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Direct Electrochemistry and Electrocatalysis of Hemoglobin Immobilized on Eggshell Membrane Modified Glassy Carbon Electrode

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Eggshell membrane is a kind of naturally occurring organic material, which can be exploited as protein immobilization matrix. In this paper, eggshell membrane was effectively attached onto the surface of support substrates such as glass slide and glassy carbon electrode. Scanning electron microscopy, cyclic voltammetry and electrochemical impedance spectroscopy were applied to characterize the supported eggshell membrane. Hemoglobin was immobilized in eggshell membrane and direct electron transfer was realized between protein and eggshell membrane modified electrode. Outer shell membrane could effectively facilitate direct electrochemistry of hemoglobin compared with the whole eggshell membrane. A pair of well-defined redox peaks of hemoglobin was obtained at outer shell membrane modified electrode. Hemoglobin immobilized in outer shell membrane showed a surface-controlled electrochemical process. Hemoglobin could still retain its catalytic activity for electrochemical reduction of O_2 and H_2O_2. It is expected that eggshell membrane may find more potential applications in biosensors and biocatalysis.

Keywords: Eggshell membrane, Hemoglobin, Electron transfer, Electrocatalysis.

INTRODUCTION

Nowadays, studies of direct electron transfer between redox-active proteins and underlying electrodes are attracting more and more interest of scientists. These researches can fundamentally provide insight into physiological electron transfer processes and establish a foundation for fabricating new kinds of third-generation biosensors, biofuel cells, biomedical devices and enzymatic bioreactors, *etc*[1,2]. It's well known that proteins exhibit a rather slow rate of heterogeneous electron transfer at conventional electrodes because of electroactive prosthetic groups embedded deeply in the protein structure, adsorptive denaturation of proteins onto electrodes and unfavorable orientations at electrodes[3,4]. Efforts are accessible through specific modification of the electrode surfaces. It has been reported that some biomaterials or bio-inspired membranes, *e.g.* yeast cell[5], silk fibroin[6,7], collagen or grafted collagen[8,9], cellulose[10,11], alginate[12], chitosan[13,14], gelatin[15,16] and biomembrane-like films[3,4,17] were employed to modify electrode as platforms for the immobilization of proteins. These biocompatible materials could provide a more acceptable microenvironment for proteins, in which protein electrochemistry was mostly achieved compared with that at bare electrode.

Hemoglobin consisting of four polypeptide chains each with a heme group is well known as a physiologically oxygen transport protein. It can be used as an ideal model molecule for investigating electron transfer of heme proteins due to its commercial availability and well-known structure. The electron-transfer reactivity of hemoglobin is physiologically hampered and great attempts have been made to enhance it without the use of any mediators or promoters in the past thirty years[18-20]. In order to obtain protein electrochemistry and retain protein bioactivities, it is significant to find special materials or suitable methods for immobilization proteins on the electrode surface. A popular concept is the incorporation of hemoglobin into stable films such as surfactant or lipid films[3,4], hydrogel polymers or biopolymers[14], DNA film[21], layer-by-layer assembly films[22-24], mesoporous materials[24-26], which are generally used in the study of protein electrochemistry.

Eggshell membrane, a naturally occurring collagenous organic material, resides between egg white and inner surface of eggshell. It is known that eggshell membrane is composed of three membranes, namely, a thick outer shell membrane attached to eggshell, a thin inner shell membrane (ISM) and a limiting membrane that surrounds egg white, from outside to inside[27]. Eggshell membrane possessing high surface area and an intricate lattice network of stable and highly cross-linked fibers results in various applications such as used for the development of biocompatible hybrid materials and templates[28,29] and as adsorbent and immobilization support[30-32]. To our best

of knowledge, the literature dealt with eggshell membrane as proteins immobilization platform to realize the direct electrochemistry of proteins has not been reported up to now.

In this paper, eggshell membrane could be effectively adhered to the surface of solid supports such as glass slide and glassy carbon electrode. Scanning electron microscopy, cyclic voltammetry and electrochemical impedance spectroscopy were used to characterize the eggshell membrane constructed on substrates. Eggshell membrane was employed as an available and convenient supporting matrix to immobilize bioactive protein. Direct electrochemistry of hemoglobin was realized at stable thin film of eggshell membrane modified glassy carbon electrode and hemoglobin could still retain its activity to enhance electrochemical reduction of O_2 and H_2O_2.

