Int. J. Electrochem. Sci., 13 **(2018)** 7129 – 7140, doi: 10.20964/2018.07.69

International Journal of
ELECTROCHEMICAL
SCIENCE
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A simple and Novel Electroanalytical Method for Determination of Brain Serotonin Based on the MWNTs/Al_2O_3/chitosan SPE

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Received: 13 April 2018 / *Accepted:* 22 May 2018 / *Published:* 5 June 2018

In this work, a simple and novel method for determination of brain serotonin (5-HT) in depression mice was proposed with modified screen-printed electrode based on MWNTs/Al_2O_3/chitosan. The modified SPE displayed excellent electrocatalytic activity towards the oxidation of 5-HT in real brain tissue samples. Square wave voltammetry (SWV) was employed to optimize the experimental conditions. The proposed sensor showed voltammetric responses with high and good sensitivity for 5-HT in optimal conditions. The peak current of 5-HT was proportional to the concentration in the range of 0.01 μM to 1.0 μM with the detection limit 0.005 μM. Compared with the HPLC method, the newly developed method also presented good results in the detection of brain 5-HT level, which made it possible for helping depression desiease diagnosis. In conlusion, our experimental results strongly suggested that the established SPE could be used to the fast depression determination of 5-HT, which might offer a potential minotor tool for in future.

Keywords: Disposable sensor, brain serotonin, modified screen printed electrode, clinical depression diagnosis

1. INTRODUCTION

Depression was one of the major mental disorders, which was often associated with various symptoms such as low energy, changes of appetite and sleep, loss of interest or pleasure in usual

activities, feelings of guilt, sadness, hopelessness and despair, even more suicidal inclination. The World Health Organization had reported that it would become the second cause of illness-induced disability by the year 2020 [1, 2]. Many researches gave the supports that the major neurochemical process of depression was frequently related with the deterioration of monoaminergic functions which was caused by the decreased levels of the monoamine neurotransmitters such as serotonin [3, 4]. Clinical evidence also suggested the alterations of monoamine neurotransmitters in central nervous monoaminergic systems played an important role in the pathophysiology of depression [5]. As a monoamine neurotransmitter of the central nervous system, serotonin (5-hydroxytryptamine, 5-HT) was synthesized in serotonergic neurons and associated with the regulation of sleep, mood and so on [6, 7]. It had been confirmed that the levels of 5-HT of the animal brain and blood samples were decreased in depression [4, 8]. Therefore, it was of great importance and necessity to develop a method for determination of 5-HT for depression auxiliary diagnosis.

Various methods for 5-HT detection in depression were developed as following, high performance liquid chromatography [9], gas chromatography [10], fluorimetry [11], spectrophotometry [12]. However, most assays above showed different disadvantages such as complicated pretreatment, high costs and time-consuming [9-12]. Due to the sensitive response and low costs, electrochemical analytical method was applied for the rapid monitor in the environment and biological samples [13]. As previous researches [14, 15], screen-printed electrode (SPE) was widely developed and the disposable neurotransmitter sensor was also fabricated for determination of the animal cerebrospinal fluid. But, the application of modified SPE in depression model had not been reported by so far. Hence, the goal of the present study was to establish a simple and novel electroanalytical method for rapid depression auxiliary diagnosis without the complicated pretreatment steps. At the same time, HPLC was employed as the comparison to verify the technology promising of modified.

As excellent electrochemical catalytic conductor, the multi-walled carbon nanotubes (MWNTs) and metal nanoparticiles were often used to modify sensors to enhance the sensitivity and increase the electrochemical signal [16]. MWNTs were reported to successfully synthesize biosensor which presented good detection sensitivity and low limit. Al_2O_3 used in our study was the metal nanoparticles, which was the ideal catalyst to catalyze the electrochemical oxidation in the sensor detection. Different sensors modified with Al_2O_3 were developed for biological determination by other scholar [17, 18], such as the gas sensor. However, the proposed sensors presented excellent catalytic performance and increased the selective sensitivity, too. Chitosan played a key role in sensor, biology, food chemistry pharmacy due to its special structural and functional properties including the biocompatibility, hydrophilicity, good adhesion, chelating and adsorptive properties [19]. Based on the researches, the functional groups of chitosan such as $–NH_2$ and –OH, were often used in the adsorption of metals or enzyme immobilization, which the protonation of chitosan made it possible for promoting the ion exchange [20]. Thus, MWNTs, Al_2O_3 nanoparticles and chitosan were recommended to perform in the experiments.

