Int. J. Electrochem. Sci., 3 **(2008)** 519 - 527

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

A Third-Generation Biosensor Based on the Enzyme-Like Activity of Cytochrome *c* on a Room Temperature Ionic Liquid and Gold Nanoparticles Composite Film

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Received: 9 December 2007 / *Accepted:* 29 January 2008 / *Online published:* 20 February 2008

A composite film constructed from gold nanoparticles (GNPs) and a room temperature ionic liquid (RTIL), 1-ethyl-3-methyl imidazolium tetrafluoroborate ([EMIm][BF_4]), was prepared directly on the surface of a basal plane graphite (BPG) electrode through a simple dropping technique to form a RTIL/GNPs modified electrode. The results from cyclic voltammetry (CV) suggested that Cytochrome c (Cyt c) could be tightly adsorbed on the surface of the modified electrode. Further, the electrode displays a good promotion for the redox of Cyt c. Direct electrochemical response of Cyt c on the modified electrode can be achieved and a couple of well-defined and nearly reversible cyclic voltammetric peaks of Cyt c can be observed in a phosphate solution. Cyt c adsorbed on the modified electrode surface shows an enzyme-like activity for the reduction of oxygen O_2. The reduction peak currents were proportional linearly to the concentration of oxygen. A third generation biosensor based on the direct electrode transfer of Cyt c can be constructed for the determination of O_2.

Keywords: Room temperature ionic liquid, [EMIm][BF_4], Gold nanoparticles, Cytochrome c, Biosensor

1. INTRODUCTION

Room temperature ionic liquids (RTILs) are air and water stable salts, composed of an organic cation and either an organic or an inorganic anion. They have unusual properties such as non-volatility, nonflammability, low viscosity, and chemical and electrochemical stabilities [1]. These attractive characteristics have led many scientists to exploit RTILs as electrolytes in batteries [2], fuel cells [3], double-layer capacitors [4], dye-sensitive solar cells [5], and actuators [6]. Although ionic liquids have not been studied systematically, work on the application of RTILs in electroanalytical chemistry field has already attracted much attention. RTILs can be used as solvents for directly investigating the

electrochemical reaction mechanisms [7], for example, the electrochemical behaviors of oxygen in RTIL [8], and performing the electrochemical measurements [9]. Besides, RTILs have been used as modifiers to modify the electrode [10] and to construct new gaseous sensors [11] and biosensors [12, 13].

Gold nanoparticles have been extensively used as an immobilized matrix for retaining the bioactivity of macromolecules, such as proteins [14] and enzymes [15], and promoting the direct electron transfer of the immobilized proteins [16, 17]. It could be used to constructed composite films on the surface of electrode through embedding GNPs in polymer, such as polymerized o-phenylenediamine [18], PMMA[19], Nafion [20] etc, and in sol-gel [17, 21-24]. In this work, a room temperature ionic liquid, [EMIm][BF_4], was selected to construct the composite film with GNPs on the surface of BPG electrode. The direct electrochemistry of Cyt c was achieved on this modified electrode.

Cyt c plays an important role in the biological respiratory chain, whose function is to receive electrons from Cyt c reductase and deliver them to Cyt c oxidase. So, the electrochemical study of Cyt c is very important[25]. Due to the difficulty of direct electron transfer between Cyt c and a bare electrode, some modified electrodes were used as a tool to investigate the direct electrochemical property. The modifiers of these modified electrodes are organic [26], or inorganic [27] compounds. They were found to promote the direct electron transfer of Cyt c at electrode surfaces. Up to today, the interaction between promoter and Cyt c is still a vital topic of investigation.

2. EXPERIMENTAL PART

2.1. Apparatus and reagents

Electrochemical experiments were carried out with a CHI660 electrochemical analyzer (CHI, USA) with a conventional three-electrode cell. The RTIL/GNPs modified BPG electrode (Φ=5.3 mm) was used as working electrode. A platinum wire electrode and Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively. The UV-vis spectra were obtained using a U-3010 spectrophotometer (Hitachi, Japan). The room temperature ionic liquid, 1-ethyl-3-methyl imidazolium tetrafluoroborate ([EMIm][BF4]), used in this study was obtained from T. Ohsaka's Laboratory in Tokyo Institute of Technology, Japan. Cyt c was obtained from Sigma Chemical Company and used without further purification. 1.2mg/ml Cyt c-phosphate buffer solutions (PBS) was stored at temperature of 4^{o}C as stock solution. Other chemicals were of analytical grade and used without further purification. All solutions were made up with doubly distilled water and deaerated with high purity nitrogen before performing electrochemical measurements. All electrochemical experiments were carried out at room temperature.

