IMPROVED ENZYME IMMOBILIZATION FOR ENHANCED BIOELECTROCATALYTIC ACTIVITY OF CHOLINE SENSOR

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ABSTRACT

A highly sensitive and stable choline sensor based on the synergetic effect of multi-walled carbon nanotubes and ZnO nanoparticles has been developed. This nanomaterials-based choline sensor was highly sensitive and showed good stability over a relatively long-term storage (90 days). The sensor prepared showed a linear response range of 1.0 μM to 0.8 mM, a sensitivity of 178 $\mu AmM^{-1}cm^{-2}$ and a detection limit of 0.3 μM.

KEYWORDS

Choline Biosensor, Choline Oxidase, Multi-Wall Carbon Nanotubes, ZnO Nanoparticles, Synergic Action

INTRODUCTION

The determination of choline is important in biological sciences of human bile, serum, amniotic fluid, brain extracts and pharmaceutical products. Among the detection methods reported, amperometric biosensors based on choline oxidase (ChOx) is one of the most promising methods for the detection of choline in clinical, industrial and environmental areas, as it offers a simple, inexpensive and rapid operation [1–4]. A variety of different immobilization techniques have been reported to construct choline amperometric biosensors [5, 6]. Carbon nanotubes (CNTs) have been incorporated in electrochemical sensors to decrease the over potential and improve the sensitivity. Recent studies of CNTs have focused on depositing metal or metal oxide nanoparticles (NPs) onto the nanotube's surface to enhance the performances of the biosensors. Qin et al. [7, 8] developed a choline biosensor based on the nanocomposite film composed of ChOx, MWCNTs, gold NPs and poly(diallyldimethyl ammonium chloride) (PDDA) for the specific detection of choline. Dai et al. [9，10] reported an electrochemiluminescent sensor, in which biocompatible titanate nanotubes and ChO_X, were immobilized on a chitosan modified glassy carbon electrode. Zhang et al. [11] fabricated a biosensor with supramolecular architecture, in which ChOx and horseradish peroxidase were assembled onto the polymer of thiolated ß-cyclodextrin and platinum NPs modified gold electrode through 1-adamantane carboxylic acid coupling. Those sensors, however, hold less than 90% of the initial sensitivity even after 30 days, exhibiting poor long-term stability. To solve this problem, Shimomura et al. [12] immobilized ChOx within mesoporous hybrid membranes, which can make the enzyme more mecanically robust and stable, but the response time increased up to 2 min.

ZnO is a wide-used semiconductor with a band gap of 3.37 eV and possesses attractive electronic and optical properties. Nanostructured ZnO has shown a great potential for sensor applications due to its biocompatibility and high electron mobility，such as the ZnO nanocomb glucose biosensor [13], the ZnO:Co nanoclusters based glucose sensor [14], the ZnO nanorod array based glucose sensor [15], the tyrosinase biosensor based on biofunctional ZnO nanorod microarrays [16], the cholesterol biosensor based on well-crystallized flower-shaped ZnO nanostructures [17], the glucose biosensor based on ZnO hollow nanospheres [18], the differences between the transferred ZnO biosensor and the grown ZnO biosensor [19]. In addition, ZnO with a high isoelectric point (IEP ~ 9.5) should be suitable for the adsorption of proteins or enzymes with low IEP [20]. We thus assumed that the positively charged ZnO NPs could not only provide a friendly microenvironment for the negatively charged ChOx (IEP=4.1) to retain its activity but also could promote the fast electron transfer between the ChOx and the electrode. Yang et al. [21] immobilized acetylcholinesterase (AChE) into a ZnO membrane deposited on a Pt electrode and developed an acetylcholine and choline sensor. Sinha et al. [22] reported a disposable AChE biosensor based on zinc oxide sol–gel for the detection of pesticides. However, they used ZnO film and ZnO sol gel, but were not ZnO NPs. Therefore, further studying on the application of ZnO NPs and MWCNTs is valuable to extend its application.

