-electrophoresis from an aqueous suspension. This layer is used as a support for enzyme deposition and increasing the effective surface area of the sensor, which leads to an increase in the sensitivity. The third step involves the deposition of glutamate oxidase(GluOx) on the previous deposited carbon nanotubes by means of AC-EPD. In order to increase the stability and the linear range of the sensor, a final step involves the application of a thin polyurethane (PU) outer membrane.

2. Experimental

2.1. Materials

Ultrapure water milliQ grade with a resistance of 18.2 MΩcm was used for all the experiments. L-Glutamate oxidase (GluOx) recombinant, expressed in *Escherichia coli*, lyophilized powder, $\geq$5 units/mg and L-glutamic acid or glutamate (Glu) $\geq$99.5% was purchased from Sigma–Aldrich. Multiwalled carbon nanotubes (MWCNT) (diameter 30 $\pm$ 1.5 nm and length < 1 μm) $\geq$95% were purchased from www.CheapTubes.com. Pyrrole (Py) reagent grade 99% was obtained from Acros. L-ascorbic acid (AA) 99% from Acros, uric acid (UA) 99% and acetaminophen (AP) were purchased from Sigma–Aldrich. Phosphate salts (NaH_2PO_4 and Na_2HPO_4) and sodium chloride analytical grade were purchased from Acros Organic. The buffered saline pH 7.4 was prepared from phosphate salts (0.1 M) and sodium chloride (0.15 M). Polyurethane selectophore grade for biosensors and chemosensors was purchased from Sigma–Aldrich. Platinum (Pt) wire 250 μm in diameter is used for the sensor preparation. It is 99.99% pure purchased from Goodfellow.

2.2. Equipment

A potentiostat (CMS 100, GAMRY) connected to a computer was used for the electrodeposition of pyrrole and for the testing of the sensors (amperometry). AgCl/Ag was used as a reference electrode. For the testing of the sensors, the polarization was set at +0.6 V vs. AgCl/Ag.

The setup used for the AC-EPD of MWCNT and GluOx enzyme was previously described (Ammam and Fransaer, 2009a,b). Briefly it consisted of an arbitrary wave form generator (ww5061, Tabor electronics) connected to a bipolar high-voltage operational amplifier (BOP 1000 M, Kepco). The applied wave form was monitored using a digital oscilloscope (Explorer III oscilloscope, Nicolet Instrument Corporation). The AC-signal is composed of two triangular waves of opposite amplitude but with different height and duration. The half-cycle with high amplitude section and low duration time corresponds to the negative part of the AC-signal. However, the area of both triangular waves is equal, so that the signal has no net DC component. The outputs of the amplifier are connected to a small electrochemical cell with a capacity of 1 mL containing the biosensor electrode and a platinum counter electrode. The biosensor electrode is connected to the electrode whose polarity with respect to the counter electrode changes positively for a longer time at low amplitude and becomes negative for a short duration at high amplitude as previously demonstrated (Ammam and Fransaer, 2009a).

Scanning electron microscopy (SEM) was carried out using an XL 30 FEG from FEI.

2.3. Sensor preparation

A 4 mm long platinum wire (250 μm diameter) was connected to an insulated copper wire to provide electrical connection. A 4 mm shrink tube was used to insulate the copper–platinum junction. The platinum end was cut and adjusted to 1 mm length, and the tip was sealed with acrylic glue yielding an active sensing area of $\sim$0.78 mm^2. The sensing cavity was cleaned by dipping in a mixture of nitric acid (7%) and hydrogen peroxide (30%) for a few seconds, and then rinsed abundantly with ultrapure water and acetone. The procedure for preparing a GluOx sensor is shown in Fig. 1A.

Step 1. The Pt electrode was immersed in 1 mL phosphate buffer solution pH 7.4 containing 6 μL pyrrole; the polarization was set at +1.1 V vs. AgCl/Ag for 25 min. The sensor was then removed and rinsed with ultrapure water.

Step 2. The Pt/PPy was immersed in 0.5 mL MWCNT suspension prepared as follows: 5 mg of MWCNT were dissolved in 0.5 mL ultrapure water and the mixture was ultrasonically stirred for a few seconds. Thereafter, an asymmetrical AC-signal at 30 Hz frequency and 500 V_{p-p} amplitude was applied to the Pt/PPy electrode for 4 min during which a layer of MWCNT was deposited on the PPy layer. The sensor electrode was then removed and rinsed with ultrapure water.

