Biosensors and Bioelectronics 24 (2008) 976–980

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Fabrication of cytochrome *c*-poly(5-amino-2-napthalenesulfonic acid) electrode by one step procedure and direct electrochemistry of cytochrome c

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ARTICLE INFO

Article history:
Received 17 April 2008
Received in revised form 28 July 2008
Accepted 29 July 2008
Available online 8 August 2008

Keywords:
Cytochrome *c*
PANS
One step procedure
Superoxide radicals

ABSTRACT

Herein, we reported for the first time one step procedure for the preparation of cytochrome *c* (cyt c)-poly (5-amino-2-napthalenesulfonic acid) (PANS) modified glassy carbon electrode by cyclic voltammetrically (CV). Hereafter, we called the above modified electrode as cyt c-PANS electrode. The presence of cyt c on modified electrode was investigated with electrochemical quartz crystal microbalance (EQCM), CV, and superoxide radicals reaction studies. The reaction between cyt c in the modified electrode and superoxide radicals in solution, was exemplified by cyclic voltammetric measurements. Surface morphology of the modified electrode was investigated by using atomic force microscopy (AFM). The modified electrode showed a pair of well defined redox peak in PBS solution, pH 6.7. The modified electrode utilized for electrocatalytic reduction as well as amperometric determination of hydrogen peroxide (H_2O_2). The detection limit and linear range for H_2O_2 were 5 and 50 μM to 7 mM, respectively.

1. Introduction

Cytochrome *c* (cyt c) is one of the well studied redox protein. It contains one Fe(III) redox center located in a haem unit which is approximately spherical in shape with 34 Å diameter and 12,384 Da molecular weight. However, on metal surfaces it usually shows a short-lived, transient response (Eddowes and Hill, 1977; Yeh and Kuwana, 1977), because cyt c molecule lacks direct electrical contact with the electrode surface due to the non-appropriate orientation of the heme site in respect to the electrode surface lead to poisoning and deactivation of a bare electrode (Szucs et al., 1992; Fedurco, 2000; Tarlov and Bowden, 1991). In order to improve the direct electron transfer (ET) between an electrode and redox protein, an important step for electrochemical process, requires interfaces that exhibit reasonably fast ET kinetics. The desired interfacial properties are accessible only through electrode modification (Armstrong et al., 1988; Harmer and Hill, 1985). There are different strategy employed to modify the electrode in order to improve interfacial properties between cyt c and electrode namely by means of formation of self assembled monolayer, DNA modification, polymer modification, deposition of metal nano particles, etc.

There are number of modified interfaces available to immobilize cyt c such as $-CO_2H$ terminated self assembled monolayers (SAMs) through electrostatic interactions between carboxylate and the exterior of the protein (Collinson et al., 1992; Yamamoto et al., 2001; El Kasmi et al., 1998), layer by layer surface (Lvov et al., 1998), or multilayer film surface (Beissenhirtz et al., 2004), on the surface of DNA modified electrode (Lisdat et al., 2001, 1999a; Chen and Chen, 2003; Liu et al., 2003), by covalent linkage (McNeil et al., 1995; Koh et al., 2008), protein modification (Heller, 1990; Dronov et al., 2008), and on the surface of inorganic porous material (Xu et al., 2003; Yu and Ju, 2002). Although these new materials have been proven to be excellent as the immobilization matrices due to their high stability and good absorbability, some inherent defects are inevitable for the application of these multilayer and inorganic porous materials in electrochemical sensing such as low conductivity in inorganic material and in thicker films or multilayers, generally only protein molecules near the electrode surface are electroactive.

In order to improve good communication between cyt c and electrode surface, cyt c has been immobilized on the conducting polymer modified electrode (Bartlett and Faington, 1989; Caselli et al., 1991) that resulted good electron transfer. Since polymer modified electrodes are stable and amount on the electrode surface can be varied in a controlled manner. Recently, Jiang et al. (2006) immobilized cyt c on surface of poly(aniline-co-*o*-aminobenzensulfonic acid) (PANABS) through electrostatic interaction between cyt c and sulfonic group of PANABS. The above all reported the literature need two steps to achieve cyt c modified electrode. The two steps are (i) first preparation of modified or interface surface and (ii)

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doi:10.1016/j.bios.2008.07.063

immobilization or entrapment or covalent linking of cyt c. The enzyme or heme protein entrapment or immobilization on pre-prepared film is a more complicated and time consuming technique, which limits the heme quantity to a few monolayers and makes it possible to reduce the activity (Gooding et al., 1999). Therefore, to retain the heme protein activity and to limit the time consumption during preparation of cyt c electrode, we have prepared cyt c-PANS electrode by one step procedure. To the best of our knowledge, there is no such literature available for one step preparation of cyt c electrode.