In our work, firstly, disposable MWNTs/Al_2O_3/chitosan SPE was synthesized for the detection of 5-HT. Secondly, the proposed sensor were performed to monitor the plasma concentration of 5-HT during the depression model in detail. In addition, the HPLC was also employed for the

comparison. It was the first time to report the MWNTs/Al_2O_3/chitosan modified SPE was used for the real biological determination in depression mice model.

2. REAGENTS AND METHODS

2.1. Reagents and apparatus

The standard substance of serotonin (5-HT) was purchased from Sigma in present study. Nano materials of MWNTs and Al_2O_3 nanoparticals were respectively employed from Chengdu Organic Chemicals Co. (Sichuan, China) and Aladdin Chemistry Co. (Shanghai, China). Furthermore, the 1% chitosan solution was obtained by dissolving 0.1g chitosan (Shanghai Kerui) in 10ml 1% acetic acid. The stock solution was obtained with dissolving 5-HT (1mM) in doubled distilled water and kept at -20℃. The buffer solutions (such as Tris-HCl, 0.05 M, pH 7.5) were prepared as supporting electrolyte. Last, other chemicals and solvents in our experiment were the analytical grade.

According to the previous research [8], the screen-printed electrodes (SPE) were used as the three electrode system instead of glass carbon electrode. The experiments were finished with the EC 570 electrochemical workstation (Gaoss Union Technology, Wuhan, China) for detection the voltammograms based on SPE. In addition, the SEM instrument (Quanta 200, Holland) was employed to confirm the characterization of modified SPEs.

2.2. Development of MWNTs/Al_2O_3/chitosan SPE

Modified SPE with MWNTs/Al_2O_3/chitosan was prepared as following report [8]. First, MWNTs SPE was obtained with dropping the MWNTs solution (2mg/mL) on the carbon working electrode, which was air-dried at room temperature for 30 min. Then, Al_2O_3 nanoparticles suspension solution (2mg/mL) was casted on the surface of MWNTs SPE for developing the MWNTs/Al_2O_3 SPE. Then, the MWNTs/Al_2O_3/chitosan SPE was fabricated through coating the working electrode surface of MWNTs/Al_2O_3 SPE with 1μl chitosan solution. Last, in order to investigate the superior performance, the different SPEs such as bare SPE, MWNTs SPE, MWNTs/Al_2O_3 SPE and MWNTs/Al_2O_3/chitosan SPE, were all used for the comparison.

2.3. Animal treatment and real sample administration

Male Kunming mice (25±3 g) were purchased from purchased from Laboratory Animal Center of Wuhan University. And the certification numbers are No. 42000600002002 and No. 00012919, SCXK 2008-00045, China. The animals were maintained for one week under laboratory conditions with controlled temperature (25±2 ◦C), humidity (50±10%) and the 12h/12h light/darkcycle schedule (light on at 8:00 am). All mice were given a standard chow and water during the study. Mice were randomly divided into two groups including control group and model group. The model group was exposed to the chronic unpredictable mild stress (CUMS) for depression model, which the CUMS procedure was performed as described by literatures with a little modification [2,5]. In summary, the stress protocol consisted of food deprivation (24h), water deprivation (24h), a soiled cage (100ml

water in sawdust bedding), 1-min tail pinch (1 cm from the end of the tail), 5-min cold swimming (at 4 ℃), two periods of 45◦ cage tilt (7 and 17 h), two periods of continuous overnight illumination and dark in day (12 and 12 h). The stressors were scheduled in a semirandom order, so that they were unpredictable for the animal. One of these stressors was given every day for 4 weeks.

The open-field test and tail suspension test were the most widely used models for assessing depressant activity in animal [2,5]. The apparatus of open- field test consisted of a wooden box measuring 40×60×50 cm^3, which the floor of the arena was divided into 12 equal rectangles. The mouse was placed in the left corner of the field at the start, which could freely explore the arena. Then, the number of rectangles crossed with all paws (crossing) was counted in a 6-min session. While, tail suspension test was measured as following: a mouse was suspended 50 cm above the floor by placed approximately 1 cm from the tip of the tail. As well, the immobility time was recorded during a 6 min period.

At the end of the experiment, the open-field test and tail suspension test were assessed and the brain samples of the sham control group (S) and CUMS model group (M) were collected for the detection of 5-HT.

