

Direct Electrochemistry of Cytochrome c on EDTA-ZrO₂ Organic-inorganic Hybrid Film Modified Electrodes

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A composite film of ethylenediamine tetraacetic acid (EDTA)-ZrO₂ organic-inorganic hybrid was prepared based on the chelation between Zr(IV) and EDTA. The direct electrochemical behavior of cytochrome c (cyt. c) at the hybrid film modified glassy carbon electrodes was investigated. The immobilized EDTA can promote the redox of heme in horse heart cyt. c which gives rise to a pair of reversible redox peaks with a formal potential of 40 mV (vs. SCE). The peak current increased linearly with the increase of cyt. c concentration in the range of 1.6×10^{-6} — 8.0×10^{-5} mol·L⁻¹ with the correlation coefficient of 0.996. Further investigation shows that metal ions can impede the electron transfer of cyt. c. The impediment capability of metal ions depends on their coordination capability with EDTA and their valence number.

Keywords direct electrochemistry, cytochrome c, EDTA-ZrO₂ organic-inorganic hybrid, metal ion

Introduction

Cytochrome c (cyt c) is probably the most thoroughly studied redox protein. It contains one Fe(III) redox center located in a haem unit which is approximately spherical shape with 3.4 nm diameter and 12384 Dalton molecular weight. On metal electrode surfaces it usually shows a short lived, transient response. Many factors can impede direct electron transfer between electrodes and cyt. c, including adsorption onto electrode surfaces of macromolecular species (impurities) or denatured forms of themselves, and unfavorable approaches to the electrode.¹ Since the pioneer work of Yeh and Kuwana² that reported the direct electrochemistry of cyt. c at an oxide electrode, and Eddowes and Hill³ found its essentially reversible redox response at a 4,4'-dipyridyl modified gold electrode, a significant advance has been made in unmediated electrochemistry of cyt. c. Special attention has been focused on the nature of the electrode and electrode/solution interface. Negatively charged polyester sulfonic acid,⁴ nucleic acids,⁵⁻⁷ DNA-carbon nanotube composite⁸ *etc.* have been used to modify electrode surfaces to promote the direct electron transfer of positively charged cyt. c (it has nine positive charges at pH 7.0⁹). Among these systems, it is generally accepted that electrostatic interaction is an important factor to guide cyt. c into contact with regions

of opposite charge in electrodes. As a consequence, the presence of charges in the close vicinity of the electrode surface appears as crucial in determining electron transfer kinetics.

Organic-inorganic hybrid materials, in which organic and inorganic components are chemically bonded each other, have extensively been studied because the blending of organic and inorganic components allows the development of materials with novel properties.¹⁰ They are a kind of very promising materials of biosensors.¹¹ The sol-gel technique is known to be a practical method for preparing the hybrid materials.¹¹ Zirconia is an inorganic oxide with the thermal stability, chemical inertness, lack of toxicity¹² and affinity for the groups containing oxygen.¹³⁻¹⁵ While EDTA is a well-known organic molecule used as a chelating reagent to effectively chelate cations in solutions. And it could be doped in silica gels to form hybrid for the determination of Cr(VI).¹¹ In this work, based on the chelation between Zr(IV) and EDTA, the EDTA-ZrO₂ organic-inorganic hybrid film modified electrode has been prepared by sol-gel method. This negatively charged film was used to promote the electrochemical response of cyt. c. The effect of metal ions such as Ca²⁺ and La³⁺ on the response of cyt. c was investigated in detail.

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Experimental

Apparatus and chemicals

Horse heart cyt. c was obtained from Sigma Co. and used as received. The chemicals, zirconium oxychloride, ethylenediamine tetraacetic acid disodium salt (EDTANa₂•2H₂O), CaCl₂, and LaCl₃ from Second Chemical Reagent Factory of Shanghai, were of analytical reagent grade. All solutions were prepared with doubly distilled water.

Electrochemical experiments were measured on a CHI660 electrochemical workstation (CH Instruments, USA) with a three-electrode system. A platinum wire was used as an auxiliary electrode, a saturated calomel electrode (SCE) as reference electrode, against which all potentials were recorded, and a glassy carbon disk electrode with the diameter of 3.0 mm as a working electrode. All the measurements were carried out at 25 ± 0.5 °C in conventional electrochemical cell. All experimental solutions were de-aerated by bubbling nitrogen for 15 min, and a nitrogen atmosphere was kept over the solution during measurements. Zeta potential measurement was carried out with a ZetaPlus instrument (Brookhaven Instruments Corp., USA). A model XSAM 800 X-ray photoelectron spectrometer was used to examine the EDTA-ZrO₂ sample. The binding energy E_b scale was calibrated with respect to the carbon 1s peak at 285.0 eV, the aluminum K α 1.2 line ($h\nu=1486.6$ eV) was used as the X-ray excitation source and an EDTA-ZrO₂ film sample on a glassy plate surface. Spectra were recorded in fixed analyzer transmission mode (FAT) to achieve maximum instrumental resolution. The instrument was operated under a vacuum of 2×10^{-8} Torr in the analysis chamber. High resolution spectra were recorded with a channel width of 0.1 eV.

