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Overoxidized polypyrrole/multi-walled carbon nanotubes composite modified electrode for *in vivo* liquid chromatography–electrochemical detection of dopamine

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ABSTRACT

Overoxidized polypyrrole/multi-walled carbon nanotubes (OPPy/MWNTs) modified electrode has been developed for sensitively detecting dopamine (DA). OPPy films developed outside MWNTs might have a porous morphology. Thus, OPPy/MWNTs films developed by this method do not reject ascorbic acid (AA). However, OPPy/MWNTs modified electrode shows largely enhancing oxidative current responses of DA. When combined with liquid chromatography, it not only obtains a low detection limit of 7.5×10^{-10} mol L^{-1} for DA, but also improves the selectivity of DA detection. Mechanisms for the enhancement are also well discussed in this paper. With this approach, microdialysis has been employed for successful assessment of DA in rat striatum.

1. Introduction

Dopamine (DA) is well known as one of the important neurotransmitters. It is released from brain neurons to extracellular fluids and plays an important role in various biological, physical and pharmacological processes [1]. Some diseases are related to the change of dopamine levels [2]. Parkinson's disease is one of them, which is characterized by a severe depletion of the *in vivo* dopamine pool. Thus it is important to develop methods for very low concentration of dopamine. Now there are a variety of methods available, such as fluorimetry [3], UV–vis [4], chemiluminescence [5,6], LC–MS/MS [7]. However, all these approaches have some shortcomings, such as complicated pretreatment, high cost, need for derivation, time consumption, and poor sensitivity. In this case, one of the better choices remains HPLC with electrochemical detection. In order to improve analytical performance and get lower detection limit, many efforts have been made by using chemically modified electrodes (CMEs) [8–14], and one of them is based on carbon nanotubes [8].

Since the existence of carbon nanotubes (CNTs) was reported in 1991, they have attracted much attention because of their high surface area, high electrical conductivity, good chemical stability, and mechanical strength. Unique properties of CNTs make them extremely attractive for electrochemical sensor and biosensors. Recent reports demonstrated that they showed high electrochemical reactivity and minimization of surface fouling if employed to improve the electrochemical response of some important bioactive substances. For example, they had been successfully used in oxidation of dopamine [15], electrochemistry of protein [16], and in electrocatalysis of NADH [17]. Our previous studies had also showed an excellent electrochemical reactivity for oxidization of thiol compounds [18]. All these indicate that the performance of carbon nanotubes is superior to many other carbon electrodes in terms of reaction rates and reversibility. However, there is a problem needed to be solved. That is, purified carbon nanotubes flocculate rapidly in aqueous or common organic solutions, thus hindering their further manipulation and application [19]. To overcome this problem, one of the best ways is to fabricate carbon nanotubes composites, as they can improve their processability and allow their unique properties to be coupled into other materials [20–22].

Overoxidized polypyrrole (OPPy) films are cation perm-selective films and have been found losing the electrical conductivity because of introducing oxygen-containing groups into the polypyrrole backbone. Many reports have demonstrated that OPPy films have widened applications of polypyrrole films in electroanalytical chemistry [23]. Usually OPPy-based modified electrodes have lower background currents [24]. Therefore, we hypothesize that OPPy films used for carbon nanotubes composite modified electrode might improve the analytical performance for detection of dopamine. The proposed modified electrode might have some properties combining the excellent electrochemical reactivity of carbon nanotubes with cation perm-selectivity of OPPy films. Thus a low detection limit might be achieved for dopamine. With this

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approach, the level of dopamine in rat brain might be easily determined when coupling with HPLC and microdialysis.

2. Experimental

2.1. Reagents

Multi-walled carbon nanotubes (MWNTs) with a diameter of 10–30 nm and a length of 1–10 μm was obtained from Sun Nanotech Co. Ltd., China. DA, norepinephrine (NE), and 3,4-dihydroxyphenyl acetic acid (DOPAc) were purchased from Sigma (St. Louis, MO, USA). Pyrrole was obtained from Fluka (Fluka Chemie AG, Switzerland). Usually pyrrole was purified by double distillation and stored at −20 °C. For experiments, prepared pyrrole solutions need to be protected from light. Ringer's solution for microdialysis experiments consists of 140 mmol L^{-1} NaCl, 1.0 mmol L^{-1} $MgCl_2$, 1.2 mmol L^{-1} $CaCl_2$ and 5.0 mmol L^{-1} $NaHCO_3$, pH 7.4. All other reagents were of at least analytical-reagent grade, and double-distilled deionized water (ddH_2O) was used for all solutions.

