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Short communication

On-line microdialysis system with poly(amidoamine)-encapsulated Pt nanoparticles biosensor for glutamate sensing *in vivo*

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ABSTRACT

In this work, an amine-terminated poly (amidoamine) dendrimer containing Pt nanoparticles (PAMAM/Pt) nanocomposite was synthesized and a novel amperometric H_2O_2 biosensor based on PAMAM/Pt and MWCNTs was developed. The resulting film of MWCNTs/PAMAM/Pt was characterized by transmission electron microscopy (TEM), linear sweep voltammetry (LSV) and amperometric *i–t* curve. It demonstrates excellent electrocatalytic responses toward the reduction of H_2O_2 at −200 mV (vs.SCE) without HRP participation. Immobilized with glutamate oxidase (GlutaOx), an effective glutamate biosensor, was fabricated, and the *in vivo* detection for glutamate was realized combining with the on-line microdialysis system. The glutamate biosensor showed good linear range from 1.0 μM to 50.0 μM with the detection limit of 0.5 μM (S/N = 3). The basal level of glutamate in the striatum of rat was detected continuously with this on-line system and was calculated to be 5.80 ± 0.12 μM (n = 3). This method was proved to be sensitive and selective and may be feasible in the further application of physiology and pathology.

1. Introduction

As an important neurotransmitter in the function of mammalian central nervous system, glutamate has been heavily implicated in many neurological disorders, such as schizophrenia, Parkinson's disease and epilepsy [1–3]. Thus, precise monitoring of glutamate in the extracellular space of brain would contribute significantly to the fundamental understanding of the role of glutamate in these disorders. The task is actually full of challenge because the concentration of glutamate in extracellular space is in the range of 4 and 350 μM [4], far below that of AA, which exists at higher concentrations (100–500 μM) [5], becoming the biggest interference in the detection of glutamate. Among various detection methods for glutamate [6], the employment of an electrochemical enzymatic biosensor has been proved to be a suitable approach and has been widely used with microdialysis technique [7,8]. Due to its short analytical time, high sensitivity and specificity to provide near real-time measurements, microdialysis sampling can provide both the temporal and chemical information that we need to fully elucidate the biochemical processes [9].

With its various intrinsic properties, including highly ordered structure, narrow size distribution, uniform dense terminal functional groups and porosity [10], dendrimers have been used as templates for the synthesis of encapsulated nanoparticles (NPs). Poly (amidoamine) (PAMAM), in particular, has been widely used to prepare and accommodate catalytic nanoscale metallic particles as Crooks and co-workers demonstrated [11,12]. The ability of high-generation PAMAM dendrimers to host Pd, Au, Ag, and Pt NPs is attributable to the spherical structure of the dendrimer, which contains void spaces and functional groups that are able to complex with metal ions [13,14].

In this work, we prepared the PAMAM-encapsulated Pt/MWCNTs based glutamate biosensor and an on-line microdialysis system for glutamate determination *in vivo* was constructed as well. The PAMAM/Pt nanocomposite realized the mediatorless on-line detection of glutamate at a low potential of −200 mV (vs. SCE) in the striatum of rats. The performance of the glutamate biosensor with respect to sensitivity, stability, selectivity, linear range, detection limit and the construction of on-line microdialysis system was presented and discussed herein.

2. Experimental

2.1. Reagents and apparatus

Amine-terminated PAMAM of G 3.0 was a gift from Polymeric Chemistry and Physics, Department of Chemistry of East China Normal University (Shanghai, China). H_2PtCl_6 was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Glutamate oxidase (GlutaOx; EC 1.4.3.11, 8.0 units/mg) and glutamate were obtained from Sigma Co. Artificial cerebrospinal fluid (aCSF: 126 mM NaCl, 2.4 mM KCl, 0.5 mM KH_2PO_4, 0.85 mM $MgCl_2$, 27.5 mM $NaHCO_3$, 0.5 mM Na_2SO_4, 1.1 mM $CaCl_2$, pH 7.4) was prepared with doubly

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distilled water, filtered (filter pore size 0.22 μm, Millipore, Bedford, MA) and used as perfusion solution for on-line measurement. All other solutions were prepared with doubly distilled water.