2.2. Preparation of gold nanoparticles

Spherical colloidal GNPs were prepared by Sodium citrate reduction of $HAuCl_4$ in aqueous solution [28]. The average nanoparticle diameter is 20 nm as measured by TEM [21].

2.3. Preparation of electrode

The BPG electrode was polished with abrasive paper before each experiment, and then washed with doubly distilled water and ethanol in an ultrasonic bath successively, and allowed to dry at room temperature.

50μL of as-prepared gold nanoparticles solution was diluted in 5mL doubly distilled water. Then, 100μL diluent was mixed fully with 50μL RTIL to form a mixture solution. 5μL of the mixture solution was dropped on the surface of BPG electrode and then was left to dry at room temperature to form a modified electrode, which is noted as RTIL/GNPs/BPGE. Cyt c /RTIL/GNPs/BPGE was prepared by following process: the RTIL/GNPs/BPGE was placed in a Cyt c solution (1.2mg/ml, 0.1M, pH 7.0 PBS) for 5 hours. Then, the electrode was removed from the solution, washed with double-distilled water and stored in pH 7.0 PBS at about 4°C. in addition, GNPs/BPGE and Cyt c/GNPs/BPGE were prepared as the similar process except including RTIL.

3. RESULTS AND DISCUSSION

3.1. Electrochemical impedance characterization of Cyt c immobilization

Figure 1 shows the results of AC impedance spectroscopy of bare BPG, GNPs/BPGE and RTIL/GNPs/BPGE electrode in the presence of equimolar $Fe(CN)_6^{3-/4-}$. Compared with that of the GNPs/BPG electrode, the semicircle of the RTIL/GNPs/BPG electrode obviously decreased, suggesting that the impedance of RTIL/GNPs/BPG electrode is smaller than that of GNPs/BPG electrode. On the other hand, it was RTIL that decreased the impedance of electron transfer between GNTs and BPG electrode.

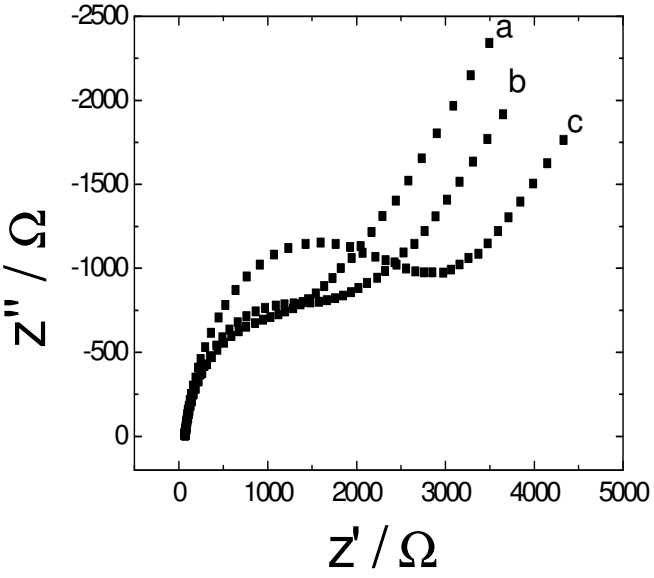

Figure 1. The Nyquist diagram (z" vs. z') in the presence of 1.0×10^{-3} M $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.1 M KCl solution. (a) Bare BPG electrode, (b) RTIL/GNPs/BPG electrode, (c) GNPs/BPG electrode

3.2. Direct electrochemical response of Cyt c on GNPs/RTIL/BPGE

Figure 2 shows the typical cyclic voltammograms (CVs) of Cyt c/RTIL/GNPs/BPGE electrode in 0.1M, pH 7.0 phosphate buffer solution. The Cyt c/RTIL/GNPs/BPGE gives a couple of stable and well-defined redox peaks, where anodic and cathodic peak potentials are located at -259 and -327 mV, respectively (curve c in Fig. 2). The separation of anodic and cathodic peak potentials (ΔE) is 68 mV, indicating a fast electron transfer reaction. However, at the RTIL/GNPs/BPG electrode (curve a in Fig. 2), no redox peaks can be observed. On the other hand, Cyt c/GNPs/BPGE also gives a couple of redox peaks in the same potential range. But its peak currents, both cathodic and anodic peak currents were obviously smaller than that given from Cyt c/RTIL/GNPs/BPGE.