2.2. Apparatus

Electrochemical experiments were performed on a CHI-830 Electrochemical detector (CH Instruments, USA) with a three-electrode system. For cyclic voltammetry experiments, a glassy carbon electrode (diameter 3 mm, BAS Co., Japan) was served as the working electrode. A saturated calomel electrode (Model 232C, Jiangsu Electroanalytical Instruments Factory, China) and a platinum electrode (Model 213, Jiangsu Electroanalytical Instruments Factory, Jiangsu, China) were used as the reference electrode and the counter electrode, respectively.

Liquid chromatographic experiments were conducted on a HP1090 liquid chromatography (Hewlett Packard Company, USA), a Zorbax ODS column (4.6 mm i.d. × 250 mm, 5 μm particle, Dupont Instruments, USA) and a CHI-830 Electrochemical detector with a homemade thin-layer radial flow cell. In this detective system, a glassy carbon disc electrode (diameter 5 mm, BAS Co, Japan) or chemically modified electrodes was used as the working electrode, the stainless steel frame served as the counter electrode, and all potentials were referenced to an Ag/AgCl (saturated KCl) electrode.

Microdialysis was accomplished on a CMA/101 microdialysis pump (CMA Microdialysis AB, Stockholm, Sweden) and a PES 12 microdialysis probe with a membrane diameter of 0.5 mm and a length of 2.0 mm (BAS Co, Japan). The probe was perfused with Ringer's solution at a rate of 1.0 μL min^{-1}.

Fourier transform (FT) IR spectra were recorded on a NEXUS 670FT IR spectrometer (Nicolet Co., USA).

2.3. Preparation of OPPy/MWNTs modified electrodes

MWNTs functionalized with carboxylic acid groups were prepared by refluxing with HNO_3 for 4–5 h. The nitric acid was then removed by washing with double-distilled deionized water until the pH of the suspension was nearly 7.0. FTIR spectra of MWNTs showed the appearance of peaks at 1716 and 1575 cm^{-1}, which corresponded to $\nu_{(C=O,-COOH)}$ and $\nu_{(C=O,-COO^-)}$, respectively. It meant that –COOH and $–COO^-$ were present on the surface of MWNTs, which was in accordance with literature [25,26].

Prior to preparation of the OPPy/MWNTs CME, a glassy carbon (GC) electrode was polished with 300 nm alumina, and sonicated sequentially in acetone, NaOH (1 mol L^{-1}), HNO_3 (1:1, v/v) and ddH_2O. Then polypyrrole/multi-wall carbon nanotubes (PPy/MWNTs) composite modified electrodes were first prepared by electro-copolymerizing functionalized MWNTs and pyrrole, which were prepared in 0.2 mol L^{-1} KCl solution (pH 7.4) containing 0.2 g mL^{-1} MWNTs and 0.1 mol L^{-1} pyrrole by scanning GC electrodes from 0 to 1.0 V and back at 0.1 V s^{-1} for 7 times. Afterwards, PPy/MWNTs CMEs were further overoxidized in stirred 1.0 mol L^{-1} NaOH by scanning them between 0 and 1.0 V at 0.1 V s^{-1} for 60 times.

2.4. In vivo microdialysis experiment

All procedures involving animals were conducted with approval of the Institutional Animal Care and Use Committee of East China University of Science & Technology. A male Sprague–Dawley rat (200 g) was anesthetized with 1.5 g kg^{-1} urethane and fixed in a Narishige SN-2 stereotaxic frame (BAS Co., Japan). Then a hole was drilled through the skull and a microdialysis probe was slowly implanted into the striatum at coordinates relative to bregma (x = +3.0, y = +0.6, z = −5.8 mm [27]). After an equilibration period