In this present work, we report for the first time, that ANS polymerized electrochemically and during polymerization cyt c was entrapped. In this way, we achieved cyt c-PANS electrode by one step procedure. The presence of cyt c on modified electrode was investigated with electrochemical quartz crystal microbalance (EQCM), CV, and superoxide radicals reaction studies. The modified electrode exhibited electrocatalytic as well as good amperometric response towards H_2O_2 with the detection of limit of 5 μM.

2. Experimental

2.1. Reagents and solutions

ANS, bovine heart cytochrome *c* (cyt c), peroxidase and catalyse were purchased from Sigma–Aldrich. Stock solutions H_2O_2 were prepared each time freshly from 34% solution (purchased from Wako). All reagents were of analytical grade and used without any further purification. Solutions were prepared with doubly distilled water. High purity nitrogen was used for deaeration. The buffer and sample solutions were purged with highly purified nitrogen for at least 10 min prior to the experiments. Nitrogen atmosphere was maintained over the solutions during experiments.

Superoxide radical was generated according to the previously reported literature (McNeil et al., 1989).

2.2. Apparatus

Electrochemical experiments were performed with CH Instruments (Model CHI-400) using CHI-750 potentiostat. Glassy carbon electrode (geometric area = 0.07 cm^2) obtained from BAS served as a working electrode. Pt wire act as counter electrode and Ag/AgCl with the saturated KCl solution used as reference electrode. All the potentials given in this paper were referred with respect to Ag/AgCl (saturated KCl solution) reference electrode. The EQCM experiment was performed using CH instruments EQCM oscillator. An Au coated working electrode (area 0.196 cm^2, 8 MHz, AT-cut quartz crystal) was used.

Ultraviolet visible (UV–vis) spectra were recorded on a model U-3300 UV–vis spectrophotometer (Hitachi). Films of cyt c-PANS and PANS electrode were prepared by cyclic voltammetrically, respectively. Also, cyt c solution in pH 6.7 PBS was used as control.

2.3. Fabrication of cyt c-PANS electrode

Prior to modification, glassy carbon electrode (GCE) was polished with 0.05 μm alumina on Buehler felt pads and then ultrasonically cleaned for about a minute in water. Finally, the electrode was washed thoroughly with double distilled water and used. After being cleaned, the electrode was immersed into 0.05 M PBS solution, pH 6.7 containing 0.5 mM ANS and 2 mg/ml cyt c and the potential of working electrode was cycled between −0.5 and 0.8 V at the 100 mV s^{-1} for 260 s to fabricate cyt c-PANS electrode.

3. Results and discussion

3.1. Fabrication of cyt c-PANS electrode by one step procedure

Fig. 1 shows the CVs obtained from solution containing ANS monomer and cyt c in 0.05 M PBS solution, pH 6.7. During potential scanning of the electrode from −0.5 to 0.8 V at the scan rate of 100 mV s^{-1}, the anodic peak noticed at more positive potential in the first sweep corresponds to oxidation of ANS. In the subsequent sweeps, the redox peak appeared at around 0 V (versus Ag/AgCl) and the continuous increase of peak currents was observed. These observations can be ascribed as that cyt c was entrapped during oxidation followed by polymerization of ANS on the electrode surface. It might be due to electrostatic interaction between negatively charged sulfonic acid group of ANS and the positively charged cyt c (Armstrong et al., 1988). It is well known that cyt c has nine positive charges at pH 7.0 (Armstrong et al., 1988), indicating that amino groups in cyt c were protonated in pH 6.7 PBS solution and then interacted with sulfonic group of ANS to form organic complexes. Jiang et al. (2006) were observed similar type of interaction between cyt c and PANABS modified electrode. For comparison, we have given CV of PANS electrode in the absence of cyt c, is shown in Fig. 1B.

