Short communication

Direct electron transfer of glucose oxidase immobilized in ionic liquid reconstituted cellulose–carbon nanotube matrix

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ABSTRACT

A conductive cellulose – multiwalled carbon nanotube (MWCNT) matrix with a porous structure and good biocompatibility has been prepared by using room temperature ionic liquids (1–ethyl–3–methylimidazolium acetate) as solvent. Glucose oxidase (GOx) was encapsulated in this matrix and thereby immobilized on surface of a glassy carbon. The direct electron transfer and electrocatalysis of the encapsulated GOx has been investigated using cyclic voltammetry and chronoamperometry. The GOx exhibited a pair of stable, well defined and nearly symmetric reversible redox peaks. The experimental results also demonstrate that the immobilized GOx retains its biocatalytic activity toward the oxidation of glucose, which can be employed to determine the glucose concentration. The results showed that the bioelectrode modified by the reconstitute cellulose–MWCNT matrix has great potential for use as a biosensor and other bioelectronics devices.

*Keywords***:** Cellulose; Ionic Liquids; Direct electron transfer; Glucose oxidase; Carbon nanotube

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1. Introduction

The investigation of direct electron transfer between redox enzymes and electrodes is important in the development of electroanalytical applications and of bioelectrocatalytic devices. Such bioelectronic devices require an optimized procedure for enzyme immobilization e.g. operational simplicity, low fabrication expense and enzyme activity in the long term. Electrode material with special physical and chemical properties, biocompatibility and low cost are important factors considered for immobilization of enzymes [1]. Enzymes are typically immobilized using a range of different strategies involving materials such as inorganic supports and synthetic polymers e.g. Fe_2O_3– or Au–nanoparticles, Nafion–nanotube composites, self–assembled material layers, and conducting polymers [2, 3]. These synthetic materials are easily to process, however, they are less suitable for enzyme immobilization due to undesirable surface characteristics and low biocompatibility, which increase the denaturation of the protein [4]. In contrast, naturally occurring macromolecular soft materials are pertinent to enzyme immobilization technologies owing to their biocompatibility. Cellulose, which is present in the cell walls of plants, is the most abundant and renewable biopolymer on earth and has many advantages when used as an enzyme immobilization material because of its biocompatibility, chemical stability, mechanical and physical properties, availability from renewable natural resources, and low cost [5]. Cellulose derivatives, such as cellulose nitrate, cellulose acetate, carboxymethyl cellulose, have been used as carriers for immobilized enzymes [6-8]. However, the application of natural cellulose has been hindered by its poor solubility, due to the presence of strong inter– and intra–molecular hydrogen bonding.

Recent studies have showed that a number of room temperature ionic liquids (RTILs), e.g. 1–ethyl–3–methylimidazolium acetate ([EMIM][CH_3COO]) and 1–butyl–3–methylimidazolium chloride (BMIMCl), exhibit good dissolution power for cellulose, which can then be reconstituted into a variety of forms such as membranes, beads and hollow fibers [9, 10]. [EMIM][CH_3COO] has also been reported to be an enzyme–friendly co–solvent for organic reactions [11]. Turner et al. have developed cellulose–RTIL composite materials for the immobilization of laccase with the retention of catalytic activity using BMIMCl [12]. In the field of bioelectrochemistry, however, RTIL–reconstituted cellulose materials have not been given the appropriate level of attention.

Due to their outstanding physicochemical properties, carbon nanotubes (CNTs) are attracting considerable attention for the development of bioelectrochemical devices. However, the poor dispersibility of carbon nanotubes has traditionally been a major technical barrier to their application. Some researchers have shown that RTILs can assist in the dispersion of carbon nanotubes and impart useful properties in their application in bioelectronics [13-18].

In this communication, we report a simple method to immobilize enzymes in a cellulose – multiwalled carbon nanotube (MWCNT) matrix reconstituted by [EMIM][CH_3COO], and on the subsequent use of this matrix to modify electrodes with glucose oxidase (GOx) as a model enzyme. The direct electron transfer between the active site of GOx and electrode has been achieved, while its behaviour as biosensor and the enzyme's stability were investigated by electrochemical techniques.