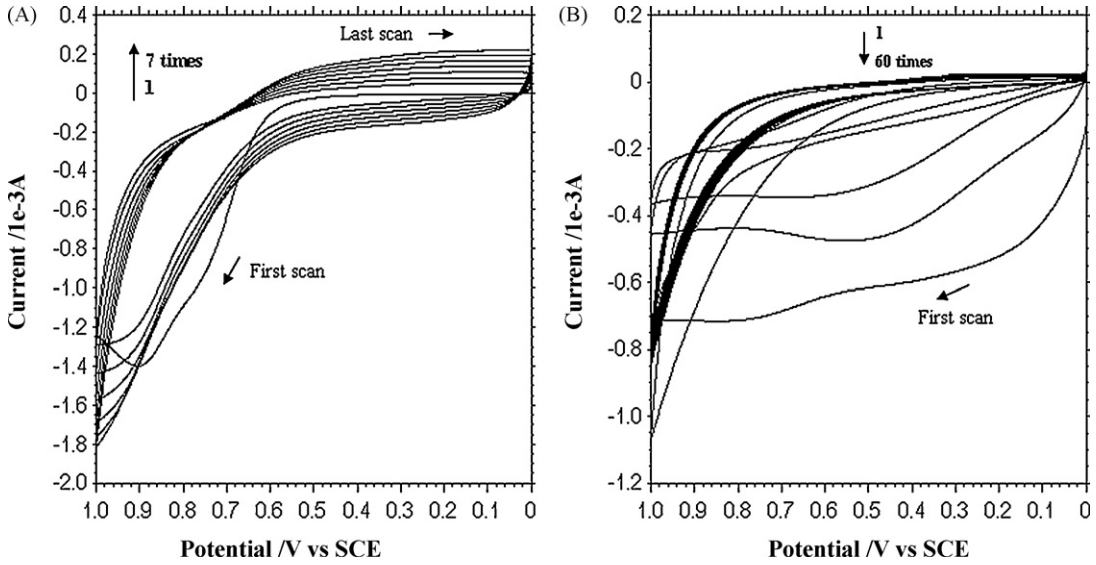

Fig. 1. Preparation of the OPPy/MWNTs CME. (A) Cyclic voltammograms of co-electropolymerization of pyrrole and MWNTs at a GC electrode, which was carried out in 0.2 mol L^{-1} KCl solution (pH 7.4) containing 0.2 g mL^{-1} MWNTs and 0.1 mol L^{-1} pyrrole by cycling the potential between 0 and 1.0 V at 0.1 V s^{-1} for 7 times. (B) Cyclic voltammograms of overoxidation of the PPy/MWNTs CME in stirred 1.0 mol L^{-1} NaOH. The electrode was scanned from 0 to 1.0 V and back at 0.1 V s^{-1} for 60 times.

for 60 min, samples were collected continuously in a 25-μL sample receiver and injected into the HPLC system.

3. Results and discussion

3.1. Preparation of OPPy/MWNTs CMEs

OPPy/MWNTs composite modified electrodes are prepared based on overoxidation of PPy/MWNTs CME. First, PPy/MWNTs composite modified electrode is fabricated by co-electropolymerizing pyrrole and MWNTs. As shown in Fig. 1A, its voltammetric behavior is quite similar to that for pyrrole electropolymerization [24]. And then overoxidization of PPy/MWNTs films for fabricating OPPy/MWNTs CMEs is carried out in 1.0 mol L^{-1} NaOH by employing cyclic voltammetry with an oxidation limit of +1.0 V vs. SCE. As shown in Fig. 1B, during its overoxidation the amplitude of currents decreases with each successive voltage scan, indicating that films lost their conductivity. And 60 times are enough for oxidizing PPy/MWNTs films. As for overoxidation of PPy/MWNTs films, it should be noted that the voltammetric behavior is also quite similar to that for overoxidation of PPy films. Here, OPPy films are more like supporting material for loading MWNTs.

3.2. Characterization of OPPy/MWNTs CMEs

Fig. 2 (curve a) shows a typical cyclic voltammogram of a MWNTs CME in 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). The CV curve shows a redox couple centered at −0.05 vs. SCE, corresponding to the redox of carboxylic acid groups [26]. However, this redox peaks decrease greatly for the OPPy/MWNTs CME (Fig. 2, curve b). We did not know the exact reason but it might be due to the difference in amount of MWNTs loaded on the surface of electrodes.