Preparation of ZrO₂ and EDTA-ZrO₂ hybrid sol-gel solutions

ZrO₂ sol-gel was prepared according to the established method.¹⁶ Briefly, 0.10 mol•L⁻¹ NH₃•H₂O was added gradually into 45 mL of 1.0×10^{-2} mol•L⁻¹ ZrOCl₂•8H₂O solution under vigorous stirring until the solution pH was *ca.* 9.5. A ZrO₂ gel could be seen as white flocculus at the bottom. The underlayer flocculus was then transferred into a 10 mL centrifugal tube to separate for 3 min at a rate of 2500 cycles per second. Then the underlayer gel was washed with 8 mL of pH 7.0 doubly distilled water. The centrifugal separation and washing process were repeated 4 times. Finally, a sol-gel containing 0.3 mol•L⁻¹ ZrO₂ with the weight percent of ZrO₂ being 3.69% at pH 7.0 was obtained. EDTA-ZrO₂ hybrid sol-gel was obtained by mixing 0.3 mol•L⁻¹ ZrO₂ sol and EDTANa₂ solution (1×10^{-2} mol•L⁻¹) with 1 : 4 in the help of ultrasonic irradiation.

Preparation of ZrO₂ and EDTA-ZrO₂ modified electrode

The glassy carbon electrodes were firstly polished with 1700 diamond paper, followed by 0.2, 0.05 μ m

alumina on chamois leather, rinsed thoroughly with doubly distilled water after each polishing step, then washed successively with doubly distilled water in an ultrasonic bath before use. 20 μ L of EDTA-ZrO₂ hybrid sol-gel and 20 μ L of ZrO₂ sol-gel were added on two bare GC electrodes, respectively, and dried at 4 °C for 8 h before use.

Results and discussion

The promotion of EDTA-ZrO₂ modified membrane to the direct electron transfer of cyt. c

The electrochemical behavior of cyt. c was studied at bare, ZrO₂ and EDTA-ZrO₂ modified glassy carbon electrodes by means of cyclic voltammetry. A well-defined and reversible voltammetric response with a formal potential of 40 mV and a peak potential difference of 70 mV were observed (shown in Figure 1). While at a bare or ZrO₂ modified glassy carbon electrode in the same cyt. c solution, and under identical experimental conditions, no any voltammetric signal occurred, revealing that the redox response of cyt. c resulted from the presence of negatively charged EDTA in the modified layer.

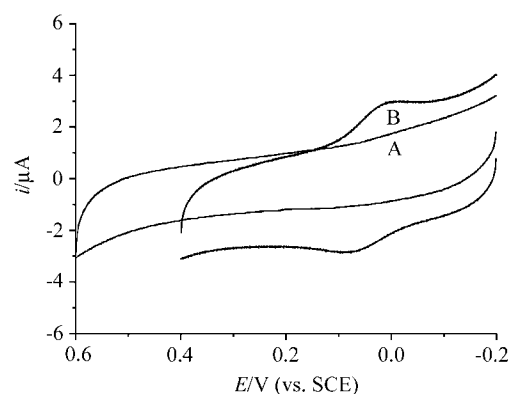


Figure 1 Cyclic voltammograms of 80 μ mol•L⁻¹ cyt. c in 0.1 mol•L⁻¹ pH 7.0 PBS at (A) a ZrO₂ modified GC electrode with the scan rate of 50 mV•s⁻¹. (B) an EDTA-ZrO₂ modified GC electrode with the scan rate of 100 mV•s⁻¹.

In order to elucidate the effect of EDTA on the electron transfer of cyt. c, the effect of EDTA in solution was further studied. When the ZrO₂ modified GC electrode was exposed to cyt. c solution in the presence of EDTA, no redox peaks were observed (not shown). It means that only immobilized EDTA can facilitate the redox of cyt. c. In order to elucidate the promotion mechanism of the hybrid film to the electron transfer of cyt.c, ζ potentials of ZrO₂ nanoparticles were studied in the presence and absence of EDTA. As 8×10^{-4} mol•L⁻¹ EDTA was added into ZrO₂ nanoparticle solution, the ζ potential was changed from 6.33 mV to -33.2 mV, indicating the formation of negatively charged EDTA-ZrO₂ hybrid. As we all known, under the condition of physiological pH, the charge on cyt. c

in its oxidation state (III) is +9, due to an excess of positively-charged groups, mainly lysines. So there is an electrostatic interaction between positively-charged cyt. c and negatively-charged EDTA-ZrO₂ hybrid film at the electrode surface, which facilitates the redox center of cyt. c proximal to the electrode surface, and the rapid electron transfer can take place on the ZrO₂-EDTA modified electrode. XPS experimental results also proved the existence of EDTA in the hybrid film based on the binding energy peaks of C1s at 283.75 eV and N1s at 398.55 eV (not shown).