2.4. Electrochemical procedure

All electrochemical experiments in present were achieved at room temperature. First, the appropriate 5-HT stock solution was dropped into the Tris-HCl (0.05mol L^{-1}, pH 7.5) buffer solution. CV and SWV were respectively performed to record the electrochemical behavior of 5-HT in the potential range from -0.2 to 0.8V and -0.1 to 0.6V versus the Ag/AgCl reference electrode. Next, the developed electrochemical method by modified SPEs was applied to detect 5-HT level of depression rats. All real samples were diluted with the equal amount of Tris-HCl buffer solution for the direct electrochemical assays with no pretreatment. The diluted real samples were directly added on modified SPE surface, which was immediately scanned with electrochemical SWV.

Furthermore, based on the researches [9], HPLC analysis method was employed to monitor the brain 5-HT content of depression rats as comparison. However, HPLC condition was set with a little change as reports [21, 22]. The liquid chromatographic system equipped with a LC-10Avp plus HPLC module (Shimadzu, Japan) was provided to perform the chromatographic analysis, which the Agilent C18 column (250 · 4.6 mm, DI. 5 μm) was used for the chromatographic separation. Meanwhile, the mobile phase for the analytical column was prepared by mixing acetate buffer and UV-grade acetonitrile (pH 4.0) (v/v 1:9). The chromatograms were monitored by fluorescence detector with the excitation wavelength 254nm and emission wavelength 340nm.

3. RESULTS AND DISCUSSION

3.1. Characterization of modified screen-printed electrode (SPE)

In order to further recognizing the surface characterization of modified SPEs, SEM was employed to evaluate the surface topographies as shown in Figure 1. The SEM image presented the bare SPE (Fig1 A), MWNTs SPE (Fig1 B), MWNTs/Al_2O_3 SPE (Fig1 C) and MWNTs/Al_2O_3/chitosan

SPE (Fig1 D). The surface of bare SPE contained a large amount of sheet graphite particles, which showed small surface area and low catalytic activity [8]. Then, as the materials of electrocatalytic activities [16], multi-walled carbon nanotubes (MWNTs) were applied to many fields such as environmental detection sensors.