On the other hand, different background currents can also be observed for MWNTs CMEs, OPPy/MWNTs CMEs and GC electrodes. Fig. 2 shows that the background current of an OPPy/MWNTs CME is a little larger than that of a GC electrode (curve c). However, the background current of an OPPy/MWNTs CME decreases a lot when comparing with a PPy/MWNTs CME (data not shown). This phenomenon could also be observed for preparation of OPPy films on other carbon electrodes. Usually, typically high background charging currents of PPy films can be returned to the level of bare

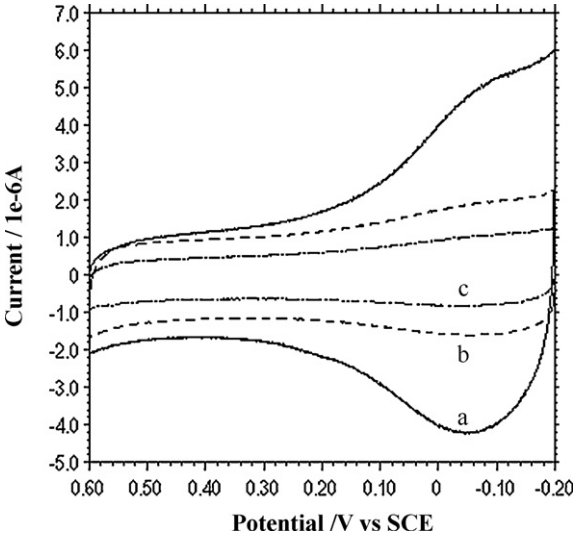

Fig. 2. Cyclic voltammograms of the MWNTs CME (a), OPPy/MWNTs CME (b) and GC electrode (c) in 0.1 mol L^{-1} phosphate buffer solution (pH 7.0). Scan rate: 0.1 V s^{-1}; initial potential: −0.2 V vs. SCE.

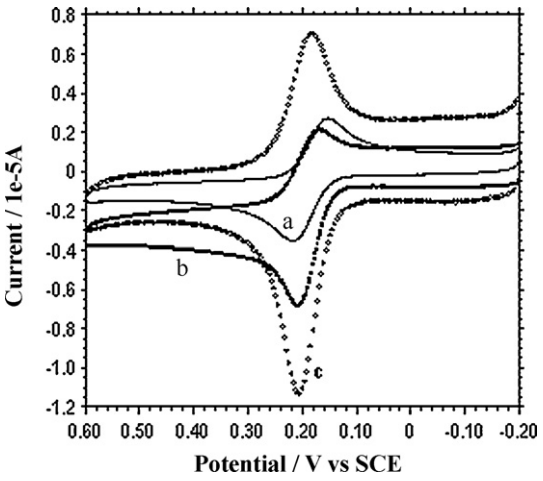

Fig. 3. Cyclic voltammograms of 1×10^{-4} mol L^{-1} DA at the GC electrode (a), OPPy CME (b) and OPPy/MWNTs CME (c) in 0.1 mol L^{-1} PBS solution (pH 7.0). Scan rate: 0.1 V s^{-1}; initial potential: −0.2 V vs. SCE.

electrodes after overoxidation. Brajter-Toth et al. attributed this to a loss in conductivity during the overoxidative process. Obviously, elimination of high background currents typical of PPy composite modified electrodes would also enhance analytical usefulness of these composite films, making lower detection limits attainable [23].

3.3. OPPy/MWNTs CMEs enhance electrochemical oxidation of DA

Once upon addition of dopamine, a very nice pair of redox waves appears at +0.2 V vs. SCE for OPPy/MWNTs CMEs, OPPy CMEs and bare GC electrodes. As shown in Fig. 3, ΔEp values of 60, 32, 28 mv are observed for the GC electrode, OPPy CME, OPPy/MWNTs CME, respectively. That is, DA shows smaller peak-to-peak separation at the OPPy/MWNTs CME than at the GC electrode, which means that OPPy/MWNTs CMEs increase the electron transfer kinetics a little in comparison to GC electrodes. In addition, among these three electrodes the faradic current of DA is the largest at the OPPy/MWNTs CME. These data suggest that OPPy/MWNTs CMEs have the ability to enhance current responses of DA. We attributed all these to MWNTs loaded in films and OPPy films.