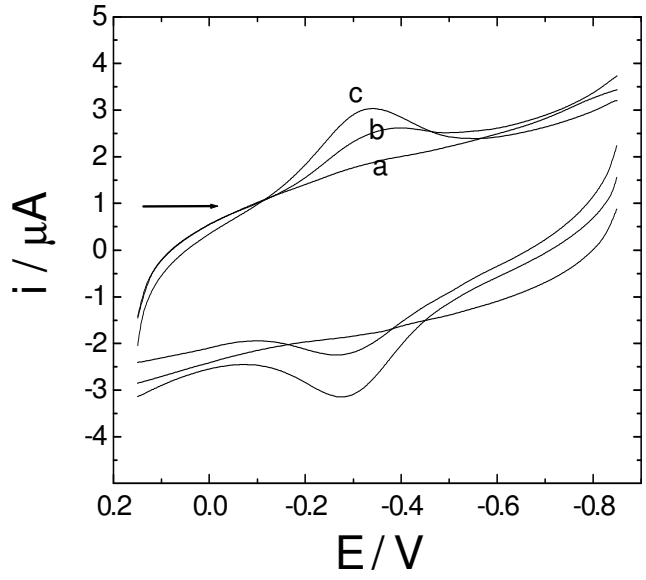

Figure 2. Cyclic voltammograms of (a) bare GNPs/BPG/BPG electrode (b) Cyt c/ GNPs/BPG electrode (c) Cyt c/RTIL/GNPs/BPG electrode in 0.1M pH=7.0 PBS with a scan rate of 100mV/s.

Above results display that Cyt c could be adsorbed on the surface of RTIL/GNPs/BPG electrode when the RTIL/GNPs/BPG electrode was dipped into the Cyt c solution. The effect of adsorption time of Cyt c on the RTIL/GNPs/BPG electrode was also investigated. The results suggested that both anodic and cathodic peak currents increase with increasing the time that the RTIL/GNPs/BPG electrode dipped into Cyt c solution. However, when the time is more than 5 hours, no dramatical change of the redox peak currents can be observed, which means that the adsorption of Cyt c on the surface of RTIL/GNPs/BPG electrode reaches a saturated state.

Figure 3 shows the UV-vis absorption spectra of Cyt c adsorbed on the surface of RTIL/GNPs composite film. It can be seen from figure 3 that the UV-vis absorption peak of Cyt c is almost unchanged after being adsorbed on RTIL/GNPs composite film, suggesting that the active structure of Cyt c adsorbed on the surface of RTIL and GNPs composite film was remained.

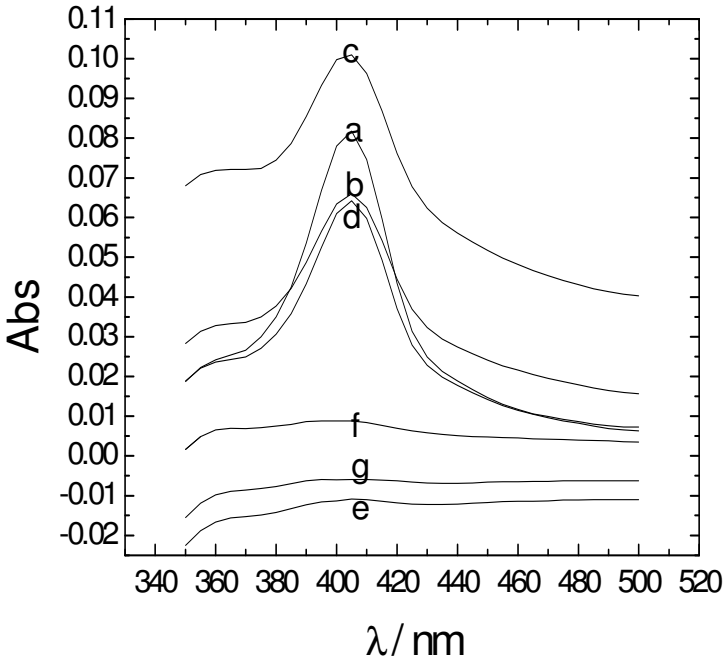

Figure 3. Absorption spectra of (a) Cyt c, (b) Cyt c on RTIL film, (c) Cyt c on GNPs film, (d) Cyt c on RTIL/GNPs composite film, (e) RTIL film, (f) GNPs film, (g) RTIL/GNPs composite film.

