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A REAGENTLESS BIOSENSOR OF NITRIC OXIDE BASED ON DIRECT ELECTRON TRANSFER PROCESS OF CYTOCHROME C ON MULTI-WALLED CARBON NANOTUBE

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1. ABSTRACT

Direct electron transfer between Cytochrome c (Cyt.c) and electrode can be achieved through immobilizing Cyt.c on the surface of multi-walled carbon nanotubes (MWNTs). Under the condition of cyclic potential scans, Cyt.c can be adsorbed on the surface of MWNTs that were modified on a glassy carbon (GC) electrode to form an approximate monolayer. The redox characteristic and bioactivity of Cyt.c could be remained after it was adsorbed on MWNTs' surface. This provides a way to construct a new biosenser based on the activity of Cyt.c. Further investigation displayed that Cyt.c adsorbed on MWNTs showed an enzyme-like activity to catalyze the reduction of nitric oxide (NO). Due to catalyzing by Cyt.c, the reduction of NO in aqueous solution was achieved, which reductive potential appeared at -0.747V (vs. SCE). The peak currents were linearly proportional to concentration of NO in the range from 2 to 48 μmol/l with a limit of detection of 1.3 μM. The biosensor showed a good stability and excellent repeatability.

2. INTRODUCTION

In recent years, direct electrochemistry of metalloproteins and metalloenzymes arouse many scientists' interest because of its potential application in the study of the redox and electron transfer properties of biomolecules(1~2), and in fabricating mediator-free or the third generation biosensors (3). Cytochrome c (Cyt.c) is a biologically important redox protein that is involved in electron transfer reactions in the mitochondrial respiratory chain(4). Cyt.c contains one Fe(III) redox center located in a haem unit which is approximately spherical shape with 34Å diameter and a molecular weight of 12,384. In electrochemistry, it is generally difficult for Cyt.c to transfer electron with a conventional electrode. So, various modified electrode were proposed to investigate its direct electrochemistry(5-17). Carbon nanotube, a kind of inorganic material with a nanostructure, is promising as an immobilization substance due to its significant mechanical strength, high surface area, excellent electrical conductivity, and good chemical stability(18). The subtle electronic properties indicates that it can promote the electron-transfer reactions of biomolecules when used as an electrode modifier (12). In this paper, Cyt.c was adsorbed on the surface of multi-walled carbon nanotubes (MWNTs) and the direct electron transfer between Cyt.c and MWNTs modified electrode was observed. Further investigation suggested that Cyt.c adsorbed on MWNTs still showed a bioactivity to catalyze the reduction of nitric oxide (NO).

Nitric oxide, freely diffuses through cell membranes, has been recognized as a ubiquitous signal transduction molecules(19,20). It plays fundamental role in many biological and physiological processes. An

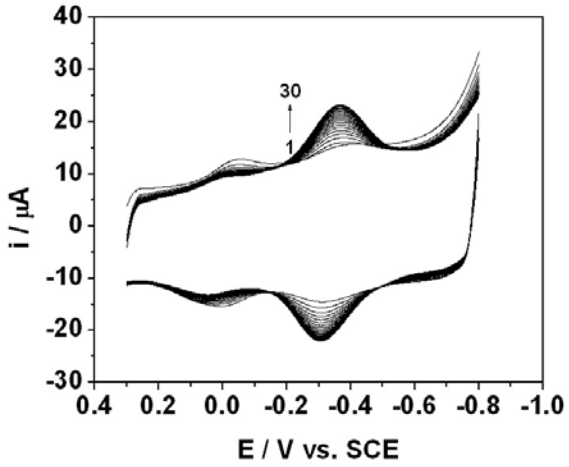

Figure 1. Multicyclic voltammograms of a MWNTs modified GC electrode in 0.1 M phosphate buffer (pH7.0) containing 9.0×10-5 mol/l Cyt.c. Scan rate: 50 mV/s.

excess amount of NO has shown to cause some human diseases(21,22). In order to clarify the function and to control the concentration in biological systems, selective and sensitive detection of NO is of great importance. However, the detection and quantification of NO is particularly difficult because of its low concentration, short half-time, low stability, and high reactivity with superoxide and other very active free radicals present in biological systems (23). Here, it was found that Cyt.c abosbed on MWNTs had an excellent electrocatalytic activity to the reduction of NO. Further, a novel reagentless biosensor, which can be used to detect NO, was constructed.

3. EXPERIMENTAL PROCEDURES

3.1. Apparatus and reagents

Expectrochemical experiments were carried out with a CHI660 electrochemical analyzer (CHI, USA) with a conventional three-electrode cell. A Cyt.c/MWNTs modified GC electrode was used as the working electrode. A saturated calomel electrode (SCE) and a platinum electrode were used as the reference and the counter electrode, respectively.