TEM images were taken on JEOL 2010 operating at 200 KV. Off-line and on-line electrochemical experiments were performed on CHI 832 electrochemical workstation (CH Instruments) with a bare or modified glassy carbon electrode (GC) as working electrode, a platinum wire as counter electrode and a saturated calomel electrode (SCE) as reference electrode. The thin layer electrochemical flow cell, with an inner volume of 0.085–0.34 μL, consisted of a thin layer flow block with a 25-μm gasket, GC (3 mm in diameter) as working electrode, stainless steel as counter electrode, and SCE as reference electrode and was integrated with microdialysis to form an on-line analytical system. Standard solutions or brain microdialysates were delivered from gas-impermeable syringes (BAS) and pumped through a liquid switch (CMA 110, Solna, Sweden) by a microdialysis pump (CMA 402, Solna, Sweden) at 2.0 μL/min.

2.2. Preparation of the GlutaOx/MWCNTs/PAMAM/Pt/Nafion biosensor

PAMAM-templated Pt NPs was prepared by a simultaneous addition of 0.3 mM of PAMAM and 0.3 mM of H_2PtCl_6 into 2.5 mL water under vigorous stirring for 10 min, followed by a single reduction step using formic acid as the reducing agent until the color of the solution changed from colorless to pale dark. The solution was kept stirring for 3 h to make sure that Pt^{4+} was reduced completely. The as-prepared PAMAM/Pt nanocomposite was kept at 4°C before use.

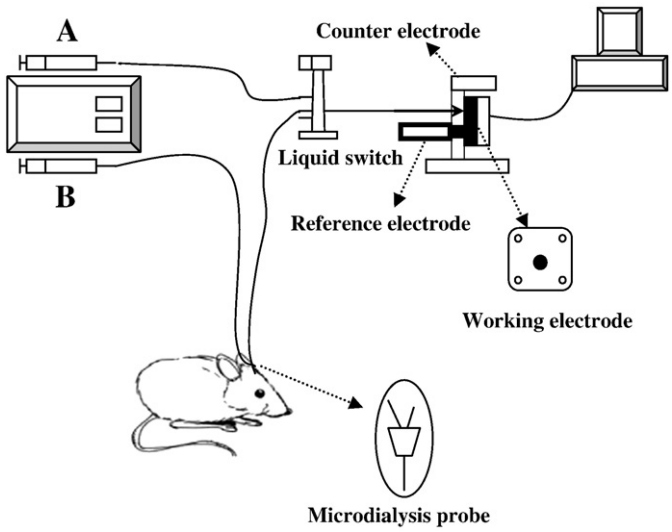
To prepare the glutamate biosensor, 1 μL of 1 mg mL^{-1} MWCNTs-DMF suspension solution was firstly dropped onto the surface of cleaned GC and allowed to dry at ambient temperature. Then, 4 μL of the PAMAM/Pt nanocomposite was fabricated on the surface of MWCNTs-coated GC. After drying, 2.0 μL of GlutaOx was mixed with 0.5 μL of 0.4 polyethyleneimide (PEI) solution and then 2.0 μL of the mixture was dropped onto the electrode. PEI was employed to stabilize the enzyme. After being air-dried for 3 h, the prepared electrode was cross-linked in a closed vessel containing 25% glutaraldehyde and water vapor for 30 min. Finally, 2.5% Nafion was dropped onto the electrode to prevent the potential interferences. The electrode was dried at room temperature for 2 h, and stored at 4 °C until use.

