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Direct electrochemistry behavior of cytochrome *c*/L-cysteine modified electrode and its electrocatalytic oxidation to nitric oxide

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Abstract

Cytochrome *c* (Cyt *c*) was successfully immobilized on L-cysteine modified gold electrode by multicyclic voltammetry method. The electrochemical behavior of Cyt *c* on the L-cysteine modified electrode was explored. In 0.10 M, pH 7.0 phosphate buffer solution (PBS), Cyt *c* showed a quasi-reversible electrochemical redox behavior with E_{pc}=0.180 V, E_{pa}=0.208 V (versus Ag/AgCl). The Cyt *c*/L-cysteine modified electrode gave an excellent electrocatalytic activity towards the oxidation of nitric oxide, and the catalysis currents were proportional to the nitric oxide concentration in the range of 7.0×10^{-7} to 1.0×10^{-5} M, the linear regression equation is I (μA)=−0.124–0.003C_{NO} (μM), with a correlation coefficient 0.996, The detection limit was 3.0×10^{-7} M (times the ratio of signal to noise, S/N=3).

Keywords: Cytochrome *c*; L-Cysteine; Nitric oxide; Electrocatalysis

1. Introduction

Cytochrome *c* is a kind of stable hemoprotein containing covalently bound heme as a prosthetic group. Its Fe(III)/Fe(II) redox center located in a heme unit which is approximately spherical in shape with 34 Å. In 1977, Hill [1] used gold and tin-doped indium oxide electrode, respectively, and made cytochrome *c* show virtually reversible electrochemistry property. After that time, the direct electrochemical behavior of Cyt *c* has been studied extensively. Many electrode materials [2–4] have been used to improve the electrochemical properties of cytochrome *c* on the electrode. Some researchers adopted glutathione [5], cardiolipin [6], TiO_2 phytate [7], and fullerene [8] as surface modifier to prepare the modified electrode. These modified electrodes were applied to explore the direct electrochemical behavior of cytochrome *c* and some satisfactory results were obtained. McNeil et al. [9] have developed a superoxide sensor based on Cyt *c* immobilized on short-chain thiol modified gold electrode. The sensor signal is proportional to the real $O_2^{\cdot-}$ concentration that fits well with the mathematical model for in vitro studies. B. Ge et al. [10] developed a long-chain mixed thiol (MUA:MU) modified electrode. They showed how the rate constant of the immobilized cytochrome *c* on the electrode and $O_2^{\cdot-}$ in solution was increased, then they got a sensitivity sensor of $O_2^{\cdot-}$. Chen et al. [11] deposited cytochrome *c* on DNA modified glassy carbon electrode and studied the electrocatalytic oxidation properties of Cyt *c* to ascorbic acid, NH_2OH, N_2H_4, and SO_3^{2-}. They concluded that cytochrome *c* shows a well-electrocatalytical property active in the above-mentioned compounds.

Nitric oxide (NO), a potentially toxic molecule, has been implicated in a wide range of physiological function and is considered as a new messenger in the brain. It has been found that measuring NO in the biological model is very difficult because of its assumed low stability and high fugacity. Some methods for determining NO were proposed with chemiluminescence [12], diazotization [13], electron paramagnetic resonance (EPR) [14], and electrochemical methods [15]. Among them, electrochemical methods show great significance to determination, not only in that they are directive, simple and rapid, but also in that they are applicable in vivo.

In this paper, cytochrome *c* was immobilized successfully on L-cysteine modified electrode by multicyclic voltammetry

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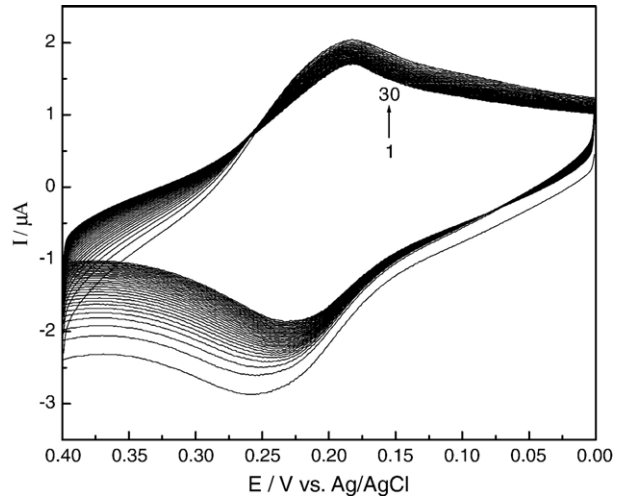