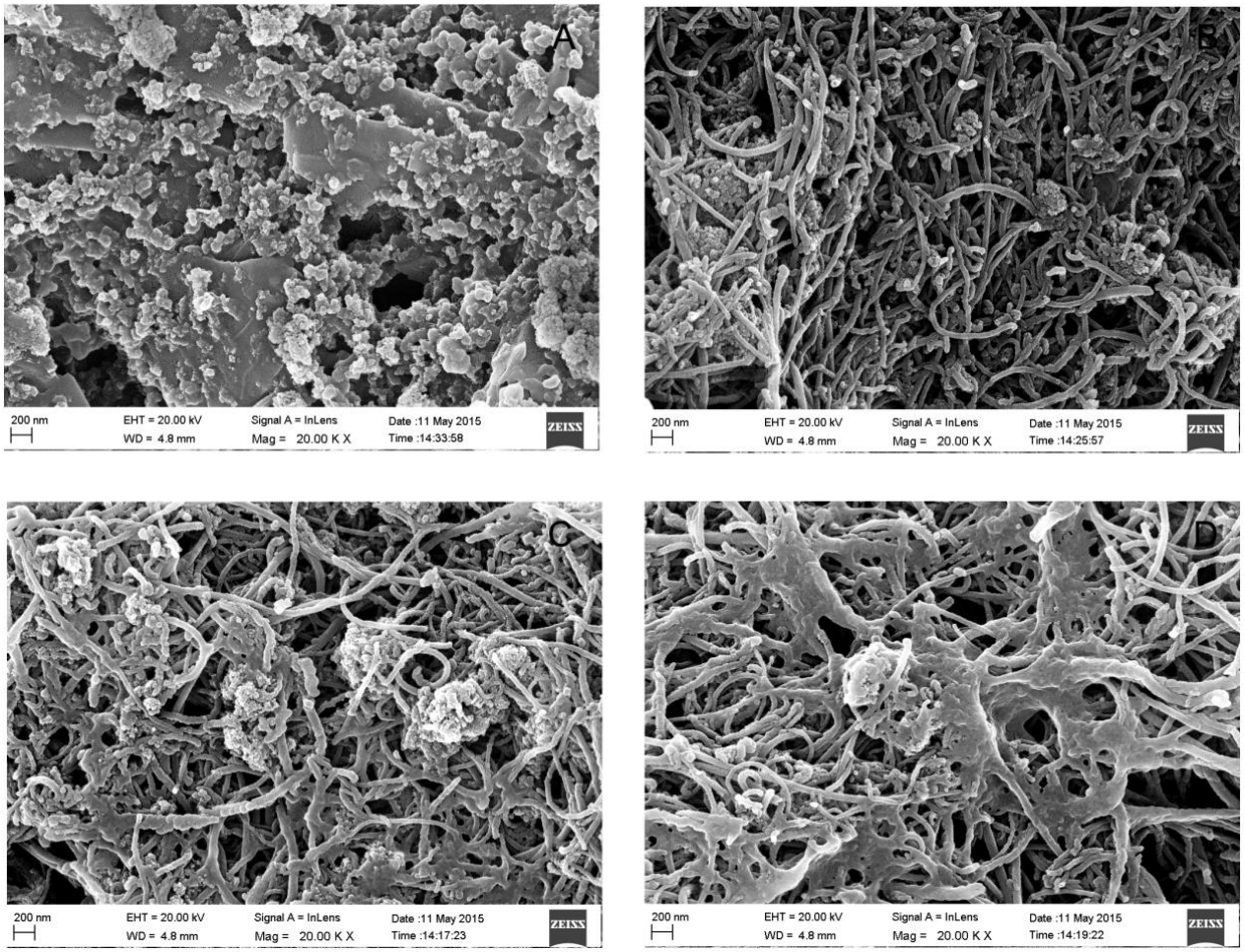

Figure 1. The morphology characterization of bare SPE (A), MWNTs SPE (B), MWNTs/Al_2O_3 SPE (C) and MWNTs/Al_2O_3/chitosan SPE (D) with SEM

Thus, MWNTs SPE was obtained (Figure1B), which increased the surface area and the binding site like other reports [16]. However, the MWNTs/Al_2O_3 SPE assembled the excellent property of MWNTs and Al_2O_3 nanoparticles including the enhancing the electrochemical signal and promoting the electron transfer rate [17，18], which provided more catalytic site. However, according to the previous study [23], the MWNTs/Al_2O_3 film was unstable because of none physical combination. Thus, in order to increasing the stability of modified SPE, the chitosan was recommended, which could be connected to the MWNTs/Al_2O_3 film through the amino groups [18]. So MWNTs/Al_2O_3/chitosan SPE (Figure1D) was fabricated to increase the sensitivity, which the composite film was more stable and provided the surface space and catalytic binding sites.

3.2. Electrochemical behavior of 5-HT at MWNTs/Al_2O_3/chitosan SPE

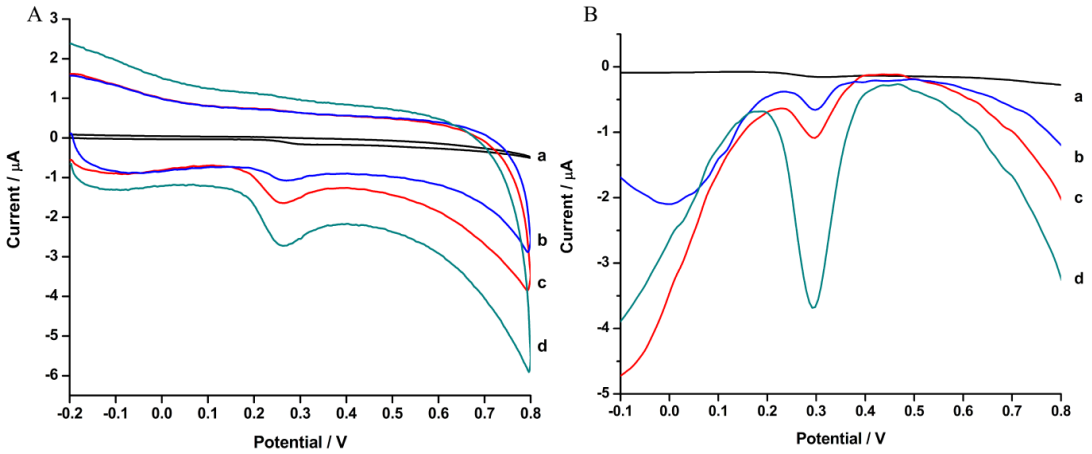

Figure 2. A CV curves of coexisted 5 μM 5-HT at bare SPE (curve a), MWNTs SPE (curve b), MWNTs/Al_2O_3 SPE(curve c) and MWNTs/Al_2O_3/chitosan SPE (curve d) in buffer solution (0.05M Tris-HCl, pH 7.5), scan rate: 100mV/s. **B** SWV curves of 2μ M 5-HT at bare SPE (curve a), MWNTs SPE (curve b), MWNTs/Al_2O_3 SPE(curve c) and MWNTs/Al_2O_3/chitosan SPE (curve d) in 0.05 M Tris - HCl (pH 7.5), scan frequency: 20Hz.