2.3. Animal surgical procedures

Male Sprague–Dawley rats (weight ranges from 200 to 250 g) were purchased from Shanghai SLAC Laboratory animal Co. Ltd and acclimatized for 4 days. Then the rats were anesthetized with chloral hydrate (initial dose of 300 mg/kg (i.p.) with additional doses of 50 mg/kg (i.p.) as needed to maintain anesthesia) and wrapped in a homeothermic blanket (Beijing Tide-Gene Biotechnology Development Center). The rats were placed in a stereotaxic frame (Beijing Tide-Gene Biotechnology Development Center) with the incisor bar set at 5 mm above the interaural line and appropriately placed holes were drilled through the skull. The microdialysis probe (CMA/110/111 Tub) was implanted in the striatum at the site of 2.5 mm anterior to bregma, 2.5 mm lateral from midline, and 7.0 mm below dura. In order to reduce the injury to the rat, the microdialysis probe should be implanted into the striatum of rats slowly within 30 min with special care.

2.4. In vivo experimental design

The *in vivo* experiment was performed as Scheme 1 shows. Firstly, aCSF solution in syringe A was pumped into the on-line microdialysis system at 2.0 μL/min and flowed to the thin layer electrochemical flow cell. After a stable baseline was achieved, the syringe selector was switched to syringe B. Several minutes later, the current started to increase slowly and then reached a plateau, which was ascribed to the biosensing of glutamate in the striatum of rat. Then we switched the selector to syringe A again. The current response started to decrease again and returned to the previous level of background current within several minutes.



Scheme 1. Schematic presentation of the on-line microdialysis system with glutamate oxidase modified biosensors for continuous monitoring of glutamate.

Before on-line electrochemical measurements, the microdialysis probe was implanted into the striatum of rats and perfused with aCSF solution at 2.0 μL/min for at least 90 min for equilibration and the injury recovery. The on-line electrochemical biosensor was calibrated with standard solutions of glutamate before and after on-line measurements to ensure there is no obvious change in the sensitivity after the measurements of the brain microdialysis.

Basal level of glutamate in the striatum of rats was expressed as absolute concentrations and the data were presented as means ± S.D. ($n = 3$). Three rats at least were employed for *in vivo* experiments.

3. Results and discussion

3.1. Characteristics of PAMAM/pt and MWCNTs/PAMAM/pt nanocomposites

Fig. 1A and B depict the typical TEM images of PAMAM/Pt and MWCNTs/PAMAM/Pt, respectively. The as-prepared dendrimer- encapsulated Pt NPs shown in Fig. 1A appeared as spherical shapes with the diameter ranging from 3 to 10 nm, most of which corresponds to 5 nm. Pt NPs were equably dispersed in PAMAM, which may result in a large effective Pt surface area and good electrocatalytic property for the electroactive molecules. Fig. 1B shows the surface morphology of the prepared MWCNTs/PAMAM/Pt nanocomposite. It is clear that the Pt NPs are uniformly attached on the surface of MWCNTs with a narrow size distribution, which confirms the strong coordination between Pt NPs and covalent-bonded dendrimers on MWCNTs.

3.2. Electrochemical behaviors of MWCNTs/PAMAM/Pt/Nafion electrode to H_2O_2

The electrocatalytic activity of MWCNTs/PAMAM/Pt film toward H_2O_2 reduction was investigated herein. Fig. 2 shows the linear sweep voltammetry (LSV) in aCSF (pH 7.4) solution with and without H_2O_2 for bare and modified electrodes. No catalytic current could be detected in 0.5 mM H_2O_2 solution at bare GC electrode (curves a, b). However, for MWCNTs/PAMAM/Pt modified electrode, an addition of