Step 3. Pt/PPy/MWCNT was then immersed in the GluOx solution (0.2 mg GluOx/0.5 mL ultrapure water) and the electrophoretic deposition of the enzyme was carried out at 30 Hz and 250 V_{p-p} for 30 min. Next, the sensor was rinsed with ultrapure water and then tested in 5 mL phosphate buffer solution pH 7.4 by injecting 10 μL of uric acid (250 μM), ascorbic acid (250 μM), acetaminophen (250 μM) and several injections of glutamate (20 μM).

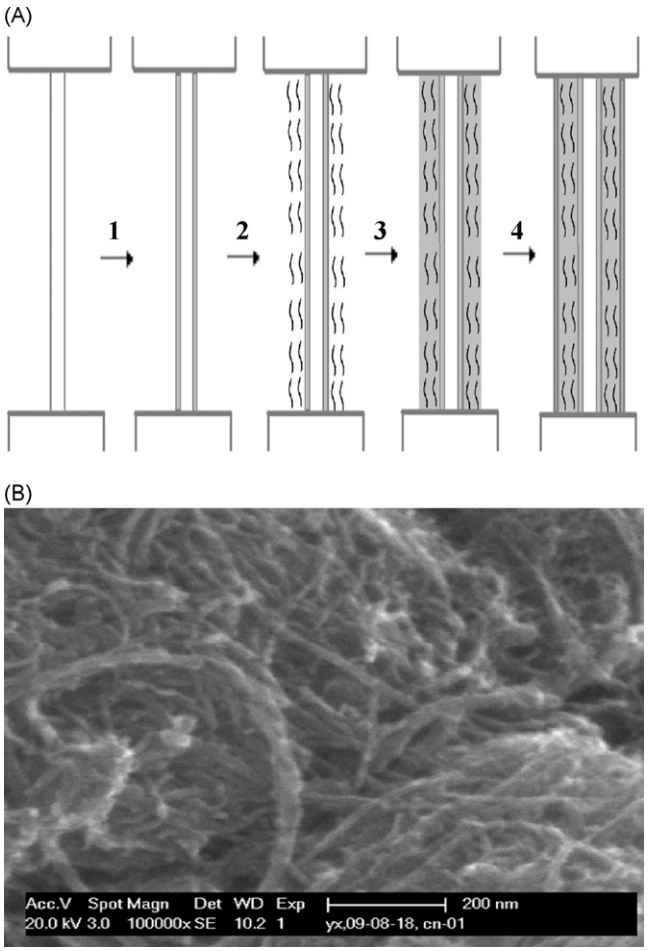

Fig. 1. (A) Schematic representation of the glutamate sensor preparation. Step 1: electropolymerization of polypyrrole; step 2: AC-EPD of MWCNT; step 3: AC-EPD of GluOx; step 4: spray coating with polyurethane outer layer. (B) Typical SEM image of the platinum electrode modified with PPy/MWCNT/GluOx.

Step 4. The sensor with an outer layer of polyurethane (PU) is prepared following the procedure: after the preparation of the sensor Pt/PPy/MWCNT/GluOx, the sensor electrode is rinsed with ultrapure water and air dried. Next, a polyurethane membrane was applied as follow: under constant stirring, 13.4 g of tetrahydrofurane (THF) extra dry (water < 50 ppm) and 0.24 g of dimethylformamide (DMF) were added to 0.424 g of polyurethane. Next, the mixture is stirred for a few minutes and transferred to a small perfume bottle which was used as a spraying device. The sensor electrode Pt/PPy/MWCNT/GluOx prepared previously is then fixed at a distance of 15 cm from the spraying bottle and sprayed three times with the freshly prepared PU mixture. The sensor electrode is left to dry at room temperature for at least 24 h. The number of sprays and the time between successive sprays are important features. The time between successive sprays should be as short as possible.