CV and SWV methods were used to investigate the electrochemical behavior of 5-HT at different composites modified SPE. It could be seen that one oxidation peak of 5-HT appeared at different SPEs. As exhibited in Figure 2A, an overlapped CV peak at 0.303V for 5 μM 5-HT was observed at bare SPE (curve a). However, good current response (curve b) was gotten for 5-HT at MWNTs SPE because of large surface area and sensitive electro conductivity of MWNTs. Besides, the background current also greatly enhanced. In order to further increasing the response sensitivity, MWNTs/Al_2O_3 SPE was proposed, and the higher peak current of 5-HT was obtained as curve c (Figure 2A), which was thought to be attribute to the catalytic effects of Al_2O_3 nanoparticles. However, the enhancement was not more than 3 times as prospection compared to curve b. As the fixed material, chitosan was applied to developed the MWNTs/Al_2O_3/chitosan SPE. Compared with MWNTs/Al_2O_3 SPE, better peak shape of 5-HT at prepared sensor was observed more than 3 times. Meanwhile, CV curve d (Figure 2A) strongly indicated that MWNTs/Al_2O_3/chitosan film presented the advantageous properties such as high surface area, more stable subtle electronic ion-exchange characters and so on. In conclusion, MWNTs/Al_2O_3/chitosan SPE greatly improved the detection sensitivity of 5-HT.

Figure 2B showed SWV curves of 5-HT at different SPEs. The oxidation peak potential appeared at 0.283V for 2 μM 5-HT. It could be seen that the peak currents obtained from the MWNTs SPE and MWNTs/Al_2O_3 SPE (curve b and curve c) were enhanced by more than 3 folds in comparison with bare SPE (curve a). Meanwhile, the oxidation peak current (curve d) of SWV from the MWNTs/Al_2O_3/chitosan SPE was 10 folds higher than bare SPE, which the result was similar to the CV result. Thus, Thus, SWV with scan increment of 5 mV, pulse amplitude of 25 mV and scan frequency 20Hz was selected for the further study.

3.3. Effect of pH on the electrochemical oxidation of 5-HT

The effect of pH on the on the electrochemical oxidation of 5-HT at MWNTs/Al_2O_3/chitosan SPE was investigated by SWV measurement. SWV currents of 5-HT were recorded in the pH range of 6.5-9.0, which the peak currents increased with the increase of pH value from 6.5 to 7.5, and reached the maximum at pH 7.5. Then, the peak current decreased with pH increasing instead (Figure 3A). Furthermore, it was also discussed the correlation about the pH value and the peak potential (Ep). As observed in Figure 3B, the oxidation peak potential of 5-HT shifted to negative values when pH increased. Unlike peak currents, the oxidation peak potential (Ep) of 5-HT complied with the following equation: Ep=0.6889-0.0522pH (R=0.9919), which the slope of 52 mV/pH suggested that two protons were involved in the oxidation of 5-HT (Figure 3B). The experiment result suggested that the number of protons in the process was equal to the number of the transferred electrons that was in agreement with the known literature reports [24, 25].

3.4. Effect of accumulation time on the electrochemical oxidation of 5-HT

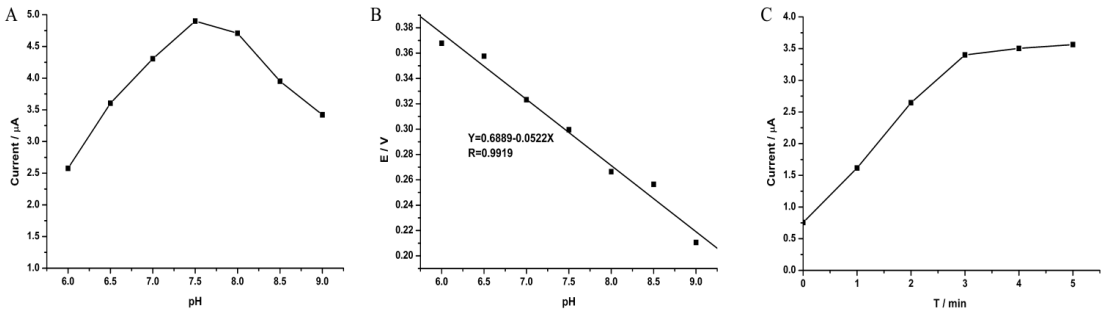

Figure 3. Effects of pH and accumulation time on the electrochemical response of 2 μM 5-HT in buffer solution (0.05M Tris-HCl, pH 7.5) at MWNTs/Al_2O_3/chitosan, the current of 5-HT response to pH (A), the relationship of the peak potential of 5-HT against pH (B) and the current of 5-HT to accumulation time (C).