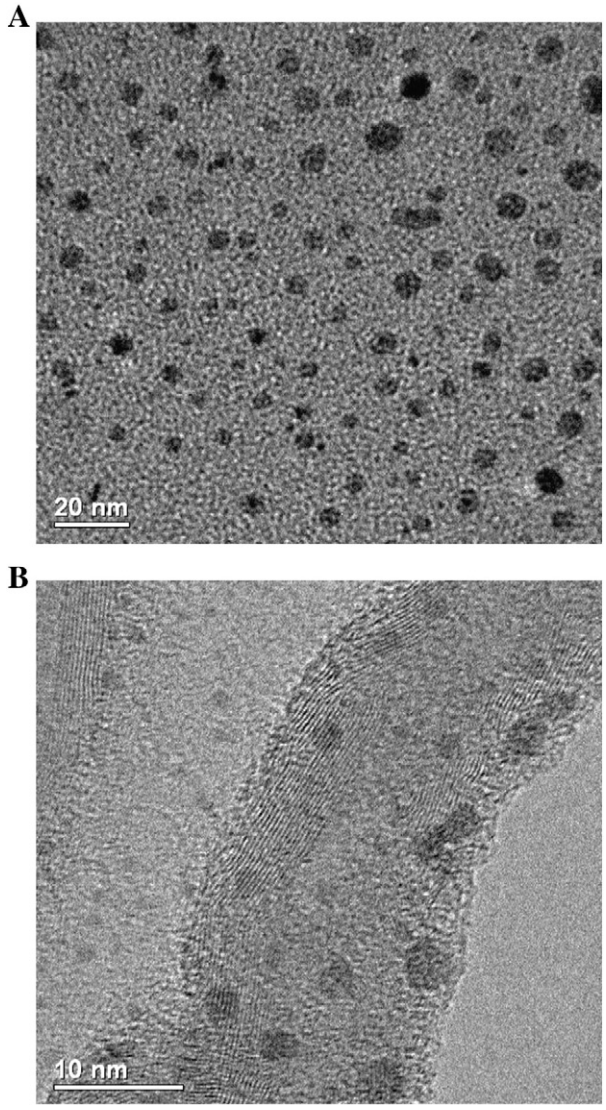


Fig. 1. TEM micrographs of (A) G 3.0 PAMAM/Pt nanocomposites and (B) MWCNTs/PAMAM/Pt composite. [Pt^{4+} ion]: [PAMAM] = 0.1:1.

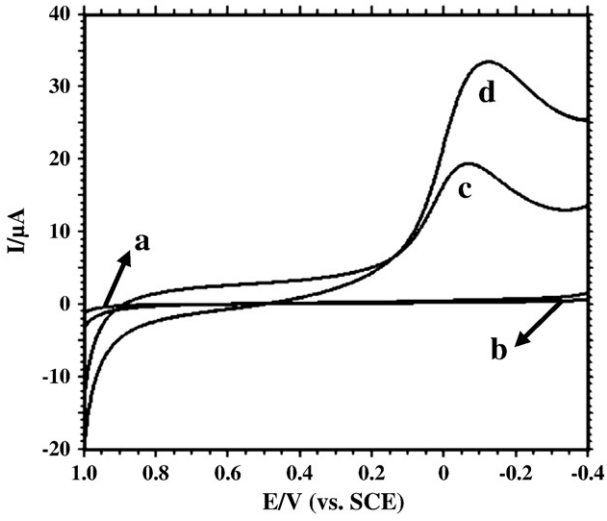


Fig. 2. Linear sweep voltammograms of (a, b) bare electrode and (c, d) MWCNTs/PAMAM/Pt/Nafion modified electrode in (a, c) aCSF (pH 7.4) and (b, d) 0.5 mM H_2O_2 solution. Scan rate: 100 mV s^{-1}.

0.5 mM H_2O_2 obviously increases the cathodic currents at the reduction potential about −150 mV (vs.SCE) (curves c, d), which indicates the good electrocatalytical activity of the immobilized MWCNTs/PAMAM/Pt film for the reduction of H_2O_2. The reduction potential of −150 mV is much more positive than that at Ag NPs (−0.68 V vs.SCE) or other material modified GC [15–17], suggesting a faster electron transfer rate and higher electrocatalytic activity toward H_2O_2 at the MWCNTs/PAMAM/Pt/Nafion modified electrode. Moreover, as we know, the typical bioelectrocatalytic reduction of H_2O_2 through the direct electron transfer of HRP normally takes place at −300 mV (vs. Ag/AgCl), which introduces great interferences from O_2 and AA. The excellent electrocatalytic property of the MWCNTs/PAMAM/Pt/Nafion film herein is prominent for constituting an analytical protocol for selective determination of H_2O_2.