3. Results and discussion

3.1. AC-EPD of GluOx

We have previously reported a highly sensitive glucose biosensor based on AC-EPD of glucose oxidase deposited on the transducer platinum electrode (Ammam and Fransaer, 2009b). The glucose sensor consists only of an enzyme layer deposited by asymmetrical alternating current electrophoretic deposition. The high sensitivity of the sensor was attributed to the deposition of a thick and compact enzyme layer of about 7–10 μm thickness. It is common knowledge that the enzyme might dissolve if it is deposited on the electrode surface without additional stabilizers. To prevent this from happening, several procedures have been reported in literature such as cross linking with glutaraldehyde (Geise et al., 1991) and cross linking by electropolymerization (Chen et al., 2002). The mechanisms of enzyme deposition under AC-polarization are not yet fully understood. Therefore, it is possible to suggest that the insolubility of the thick enzymatic layer deposited by means of AC-EPD is related to the small amount of the generated AC-electrolysis, which denaturalizes a small amount of the deposited enzyme. Other scenarios such as possible electropolymerization within the denaturized enzyme under AC-polarization are also possible. This may hold or cross link the active enzyme, thus preventing the enzyme layer from wandering away. On the other hand, because the enzyme layer is thick and compact, it screens out a substantial part of the interferences, leading to a reasonable selectivity without using any permselective membrane. The optimal concentration for the manufacturing of the glucose sensor was found to be 50 mg/0.5 mL solution. The use of similar concentrated solution to manufacture a glutamate biosensor would be prohibitively expensive. On the other hand, we have observed that using low enzyme concentration (0.2 mg GluOx/0.5 mL solution) results in a high current response to glutamate compared to other electrochemical deposition methods (Chen et al., 2002), but does not screen out the interferences. Fig. 2 shows the comparison between current–time curves of GluOx deposited by means of AC-EPD from ultrapure water at 30 Hz, 250 V_{p-p} for 30 min (Fig. 2A) and GluOx electrodeposited from phosphate buffer solution pH 7.4 at +1.3 V vs. AgCl/Ag for 90 min (Fig. 2B). As can be seen, the current response to 20 μM glutamate injection is nearly 600 times larger for the enzyme deposited by AC-EPD compared to conventional electrodeposition even though the deposition time is three times smaller. However, the selectivity is bad as practically no decrease in the current response to the injection of 250 μM uric acid, ascorbic acid or acetaminophen is observed compared to the bare Pt electrode as shown in Table 1A. This is due to the limited thickness of the deposited enzyme layer caused by the low enzyme concentration (0.2 mg GluOx/0.5 mL solution) used in this study, which is 250 times lower than the concentration used for the manufacturing of the glucose sensor reported previously (Ammam and Fransaer, 2009a,b).

3.2. Pt/PPy/MWCNT/GluOx electrode

In order to keep the advantages of the AC-EPD process for GluOx deposition, our strategy was to deposit first a permselective membrane for the rejection of interferences, then deposit a layer of GluOx by means of AC-EPD as previously described. Our results showed that AC-EPD of MWCNT and GluOx were greatly influenced by the monomer employed. We found polyphenol very selective for interference rejection, but it lowers the AC-EPD rate of MWCNT and GluOx. With polyphenol, a typical non-conducting polymer (Eddy et al., 1995), a substantial part of the applied electric field is screened out by the membrane, leading to a decrease in the particle electrophoresis. Using polypyrrole which has a high electrical conductivity (Ateh et al., 2006) as permselective membrane, we have observed an enhancement in the rate of AC-EPD of MWCNT and GluOx. The selectivity of the polypyrrole film is greatly influenced by the deposition time, e.g. film thickness. Table 1B shows the current response to 250 μM ascorbate and hydrogen peroxide injections as a function of the deposition time of polypyrrole. Thicker films are known to exclude a high amount of interference, but they will also lower sensitivity to the analyte, e.g. hydrogen peroxide issued from the enzymatic conversion of

Table 1
(A) Current response to 20 μM glutamate and 250 μM interferences (AP, UA, AA) on the bare platinum electrode (control) and a platinum electrode modified with glutamate oxidase using AC-EPD at 30 Hz, 250 V_{p-p} for 30 min. (B) Current response to 250 μM ascorbic acid and 250 μM hydrogen peroxide as a function of deposition time of polypyrrole.