2. Experimental section

2.1 Chemicals, reagents and pretreatments

Glucose oxidase (GOx, EC 1.1.3.4, 200 units mg^{-1}, from Aspergillus niger), Microcrystalline cellulose and 1–ethyl–3–methylimidazolium acetate ([EMIM][CH_3COO]) were purchased from Sigma–Aldrich and used with no additional purification. Multiwalled carbon nanotube (MWCNT, Nanocyl–3100 series with an average diameter of 10 nm) were refluxed in HNO_3 (aq, 2.6 M) for 10 h, followed by precipitation, rinsing with distilled water to eliminate the residual HNO_3, and drying in a vacuum oven at 80°C for 12 h [19]. D–Glucose solutions were prepared the day before use and allowed to stand overnight to allow equilibration of the monomers. All solutions were prepared with ultrapure water (18.2 MΩ cm^{-1}) from a Select Fusion system (Purite Corporation).

2.2 Electrode modification

Glassy carbon (GC, 3 mm diameter) was sequentially polished with 1.0 and 0.5 μm alumina slurry and then washed ultrasonically in ultrapure water for a few minutes. The cellulose–MWCNT–GOx modified GC electrodes were prepared as follows: the cellulose (3.0% mass)–[EMIM][CH_3COO] solution was obtained by thoroughly mixing cellulose and [EMIM][CH_3COO] and then heating to 70°C for 1 h in an ultrasonic bath until an optically clear solution was obtained. The MWCNT (1.0% mass) were then suspended in the [EMIM][CH_3COO]–cellulose solution by grinding in an agate mortar for 15 min under high purity nitrogen to prevent the [EMIM][CH_3COO] from absorbing moisture. GOx (3.3% mass) was finally added to the resulting mixture to afford the target GOx–cellulose–MWCNT–RTIL dispersion. A 10 μL aliquot of this solution was evenly spread on the GC surface and the modified electrode was then immersed in deionized water, to remove the [EMIM][CH_3COO] by dissolution, leaving the cellulose–MWCNT matrix with encapsulated GOx on the surface; the [EMIM][CH_3COO] can be recovered by distillation for reuse. The electrode was dried at room temperature and stored dry in air at 4°C until required.

2.3 Apparatus and electrochemical measurements

A Hitachi S2300 SEM was used to characterize modified electrode with an acceleration voltage of 5.0 kV. Electrochemical measurements were carried out using an Autolab PGSTAT (EcoChemie, Netherlands) in a three–electrode cell with 25 mL volume. The cellulose–MWCNT–GOx modified GC was used as a working electrode, a Pt–wire served as a counter electrode and the reference electrode was an Ag/AgCl type (3 M NaCl, +0.196 V vs. SHE at 298 K). The electrolyte was aqueous citrate phosphate buffer (0.2 M), which was purged with either high purity nitrogen or air for testing. All electrochemical experiments were carried out at 22 ± 1 °C.

3. Results and discussion

3.1. Characteristics of the cellulose – MWCNT matrix

Fig. 1 shows a SEM image of the RTIL reconstituted cellulose–MWCNT matrix which has a porous and three-dimensional structure. This electrically conductive, porous and biocompatibable microenvironment is a candidate for an enzyme immobilization host with the potential for enhanced direct electron transfer between the active site of enzyme and long–term electrode stability.

<Fig. 1 near here>

3.2 Direct electron transfer of GOx

GOx is of considerable commercial importance and has wide-ranging applications in the food and fermentation industry and clinical analysis [20-23]. GOx is an enzyme with a molecular weight of 160 kDa and contains flavin adenine dinucleotide (FAD) as the redox prosthetic group, which can catalyze the oxidation of β-D-glucose in the presence of molecular oxygen. However, from a bioelectrochemical perspective, the FAD in the GOx is deeply embedded within a protective protein shell and the direct electron transfer reaction between GOx and electrode does not occur easily [24].