EXPERIMENTAL

Hemoglobin (human) was purchased from Sigma (USA) and used without further purification. Hydrogen peroxide (H_2O_2, 30 %) solution was purchased from Beijing Chemical Reagent Company (Beijing, China) and a fresh solution of H_2O_2 was prepared daily. The eggshell membrane was obtained from the commercially available hen eggs. All other reagents were of analytical reagent grade and used as received. All aqueous solutions were made with deionized water, which was further purified with a Milli-Q system (Millipore).

Separation of eggshell membrane and preparation of the enzyme electrode: An eggshell membrane was carefully peeled off from a broken fresh eggshell after the albumen and yolk had been emptied, in which the inner shell membrane and limiting membrane could be manually removed. The peeled membrane was immersed in 1 mol L^{-1} HCl to dissolve residual eggshell, followed by washing with deionized water. The membrane was placed on the surface of a freshly polished glassy carbon electrode and cut to fit the diameter of the electrode. Then 10 µL of hemoglobin solution with the concentration of 3 mg/mL dissolved in 10 mmol L^{-1} phosphate buffer solution (PBS, pH = 7) was dropped on the modified electrode and a small beaker was covered over the electrode to serve as evaporating room. Before electrochemical measurements, as-prepared enzyme electrode was immersed in buffer solution for 0.5 h to remove the redundant protein. After hemoglobin was immobilized, the membrane changed colour from white to yellow brown, which is the colour of protein solution. Unless otherwise stated, the membrane modified on glassy carbon electrode in this study is outer shell membrane. The final electrode was denoted as hemoglobin-outer shell membrane/GC electrode.

Characterization of eggshell membrane: The eggshell membrane placed on glass slide substrate and coated with a thin layer of gold using a spray gun was characterized with a XL 30 ESEM FEG scanning electron microscopy.

Electrochemical measurements: All cyclic voltammetry experiments were performed on CHI900 electrochemistry station (CHI, USA). Impedance spectroscopy was carried out with an autolab PGSTAT30 (Eco Chemie B.V., Utrecht, the Netherlands) in the frequency range from 10 kHz to 0.1 Hz with signal amplitude of 10 mV. The standard three-electrode system was used for the measurements with a platinum wire as counter electrode, an Ag/AgCl (KCl-saturated) electrode as reference electrode with respect to which all potentials were reported and a glassy carbon electrode as working electrode. The 10 mmol L^{-1} phosphate buffer solution containing 0.1 mol L^{-1} KCl used as electrolytes was purged with purified nitrogen for at least 20 min to remove oxygen. Nitrogen atmosphere environment was kept during electrochemical measurements.

RESULTS AND DISCUSSION

Characterization of eggshell membrane: With outer shell membrane as inner leaflet attached to support substrate and inner surface of eggshell membrane faced out, eggshell membrane can stably adhere to the surface of clean glass slide. SEM images in Fig. 1 illustrate the morphology of eggshell membrane. The layered structure of eggshell membrane could be obviously observed. The left bottom side of the Fig. 1a corresponds to the outer surface of eggshell membrane, namely outer shell membrane and the right upper side shows the inner surface of eggshell membrane. The images of different layered structure with enlarged scale are displayed in Fig. 1b and 1c, respectively. The outer shell membrane shown in Fig. 1b reveals a network with macropores or open voids composed of interwoven and coalescing shell membrane fibers ranging in diameter from 0.5 to 3 µm. The inner surface of eggshell membrane shown in Fig. 1c has a relatively smooth surface and the fibers and marcopores is indistinct compared with that of the outer surface.

Characterization of outer shell membrane modified electrode: Electrochemical methods could usually be used for determining the change of electrode surface resulting from membrane modification[17,33,34]. Theoretically speaking, the electron transfer rate and resistance of an electroactive probe at the electrode surface is related to the properties of electrode surface. In this section, cyclic voltammetry was applied as a convenient and informative electrochemical method for the examination of membrane formed on the surface of glassy carbon