(A)

Analyte	20 μM Glu	250 μM AP	250 μM UA	250 μM AA
I (nA) (Pt)	0	532.2	112.5	1050.3
I (nA) (Pt/GluOx)	63.6	478.3	91.7	873.8

(B)

PPy deposition time (s)	Response to 250 μM H_2O_2 (nA)	Response to 250 μM AA (nA)
0	1878.3	1050.3
200	388.1	35.2
400	274.6	13.7
1000	241.1	0
1500	184.2	0

glutamate by GluOx (Eq. (1)). As a matter of fact, it can be seen from Table 1B that with increasing deposition time, the current response to ascorbate and hydrogen peroxide decreases. Using a deposition time of 1500 s, the response to the interference is zero,

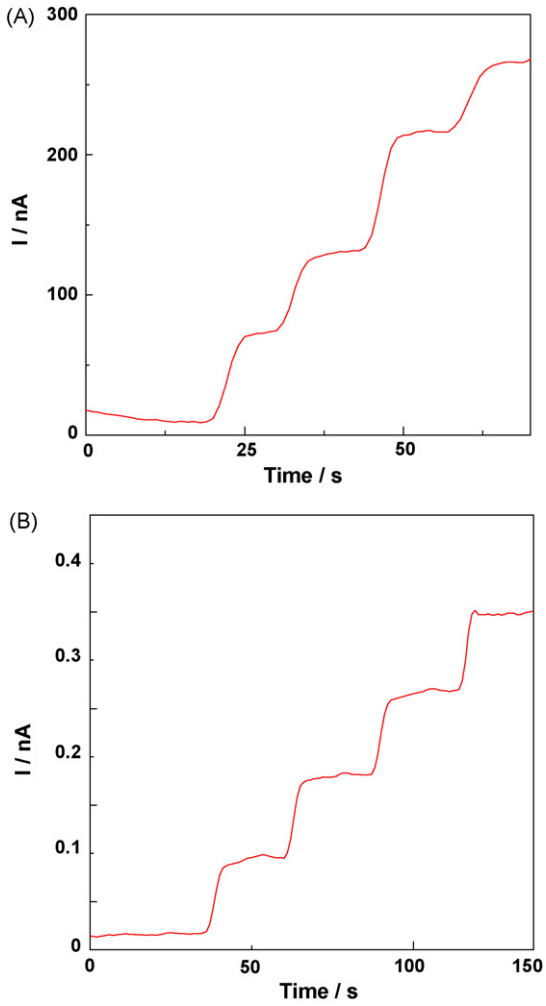

Fig. 2. Current response to successive injections of 20 μM glutamate (A) platinum electrode 0.78 mm^2 modified with GluOx deposited by AC-EPD process at 30 Hz, 250 V_{p-p} for 30 min (0.2 mg GluOx/0.5 mL ultrapure water) and (B) platinum electrode modified with GluOx by electrodeposition at +1.3 V vs. AgCl/Ag for 90 min (0.2 mg GluOx/0.5 mL buffer solution, pH 7.4).

but the current response to hydrogen peroxide is still significant. With only Pt/PPy, it is observed that complete screening of ascorbate is obtained with deposition times lower than 1000 s. However, with the Pt/PPy/MWCNT/GluOx sensor, it is observed that 1000 s deposition time is not enough to screen out all the interferences, and the deposition time should be at least 1500 s. This implies that part of the deposited PPy might be dissolved during the AC-EPD of MWCNT and GluOx.

In order to "compensate" the decrease of the current response to hydrogen peroxide due to presence of the polypyrrole inner membrane, a layer of MWCNT was deposited on top of the polypyrrole inner layer using AC-EPD. This layer is used as a support for GluOx deposition, and because MWCNT increase the sensor surface area, this will boost the sensitivity to glutamate. For AC-EPD of MWCNT and GluOx, applied parameters such as deposition time, applied amplitude and frequency have been shown to influence the sensor response to glutamate. AC-EPD of MWCNT from ultrapure water is observed to be optimal at 30 Hz, 500 V_{p-p} and deposition times of 3–4 min. On the contrary, for much longer deposition times, a decrease in the current response to glutamate was observed. In other words, if the MWCNT layer is thick, the GluOx will not be fully accessible to deposit on all of the MWCNT, but will probably be limited to the last layer of the deposited MWCNT. The morphology of the Pt/PPy/MWCNT/GluOx electrode is shown in Fig. 1B. The image indicates that the MWCNT are uniformly distributed on top of the Pt/PPy electrode exhibiting a three-dimensional structure. This uniform structure of the MWCNT significantly increases the effective surface area of the sensor for GluOx deposition, thus increasing the sensitivity. The optimal parameters for glutamate oxidase deposition were found to be 30 Hz and 250 V_{p-p}. For GluOx, longer deposition times are required because this permits the maximum deposition of enzyme on the MWCNT, leading to a higher current response. On the other hand, no difference was observed between the morphology of Pt/PPy/MWCNT and Pt/PPy/MWCNT/GluOx electrodes determined by SEM as was seen for glucose oxidase, where a thick layer could be distinguished even at low magnifications (Ammam and Fransaer, 2009a). This is due to the much lower GluOx concentration used in this study.