Carbon nanotubes can act as a promoter to enhance the electrochemical reaction, increasing the rate of the heterogeneous electron transfer [28]. On the other hand, it is also believed that the increased surface area provided by carbon nanotubes plays an important role in the current enhancement. Actually, more recently Compton's group demonstrated significantly that both CNT-based electrodes and edge-plane pyrolytic graphite electrodes showed similar electrocatalytic activity towards a range of redox systems. Key structural features of CNTs for electrochemical reactions are the edged plane-like nanotube ends rather than nanotube bodies themselves [29]. This can be further confirmed by our experiments. MWNTs used in these experiments are all functioned with –COOH groups by refluxing with HNO_3 for 4–5 h, which also helps obtaining more edged plane-like nanotube ends, whereas MWNTs without refluxing do not enhance the current response of DA (data not shown). That is, in a number of case molecules can interact with carbon nanotubes in a way that well polished "traditional" carbon electrodes cannot, and it is this combination of edge plane-like electro-reactivity with large surface area of CNTs that lead to significantly enhancing the response of DA.

Certainly, OPPy films outside MWNTs also play an important role in enhancing the current response of DA. OPPy films, as mentioned before, are derived from PPy films. When PPy films are overoxidized,

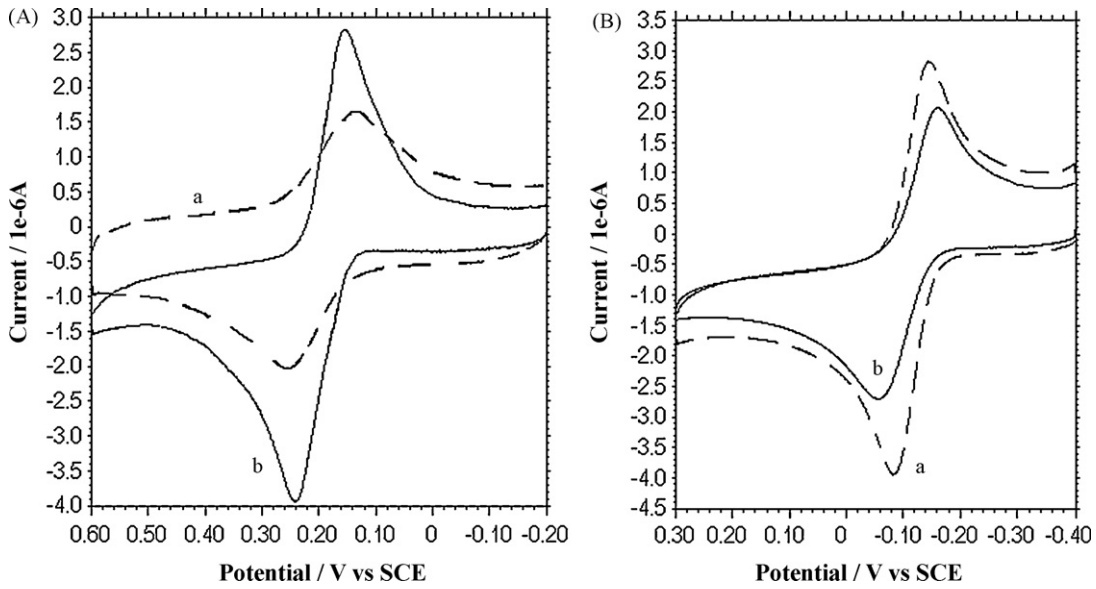

Fig. 4. Cyclic voltammograms of 5 mM $Fe(CN)_6^{3-}$ (A) and 5 mM $Ru(NH_3)_6^{3+}$ (B) in 0.1 mol L^{-1} PBS solution (pH 7.0) at the OPPy/MWNTs CME (dashed curve, a) and GC electrode (solid curve, b). Scan rate: 0.05 V s^{-1}.

films lose the conjugated structure and electrical conductivity, and then convert into non-electronic but pure ionic conductor [23]. That is to say, OPPy films can be regarded as negatively charged polymer films. So they can effectively attract the positive cation DA to films in the neutral aqueous. Similar results can also be obtained by Yao's group. They reported that OPPy/CNTs showed a notably larger affinity to DA molecules by electrochemical quartz crystal impedance method [30].

3.4. Selectivity of OPPy/MWNTs CMEs

Since OPPy films are cation perm-selective, it is quite natural for us think that an OPPy/MWNTs composite modified electrode has the same characteristic. To test the selectivity of this CME, $Fe(CN)_6^{3-}$ and $Ru(NH_3)_6^{3+}$ are chosen as electrochemical probes because of their similar size and fast kinetics on carbon electrodes, but with opposite charges. A well behaved redox wave can be obtained for both probes (shown in Fig. 4). However, compared with the GC electrode, the redox reactions of $Fe(CN)_6^{3-}$ are significantly inhibited, whereas the current of $Ru(NH_3)_6^{3+}$ increases obviously at the OPPy/MWNTs CME. It means that OPPy/MWNTs composite films repel anions in some degree. However, when comes to ascorbic acid (AA), one of the interference of anions for determination of DA, these composite films do not decrease the responses of AA, instead they help reduce its oxidation potential (0.1 V vs. SCE, PBS pH 7.0, shown in Fig. 5). Similar results can also be obtained from OPPy/SDS-SWNTs modified GC electrode [31].