Fig. 1. Multicyclic voltammograms of a L-cysteine modified gold electrode in 0.10 M phosphate buffer (pH 7.0) containing 1.0×10^{-5} M Cyt *c*. Scan rate, $v=50$ mV/s.

methods to fabricate Cyt *c*/L-cysteine modified gold electrode. The electrochemical behavior of cytochrome *c* on L-cysteine modified electrode was explored by using cyclic voltammetry and amperometry. The related electrochemical parameters were obtained. The Cyt *c* on L-cysteine modified electrode gave an excellent electrocatalytic activity towards the oxidation of nitric oxide, which can be applied in the detection of NO.

2. Experimental

2.1. Apparatus and chemicals

Cytochrome *c* from horse heart (Cyt *c*) and L-cysteine were purchased from Rche. NO solution was prepared as reference [16], typically containing NO ≈ 1.4 mM. Other chemicals were of analytical reagent grade. Phosphate buffer solutions (PBS) were prepared with 0.10 M KH_2PO_4–K_2HPO_4. All aqueous solutions were prepared with double distilled, de-ionized water.

Electrochemical experiments were performed with CHI660A electrochemical analyzer (Shanghai Chenhua apparatus, China) with conventional three-electrode cell. The working electrode was L-cysteine modified gold electrode and Cyt *c*/L-cysteine modified gold electrode (2.0 mm in diameter), the reference electrode was an Ag/AgCl electrode (saturated KCl) and a platinum electrode was used as the auxiliary electrode. Prior to experiment, solutions were purged with purified nitrogen for 15 min to remove oxygen. All measurements were performed at room temperature.

2.2. Electrode modification

The gold electrode was polished by 1500 diamond paper, followed by 0.2 μm alumina on chamois leather to mirror, then

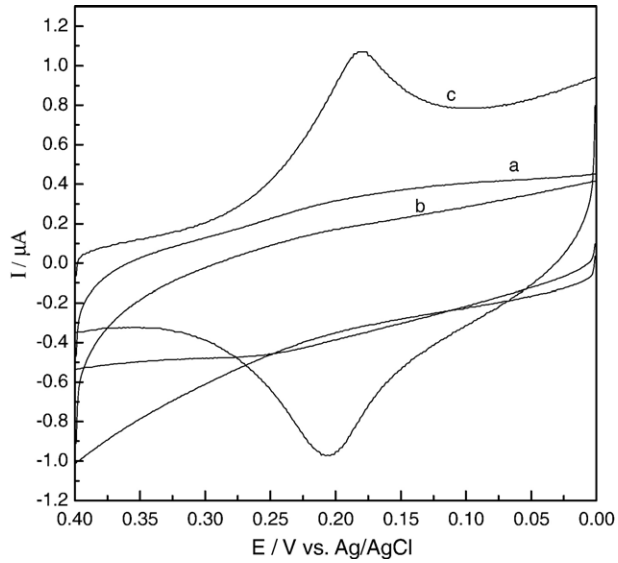

Fig. 2. Cyclic voltammograms of (a) bare gold electrode (b) L-cysteine modified gold electrode and (c) Cyt *c*/L-cysteine modified gold electrode in a phosphate buffer solution (pH 7.0). The scan rate $v=50$ mV/s.