The deposition of the three layers (PPy, MWCNT and GluOx) under the parameters listed above gives the highest ratio of enzyme activity (measured by the current response due to peroxide oxidation issued from the injected glutamate following Eq. (1)). As shown in Fig. 3A, current responses to glutamate up to 3.84 nA/(μM mm^2) have been observed using only 0.2 mg GluOx dissolved in 0.5 mL ultrapure water. This sensitivity is higher than most of the reported glutamate oxidase sensors, where the sensitivity varies between 1.35 pA/(μM mm^2) and 1.46 nA/(μM mm^2) (Kwon et al., 2005; Na Nakorn et al., 2003; Cooper and Pritchard, 1993; Cooper et al., 1995). This would be attributed mainly to the uniform distribution of the deposited MWCNT and to the high amount of the GluOx deposited by AC-EPD. As can be seen from Fig. 3A, thanks to the PPy inner layer, the electrode has negligible response to 250 μM UA, AA and AP. Oxidizable species such as ascorbate, urate and acetaminophen can interfere with the detection of the hydrogen peroxide produced by the enzymatic reaction. The selectivity of a biosensor is measured by the ratio of the current response to glutamate to the current response to interferences. From Fig. 3A, it will be noted that ratio of the response I_{Glu}/I_{interf} is ~70, which is due on the one hand to the high enzyme activity of glutamate oxidase deposited on the carbon nanotubes using AC-EPD process and, on the other hand, to the exclusion of interferences by the polypyrrole inner membrane. The response time of the glutamate sensor prepared in this study is less than 8 s (Fig. 3A), suggesting that the electrode responds rapidly to the change of the analyte concentration. Fig. 3A also shows that the GluOx sensor is linear up to 140 μM and attains a saturation level at higher concentrations as expected by Michaelis–Menten type