Generally, it is believed that the selectivity of OPPy/MWNTs CMEs is determined by OPPy films outside MWNTs. Many reports demonstrated that OPPy films had cation perm-selectivity, because of the introduction of carbonyl groups into the polymer backbone. The high electron density of carbonyl groups acted as a barrier to hinder the diffusion of anions in films. However, anions such as AA cannot be rejected by OPPy/MWNTs films, even $Fe(CN)_6^{3-}$ is only partially rejected. This may be due to the morphology and properties of OPPy films outside MWNTs. Brajter-Toth's group had demonstrated that differences in response to ionic probes could arise from the porosity of membranes, which affected the electron density within films due to the spacing of carbonyl groups introduced during overoxidization; and the substrate under OPPy films played a definite role in the porosity of the polymer layer [10]. In this case, OPPy films developed on MWNTs may have a larger porous morphology. Therefore, AA can permeate OPPy films to reach the surface of MWNTs easily, and an electrochemical response can be achieved. If the selectivity of films need to be improved, compact ultrathin OPPy films may be required, which can be prepared by taking advantage of repeatedly polymerizing pyrrole (PY) and overoxidizing it to OPPy [24]. Nevertheless, in this paper liquid chromatograph can be employed for determination of DA in the presence of AA, and there is no need for compact ultrathin OPPy films.

3.5. Electrochemical detection of DA in liquid chromatography

Since OPPy/MWNTs CMEs enhance current responses of DA, these CMEs could be used for achieving a lower detection limit

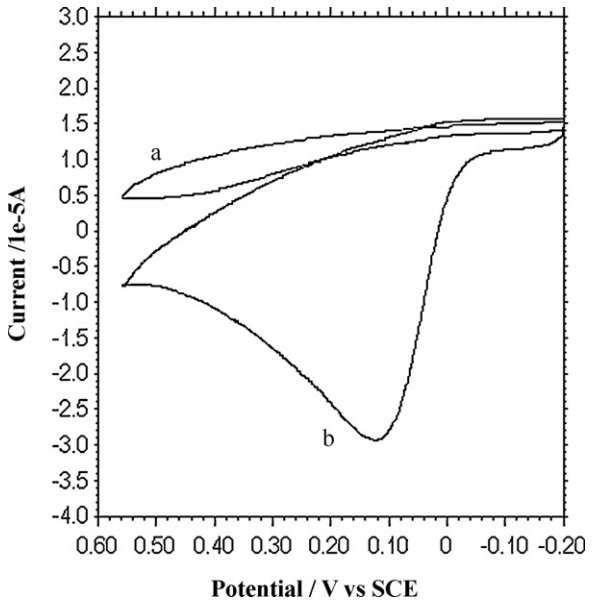

Fig. 5. Cyclic voltammograms of 5×10^{-4} mol L^{-1} AA in 0.1 mol L^{-1} PBS solution (pH 7.0) at the GC electrode (a) and OPPy/MWNTs CME (b). Scan rate: 0.1 V s^{-1}; initial potential: −0.2 V vs. SCE.

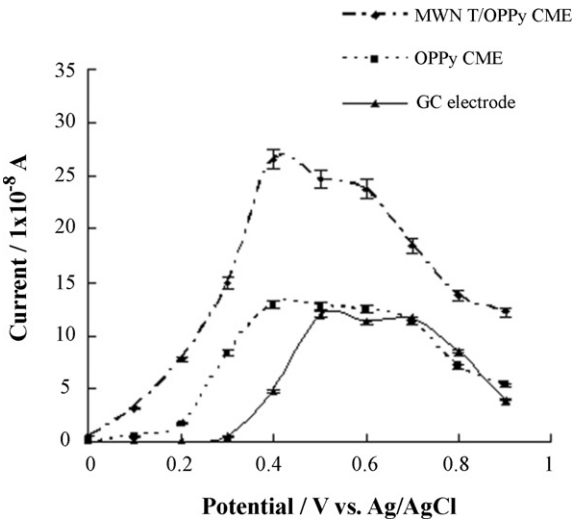

Fig. 6. Hydrodynamic voltammograms of 3.0×10^{-6} mol L^{-1} DA at the OPPy/MWNTs CME, OPPy CME and GC electrode. Mobile phase: methanol–PBS solution (0.1 mol L^{-1} and containing 1×10^{-4} mol L^{-1} Na_2EDTA, pH 5.0) (5:95, v/v); flow rate: 1 mL min^{-1}; injection volume: 20 μL.