<Fig. 2 near here>

Fig. 2A shows the cyclic voltammograms (CVs) of a cellulose–MWCNT–GOx modified electrode in a N_2–saturated buffer at increasing scan rates. The anodic and cathodic peak potentials at a scan rate of 100 mV s^{-1} are located at –357 and –386 mV vs. Ag/AgCl respectively (see Fig. 2B). The peaks are attributable to the reduction and oxidation of the $FAD/FADH_2$ electroactive centre of the GOx enzyme by a direct electron transfer process (reaction 1) [25]; redox peaks were not observed with a protein–free cellulose–MWCNT modified electrode in the potential range – 650 to 0 mV.

$$GOx(FAD) + 2e^- + 2H^+ \rightleftharpoons GOx(FADH_2) \qquad (1)$$

The redox potentials do not vary at the different scan rates, and the separation of the cathodic and anodic peak potentials for each is 29 ± 3 mV, which implies that the reaction is a reversible two–electron reversible redox process (reaction 1). The anodic and cathodic currents were both linearly proportional to the scan rate (inset of Fig. 2A), indicating that the electrode reaction is a surface confined process [26].

3.3 Effect of solution pH on the modified electrode

A series of experiments was performed to study the dependence of potential response on the solution pH. Fig. 3 shows that an increase of pH causes a negative shift in both the reduction and oxidation peak potentials. The linear regression is $E^{o\prime}$/V= –0.032 – 0.058pH, where $E^{o\prime}$ is the formal potential [$E^{o\prime} = (E_{pa} + E_{pc}) / 2$] and the linear regression coefficient R^2 = 0.997. The slope of –58 mV pH^{-1} (Fig. 2, inset) is very close to the

theoretical value of –58.5 mV pH^{-1} expected at 22°C for a reversible, 2–proton and 2–electron transfer process according to the reaction 1.

<Fig. 3 near here>

3.4 Biocatalytic activity of the modified electrode

<Fig. 4 near here>

Compared with N_2–saturated buffer (Fig. 4A, curve a), a pair of well defined GOx redox peaks were also observed in air–saturated buffer (Fig. 4A, curve c) in the absence of glucose, with an increased reduction peak current and a decreased oxidation peak current. After the addition of glucose (0.5 mM) into the buffer, the reduction peak current decreased significantly (Fig. 4, curve b). The reason is that the dissolved oxygen and GOx take part in the non–electrochemical oxidation of glucose. This phenomenon can be explained by the following, previously proposed, mechanism [27, 28]:

$$GOx(FADH_2) + O_2 \rightarrow GOx(FAD) + H_2O_2 \quad (2)$$

$$GOx(FAD) + Glucose \rightarrow GOx(FADH_2) + Gluconolactone \quad (3)$$

In the presence of oxygen, the reduced enzyme $GOx(FADH_2)$ is quickly oxidized to the oxidized form GOx(FAD) via reaction 2. The electrocatalytic regeneration of the reduced?? enzyme via reaction 1 causes the loss of reversibility and the increase in size of the cathodic peak current. As the substrate of GOx, the presence of glucose may result an enzyme–catalyzed reaction according to reaction 3 which will decrease the concentration of the oxidized form of GOx on electrode surface thus lead to the decrease of reduction peak current. These results provide evidence that the GOx, encapsulated by the RTIL formulated cellulose–MWCNT composite, still retains its biocatalytic activity towards glucose oxidation.

The decrease of the reduction peak current can be utilized as a tool to determine the glucose concentration. Fig. 5 shows a typical current–time curve of the GOx electrode on successive additions of 0.2 mM glucose when the electrode was poised at –500 mV vs Ag/AgCl in an air–saturated solution. The current values change linearly with the concentration of glucose from 0.05 to 1.0 mM with a linear regression equation of $I = 0.68 - 0.46 \times$ [glucose] and a correlation coefficient of 0.9995. The sensitivity of the cellulose–MWCNT–GOx electrode is 6.57 μA $mM^{-1}\,cm^{-2}$ which is higher than reported in earlier studies (3.3 μA $mM^{-1}\,cm^{-2}$ at chitosan modified electrodes [26] and 3.47 μA $mM^{-1}\,cm^{-2}$ at silica gel based electrodes [27]).

<Fig. 5 near here>

The apparent Michaelis-Menten constant K_m of the GOx was determined by Michaelis–Menten equation to be 1.10 mM. The value is also lower compared to previously reports

(6.8 mM [29] and 15.19 mM [30]), which indicates an enhanced enzyme affinity of GOx to glucose with the biocompatible microenvironment of the cellulose–MWCNT matrix.