The MWNTs were obtained from Chengdu Institute of Organic Chemistry of Academy of Sciences and the purity is more than 95%. Horse heart Cyt.c was obtained from Sigma Chemical Company and used without further purification. Saturated NO solutions were prepared as the previous literature (24,25) with a concentration of 1.9×10^{-3} mol/l. Other chemicals were of analytical grade and use as received. All the solutions were prepared with double distilled water and were deaerated with high purity nitrogen before experiments. All electrochemical experiments were carried out at room temperature.

3.2. Preparation of the Cyt c /MWNT/GC electrode

The GC electrode (3mm in diameter) was polished sequentially with abrasive paper (NO.6) and slurries of 0.3 and 0.05-μm alumina to mirror. Then, it was washed with double-distilled water and ethanol in an ultrasonic bath, and 15 cyclic scans were carried out in the potential range from 2.0 to –2.0V (vs. SCE) in the solution of 1.0 mol/l H_2SO_4. Acid treated MWNTs were prepared as the precious report (29). 2.5mg of acid treated MWNTs was dispersed in 10 mL acetone under the ultrasonic agitation to form a black solution. 25μl of acid treated MWNTs solution (0.25 mg/ml) was dropped on the surface of GC electrode. The solvent acetone was evaporated into air to form an MWNTs-modified electrode. The effective area of the modified electrode, calculated from the cyclic voltammogram of 1mmol/l $K_3(Fe(CN)_6)$, is ca. 0.30 cm^2 .

Cyt.c/MWNTs modified electrode was prepared by following steps: Cyt.c was dissolved in pH7.0 phosphate buffer solution (PBS) and the above-prepared MWNTs electrode was transfer into the Cyt.c solution. A consecutive cyclic scan was preformed in the potential range from +0.3 to –0.8V (SCE) up to obtained a stable voltammogram. Then, the electrode was removed from the solution, washed with double-distilled water and stored in pH 7.0 PBS at about 4ºC.

4. RESULTS AND DISCUSSION

4.1. The adsorption of cytochrome c on MWNTs

Figure 1 shows the cyclic voltammograms of Cyt.c at MWNTs-modified GC electrode under the condition of consecutive scan. It shows clearly the adsorption process of Cyt.c on the surface of MWNTs. In figure 1, two couple peaks can be observed. One couple peaks at near 0V, the peak currents decrease slightly with cyclic scans, is corresponding to the redox of carboxylic groups on the surface of acid treated MWNTs (26). Another one at near –0.32V, which peak currents increase with cyclic scans, should be corresponding to the redox of Cyt.c. The more the cycles that the modified electrode swept in the Cyt c solution was, the higher the redox peaks was, demonstrating that Cyt.c could be adsorbed onto the surface of MWNTs. When the cycle was above 70 circles, no obvious changes of peak currents could be observed from the cyclic voltammograms, indicating the adsorption of Cyt.c reached a saturated state (27).

The carboxylic acid groups were introduced onto the MWNTs surface when MWNTs were pretreated with nitric acid during the purification process (28). On the other hand, Cyt.c is a redox metalloprotein with an over charge of +7/+8 at neutral pH (16). So, Cyt.c can be effectively adsorbed on the surface of acid treated MWNTs by electrostatic interaction. And continuous cyclic potential sweep can promote the adsorption process dramatically.

4.2. Direct electrochemistry of Cyt c on the surface of MWNTs

After enough cyclic scans were performed in a phosphate buffer (pH7.0) containing 9.0×10^{-5} mol/l Cyt.c, the electrode was transfer into a blank PBS. A pair of quasi-reversible redox peaks can be observed, as shown in Figure 2. The anodic and cathodic peak potential located at –303 mV and –365mV, respectively. The heights of

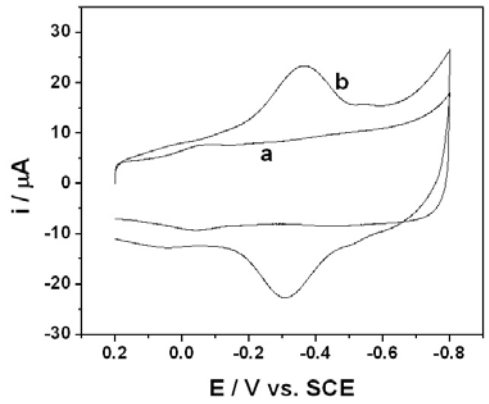

Figure 2. Cyclic voltammograms of MWNTs modified GC electrode (a) and Cyt.c/MWNTs eletrode (b) in 0.1 mol/l PBS (pH7.0). Scan rate: 50 mV/s.