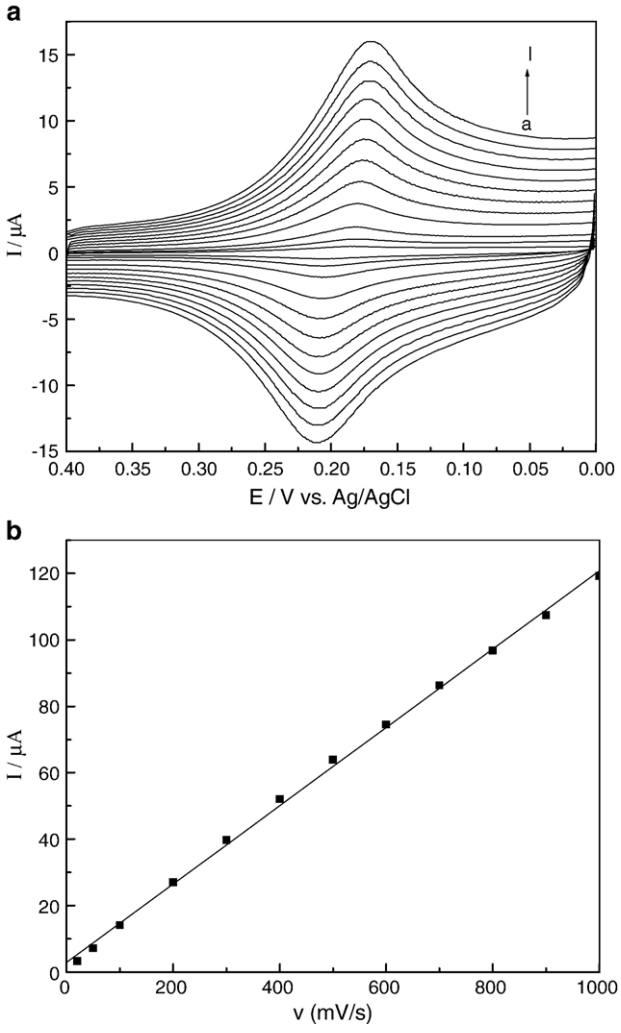

Fig. 3. (a) Cyclic voltammograms of Cyt *c*/L-cysteine modified electrode in pH 7.0, 0.10 M phosphate buffer solution at different scan rates: (a) 20, (b) 50, (c) 100, (d) 200, (e) 300, (f) 400, (g) 500, (h) 600, (i) 700, (j) 800, (k) 900, (l) 1000 mV/s. (b) Background-subtracted cathodic peak current vs. scan rate.

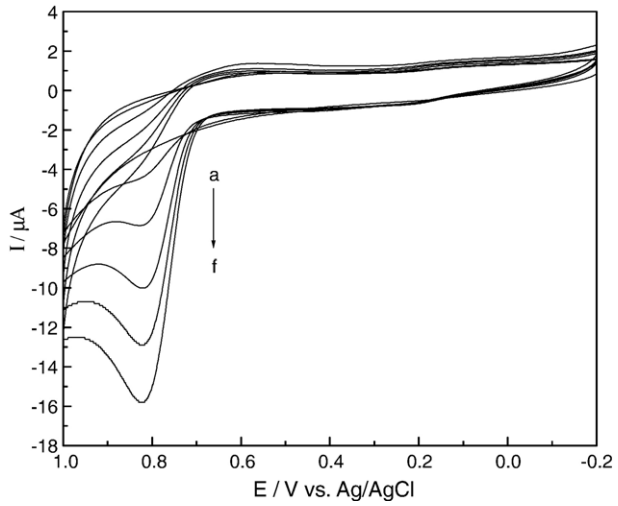

Fig. 4. Cyclic voltammograms of Cyt *c*/L-cysteine modified gold electrode in 0.10 M PBS (pH 7.0) containing NO (a) 0, (b) 2, (c) 4, (d) 6, (e) 8, (f) 10 μM). The scan rate v=50 mV/s.

cycled in 0.5 M H_2SO_4 from −0.35 V to +1.70 V for 20–50 cycles at 200 mV s^{-1} until the typical cyclic voltammogram of a clean polycrystalline gold surface was obtained. Following that, 20 μL L-cysteine solution (50 mM) was dropped onto the clean gold electrode and solvent was evaporated at 4 °C circumstance for 12 h.

Cyt *c*/L-cysteine modified electrode was prepared by the following steps: Cyt *c* was dissolved in 0.1 M (pH 7.0) phosphate buffer solution (PBS) and the above-modified electrode was transferred into Cyt *c* solution. A successive cyclic scan was performed in the potential range from +0.4 to 0 V (versus Ag/AgCl) to obtain a stable voltammogram. Then, the electrode was removed from the solution, was washed with double-distilled water and stored in pH 7.0 PBS at 4 °C.

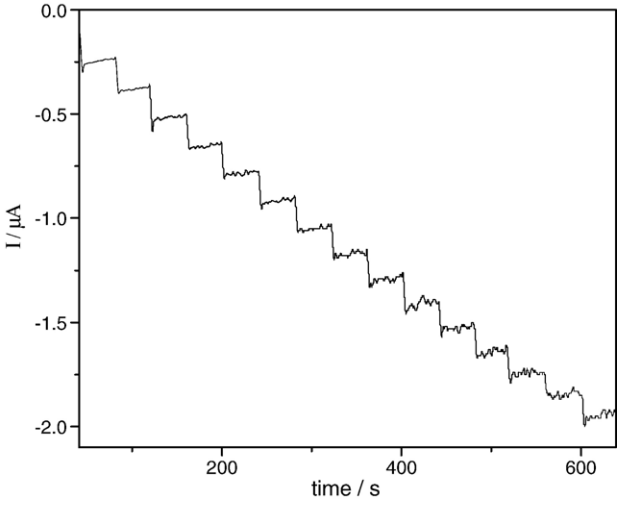

Fig. 5. Amperometric response of Cyt *c*/L-cysteine modified gold electrode to NO. Conditions: +0.824 V constant potential modulated with 50 mV pulse in the time intervals of 0.5 s, successive additions of 50 μL, 2 μM NO to 5 mL pH 7.0 PBS and the stirring rate is 200 rpm.