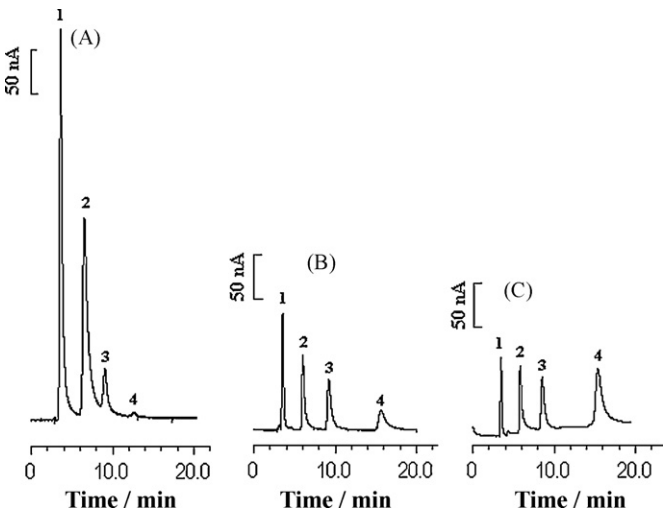

Fig. 7. Chromatograms of mixed standard solution of monoamine transmitters and their metabolites at the OPPy/MWNTs CME (A), OPPy CME (B) and GC electrode (C). The mixed standard solution: 2.5×10^{-6} mol L^{-1} NE (1), DA (2), DOPAc (3) and 5-HT (4). The working potential was set at +0.5 V vs. Ag/AgCl for GC electrode, and +0.4 V vs. Ag/AgCl for OPPy/MWNTs CME and OPPy CME; other conditions were the same as in Fig. 6.

of DA. Here liquid chromatography coupled with electrochemical detection (LC–ECD) is employed for determination of DA because this technique usually gives much lower detection limit and better selectivity than CV. Hydrodynamic voltammetry (HDV) is used for selecting the best applied potential. In this paper, a standard solution of 3.0×10^{-6} mol L^{-1} DA was repetitively injected to the LC–ECD system and the peak currents were recorded when the applied potential was increased from 0 to +0.9 V vs. Ag/AgCl by 0.1 V increments. As indicated in Fig. 6, the best applied potential of +0.4 V vs. Ag/AgCl can be chosen for detection of DA at the OPPy/MWNTs CME. However, it should be noted that at the potential of +0.4 V vs. Ag/AgCl responses of DA at the GC electrode did not reach the most until the potential is applied at +0.5 V vs. Ag/AgCl. Nevertheless, responses of DA are still lower than those at the OPPy/MWNTs CME (indicated in Fig. 6). These data suggest that the OPPy/MWNTs CME not only decreases the oxidative potential of DA when compared with the GC electrode, but also increases current responses of DA when compared with both OPPy CME and GC electrode.

Generally, CMEs can be used for improving detection limits by using CV method, but it does not mean they get the same results in liquid chromatograph as well. In this paper, OPPy/MWNTs CMEs have been explored to show steadily enhancing current responses of DA in LC–ECD. This is significantly useful for achieving the lower detection limit of DA in LC–ECD. To determine the detection limit, a series of DA solutions were tested with concentrations ranging from 1.0×10^{-9} to 1.0×10^{-4} mol L^{-1}. The results show that at the OPPy/MWNTs CME peak currents of DA are linear to their concentrations ($i_p = 0.0885C + 3 \times 10^{-11}$) ranging from 2.5×10^{-9} to 5.0×10^{-6} mol L^{-1} with correlation coefficient of 0.995. The detection limit of 7.5×10^{-10} mol L^{-1} for DA can be achieved (measured using 3σ, where σ is the standard deviation of a blank solution, $n = 11$). Obviously, this detection limit is lower than those at many CMEs in LC–ECD, such as MWNT CME [8], OPPy CME [11], ploy (para-aminobenzoic acid) CME [12], thioctic acid/iridium oxide-palladium CME [13] and Nafion [14].