3.3. Optimization of experimental parameters

The dependence of the amperometric responses of MWCNTs/PAMAM/Pt/Nafion biosensor toward 50 μM H_2O_2 on different working potentials was studied. As shown in Fig. 3A, the amperometric responses increase sharply with the increase of working potential from +50 mV to −150 mV (vs. SCE). After that, it rises a bit slowly from −200 mV to −300 mV. Considering the noise of the background and the interferences from oxygen and AA, we selected −200 mV (vs. SCE) as the optimal working potential in the following experiments.

The effects of PAMAM and $H_2P_tCl_6$ concentrations on amperometric responses toward 50 μM H_2O_2 were also investigated shown in Fig. 3B and C, respectively. We varied the concentration of PAMAM from 0.05 to 0.4 mM and $H_2P_tCl_6$ from 0.05 to 0.8 mM. With the increase of the concentrations of PAMAM and $H_2P_tCl_6$, the amperometric responses also increase and reach the maximum value at 0.3 mM for PAMAM and 0.3–0.4 mM for $H_2P_tCl_6$. Then, an obvious decrease of the responses is noted both for PAMAM and $H_2P_tCl_6$. A possible explanation is that a larger amount of dendrimers could inhibit the approach of substrate to the catalyst surface [18] and the saturation of the dendrimer structure could also lead to a lower electrocatalytic activity. Herein, 0.3 mM PAMAM and 0.3 mM $H_2P_tCl_6$ were used as the optimal concentrations through the experiments.

3.4. Amperometric detection of glutamate with on-line microdialysis system in vitro

The reduction current of glutamate was firstly calibrated *in vitro* before the start of *in vivo* experiments. Fig. 4A shows the amperometric responses of different concentrations of glutamate with the on-line microdialysis system *in vitro* at 2.0 μL/min. Well-defined steady-state currents are obtained for standard glutamate solutions which are continuously injected into the on-line microdialysis system. The current responses were in good linear with glutamate from 1.0 μM to 50.0 μM ($I\ (nA) = (0.41 \pm 0.25) + (1.74 \pm 0.02)\ C_{glutamate}\ (\mu M)$, $\gamma = 0.9997$) with a sensitivity of $1.74 \pm 0.02\ nA \cdot \mu M^{-1}$. The detection limit, based on the signal-to-noise ratio of three, was calculated to be 0.5 μM. This value was comparable to those obtained at redox hydrogel based planar Au electrodes [19] and polypyrrole film based glutamate biosensor [20].

The apparent Michaelis–Menten constant (Kapp m), an indication of enzyme-substrate kinetics, was calculated to be about 0.053 mM for glutamate in this paper, much smaller than previous relative reports [21,22]. This value was also remarkably small as compared to the free GlutaOx, which was 0.21 mM in neutral buffer solution [23]. The result demonstrates an increased bio-affinity and a higher enzymatic activity toward glutamate with the MWCNTs/PAMAM/Pt film modification.

The selectivity of the prepared biosensor for glutamate was evaluated with the on-line microdialysis system. Ascorbic acid (AA) and dopamine (DA) have always been considered to be the major