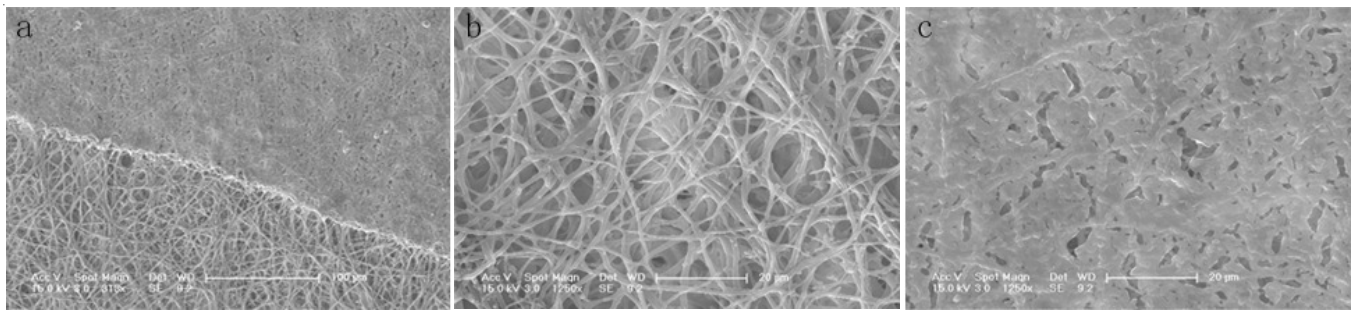

Fig. 1. Scanning electron microscopic images of the eggshell membrane: (a) Layered structure, (b) Outer shell membrane and (c) Inner surface

electrode with $Fe(CN)_6^{4-/3-}$ playing the role of electroactive species as a probe. The cyclic voltammetry responses at bare GC (curve a) and outer shell membrane modified GC (curve b) electrodes are shown in Fig. 2 in 1 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} KCl solution at a scan rate of 100 mV s^{-1}. We chose the second scan cycle of cyclic voltammograms. A well-defined reversible waves of $Fe(CN)_6^{4-/3-}$ are observed at bare GC electrode. After modified with outer shell membrane, the anodic and cathodic peaks disappeared, indicating that membrane could act as blocking layer to prevent the redox probe from reaching the electrode surface under this condition. We attributed the disappearance of peak current in Fig. 2b to the successful modification of eggshell membrane on the electrode surface.

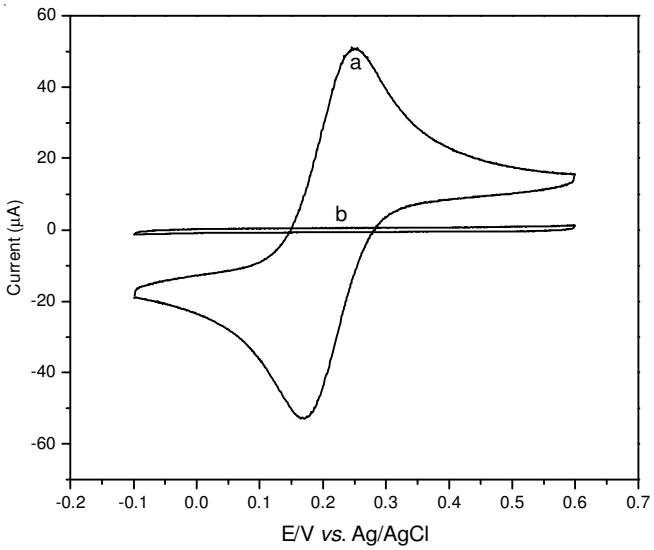

Fig. 2. Cyclic votammograms at (a) Bare glassy carbon electrode and (b) Outer shell membrane/glassy carbon electrode in 1 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ solution containing 0.1 mol L^{-1} KCl at scan rate of 100 mV s^{-1}, respectively

Simultaneously, we employed electrochemical impedance spectroscopy as an effective method for probing the feature of biomaterial modified electrode. The Nyquist plots of different electrodes are shown in Fig. 3. It could be seen from Fig. 3a that a straight line was obtained on bare glassy carbon electrode, which was attributed to the good conductivity of the electrode. With respect to the modified electrode, significant difference in impedance spectra was observed from Fig. 3b, which shows a portion of single semicircle at higher frequencies corresponding to a kinetic limiting step of electron transfer process[35,36]. The diameter of semicircle corresponded to the electron transfer resistance (R_{ct}), which controlled the electron transfer kinetics of redox probe at electrode interface and could be used to describe the interface properties of electrode[36,37]. The presence of membrane on electrode surface caused electron transfer resistance increasing with large semicircle diameter compared with that of bare glassy carbon electrode, further proving the successful modification of outer shell membrane on glassy carbon electrode. The result observed from electrochemical impedance spectroscopy was consistent with that obtained from the cyclic voltammetry measurements.