3.5 Reproducibility and stability

The reproducibility and stability of the proposed GOx electrode have also been investigated. The fabrication reproducibility of five electrodes, made independently under the same conditions, showed an acceptable reproducibility for the current determined at 0.2 mM glucose concentrations (evidence?). The stability of the electrode was tested by repeated CV scanning in a potential range from –650 to 0 mV vs Ag/AgCl at 100 mV s^{-1}; the modified electrode showed an unchanged peak current after 200 cycles (see Fig. 2B). The electrode was tested again after the bioelectrode was stored for 10 days and a decrease of only 11% in peak currents was observed.
These favorable results could be mainly attributed to two factors: firstly, cellulose, endowed with a large amount of –OH groups, provides a biocompatible environment for encapsulation of GOx; secondly, as the SEM image shown in Fig. 1, the cellulose–MWCNT matrix possesses a porous structure, allowing a large amount of enzyme to be immobilized close to the electrode surface, where direct electron communication between active site of enzyme and electrode is enabled.

4. Conclusions

This work reports a simple procedure for the preparation of a biocompatible cellulose–MWCNT matrix via the RTIL reconstitution process, and the porous matrix has been employed in the immobilization of GOx (as an example enzyme). The encapsulated GOx showed good bioelectrochemical activity, high biological affinity as well as good stability and repeatability. The simple electrode fabrication methodology and the biocompatibility of the cellulose–MWCNT matrix mean that the immobilization matrix not only can be used for GOx but also can be extended to other proteins, thus providing a promising platform for the further research and development for biosensor and other bioelectronics devices. Further investigations into electrochemical properties of the cellulose–MWCNT matrix and immobilization process with different enzymes, with the objective of developing enzymatic fuel cells, are underway.

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Captions

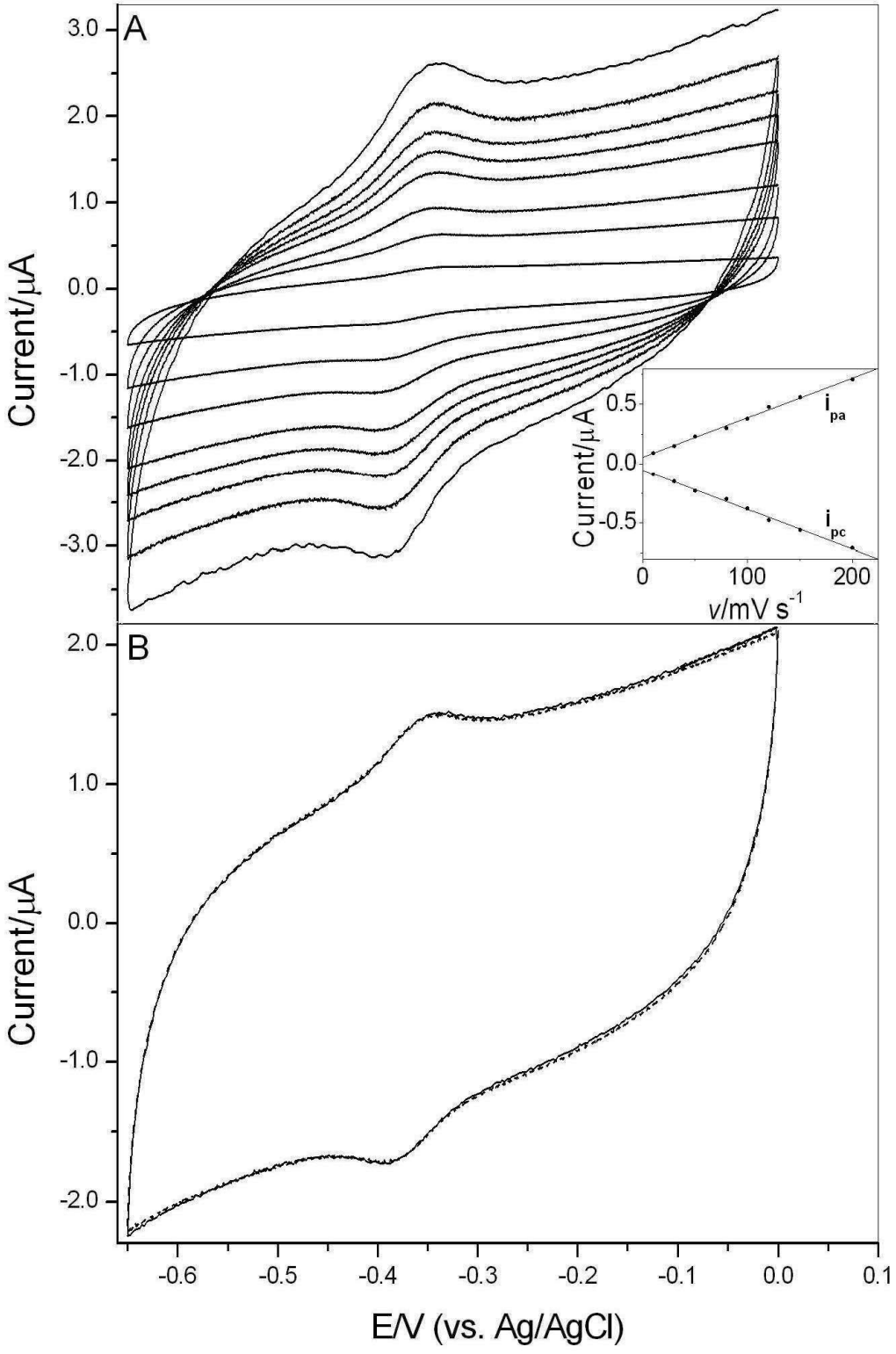
Figure 1. SEM of the cellulose–MWCNT matrix.