In order to estimate, the amount of cyt c entrapped in PANS during polymerization, we have carried out EQCM experiment and recorded the frequency changes on Au electrode surface in the presence and absence of cyt c. There was a decrease in frequency in the range of 132 Hz, which corresponds to a mass change (Δm) of ≈184 ng, in the presence of cyt c and only 60 Hz, which corresponds to mass change (Δm) of ≈84 ng, in the absence of cyt c (figures are given in supplementary file). This significant frequency and mass variation between these two electrodes confirmed and that excess frequency as well as mass change is due to the presence of cyt c while it was entrapped during

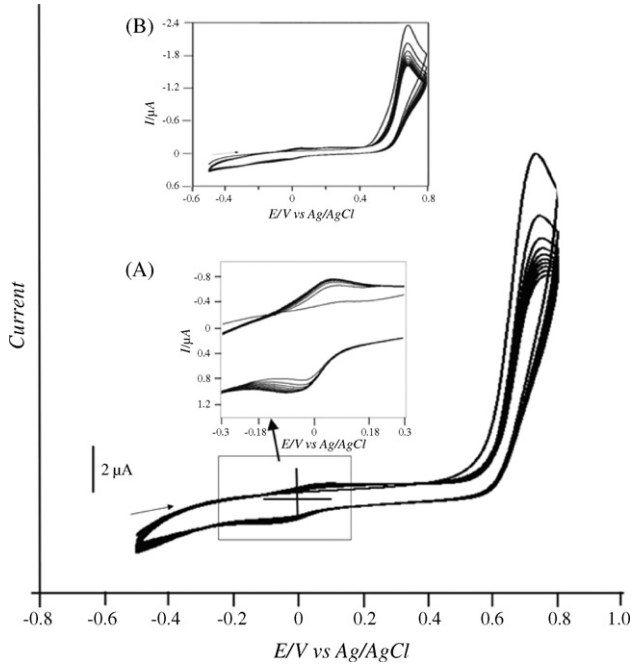

Fig. 1. CVs of cyt c-PANS electrode from solutions containing 0.5 mM ANS and 2 mg/ml cyt c in 0.05 M PBS solution, pH 6.7, 0.05 M PBS for modification. Scan rate: 0.1 V s^{-1}. (A) Magnified CV of cyt c-PANS electrode in the region of −0.3 to 0.3 V. (B) CVs of PANS electrode from solutions containing 0.5 mM ANS in 0.05 M PBS solution, pH 6.7, for modification. Scan rate: 0.1 V s^{-1}.

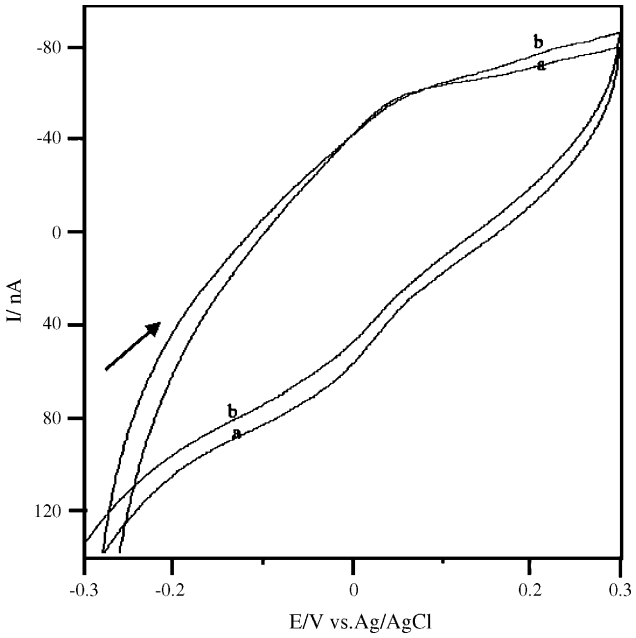

Fig. 2. CVs of cyt c-PANS electrode before (a) and during superoxide generation (b) 0.05 M PBS solution, pH 6.7, 10 μM EDTA, 100 μM hypoxanthine, 100 μM peroxidase, 4 μM catalyse. Scan rate: 5 mV s^{-1}.

polymerization. From the mass change of PANS and cyt c-PANS electrode, we measured amount of entrapment of cyt c and found to be ≈100 ng. The entrapped amount of cyt c was comparable with previously reported literature by two step protocol that cyt c/poly(terthiophene-3-carboxylic acid) {poly (TTCA)} micro electrode (Koh et al., 2008), protein modified electrode (Dronov et al., 2008), SAM/DNA/cyt c electrode (126 ng) (Ding et al., 2006) and with cyt c/C_{60}-Pd modified electrode (Δm, 135 ng) (Souzaa et al., 2005). The mass changes from the frequency were calculated by using the Sauerbrey equation (Sauerbrey, 1959; Okahata and Ebato, 1989).