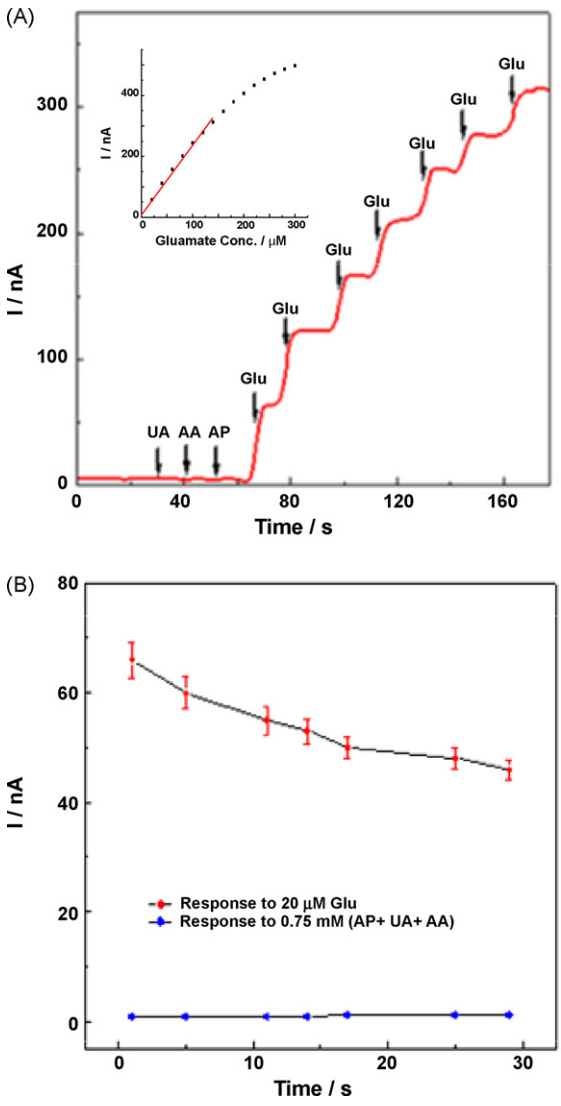

Fig. 3. (A) Typical example of the current response of a Pt/PPy/MWCNT/GluOx electrode to 250 μM AP, UA and AA and successive injections of 20 μM glutamate (PPy: 1500 s at +1.1 V vs. AgCl/Ag from buffer solution pH 7.4; MWCNT: 4 min at 30 Hz and 500 V_{p-p} from MWCNT/ultrapure water suspension; GluOx: 30 min at 30 Hz and 250 V_{p-p} from GluOx/ultrapure water solution). (B) Stability of this electrode over a period of 4 weeks.

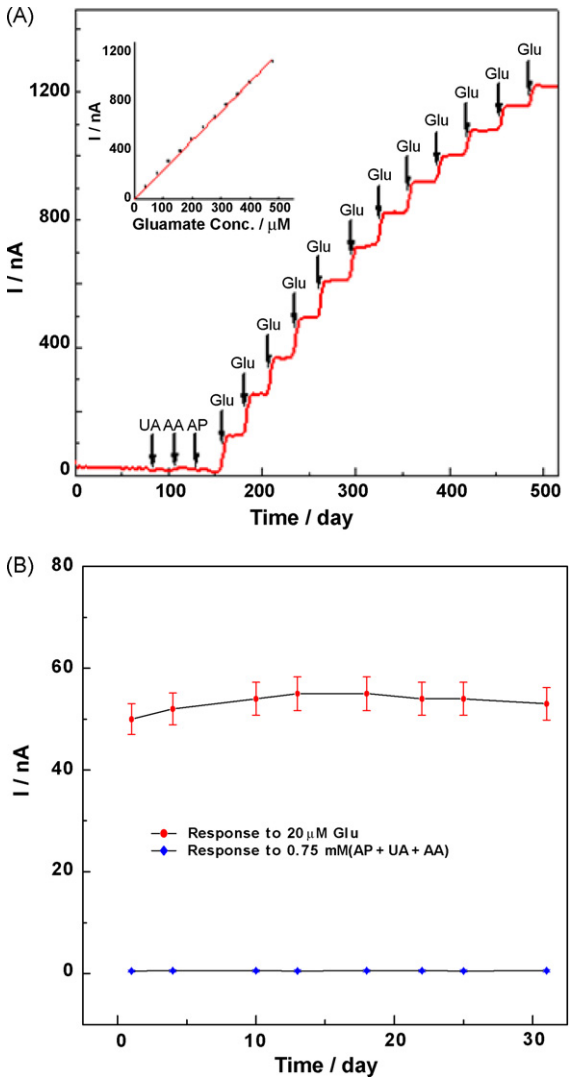

Fig. 4. (A) Typical example of the current response of a Pt/PPy/MWCNT/GluOx/PU sensor to 250 μM AP, UA and AA and successive injections of 40 μM glutamate (PPy: 1500 s at +1.1 V vs. AgCl/Ag from buffer solution pH 7.4; MWCNT: 4 min at 30 Hz and 500 V_{p-p} from MWCNT/ultrapure water suspension; GluOx: 30 min at 30 Hz and 250 V_{p-p} from GluOx/ultrapure water solution; PU: 3 sprays). (B) Stability of this sensor over a period of 4 weeks.

enzyme kinetics (Leskovac, 2003). The detection limit of the present glutamate sensor is less than 0.3 μM (S/N = 3). Table 2 gathers the main characteristics of the sensor shown in Fig. 3A.

The stability of this biosensor is explored. Fig. 3B shows the response of the Pt/PPy/MWCNT/GluOx electrode to 20 μM glutamate and 750 μM interferences for a period of 30 days. The sensor is stored under air at room temperature and the stability is checked on a regular basis. It is important to remind that the same sensor was tested each time. It will be noted that the response to glutamate is decreasing continuously and after 1 month the sensor lost ~30% of its original response. This can be attributed to the dissolution of the deposited MWCNT and enzyme. The response to the interferences remained constant during this period. This shows that the polypyrrole layer is stable and does not deteriorate.