In this work, the unique multi-layer structure (PDDA/ChOx/ZnO/MWNTs) provided a favorable microenvironment to keep the bioactivity of ChOx, which led to a highly sensitive and extremely stable choline sensor.

EXPERIMENTAL

Apparatus

Electrochemical experiments were performed with a CHI 630A workstation (CH Instruments, Shanghai, China) at room temperature, employing a conventional three-electrode system containing a 3.0 mm-diameter pyrolytic graphite (PG) disk working electrode modified with PDDA/ChOx/ZnO/MWNTs, a platinum wire as the auxiliary electrode, and the Ag/AgCl (3 M KCl) as the reference electrode. SEM, TEM and XRD images were recorded by using a JEOL JSM-6700F Electron Microscope (Japan), HRTEM (Hitachi H-9000) and a D/MAX 2550V with Cu K radiation, respectively. An Agilent series HPLC system (Agilent Technologies) equipped with a thermostated autosampler and a degasser were used for the detection of choline in the blood samples in the control experiment.

Reagents

ChOx (EC 1.1.1.17, 14 units/mg, from *Alcaligenes*), choline chloride, D_9-choline chloride and PDDA were purchased from Sigma Chemical Co. Uric acid, ascorbic acid, glucose, acetaminophen, Serine, cyteine and trichloroacetic acid (TCA) were obtained from Tianjin

Damao Chemical Reagent Co. (China). Methanol, acetonitrile and formic acid were HPLC grad and purchased from Merch Chemicals. Other reagents were of analytical-reagent grade. All solutions in the testing were prepared using doubly distilled water.

Preparation of ZnO / MWNTs / PG electrode

The pyrolytic graphite (PG) (3-mm in diameter, 1-mm in thickness) was firstly polished with metallographic abrasive paper (No. 6), slurries of 0.3- and 0.05-μm alumina, and sequentially sonicated in acetone, NaOH (1 mol L^{-1}), HNO_3 (HNO_3 : H_2O=1:1, v/v) solution and doubly distilled water. After being rinsed with double-distilled water, it was sonicated again with absolute ethanol and double distilled water for about 1 min, respectively. MWNTs were obtained by catalytic decomposition of CH_4 with La_2NiO_4 as catalyst [23].

ChOx immobilization

ZnO/MWNTs/PG electrodes with identical performance were screened out. ChOx solution was prepared by dissolving 9.62 mg ChOx in 0.5 mL of 0.05 M phosphate buffer solution (PBS, pH 7.8). 1.0 μL of such solution was dropped onto the surfaces of ZnO/MWNTs/PG electrodes. Then the electrodes were dried in air at room temperature. To improve the distributing uniformity of ChOx on the electrodes surface, this procedure was repeated four times until the desired amount of ChOx was reached. Finally, 4.0 μL of 0.5% PDDA was coated on the enzyme electrode to eliminate the possible fouling and prevented the leaching of the enzyme. The enzyme electrodes were stored in refrigerator at 4 °C before use.

Blood samples for sensor test

Preparation of blood samples was carried out according to Ref. [24]. Briefly, for determination of free choline (unbound), 1 ml of plasma was diluted with 9 ml of PBS containing 5% TCA to precipitate protein. The mixture was centrifugated at 2000 rpm for 10 min and the supernatant was collected and passed through a glass column with 80 mm in length and 6 mm in diameter in which the Dowex 1 anion-exchange resin was packed to remove interferences. 5ml of the filtrate was transferred into the electrochemical cell. Note that the original specimen was diluted ten times.

For determination of total choline (unbound and bound), 0.1 ml of plasma was diluted with 0.4 ml of 3% (v/v) H_2SO_4 and autoclaved at 121 ºC for 30 min. After centrifugation, 0.1 ml supernatant was diluted with 7.9 ml of PBS buffer solution. Then 5 ml of the diluted supernatant was transferred into the electrochemical cell. Note that the original specimen was diluted 400 times. The sensor was immersed in a 10 ml cell containing the testing solution and maintained at desired temperature by using thermostated water circulation.