3.3. Electrochemical behaviors of Cyt c on the modified electrode

Figure 4 shows the CVs of Cyt c/RTIL/GNPs modified electrode in different scan rates in the range from 100 to 500 mV/s. The peak current linearly increased with the scan rate with a correlation coefficient of 0.9996, suggesting that this is a surface-controlled process (as shown in Figure 4B). According to the slope of the Ip — ν curve and the equation of $i_p=n^2F^2\nu A\Gamma^*/4RT$ [29], the average surface concentration (Γ*) of electroactive Cyt c adsorbed on the modified electrode was calculated to be $(7.9 \pm 0.3) \times 10^{-11}$ mol/cm^2, suggesting an approximate monolayer. The plot of logarithm I_{pc} versus the logarithm of ν (scan rate) gives a linear relationship with a correlation coefficient of 0.9994 and the slope was 0.9996, which is very close to the theoretical slope of 1 for thin layer voltammetry [30]. The result revealed that Cyt c could be adsorbed on the surface of RTIL/GNPs modified electrode to form an approximate monolayer film [31].

The effect of the pH of the supporting electrolyte on the peak potentials of the Cyt c/RTIL/GNPs/BPG electrode was also investigated. The peak potentials are obviously dependent on the pH value in the range of 5.0 – 9.0. The apparent formal potentials of the Cyt c redox couple, which were estimated as the midpoint of cathodic and anodic peak potentials, had shifted linearly to the negative direction with the increase of pH value, indicating that proton is contained in the electron transfer process of Cyt c, as described in reference [32].

$$\text{Cyt c hemeFe(III)} + H^+ + e^- \rightarrow \text{Cyt c hemeFe(II)}$$

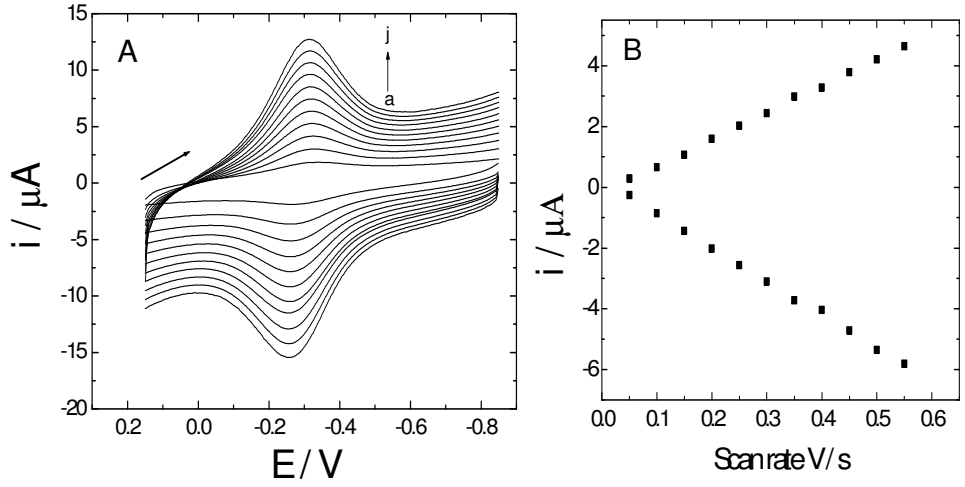

Figure 4. A) Cyclic voltammograms of Cyt c/RTIL/GNPs/BPG electrode in PBS at different scan rates: (a) 50, (b) 100, (c) 150, (d) 200, (e) 250, (f) 300, (g) 350, (h) 400, (i) 450, (j) 500 (mV/s) B) The dependence of the cathodic and anodic peak current on the scan rate (data were obtained from Fig.4A.)

3.4. Electrocatalytic activities of Cyt c/RTIL/GNPs/BPG electrode to the reduction of oxygen

Figure 5 is typical CVs of oxygen at the Cyt c/RTIL/GNPs/BPG electrode. With increasing the concentration of oxygen, the cathodic peak current of Cyt c increases and its anodic peak current decreases up to disappear, showing an obvious catalytic reaction process. The catalytic reduction peak potential of oxygen appeared at about -0.259V (vs. Ag/AgCl). However, at a bare BPG electrode [33], the reduction peak potential of oxygen is about -0.648 V (vs. Ag/AgCl) under the same conditions. Compared with bare BPG electrode, the Cyt c/RTIL/GNPs electrode decreased the reduction overpotential of O_2 by about 389 mV.