Table 2
Main characteristics of the glutamate sensor without PU outer membrane shown in Fig. 3A.

Area	0.78 mm^2
Sensitivity	3.02 ± 0.04 nA/μM Glu
Linear range	0.3–140 μM Glu
Response time	7 ± 1 s
Interferences (AP + UA + AA)	0.93 ± 0.05 nA/750 μM
Detection limit	<0.3 μM Glu

3.3. *Pt/PPy/MWCNT/GluOx electrode with a mass transfer-limiting outer membrane of polyurethane*

In order to optimize the linearity, stability and biocompatibility (for eventual in vivo applications), an outer membrane of polyurethane (PU) was applied just after the deposition of the active layers (PPy/MWCNT/GluOx). An important benefit of using the PU outer layer is that the rate of the enzymatic reaction is not controlled by enzyme kinetics, rather by the flux of substrate concentration entering the enzyme layer (Wilson and Johnson, 2008; Wilson and Ammam, 2007). Fig. 4A shows a typical current-time curve for a biosensor with a thin PU outer membrane (3 PU sprays) upon the injection of 250 μM AP, UA and AA and successive injections of 40 μM glutamate. The response to 250 μM AP, UA and AA is essentially zero. Besides the polypyrrole inner layer, the PU outer

layer also rejects part of the interferences, leading to a further decrease of the response to the interferences. Fig. 4A also shows that the presence of the PU layer makes the response to glutamate linear up to ~500 μM. Without PU outer layer and at high glutamate concentrations (>140 μM, Fig. 3A), the oxygen supply is not sufficient to support the enzymatic reaction shown in Eq. (1). This leads to the moderate linear range observed in Fig. 3A. The deposition of the outer PU membrane on top of the enzyme layer reduces the flux of glutamate reaching the enzyme layer, whereas the flux of oxygen is not materially affected. This leads to an excess of oxygen in the enzyme layer and a glutamate response that is independent of oxygen over a wide range. This results in an increase in the linear range at the expense of a decrease in the sensitivity which dropped from 3.84 nA/(μM mm^2) for the sensor without PU outer layer (Fig. 3A) to 2.5 nA/(μM mm^2) for the sensor with PU membrane (Fig. 4A). However, this sensitivity is still higher than most glutamate sensors reported in literature. The presence of the PU outer layer increases also the response time to glutamate, which rises from 7 s (Fig. 3A) to about 15 s (Fig. 4A). This was understandable because of the slow diffusion of glutamate through the PU outer layer to reach the deposited enzyme (Wilson and Johnson, 2008; Wilson and Ammam, 2007).

The application of the outer membrane has a strong influence on the stability of the sensor. As can be seen in Fig. 4B, the response to glutamate shows a small initial increase before reaching a constant value. This can be explained by a slight change in the pore size of the outer PU layer during the first few days. Thereafter, the morphology of the PU layer remains constant, leading to a constant response. Apparently, the thin PU outer layer prevents the dissolution of the deposited MWCNT and enzyme, thus providing a good stability. Finally, the reproducibility of the complete biosensor response can be estimated ~90%. The good reproducibility of the sensors is due to the reproducibility of the manufacturing process. As a matter of fact, the four deposited layers are entirely automated.

4. Conclusions

In this study we developed a fabrication process of a highly sensitive and selective glutamate oxidase sensor. The sensor consists of an electropolymerized polypyrrole inner membrane on the transducer platinum electrode for the rejection of interferences followed by a layer of multiwalled carbon nanotubes then glutamate oxidase deposited by AC-EPD process. The resulting Pt/PPy/MWCNT/GluOx sensor has a high sensitivity towards glutamate (3.84 nA/(μM mm^2)), low interference from endogenous interferences, fast response time (<8 s), a linear range of 140 μM and reasonable stability. In order to increase the linear range and the stability, a thin outer layer of polyurethane is deposited which extends the linearity to 500 μM glutamate and the lifetime of the sensor. Overall, high sensitivity, excellent selectivity, linearity and long-time stability exhibited by the complete sensor with the polyurethane outer layer make it a good candidate for glutamate measurements.

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