We further investigated whether excess mass change was due to presence of cyt c or not? In order to explicate and confirm that cyt c was entrapped during polymerization of ANS on the electrode surface, we have performed experiment to reduce oxidized cyt c by using superoxide radicals. Superoxide radicals rapidly reduce oxidized cyt c (McNeil et al., 1989). Lisdat et al. (1999a) has used this reaction to test the possibility of a signal chain from the analyte to the modified electrode. Here, we utilized this reaction to examine the presence of cyt c on modified electrode and experimental result is shown in Fig. 2. We employed the cyt c-PANS electrode towards super oxide radical reaction and observed that oxidation peak of cyt c was enhanced in the presence of superoxide which continuously reduces the electro-oxidized cyt c as result the reduction peak diminished as shown in Fig. 2(b). The catalytic effect of cyt c-PANS electrode is not so obvious compared to that of (mercaptoundecanoic acid) MUA/Cyt c modified electrode (Lisdat et al., 1999b). But entrapped cyt c is still accessible for superoxide radical and also the catalytic effect is comparable with DNA/cyt c modified electrode (Lisdat et al., 1999a). From the above experimental results, we concluded that oxidized cyt c was reduced by superoxide radicals and it could be confirmed that cyt c was entrapped in the modified electrode and the excess mass change is due to presence of cyt c. The control experiment was carried out for PANS electrode only (figure not shown) and did not respond for superoxide radicals because no cyt c on the electrode surface.

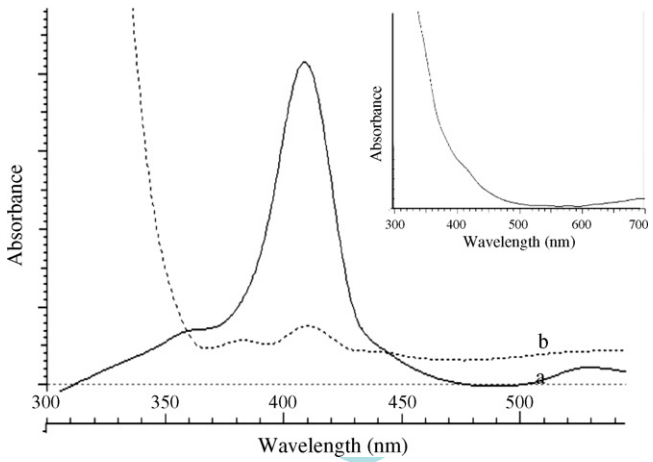

Fig. 3. The UV–vis absorption spectra for (a) cyt c in PBS solution, pH 6.7 and (b) Cyt c-PANS ITO electrode. *Inset*: UV–vis absorption spectra PANS electrode only.

3.2. UV–vis absorption spectrum

The UV–vis spectrum of cyt c shows a band at 410 nm in PBS solution, pH 6.7 (Fig. 3, curve a). The locations of Soret absorption band of iron heme may provide information about the denaturation of heme proteins. When cyt c was denatured, the Soret band shifted or disappeared (Rusling, 1998). Fig. 3, curve b shows the Soret band of cyt c-PANS electrode. Its Soret absorption band very close to natural cyt c in solution (curve a), indicating that cyt c was entrapped in the PANS film and almost retains its native structure. The smaller absorption peak of cyt c (in curve b) than cyt c in solution (curve a), might be due to presence of small quantity cyt c in the modified electrode. Inset of Fig. 3 shows UV–vis spectrum of PANS electrode.

3.3. Surface morphology of cyt c-PANS electrode

By using AFM, we investigated surface morphology of cyt c-PANS and PANS electrode, are shown in Fig. 4. AFM images of PANS (Fig. 4A) showed that it has distributed uniformly and detached from one another while cyt c-PANS electrode showed bright structure with aggregation. This might revealed that there is strong electrostatic interaction between cyt c and PANS. To support above electrostatic interaction we measured average spacing for cyt c-PANS and for PANS electrode it ranged ≈700–800 and ≈100–150 nm, respectively. The higher spacing for cyt-PANS than PANS electrode revealed that cyt c appeared on the modified electrode and more likely it might be due to electrostatic interactions between cyt c and PANS. Similar type of electrostatic interaction studies was reported between SWNTs and cyt c by using AFM spacing interaction parameter (Shi Kam and Dai, 2005). According to above results, cyt c changed the morphology of the modified electrode is shown in Fig. 4B and C.