Figure. 2. (A) Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode in anaerobotic buffer (0.2 M) at pH 6.0. The scan rates were 10, 30, 50, 80, 100, 120, 150, and 200 mV s^{-1} (inner to outer CV). The inset shows the relationship between scan rate and the cathodic and anodic peak currents. (B) Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode at pH 6.0 and a scan rate of 100 mV s^{-1} under anaerobic condition. The solid line represents the first scan cycle and the dashed line represents the CV after 200 continuous cycles.

Figure 3. Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode in aqueous citrate phosphate buffer (0.2 M) of pH 5.0, 6.0, 7.0 and 8.0 (from left to right) under anaerobic condition. The scan rate was 100 mV s^{-1}. The inset shows the dependence of $E^{o\prime}$ of GOx on pH.

Figure 4. Cyclic voltammograms of a cellulose–MWCNT–GOx modified electrode in buffer (0.2 M) at pH 6.0 and a scan rate of 100 mV s^{-1} in: (a) anaerobic aqueous citrate phosphate buffer, (b) aerobic aqueous citrate phosphate buffer and (c) aerobic buffer with the addition of 0.5 mM glucose.

Figure 5. Current (baseline subtracted?) − time curves obtained with a cellulose–MWCNT–GOx–modified electrode on addition of successive 0.2 mM glucose in air–saturated buffer at pH 6.0. The electrode was held at a potential –0.50 V vs. Ag/AgCl.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-0.5
-0.4
-0.3
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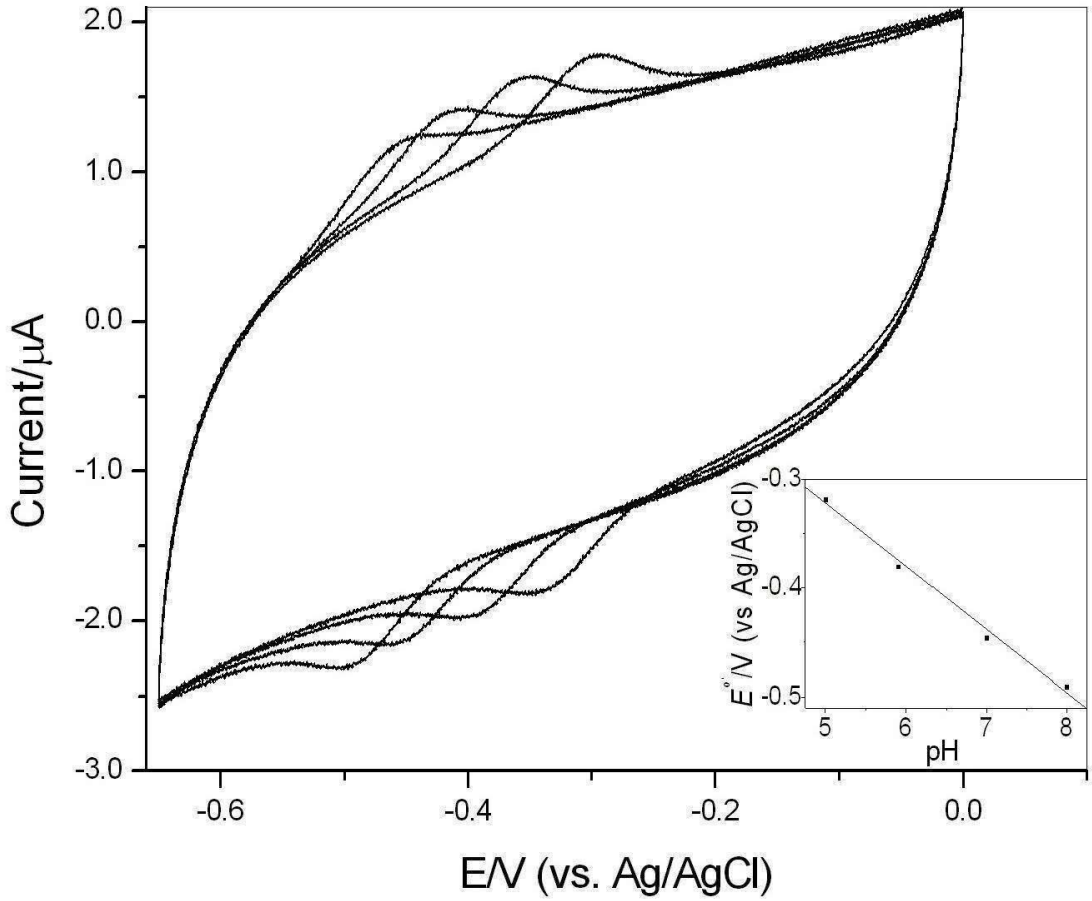
0.5
-0.5