3. Results and discussion

3.1. The adsorption of cytochrome c on L-cysteine modified Au electrode

Fig. 1 shows the cyclic voltammograms of Cyt *c* at L-cysteine modified Au electrode under the condition of consecutive scans. It shows clearly the adsorption process of Cyt *c* on the surface of L-cysteine. A couple of redox peaks can be observed on cyclic voltammograms. The cathodic and anodic peak potentials appear near 0.186 V and 0.235 V, respectively. The more the cycles that the modified electrode swept in the Cyt *c* solution was, the higher was the redox peak, demonstrating that Cyt *c* could be adsorbed onto the surface of L-cysteine modified electrode. When the cycles was above 40 circles, no obvious changes of peak current could be observed from the cyclic voltammograms, indicating the adsorption of Cyt *c* reached a saturated state [17]. During this procedure, L-cysteine plays a promoter role for the electron transfer reaction of Cyt *c*, it can provide negatively charged sites and interact with the hydrophilic surface of Cyt *c*, which has the positive charge to +8 at a neutral pH. In addition, the carboxyl and amino of L-cysteine interact with the lysine residues surrounding the heme edge of Cyt *c* [18]; therefore, Cyt *c* can be adsorbed on the L-cysteine modified electrode.

3.2. Direct electrochemical behavior of Cyt c on L-cysteine modified electrode

In pH 7.0 PBS, the electrochemical properties of Cyt *c* on L-cysteine modified electrode were studied. Fig. 2a shows the CV curves of bare Au electrode, the L-cysteine modified Au electrode (Fig. 2b) and the Cyt *c* on L-cysteine modified Au electrode (Fig. 2c) at a scan rate of v=50 mV/s. As shown in Fig. 2c, Cyt *c* on the L-cysteine modified electrode showed a pair of reversible redox peaks; its cathodic and anodic peak potential were 0.180 V and 0.208 V (vs. Ag/AgCl), respectively. The formal potential is +0.194 V and the shapes of the cathodic and anodic peaks were nearly symmetric. The contrast

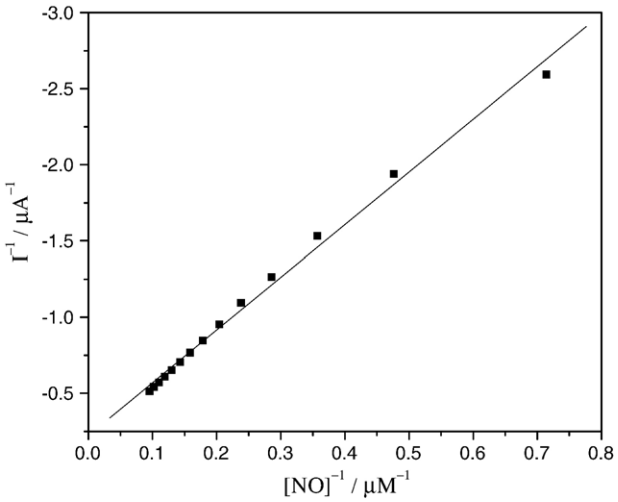

Fig. 6. Linear calibration curve for the determination of K_m.

experiment was performed in the same procedure with bare gold electrode instead of L-cysteine modified electrode. No obvious redox peak of Cyt *c* on bare gold electrode was observed (figure not shown). These experimental results indicated that Fe(III) in Cyt *c* can be reduced to Fe(II) on L-cysteine modified electrode, L-cysteine plays a promoter role for the electron transfer reaction of Cyt *c*. On L-cysteine modified electrode, Cyt *c* shows well electrochemical activation.