The ratio of EDTA to ZrO₂ greatly affects the electrode response. When the ratio is too small, there is no electrochemical response to cyt. c. With the ratio increase, the enhanced response was observed. This phenomenon also indicates that EDTA plays a key role in promoting the redox of cyt. c. As the ratio is over 4, the peak current response trends to a steady value but the background current increases (shown in Figure 2). The ratio of 4 was selected for the following experiments.

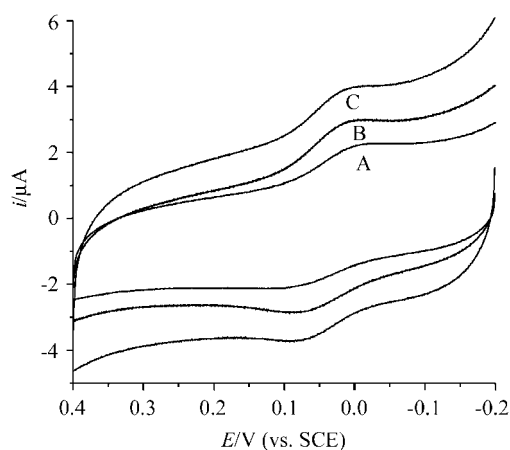


Figure 2 Cyclic voltammograms of 80 $\mu\text{mol}\cdot\text{L}^{-1}$ cyt. c in 0.1 $\text{mol}\cdot\text{L}^{-1}$ pH 7.0 PBS at EDTA-ZrO₂ modified GC electrodes with various ratio of ZrO₂/EDTA, (A) 1 : 3; (B) 1 : 4; (C) 1 : 5. Scan rate: 100 $\text{mV}\cdot\text{s}^{-1}$.

Effect of scan rate on the peak current at the hybrid film-modified electrode was investigated by cyclic voltammetry. The reduction peak current of cyt. c at the electrode increased linearly with the square root of the scan rate in the range from 10 to 180 $\text{mV}\cdot\text{s}^{-1}$ with a correlation coefficient of 0.994 (shown in Figure 3). The linear regression equation is expressed as $I_p = -0.151 + 0.122v^{1/2}$ (I_p in unit of μA and v $\text{mV}\cdot\text{s}^{-1}$). The result suggests that the electrode reaction of cyt. c be a diffusion-controlled process.

The experimental results showed that the dependence of peak current from differential pulse voltammograms on the concentration of cyt. c displayed a linear relationship in the range from 1.6×10^{-6} to 8.0×10^{-5} $\text{mol}\cdot\text{L}^{-1}$ (shown in Figure 4). The linear regression equation is expressed as $I_p = 0.0216 + 0.00889c$ (I_p in unit of μA and c $\mu\text{mol}\cdot\text{L}^{-1}$, correlation coefficient $r = 0.996$). The detection limit is 1.0×10^{-6} $\text{mol}\cdot\text{L}^{-1}$.

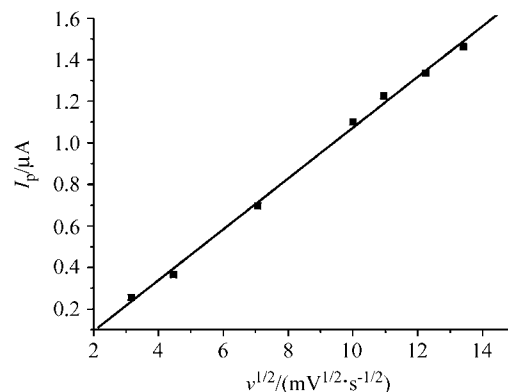


Figure 3 Dependence of the cathodic peak currents of cyt. c on the square root of the scan rates.