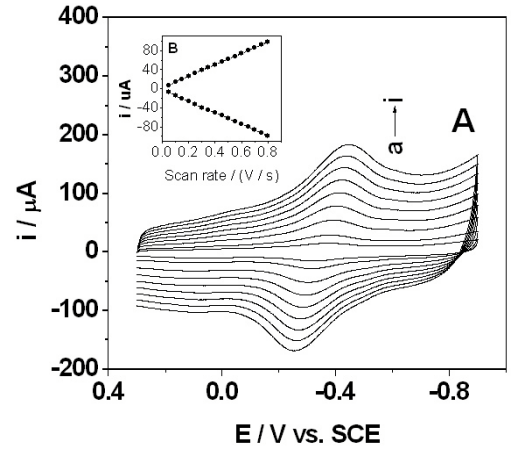

Figure 3. Cyclic voltammograms of Cyt.c/MWNTs/GCE in 0.1 mol/l PBS (pH7.0) at scan rate 50, 100,200, 300, 400, 500, 600, 700, 800 from a to i. Inset: Plot of cathodic and anodic peak current vs. scan rate.

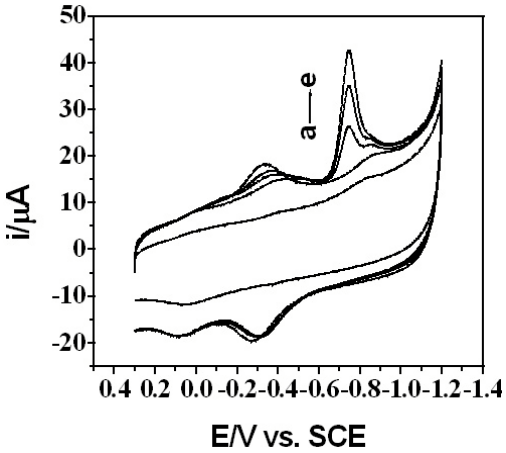

Figure 4. Cyclic voltammograms obtained at MWNTs modified electrode (a) and Cyt.c/MWNTs electrode (b, c, d, e) in 0.1 mol/l phosphate buffer (pH7.0) solution containing (a)56μM, (b)0, (c)24μM, (d)40μM, (e)56μM NO. Scan rate: 50 mV/s.

the oxidative and reductive peaks were nearly equal, and the shapes of the redox peaks were symmetric, suggesting that all electroactive Cyt.c in the film was reduced on the forward negative scan and the reduced Cyt.c was oxidated fully again on the reverse positive scan.

The effect of scan rate on peak current was investigated and the results are shown in Figure 3. As can be seen, reductive and oxidative peak currents increased linearly with scan rates in the range of 20 to 800 mV/s. Integration of reduction peaks gave approximately constant charge (Q) values with different scan rates. All these are characteristic of surface-control or thin-layer electrochemical behavior. According to following equation (29): $I_p=n^2F^2A\Gamma^*\nu/4RT$, an average surface concentration (Γ^*) of electroactive Cyt.c adsorbed on MWNTs could be calculated. In our experiments, it is $(4.1\pm0.3)\times10^{-10}$ mol/cm^2, showing an approximate monolayer adsorption. According to Laviron's equation(30), the heterogeneous electron transfer rate constant(Ks) was calculated to be 4.0 ± 0.2 s^{-1}, suggesting a fast electron-transfer kinetics process.

4.3. Electroatalytic reduction of NO on Cyt.c/MWNTs electrode

The electrocatalytic properties of Cyt c/MWNTs electrode toward NO were investigated and the results were shown in Figure 4. In a pH 7.0 phosphate buffer containing NO, a new cathodic peak appears at the potential of –0.747V. The peak currents increase with the concentration of NO (as shown in c, d, e). Obviously, no corresponding cathodic peaks can be observed at MWNTs modified GC electrode under the same conditions (as shown in Figure 4a). So, this new cathodic peak should come from the reduction of NO that was catalyzed by Cyt.c.

pH of supporting electrolyte is an important effect factor in the determination of NO. As shown in Figure 5, the cathodic peak currents decreased gradually with the increase of pH value in the range from 4.0 to 8.0. However, the peak currents kept a relative stability from pH 6.0 to 8.0. The peak high of the cathodic peak at pH 4.0 is about 1.8-fold higher than that at pH 8.0. This means that there is a higher sensitivity for the determination of NO under the condition of a lower pH. However, considering the potential biological application of this sensor, the condition of pH 7.0 was selected to investigate the characteristics of the sensor in our experiments.

The differential pulse voltammogram(DPV) is a technique with better peak shape and higher sensitivity (31). Figure 6 shows the DPV curves obtained under different NO concentration. It can be observed that the peak current increased with NO concentration. In the range of 2.0 ~ 48 μM, the peak current is proportional linearly to the concentration of NO with a correlative coefficient of 0.9977. The limit of detection is 1.3 μM when the signal-to-noise ratio is 3. The relative standard deviation of peak current was only 3.2% when seven independent solutions containing 1.0×10^{-5} M NO were determined.