3.4. Electrochemical characterization of cyt c-PANS electrode

Fig. 5 shows CVs for the cyt c-PANS electrode in 0.05 M PBS solution, pH 6.7 at different scan rates. As can be seen that cyt c-PANS electrode leads to a pair of well defined redox peaks with a formal potential of around 0 V (versus Ag/AgCl). The plot of peak current versus scan rate was linear from 50 to 1000 mV s^{-1} (inset of Fig. 5), indicating that redox process was surface confined and the cathodic and anodic peak current ratio was almost unity confirming that entrapped state of cyt c. The surface coverage, Γ of modified electrode was obtained by integrating area under anodic peak and was found to be 1.7×10^{-11} mol/cm^2. The kinetics of

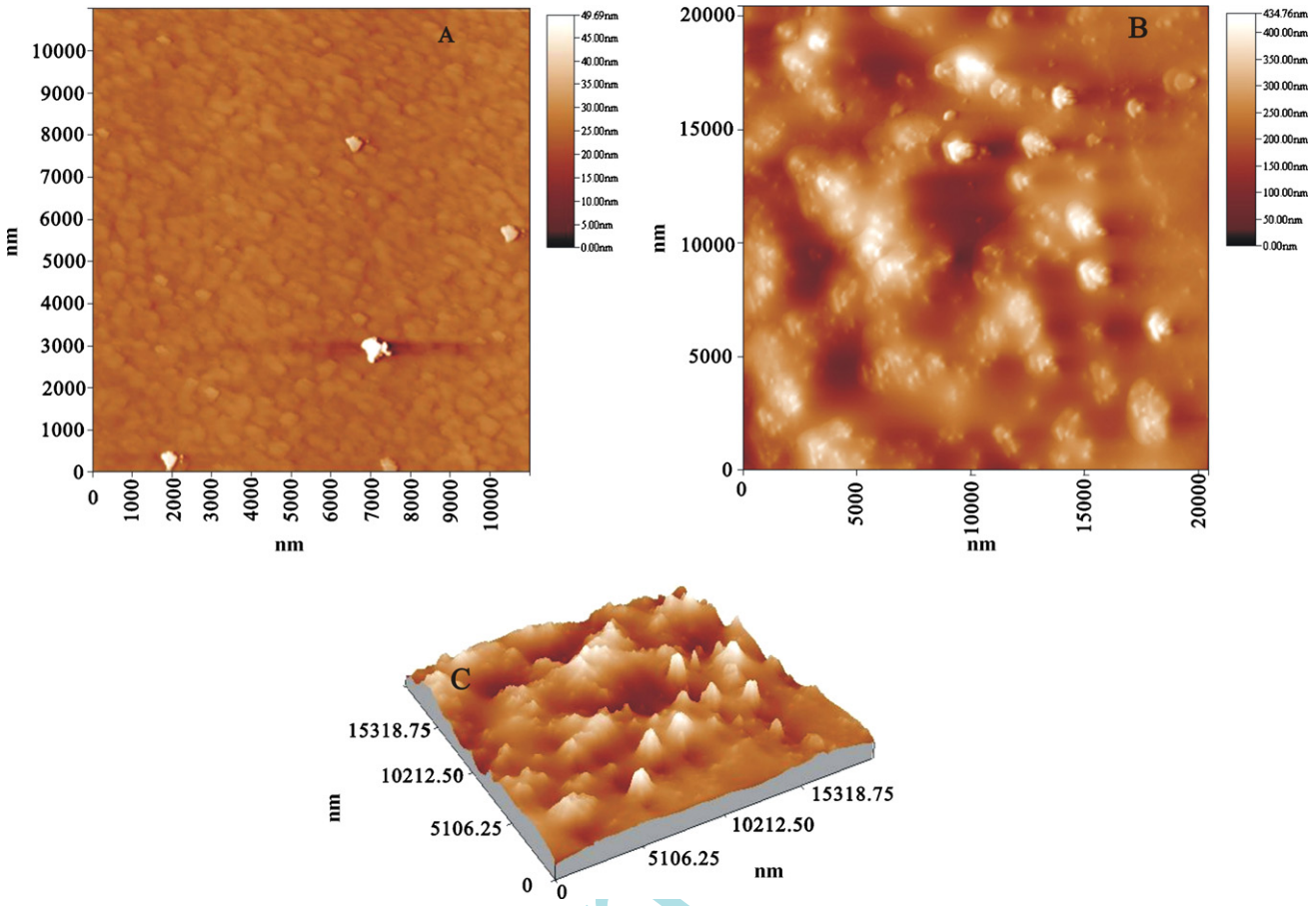

Fig. 4. Topographic tapping mode AFM (A) PANS, (B) cyt c-PANS and (C) 3D image of cyt c-PANS electrode deposited on ITO substrate.

heterogeneous electron transfer rate constant (k_s) can be calculated by using Laviron's equation (Laviron, 1979). The ΔE_p values obtained using various scan rates for less than 200 mV and the calculated k_s value was found to be 8.34 s^{-1}. The result was comparable with cyt c/PANABS electrode (12.9 s^{-1}) (Jiang et al., 2006) and higher than those of cyt c/PTTCA electrode (1.86 s^{-1}) (Koh et al., 2008), cyt c/Nb_2O_5 electrode (0.28 s^{-1}) (Xu et al., 2003). The higher electron transfer rate constant indicates that PANS is an excellent candidate to promote the electron transfer between cyt c and the electrode. Thus, PANS is good platform for the entrapment of cyt c.