The influence of accumulation time (T) on the electrochemical oxidation of 5-HT was also examined by SWV as Figure 3C. The peak current of 5-HT obviously enhanced when the accumulation time increased until 3 min. After that, the increase of peak current was no longer significant, which tended to be slow and stable. This result might be caused by the amount saturation of 5-HT adsorbed on the modified electrode surface. Thus, the accumulation time with 3 min was selected for the subsequent analysis.

3.5. Calibration curve

The ability of modified SPE to promote the electrochemical behavior of 5-HT was investigated by SWV. In order to exploring the concentration of 5-HT in the real samples, various concentrations of 5-HT in the buffer solution (0.05M Tris-HCl, pH 7.5) were examined by SWV. Calibration curves at MWNTs/Al_2O_3/chitosan were attained under the optimized procedure conditions, which the result was

showed in Figure 4. And SWV relevant parameters were set as follows: initial potential = -0.1V, end potential = 0.7 V, step width = 5 mV, amplitude = 25 mV, and frequency = 20 Hz. However, the peak currents of 5-HT increased linearly with the increase of concentrations, and the calibration equation was obtained in the range of 0.01 μM to 1.0 μM: Ip (μA) =3.76987c (μM) + 0.23189 (R = 0.9969). Based on the three times the standard deviation of the blank sample measurement, the detection limit of 5-HT was calculated to be 0.005 μM. The intra-assay reproducibility and inter-assay repeatability of 2 μM 5-HT were performed. The intra-assays RSD (%) and inter-assays RSD (%) of three samples of 5-HT were 3.1% and 4.4%, respectively. The results showed that the MWNTs/Al_2O_3/chitosan SPE was reliable and presented excellent property. Moreover, the longtime stability of modified SPE for three months was also investigated by measuring SWV responses of 5-HT. However, the average response decrease was found to be less than 5%. This good stability was mainly because of the MWNTs/Al_2O_3/chitosan composites film.

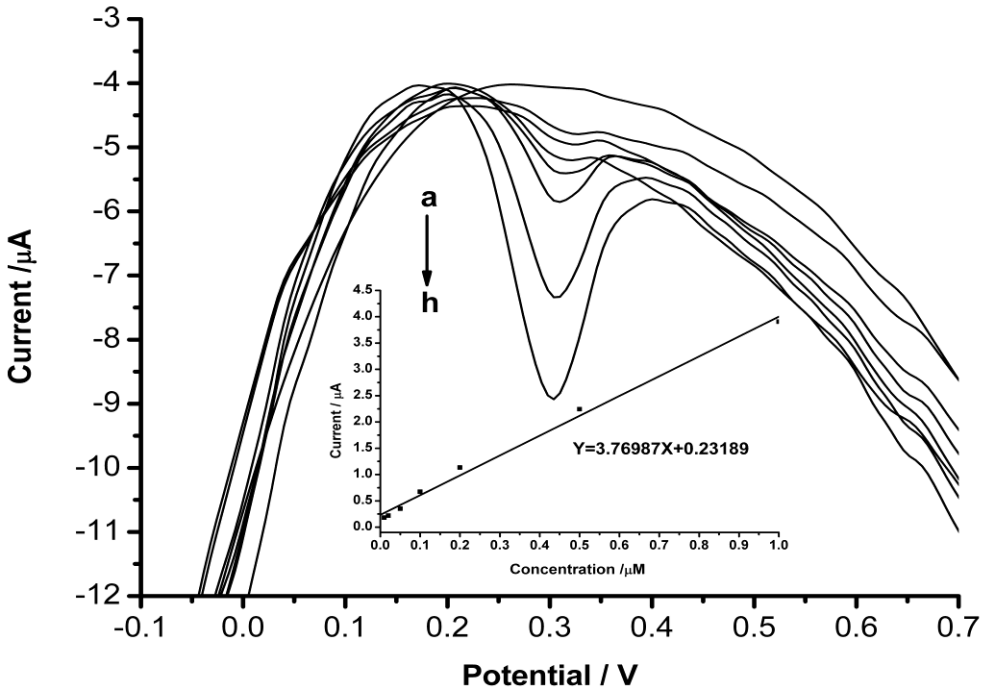

Figure 4. SWV curves of different concentrations of 5-HT in Tris- HCl buffer (0.05 M, pH 7.5), scan frequency: 20 Hz. Concentration: 0.01 μM, 0.02 μM, 0.05 μM , 0.1 μM, 0.2 μM , 0.5 μM and 1.0 μM.