Direct electrochemistry of hemoglobin immobilized in outer shell membrane: The electrochemical behaviour of

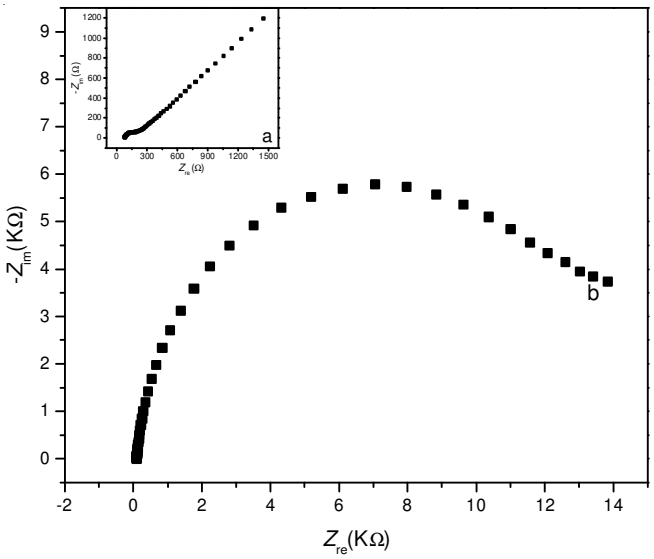

Fig. 3. Nyquist plots of (a) Bare glassy carbon electrode and (b) Outer shell membrane/glassy carbon electrode in 1 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ solution containing 0.1 mol L^{-1} KCl

hemoglobin immobilized electrodes was studied by cyclic voltammetry. Fig. 4 shows cyclic voltammograms of different electrodes in N_2-saturated PBS at scan rate of 100 mV s^{-1}. Clearly, there are no obvious redox peaks observed at the blank electrodes of bare glassy carbon electrode (curve a) and outer shell membrane/glassy carbon electrode (curve b). However, a pair of well-defined and quasi-reversible redox peaks were obtained at the hemoglobin-outer shell membrane modified glassy carbon electrode (curve d), corresponding to the electrochemical redox reaction of the immobilized hemoglobin. The results indicated outer shell membrane modified electrode could provide a favorable microenvironment that facilitates the electron transfer. We observed that when eggshell membrane possessing inner shell membrane, namely whole eggshell membrane was used to modify electrode and immobilize hemoglobin (curve c), electron transfer between hemoglobin and underlying electrode can also be achieved but not obvious

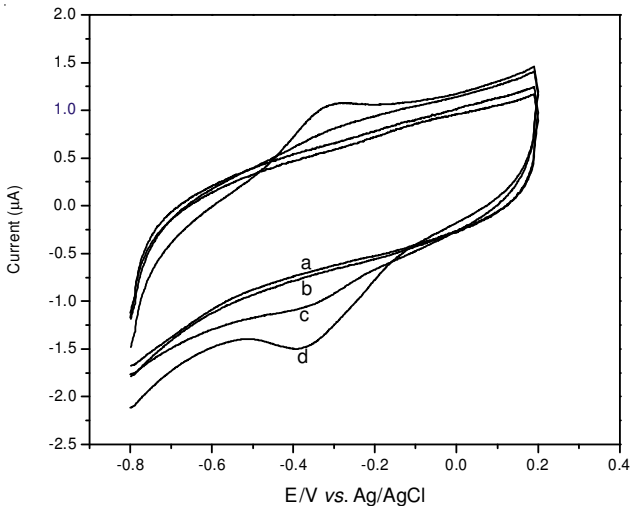

Fig. 4. Cyclic votammograms of (a) Bare glassy carbon electrode, (b) Outer shell membrane/glassy carbon electrode, (c) Hemoglobin-eggshell membrane/glassy carbon electrode and (d) Hemoglobin-outer shell membrane/glassy carbon electrode in N_2-saturated PBS, respectively. Scan rate: 100 mV s^{-1}

compared with that obtained from outer shell membrane modified electrode. It is possible that the addition of ISM and limiting membrane with poor conductivity on electrode surface results in the increase of electrode interface resistance, which attenuates the direct electron transfer between hemoglobin and glassy carbon electrode.

Fig. 5 gives the typical cyclic voltammograms of the hemoglobin-outer shell membrane/glassy carbon electrode in PBS with scan rates in the range of 50-500 mV s^{-1}. With the increase of the scan rates, the cathodic and anodic peak currents increased simultaneously. As shown in the inset of Fig. 5, the anodic peak current (I_{pa}) and cathodic peak current (I_{pc}) increased linearly with scan rates from 50 to 500 mV s^{-1}. This result reveals that the electron transfer of hemoglobin with glassy carbon electrode is governed by a surface-controlled electrochemical process, which is different from the diffusion-controlled process of DDAB-hemoglobin modified graphite electrodes[38].