Furthermore, the present OPPy/MWNTs CME shows low-noise characteristics and a stable baseline because OPPy/MWNTs films are stable over time. The repeatability of the CME was estimated by repetitive injection ($n = 8$) of 1.0×10^{-7} mol L^{-1} DA under the same conditions. The relative standard deviation (R.S.D.) of peak currents is found to be 3.5%. And the intermediate precision was evaluated by analyzing 1.0×10^{-7} mol L^{-1} DA under the same conditions on 5 different days and the R.S.D. of peak currents was 7.2%. Recoveries of DA were determined by the standard addition and they were in the range of 97.5–105% ($n = 5$). All these mean that the developed OPPy/MWNTs CME can *in vitro* sensitively detect DA with relatively high stability.

3.6. Responses of monoamine at the GC electrode, OPPy CME and OPPy/MWNTs CME in the LC–ECD

Fig. 7 gives typical responses of 2.5×10^{-6} mol L^{-1} NE, DA, DOPAc and 5-HT at the GC electrode, OPPy CME and OPPy/MWNTs CME, which shows the best performance for each electrode, i.e. the ratio of signal to noise is highest. Compared with the GC electrode and OPPy CME, current responses of NE and DA at the OPPy/MWNTs CME are improved greatly. They are consistent with results obtained from cyclic voltammetry (data not shown for NE). However, when it concerns to 5-HT, responses at the OPPy/MWNTs CME are smaller than those at the GC electrode or OPPy CME. The reason is not clear, but these data further confirm that OPPy/MWNTs CMEs can give the best performance for detecting DA in the LC–ECD.

3.7. In vivo determination of DA in rat striatum

It has already been demonstrated that OPPy/MWNTs CMEs can be used for *in vitro* detection of DA with a low detection limit. Next we like to know whether this method is good enough for *in vivo* determination of DA. Microdialysis is a very good way for *in vivo* sampling small molecules. Therefore, it is quite natural for us to couple microdialysis with this LC–ECD method for *in vivo* determining the level of DA in rat brains. Generally, the recovery of a microdialysis probe first need to be *in vitro* determined before sampling. Here the recovery of the probe is given by C_{out}/C_{in}, where C_{out} is the concentration of DA in microdialysate and C_{in} is its concentration in the medium surrounding the probe. The recovery of the probe for DA in this paper is determined to be $35.4 \pm 2.7\%$ (mean ± SD, $n = 5$) at the microdialysis rate of 1.0 μL min^{-1}, which is chosen for rapid and accurate detection of DA.

Fig. 8 shows the chromatogram of microdialysate in rat striatum. It can be found that NE cannot be completely resolved due to the similar retention time of AA. However, DA, DOPAc and UA can give

Fig. 8. Chromatograms of monoamine transmitters in rat striatal microdialysate: AA (1), NE (2), UA (3), DA (4) and DOPAc (5) were confirmed by standard addition. The working potential was set at +0.4 V vs. Ag/AgCl and other conditions were the same as in Fig. 6.

clear peak current response. The average content ($n = 4$) of DA in rat striatal microdialysate samples is 2.3×10^{-8} mol L^{-1}. Thus, DA in the rat striatum is 6.5×10^{-8} mol L^{-1}, which is consistent with the value reported by Kennedy and co-workers [32]. All these data mean that the level of DA in rat brain can be successfully determined by the method we developed.

4. Conclusions

In this paper, polypyrrole/multi-walled carbon nanotubes composite is oxidized to form overoxidized polypyrrole/multi-walled carbon nanotube composite. The resulting modified electrodes have some properties combining the excellent electrochemical reactivity of carbon nanotubes with some cation perm-selectivity of OPPy films. By using CV technology and liquid chromatography–electrochemical detection technology, OPPy/MWNTs CMEs have shown that they get the ability to enhance current responses of DA with relatively high stability. Therefore, the sensitivity is improved greatly and lower detection limit of 7.5×10^{-10} M is also obtained for determination of DA in LC–ECD. And more importantly, the level of DA in rat striatum could be easily assessed when combining with microdialysis. These studies would be valuable for making neurochemical measurements.

Acknowledgements

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