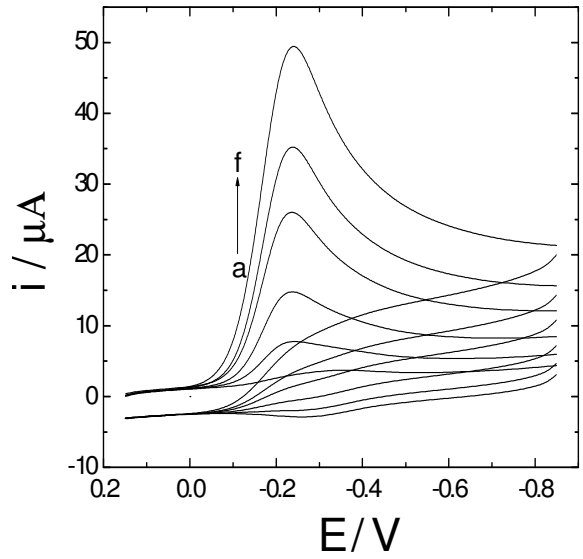

Figure 5. Cyclic voltammograms of Cyt c/RTIL/GNPS/BPG electrode in 0.1 mol/L PBS containing (a) 0, (b) 0.06, (c) 0.14, (d) 0.18 (e)0.25 and (f) 0.32 mM O_2. The scan rate was 100 mV / s.

The amperometric response of the Cyt c/RTIL/GNPs modified electrode to O_2 was recorded through successively adding O_2 to a continuous stirring PBS solution. In this process, a potential of -0.259 V was applied to the working electrode. The reductive current increases to reach a stale plateau within 5s when adding O_2 into the buffer solution. This suggested that the response of the electrode to O_2 should be a quick responsive process. The experimental results showed that the amperometric response current is linear with the concentration of O_2 in the range of 0.12 – 2.52μM. The linear equation could be described as follows: I (μA) = 0.2839 + 0.1476C (μM), with a correlation coefficient of 0.9995.

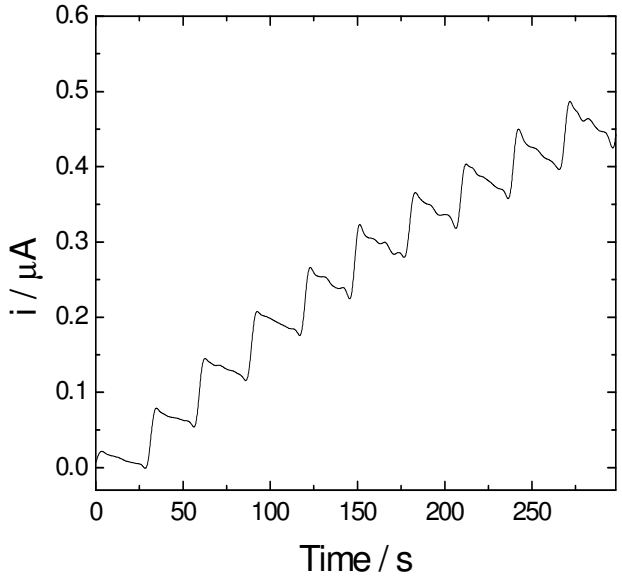

Figure 6. Amperometric response of Cyt c/RTIL/GNPs modified BPG electrode to O_2. Conditions: a -0.26 V constant potential modulated with 50 mV pulse in the time intervals of 0.5 s; successive additions of 3μL of 1.5mM O_2 to 10 mL of 0.1 M PBS, pH 7.0, and the stirring rate of solution is 400 rpm.

Cyt c can be adsorbed on the surface of RTIL/GNPs to form a stable Cyt c/RTIL/GNPs modified electrode. Even the 500 continuous cyclic scans were carried out in the potential range from 0.2 to -1.0 V with a 100 mV/s scan rate, no obvious change of CV curve of the electrode can be observed. When the Cyt c/RTIL/GNPs electrode was stored at 4^{o}C in a pH 7.0 PBS for four weeks, the original CV curve is still retained, which suggested that the electrode has an excellent stability.

4. CONCLUSIONS

The composite film of RTIL and GNPs can be constructed on the surface of a BPG electrode to form a RTIL/GNPs film modified electrode. Cyt c can strongly adsorbed onto the RTIL/GNPs film and shows a stable, approximate monolayer Cyt c film. Due to the promoting effect of RTIL/GNPs, the direct electron transfer between Cyt c and electrode was reached. Meanwhile, Cyt c adsorbed on RTIL/GNPs film shows an enzyme-like activity for the reduction of oxygen. Based on these, new

reagentless biosensor of O_2 was constructed. The further researches should enlarge the applications of RTIL in biosensor filed.

ACKNOWLEDGEMENTS
The authors thank Professor T. Ohsaka of Tokyo Institute of Technology for providing the electrode material, BPG and RTIL, [EMIm][BF_4]. This work was supported by the National Natural Science Foundation (No.20475002) and Program for Innovative Research Team in Anhui Normal University.

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