The dependence of peak currents (I_p) on the scan rate (v) was shown in Fig. 3. The slope of the plot of I_p vs. v could be observed, according to the slope of $I_p - v$ curve and the Laviron's equation [19]

$$I_p = n^2F^2v\Gamma A/4RT \quad (1)$$

The surface concentration (Γ) of Cyt *c* on L-cysteine modified electrode was estimated. Under the condition of saturated adsorption, the average surface concentration is about 9.986×10^{-7} mol/cm^2. The dynamic constants of Cyt *c* on L-cysteine modified electrode can be obtained by the following equation about the relation of peak potential (E_p) and scan rate (v) [20],

$$E_p = E_0 + \frac{RT}{\alpha nF}\ln\left(\left[\frac{RTK_s}{\alpha nF}\right]/Vs^{-1}\right) - \frac{RT}{\alpha nF}\ln\left[v/\left(mVs^{-1}\right)\right] \quad (2)$$

The heterogeneous electron transfer rate constant (K_s, s^{-1}) and electron rate coefficient α were calculated to be $K_s = 1.25 \pm 0.10$ s^{-1} and $\alpha = 0.18 \pm 0.05$, respectively. This K_s value indicated that L-cysteine was an excellent promoter for the electron transfer between Cyt *c* and the Au electrode. The plot of the logarithm of (I_{pc}, μA) versus the logarithm of [v, mV·s^{-1}] (scan rate) gives a linear relationship with correlation coefficient of 0.999 [21].

3.3. Electroatalytic oxidation of NO on Cyt c/L-cysteine modified gold electrode

In order to explore the electrochemical activity of Cyt *c* on the L-cysteine modified gold electrode, its response to the oxidation of NO was explored. Fig. 4 shows the cyclic voltammogram of the Cyt *c*/L-cysteine modified electrode in pH 7.0 phosphate buffer solution. When different concentrations of NO were added, an obvious increase of the peak current was observed at near 0.824 V (Fig. 4, curves a–f); however, no corresponding anodic peak can be observed at L-cysteine modified Au electrode under the same condition (figure not shown), which indicated that immobilized Cyt *c* on L-cysteine modified electrode exhibited excellent electrochemical activity to the oxidation of NO.

Fig. 5 shows the steady-state current response of NO. The response was linear in the NO concentration range from 7.0×10^{-7} to 1.0×10^{-5} M. The linear regression equation was I (μA) = −0.124–0.003C_{NO} (μM), with a correlation coefficient of 0.996. The response time was about 3 s and the relative standard deviation is 2.0% for seven successive determinations at NO concentration 1.0×10^{-6} and the detection limit is 3.0×10^{-7} M (three times the ratio of signal to noise).When the concentration of NO was higher than 1.0×10^{-5} M, a response plateau was observed, showing the characteristic of Michaelis–Menten kinetic mechanism, The apparent Michaelis–Menten constant (K_m), which gives an indication of the enzyme-substrate kinetics, can be obtained from the Lineweaver–Burk equation [22],

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m}{I_{max}c}. \quad (3)$$

Here, I_{ss} is the steady-state current after addition of substrate, c is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated substrate conditions. The K_m value of the modified electrode was found to be 1.4×10^{-4} M (Fig. 6). The low value of K_m indicated that the immobilized Cyt *c* possessed a high biological affinity to NO.

In most cases, the solution pH is important to the electrocatalytic behavior of proteins [4]. In this work, the effect of pH on the electrocatalytic oxidation of NO on Cyt *c*/L-cysteine electrode was tested in the pH ranges of 4.0–10.0 (figure not shown). The oxidation current peak exhibited a maximum value at pH 7.0. It is close to the pH value of the biological media. This is due to the effect of protonation states of *trans*-ligands to the heme iron and amino acids around the heme or to the protonation of water molecules coordinated to the center [23]. The stability of the electrode was also examined. When it was stored in 0.1 M PBS (pH 7.0) for at least 1 week at 4 °C, the electrode retained more than 95% of its initial response to the oxidation of NO. If the modified electrode was stored in the concentration of PBS (pH 7.0) higher than 0.2 M, the response to oxidation of NO will be reduced; probably the immobilized Cyt *c* was denatured.

4. Conclusion

The direct electron transfer of cytochrome *c* on L-cysteine modified gold electrode was achieved. L-cysteine plays a key role in promoting the electron transfer between Cyt *c* and gold electrode. Cyt *c* can strongly adsorbed on the surface of L-cysteine immobilized gold electrode. The Cyt *c*/L-cysteine modified electrode gave an excellent electrocatalytic activity towards the oxidation of nitric oxide. It could be applied for determination of micro-NO.

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