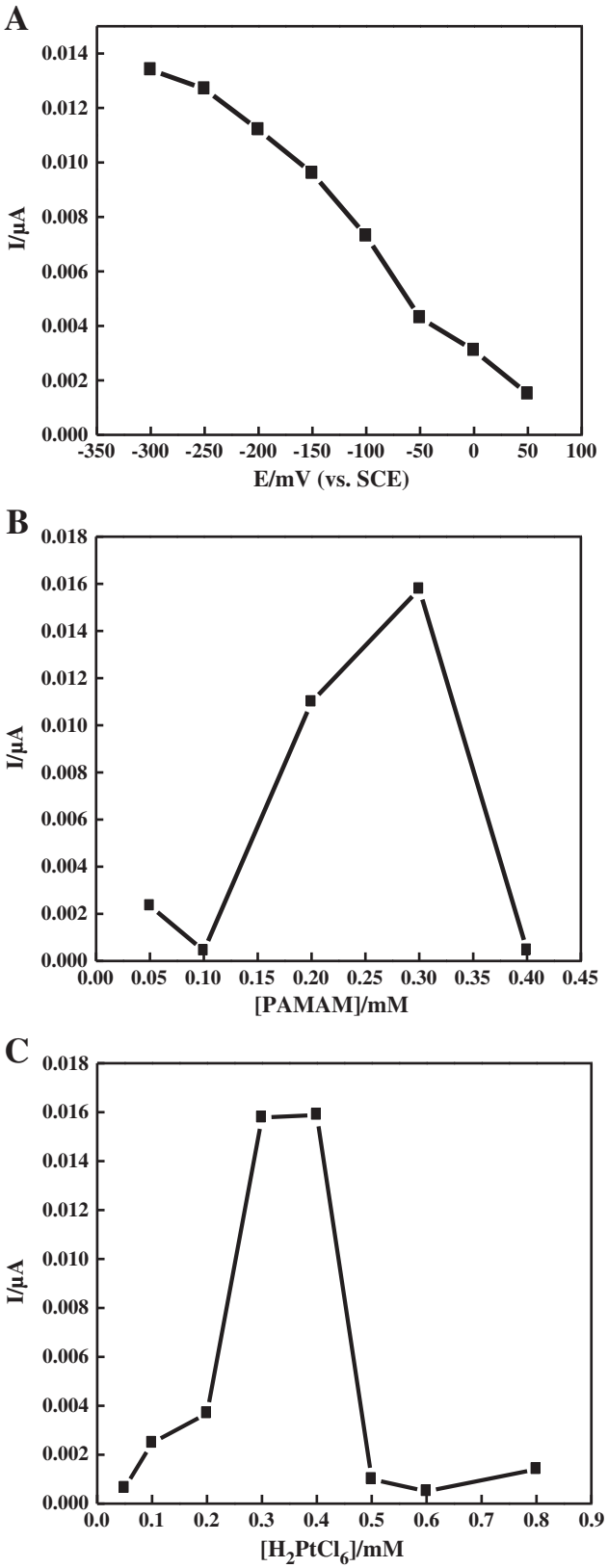


Fig. 3. (A) Dependence of the current responses to 50 μM H_2O_2 on different working potentials ranging from −300 mV to +50 mV (vs. SCE). (B) and (C) Dependence of the current responses to 50 μM H_2O_2 on the concentrations of PAMAM and H_2PtCl_6, respectively.