3.6. Effect of interferences

The effect of some common interfering species on the detection 5-HT was investigated under the optimum conditions. The data indicated that the various interferences including 200 μM glucose, 200 μM citric acid, 200 μM AA and 50μM DA did not affect the determination of 0.5 μM 5-HT. And the result was less than ±5% within the margin of error. However, the content of UA in real sample was higher than other substance such as AA and DA, and it might greatly affect the real biological monitor. Therefore, it was necessary to evaluate the interfere effect of UA on the detection of 5-HT. A oxidation peak of 200μM UA appeared at about 0.17V, which presented little interference for the

determination of 5-HT. Thus, the developed method was free from common interfering species (approximately ±5% relative error), which indicated the MWNTs/Al_2O_3/chitosan SPE presented good anti-interference capability for the determination of 5-HT in real biological sample.

3.7. Real brain sample analysis in depression rat.

After 4 weeks of CUMS, it could be observed that crossing numbers of in M group significantly reduced compared S group, and however, the immobility time of the tail suspension test in M group obviously increased than S group, which the results both presented the statistic difference ($p<0.01$) (as exhibited Figure 6A). The depression model was successfully established in terms of behavior tests. Next, the concentration of 5-HT in mice brain tissue was detected with modified SPE and HPLC for comparison. HPLC detection results were displayed as Figure 6. It was obvious to separate the major analyte 5-HT within 10 min, which the retention time of standard 5-HT was about 7 min as Figure 6B. The HPLC calibration equation was obtained with Y=4.470x+0.034(R=0.9981). The HPLC flow results of brain 5-HT in S group and M group were detected as Figure 6C and 6D, respectively, which the retention time was similar to Figure 6B. Furthermore, the developed method was also employed to determinate the 5-HT content of brain tissue in depression mice as Figure 6 E. Cruve a was the SWV curve of spiked standard brain tissue sample, and an obvious oxidation peak appeared at about 0. 3 V, which the calibration equation was displayed as follwing: Y = 3.853x + 0.215 (R=0.9930). The proposed SPE method presented good detection results of 5-HT in brain biological samples such as curve b and curve c in S group and M group. It could be observed the results of modified SPE and HPLC were similar with RSD less than 5% by two methods. The 5-HT level of M group was declined obviously compared with S group, which was in agreement with the reports [5, 21, 22]. Thus, the established method was accurate, reliable and effective during the determination of 5-HT level in depression.

Table 1 presented the analytical performance of fabricated sensor for the comparison of the sensor with similar reported serotonin sensors [8,24]. Although, the detection range of MWNTs/Al_2O_3/chitosan SPE was inferior to some sensors, the proposed sensor could be successfully applied to detect 5-HT content of brain tissue in depression mice. To our knowledge, it was the first report about the modified SPE for depression dtermination. While, other sensors couldn't achieve, which was only used for the PBS, blood sample and clinical serum.

Table1 Comparison of the detection samples and linear range obtained at the MWNTs/Al_2O_3/chitosan SPE for detection of 5-HT with other sensors.

Methods	Linear range (μM)	Detection samples	Reference
MCM-41-NH_2/GCE	0.0002-0.094	Human serum	6
MWNTs-ZnO/chitosan SPE	0.10-1.00	CFS	8
MWNTs-SiO_2-chitosan SPE	0.10-2.0	CFS	15
brain tissue in depression	0.20-2.00	Blood serum	24
AuAg NPs-GR	0.0027-4.82	Human serum	26
MWNTs/Al_2O_3/chitosan	0.01-1.0	Depression brain tissue	This work