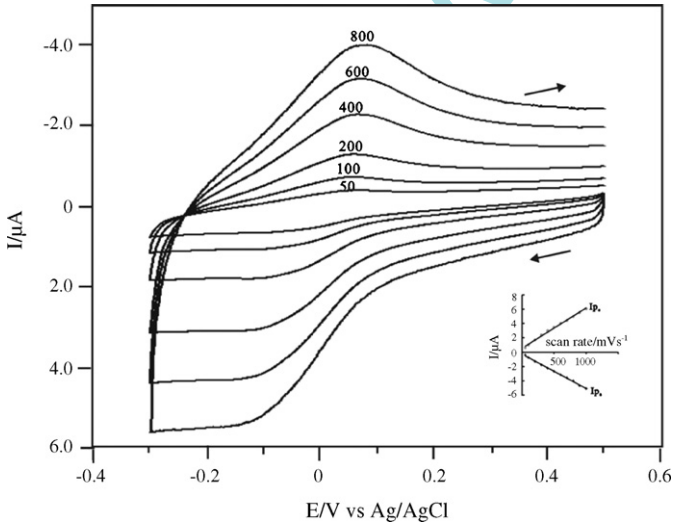

Fig. 5. CVs of cyt c-PANS electrode at different scan rate: Electrolyte: 0.05 M PBS solution, pH 6.7. *Inset*: ip_a and ip_c versus scan rate.

3.5. Catalytic activity of cyt c-PANS electrode for electrocatalytic reduction of hydrogen peroxide

Previous literatures (Xu et al., 2003; Jiang et al., 2006) showed that the cyt c "modified" electrodes display an electrochemical response to H_2O_2. Electrocatalytic activity of cyt c-PANS electrode towards substrate of medicinally important molecule (Kulys et al., 1993) such as hydrogen peroxide was examined by using CV and the results are shown in Fig. 6A. The typical catalytic reduction peak current of cyt c-PANS electrode increased and oxidation peak current decreased upon addition of 5 mM H_2O_2 into 0.05 M PBS solution [Fig. 6A(d)] compared to that of cyt c-PANS electrode in the absence of H_2O_2 [Fig. 6A(c)], indicating that cyt c-PANS electrode mediates the reduction of H_2O_2. To verify whether catalytic current was due to non-enzymatic reduction of H_2O_2, the control experiment was performed for PANS electrode only. Only small current response could be observed at the PANS electrode [Fig. 6A(b)] for H_2O_2. This indicates that catalytically active cyt c was present on the modified electrode and the catalytic current is mainly due to direct electron transfer from cyt c to the electrode.