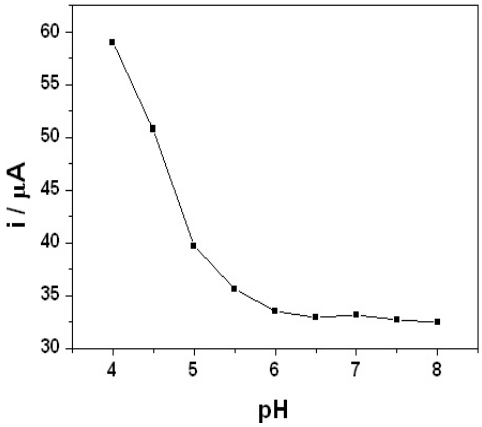

Figure 5. Relationship between the peak current of NO reduction and the pH value of supporting electrolyte solution.

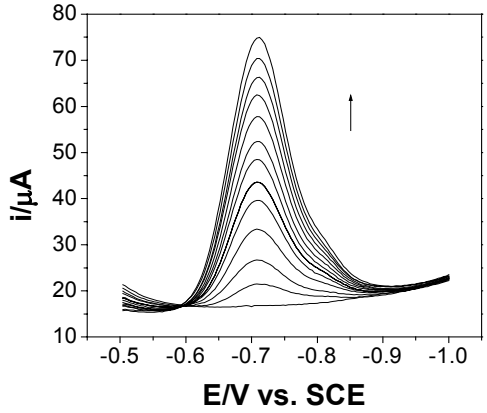

Figure 6. Differential pulse voltammograms obtained at a Cyt.c/MWNTs electrode in different NO concentration solution. Initial potential: -0.5 V, final potential: -1.0 V, pulse height: 50 mV, pulse width: 50 ms. NO Concentrations is 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 μM from a to m, respectively.

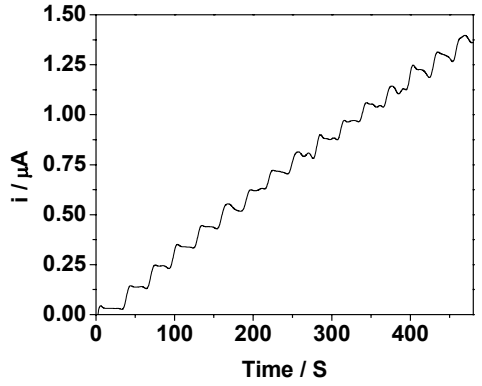

Figure 7. Amperometric response of NO at Cyt.c/MWNT modified electrode in o.1 mol/l phosphate buffer solution. Alternating current was measured at constant potential of –0.75 V modulated with 50 mV pulse in time intervals of 0.5s. Each addition of 1 μmol/l NO.

In order to investigate the response characteristic of Cyt.c/MWNTs electrode as a NO biosensor, amperometric experiment was preformed. The amperograms were recorded through successively adding NO to a continuous stirring PBS solution. In this process, a potential of –0.75V was applied to the working electrode. Figure 7 shows the amperometric response curve of Cyt.c/MWNTs modified electrode to the reduction of NO. As shown in Fig.7, the response of the electrode to NO should be a quick response process.

According to the Lineweaver-Burk equation (32):

$$1/I_{ss}=1/I_{max}+K_m^{app}/I_{max}C$$

where Iss is the steady-state current value in the presence of a catalyzed substrate, Imax is the maximum current with saturated substrate and C is the substrate concentrate. The apparent Michaelis constant (Kmapp), which is a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants, was estimated to be 96.7 μM. And a low Kmapp value indicates strong substrate binding (33). Compared with previous reports (34), this Kmapp is slightly higher than 81.4 μM.

The possible interference of foreign molecules for NO determination was also investigated. At concentrations of 20-fold AA, 30-fold Dopamine, 35-fold L-Cysteine and L-Valineas high as that of NO (10 μM), no obvious interferences could be observed. But 10-fold oxygen, 10-fold hydrogen peroxide and 5-fold nitrite would result in a relative deviation of about 5%. If the Cyt.c/MWNTs modified electrode was exposed in air at 4˚C for one month, signals only decreased about 4.0%, which suggested that the electrode has an excellent stability.

5. CONCLUSION

The experimental results revealed that carbon nanotube is an ideal electron promoter when used as electrode material. Due to its promoting effect, the direct electron transfer between Cyt.c and electrode was reached. Through a simple potential scan technique, Cyt.c can be adsorbed tightly onto the surface of MWNTs to form a stable, approximate monolayer Cyt.c film. The Cyt.c film shows a good electrocatalytic activity toward the reduction of nitric oxide. Based on these, new reagentless biosensor of NO can be constructed. The biosensor shows a stable, sensitive and fast response to NO.

6. ACKNOWLEDGEMENTS

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