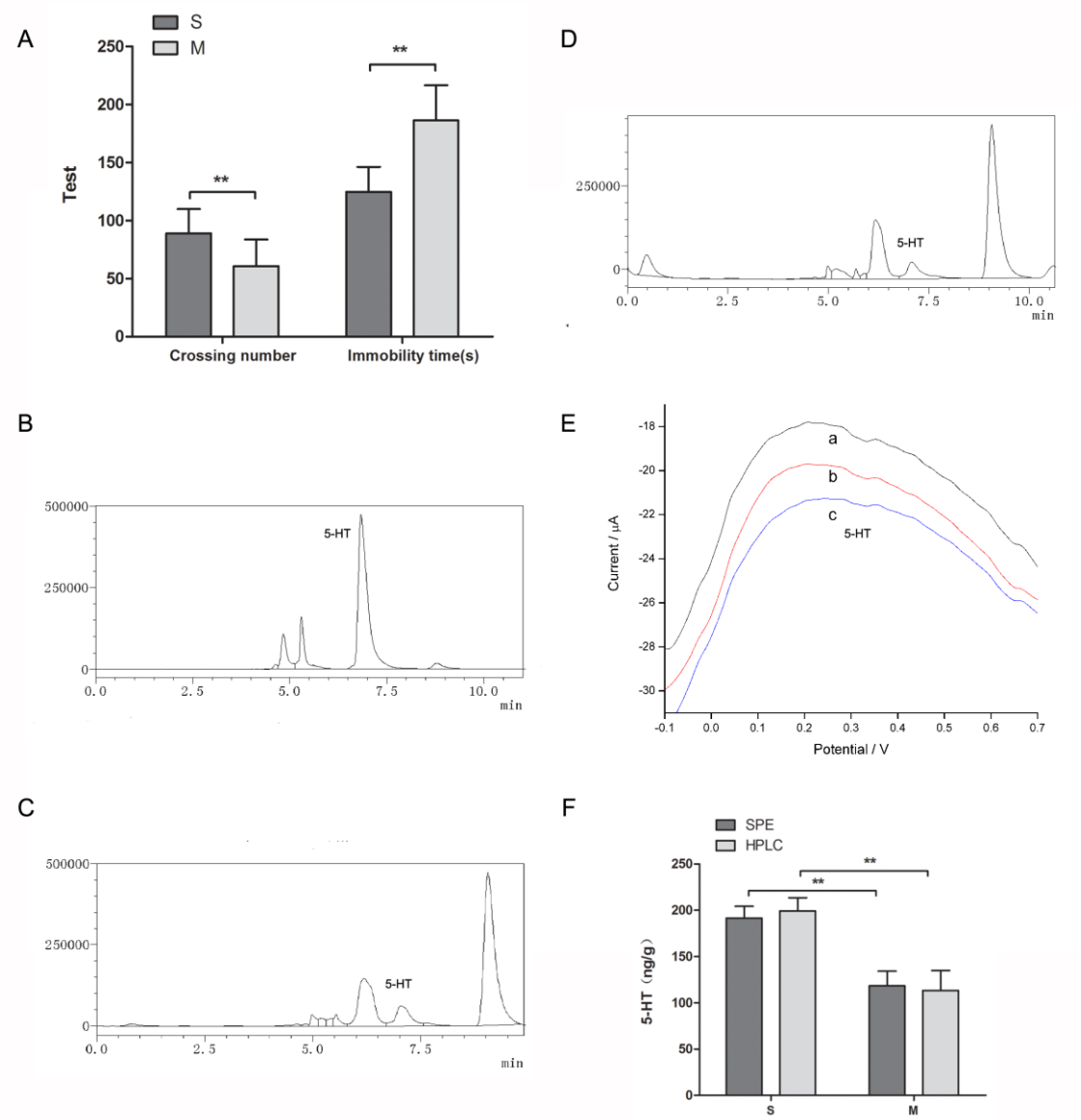

Figure 6. (A) The crossing number of the open- field test and the immobility time of the tail suspension test in S and M groups; (B) chromatograms of a standard sample spiked with 0.1 μM 5-HT (7.02min); (C) chromatograms of a brain tiusse sample in S group (7.03min); (D) chromatograms of a brain tiusse sample in M group (7.06min); (E) SWV curves of a standard sample spiked with 0.01μM 5-HT(curve a)and brain tiusse samples in S group (curve b) and M group; (F) the 5-HT contents of brain tissue in S and M groups detected with modified SPE and HPLC methods.** P<0.01, M group compared with S group

4. CONCLUSION

To our best knowledge, few researches had published about disposable sensor for detection of brain 5-HT level in depresssion rat. Our work shared a simple and novel method to monitor the brain 5-HT concentration for helping depresssion diagnosis. MWNTs/Al_2O_3/chitosan SPE was successfully fabricated and firstly used during the CUMS micemodel with good result, which the electrochemical oxidation peak of 5-HT was gotten at about 0.3 V through SWV, and the oxidation peak currents were increasing linearly with concentrations. Meanwhile, the developed method was compared with HPLC

for real sample determination. And ideal results indicated modified SPE had high sensitivity and good repeatability, which could directly determinate the brain tissue samples without complex HPLC pretreatments and provide a promising tool for fast depression determination of 5-HT. In summary, the established method offered a potential and easy extension to on-spot depression desiease diagnosis.

ACKNOWLEDGEMENTS

This research was supported by the Guangdong Provincial Medical Science and Technology Research Foundation (B2017087), Scientific Research Project of Health and Family Planning system in Shenzhen (201601030).

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