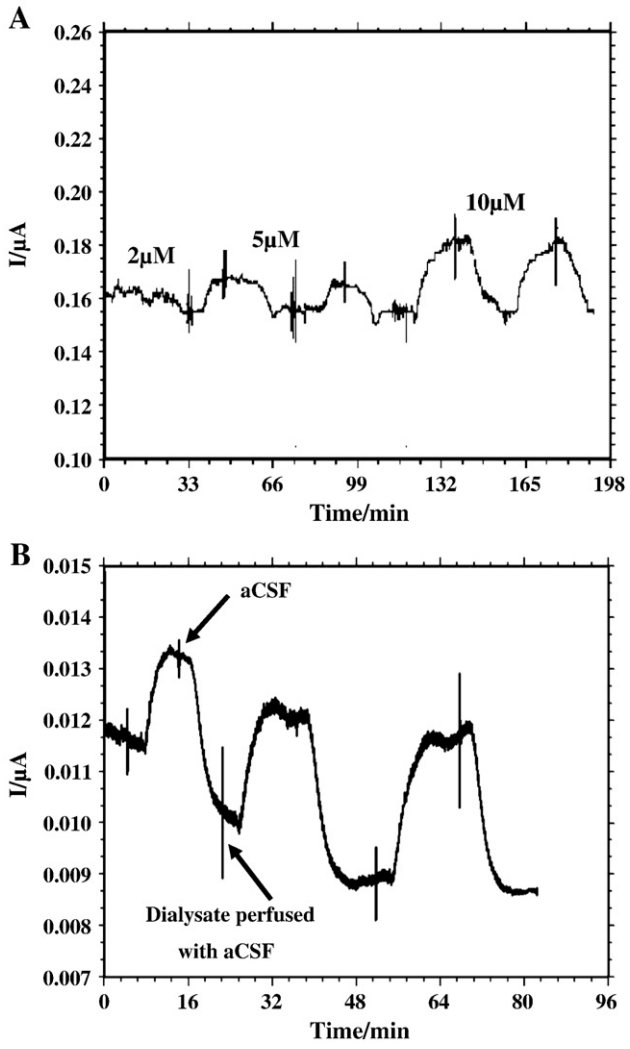


Fig. 4. (A) Current–time plots of GlutaOx/MWCNTs/PAMAM/Pt/Nafion biosensor on successive perfusions of specific concentration of glutamate (2.0, 5.0 and 10.0 μM) into the on-line microdialysis system *in vitro*. (B) Current-time plots of GlutaOx/MWCNTs/PAMAM/Pt/Nafion biosensor on brain dialysate from the striatum of anesthetic rats repeated for three times with the microdialysis system *in vivo*. Flow rate: 2.0 μL/min. Working potential: −200 mV (vs.SCE).

interferences for *in vivo* measurement of glutamate due to their high concentration in biological samples and low oxidation potentials. Our results showed that 0.2 mM of AA and 1.0 μM of DA cannot produce measurable responses, which convincingly indicated that AA and DA could not interfere the detection of glutamate (Fig not shown). It also demonstrated that the use of "artificial peroxidase" to replace "natural peroxidase" in the present work essentially offers a selective electrochemical method for on-line measurements of biological compounds.

The stability of the biosensor was checked by employing the biosensor with the on-line microdialysis system for at least 6 days (5–6 h each day) without observable day-to-day variations. The biosensor retained almost 86% of their initial activity after they were stored at +4°C for 2 weeks. The good long-term stability can be ascribed to the excellent biocompatibility of the MWCNTs/PAMAM/Pt film, which provides a favorable microenvironment for GlutaOx to maintain its bioactivity. The reproducibility of the method was also estimated through consecutively measuring glutamate (10 μM) with the microdialysis system for five times (Fig not shown) with a RSD value of 4.2% ($n=5$), indicating an excellent reproducibility under the mentioned conditions.

3.5. In vivo measurement of glutamate in the striatum of rats

After the continuous perfusion for 90 min with aCSF solution, the on-line microdialysis system can start to measure glutamate as Section 2.4 depicted. The relative recovery for glutamate was calculated to be 21% at the flow rate of 2.0 μL /min. For determination of the relative recovery, the probe is immersed in a vial filled with a known concentration of glutamate and then the probe is perfused with aCSF solution at 2.0 μL /min. The relative recovery is just the ratio between the concentration of glutamate which can be calculated from its calibration curve and the known concentration of glutamate in the vial. It can be calculated from the following equation:

$$R\,(\%) = C_{\text{out}} / C_{\text{in}} \times 100$$

where R (%) is the relative recovery in percentage, C_{out} is the concentration of the solution calculated from the calibration curve, C_{in} is the known concentration of the solution in the vial.

Fig. 4B displays the amperometric responses of brain microdialysate sampled from the striatum of anesthetic rats. The same procedure was performed three times successively on one rat. Three rats were totally investigated in this experiment. Based on our postcalibration, three data of 5.67 μM, 5.83 μM and 5.90 μM were obtained. The basal glutamate concentration in the striatum of rats was calculated to be about 5.80 ± 0.12 μM (mean ± s.d., $n = 3$). This value was comparably consistent with previous reports [24]. The above results suggest that the glutamate biosensor is sufficiently sensitive and can detect even the lower basal glutamate levels in the extracellular space of the striatum.

4. Conclusions

A PAMAM/Pt nanocomposite has been synthesized for the construction of MWCNTs/PAMAM/Pt/Nafion biosensor. Combined with microdialysis technology, an on-line microdialysis system was designed for the measurement of glutamate in the striatum of rats continuously. The low working potential, good sensitivity and selectivity of the biosensor and the continuous sampling of microdialysis technology suggested that the on-line microdialysis system, together with the glutamate biosensor, is well suitable for the further research *in vivo*.

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