RESULTS AND DISCUSSION

Cyclic voltamograms of choline

It can be seen from Figure 1 that the PDDA/ChOx/MWCNT/PG electrode exhibited good electro-catalysis to the oxidation of choline starting around 220 mV and reaching the maximum current value at 400 mV (Figure 1, curve b). After the growth of ZnO NPs, the oxidation current of choline showed a great increase (Figure 1, curve c). These results indicated that the remarkable synergistic effects of the ZnO NPs and MWCNTs promoted the direct electron transfer of ChOx greatly.

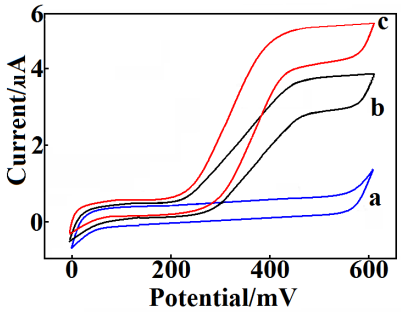

Figure 1: Cyclic voltammograms of 0.5 mM choline on PDDA/ChOx/MWCNT/PG electrode and PDDA/ChOx/ ZnO/MWCNT/PG electrode in 0.1 M PBS buffer solution with a scan rate of 100 mV s^{-1}.

Calibration curves

The response time of the choline sensor based on the PDDA/ChOx/ZnO/MWCNT/ PG electrode can be abstracted from the time dependent current curve obtained at a fixed potential of 400 mV. The response times were 10 s and 13 s for the concentration of choline changing from 0 to 0.5 mM and reverse, respectively, indicating the establishment of stable mass transport within 13 s. Fig. 2 represents the amperometric responses of a PDDA/ChOx/ZnO/MWCNT/ PG electrode at an applied potential of 400 mV to choline in 0.1 M PBS (pH7.8) under continuous stirring. Inset shows the calibration plots for choline from 1.0 to 10.0 μM. Each data point and error bar represents the mean and SD of 3 replicate measurements. The linear response range, sensitivity and detection limit (3σ) were 1.0 μM -0.8 mM, 178 $\mu AmM^{-1}cm^{-2}$ and 0.3 μM, respectively. It can also be clearly seen from the Figure 2 that the choline concentration at a half of the maximum current, which corresponds to the value of the apparent Michaelis constant (K_M^{app}), is about 0.81 mM.

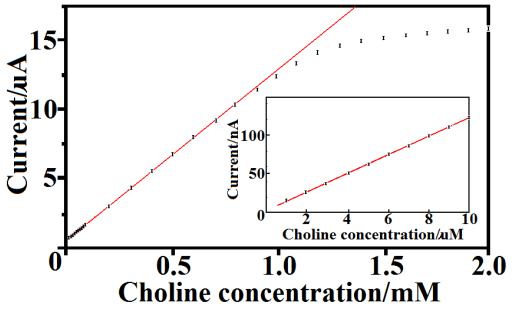

Figure 2: Amperometric responses of a PDDA/ChOx/ZnO /MWCNT/ PG electrode at an applied potential of 400 mV to choline in 0.1 M PBS (pH7.8) under continuous stirring. Inset shows the calibration plots for choline from 1.0 to 10.0 μM.

The response of the choline biosensor to 0.5 mM choline standard solution was repeatedly measured 6 times under the same conditions. The measurement precision for the sensor typically has a relative standard deviation (RSD) of ~2.36 %.