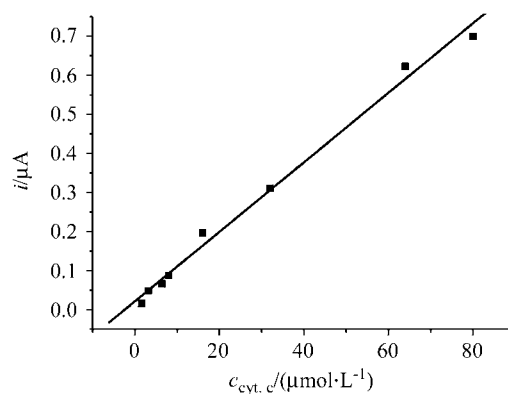


Figure 4 Dependence of the cathodic peak currents from differential pulse voltammograms on the concentration of cyt. c.

The effect of metal ions on the electrochemical behaviour of cyt. c

Because the promotion of immobilized EDTA to the direct electron transfer of cyt. c is due to the electrostatic interaction between EDTA and cyt. c, metal ions, which can coordinate with EDTA, would influence the redox behaviour of cyt. c. The studies showed that Ca²⁺ and La³⁺ ions can strongly affect the electron transfer rate of cyt. c. Figure 5 showed the dependence of ζ potentials of EDTA-ZrO₂ hybrid on Ca²⁺ concentrations. It can be seen that the ζ potentials increased with the increase of Ca²⁺ concentrations, indicating that the negative charges of the hybrid decreased with the increase of metal ion concentrations. Figure 6 showed the effect of the concentration of Ca²⁺ and La³⁺ on the response of cyt. c. At small amounts of Ca²⁺ (10 $\mu\text{mol}\cdot\text{L}^{-1}$) and La³⁺ (5 $\mu\text{mol}\cdot\text{L}^{-1}$), the cathodic peak currents of cyt. c increased. When the concentration of Ca²⁺ and La³⁺ increased continuously thereafter, the peak currents decreased gradually. This phenomenon is somewhat analogous to the results of Huang *et al.*¹⁷ However, at this hybrid film modified electrode, the effect of metal ions on the response of cyt. c was more sensitive than at cysteine-modified gold electrode. This may be due to the strong chelation between EDTA and

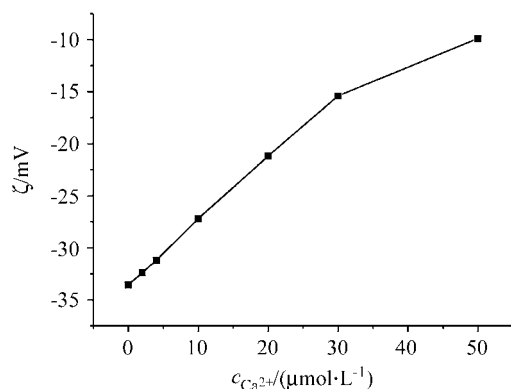


Figure 5 Dependence of ζ potentials of EDTA-ZrO₂ hybrid on Ca²⁺ concentrations.

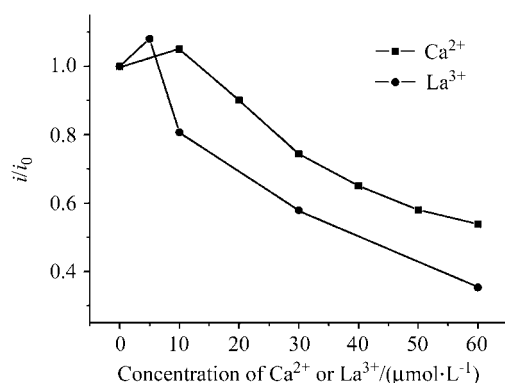


Figure 6 Relationship between the ratio of cathodic peak current of cyt. c in the presence and absence of metal ion to metal ion concentrations.

metal ions. The presence of small amounts of metal ion unlocks a partially locked surface (probably by strong adsorption of cyt. c on the electrode), rendering the entire surface electroactive, which shows a quasi-reversible electrochemical process. With the increase of the concentration of metal ions, strong chelation occurred between EDTA and metal ions, which resulted in the changes of charge on the electrode surface. The further investigations revealed that the degree of the current decrease was different from each other after the metal ions were added, under the same condition. The peak current decreased more quickly with the addition of La³⁺ than that of Ca²⁺, which indicates that the lanthanum ion has stronger interaction with the immobilized EDTA than calcium ion, and this order is consistent with their

coordination ability with EDTA. And the number of positive charge of metal ions was also an attribution to impede the arrival of cyt. c at electrode surface.

Conclusion

ZrO₂-EDTA hybrid film was successfully obtained by the sol-gel method, and the immobilized EDTA could promote the direct electrochemistry of cyt. c. Metal ions, such as La³⁺ and Ca²⁺, which are able to coordinate with EDTA can impede the electron transfer between cyt. c and the electrode surface with the order of La³⁺ > Ca²⁺, under the same conditions. Further studies will be focused on the switch effect of metal ions on the redox of cyt.c.

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