Amperometric response of cyt c-PANS electrode upon successive additions 0.5 mM H_2O_2 into stirring 0.05 M PBS solution, pH 6.7 at an applied potential of −0.05 V is illustrated in Fig. 6B(a). As H_2O_2 was added at regular interval of time into stirring buffer

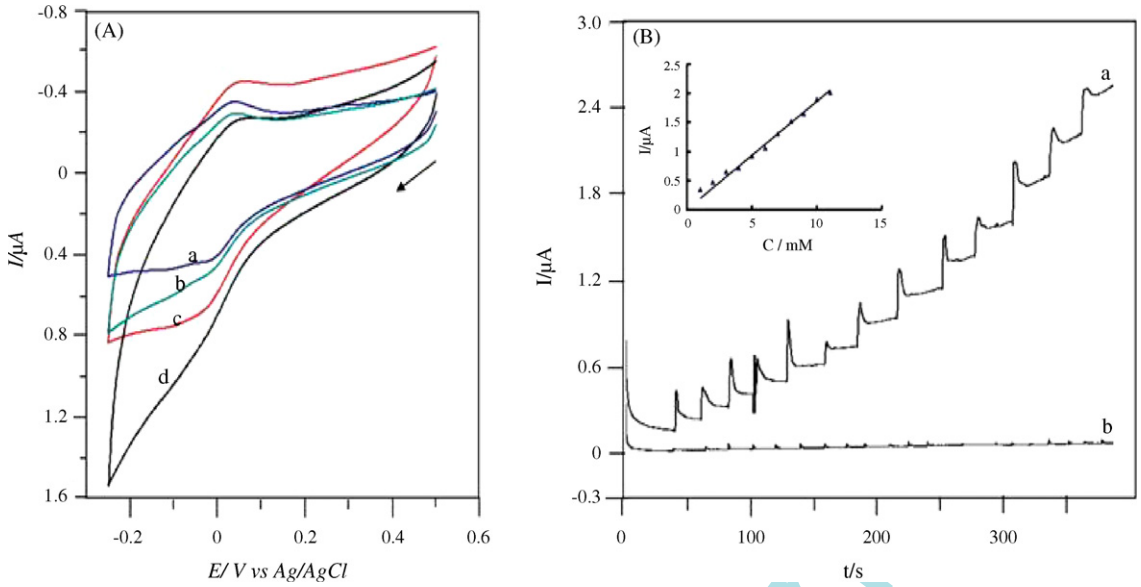

Fig. 6. (A) CVs of cyt c-PANS (c and d) and PANS electrode (a and b) in 0.05 M PBS, pH 6.7 (a and c) and containing 5 mM H_2O_2 (b and d). Scan rate: 50 mV s^{-1}. (B) Amperometric response of cyt c-PANS electrode upon successive additions 0.5 mM H_2O_2 into stirring 0.05 M PBS, pH 6.7 Applied potential: −0.05 V. *Inset*: Calibration curve.

solution, cyt c-PANS electrode responded rapidly and could attained the steady state current within less than 3 s. The control experiment was performed to PANS electrode [Fig. 6B(b)] and found that the response was negligible as compared to cyt c-PANS electrode. The cyt c-PANS electrode shows linear response towards H_2O_2 in the concentration range of 50 μM to 7 mM (inset of Fig. 4C). The detection limit was estimated to be 5 μM at the S/N of 3.

3.6. cyt c-PANS electrode reproducibility and stability

To characterize the reproducibility of the cyt c-PANS electrode, repetitive measurements were carried out in 5 mM H_2O_2. The results of 10 successive measurements show a relative standard deviation (R.S.D.) of 5%, indicating that modified electrode has an excellent reproducibility. The stability of the cyt c-PANS electrode was studied. After electrode preparation, electrode was washed in double distilled water followed by the electrode was cycled in PBS pH 6.7, potential between −0.3 and 0.3 V in 50 mV s^{-1}. The entrapped cyt c only lost 10% of its initial activity in after more than 100 successive measurements.

4. Conclusions

In this work, we reported one step procedure for the fabrication of cyt c-PANS electrode. The cyt c in the modified electrode was confirmed by using EQCM, CV, and superoxide radicals reaction studies. UV–vis spectrum revealed that cyt c could retain bioactivity on the modified electrode. The modified electrode exhibited electrocatalytic activity towards H_2O_2 and used to estimate H_2O_2 in amperometric mode.

Acknowledgements

Authors like to thank Dr. R. Thangamuthu, post doctoral fellow for reading this manuscript carefully and for fruitful discussions during preparation of this manuscript. Authors also acknowledge the Ministry of Education and NSC (ROC), Taiwan for the financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.07.063.

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