Thermal Stability

Enzymes are susceptible to thermal denaturation. However their thermal behavior will differ from that they are in the "free" state when they are immobilized onto a conducting surface [25]. Thermal stability is an important factor to evaluate the practical features of the biosensor to withstand elevations in temperature, frequently in excess of those that normally denature the native enzyme [26]. The thermal stability of the proposed sensor was investigated between 10 and 50 ºC. The amperometric responses continued to increase with the increase of temperatures and exhibited the ideal Arrhenius temperature-dependant relationship (inset of Fig. 3). The effect of temperature on the ChOx – glutaraldehyde – BSA – modified sensor was also investigated under the same condition for comparison. In this case, immobilized ChOx almost lost its most activity at 50 ºC and showed low amperometric response. We are not clear about this abnormal phenomenon and still working on it. The experiment above confirmed that the immobilization of ChOx on ZnO NPs exhibits enhances of its thermal stability.

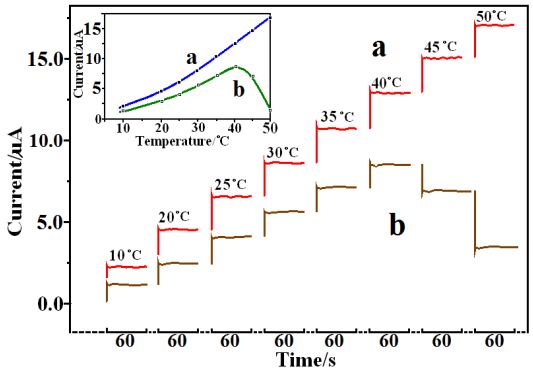

Figure 3: Amperometric responses for the proposed sensor (a) and the sensor with LOD-glutaraldehyde-BSA at 10, 20, 25, 30, 35, 40, 45 and 50 ºC in 0.5 mM choline in 0.1 M PBS (pH 7.8). Inset: plot of current vs. temperature. Measurement time is 60 s. Heating time is not marked.

Interferences

Table 1 Effect of possible interferents on the biosensor response to 0.5 mM of choline

Possible interferents	Response current/μA
0	6.61±0.16
Cysteine/0.2 mM	6.63
Glucose/7.0 mM	6.67
Serine/10 mM	6.65
Uric acid/0.1 mM	6.65
Acetaminophen/0.1 mM	6.64
Ascorbic acid/0.1mM	6.73

Table 1 shows that the amperometric responses of the sensor to possible interference species including ascorbic acid, uric acid, glucose, acetaminophen, serine and cysteine [27]. The current responses to the tested interferences were lower than 0.13 μA, less than RSD 2.36 % of 6.61 μA which was the response of the sensor to 0.5 mM of choline. Therefore, when the concentration of choline is higher than that of interferences species, their influence could be ignored, just as in the procedure for the determination of total choline. However, when the concentrations of interference species are several ten times larger than that of free choline in plasma, they have to be separated to obtain an accurate result.

Long-term stability

A decrease in response current of the choline sensor was observed during the first several days, which might arise from to the loss of un-immobilized enzymes. The response current decreased only 5.4 % of the original value over 90 days for the choline sensor (See Fig. 4). This long-term stability is attributed to the synergetic effects of MWNTs and ZnO NPs.

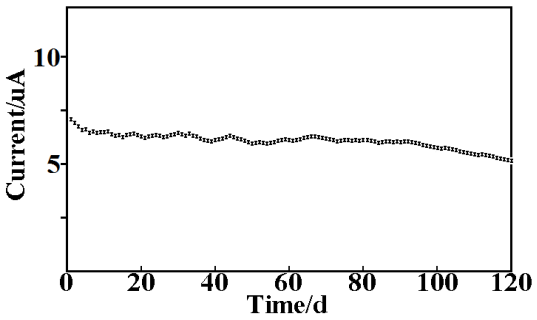

Figure 4: Long-term stability of the PDDA/ChOx/ZnO /MWCNT/ PG electrode.

CONCLUSIONS

The unique multilayer structure (PDDA/ChOx/ZnO/ MWCNT/PG) provided a favorable microenvironment to keep the bioactivity of ChOx and the excellent electrocatalytic activity of MWNTs toward H_2O_2, enabling sensitive determination of choline with good reproducibility and excellent stability.

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