Figure 2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pH

Figure 3

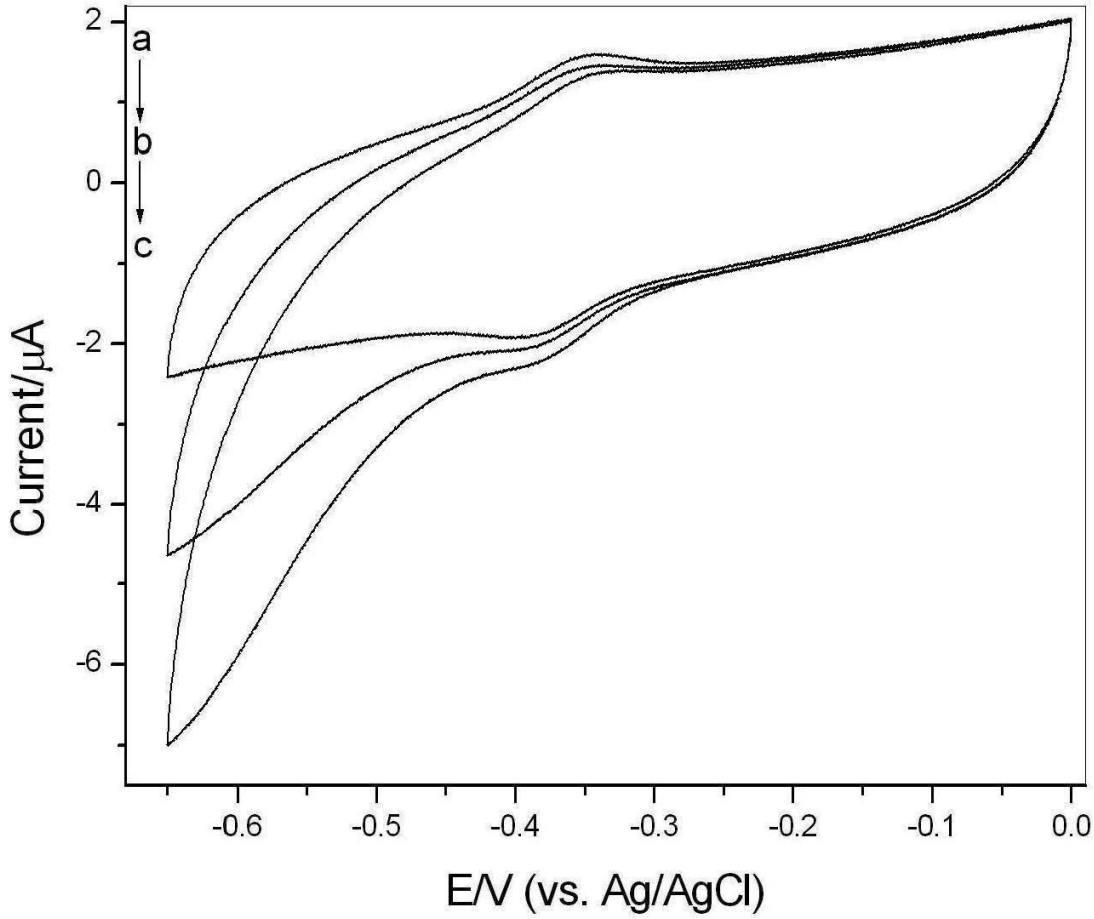

Figure 4

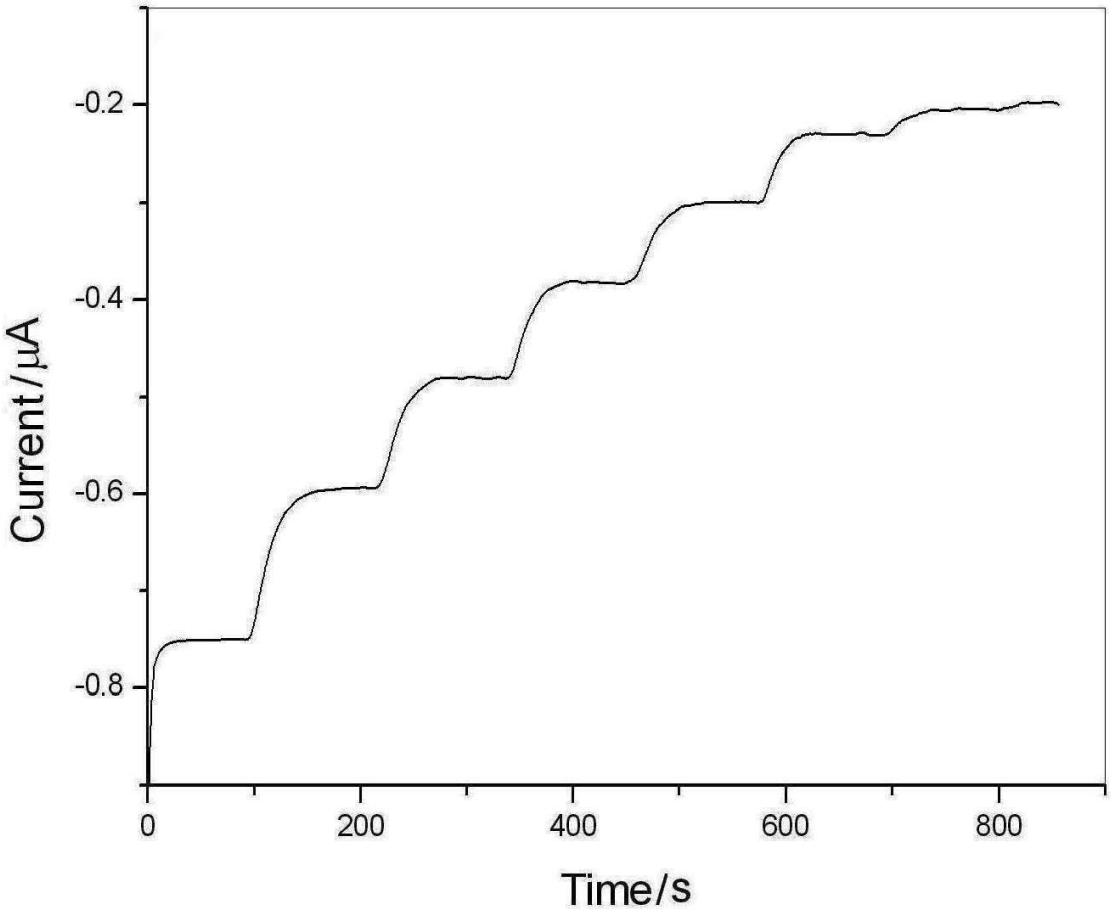

Figure 5