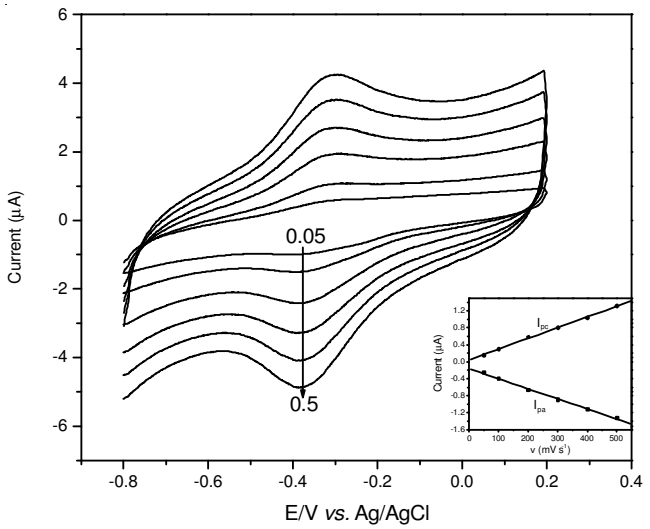

Fig. 5. Cyclic votammograms of hemoglobin-outer shell membrane/glassy carbon electrode in PBS with N_2-saturated at different scan rates from 50 to 500 mV s^{-1}, respectively. Inset: plots of cathodic (●) and anodic (■) peak currents *vs* the scan rates

Electrocatalytic properties of hemoglobin immobilized in outer shell membrane: As a tetramer heme protein, bioactive hemoglobin immobilized on an electrode surface usually has good electrocatalytic activity for O_2, TCA, NO_2^-, H_2O_2, *etc*[14]. Taking O_2 and H_2O_2 as examples in this section, we studied the electrocatalytic properties of hemoglobin-outer shell membrane modified glassy carbon electrode.

To check the bioelectrocatalytic activity of the hemoglobin to the reduction of O_2, cyclic voltammetry measurements at different electrodes were performed. Fig. 6 shows the cyclic voltammograms of the outer shell membrane/glassy carbon electrode and hemoglobin-outer shell membrane/glassy carbon electrode in 10 mmol L^{-1} PBS with N_2-saturated (curves a and b, respectively) and air-saturated (curves a' and b', respectively) solutions at scan rate of 100 mV s^{-1}. As shown in Fig. 6, hemoglobin-outer shell membrane/glassy carbon electrode in air-saturated solution (curve b') shows a large cathodic peak current of O_2 reduction at a more positive potential of -0.35 V compared to outer shell membrane/glassy carbon electrode in air-

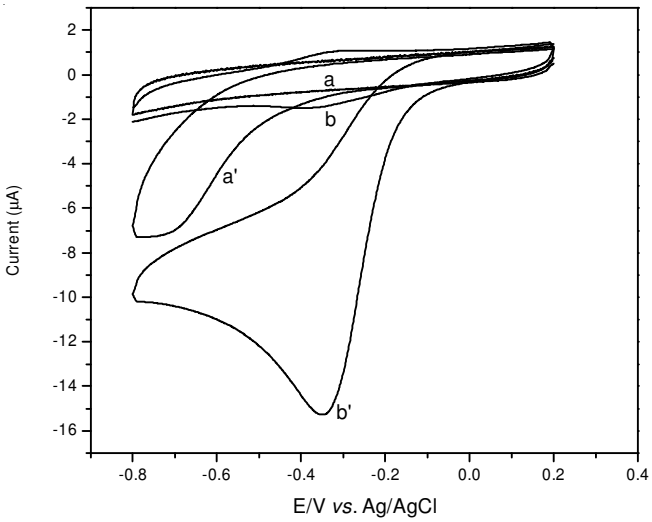

Fig. 6. Cyclic votammograms of outer shell membrane/glassy carbon electrode and hemoglobin-outer shell membrane/glassy carbon electrode in N_2-saturated (a and b) and air-saturated (a' and b') PBS at scan rate of 100 mV s^{-1}, respectively

saturated solution (curve a') and anodic peak current decreases completely compared to that in N_2-saturated solution (curve b). These results indicate that hemoglobin keeps its bioelectrocatalytic activity after immobilization in outer shell membrane.

The electrocatalytic reduction of H_2O_2 at hemoglobin-outer shell membrane/glassy carbon electrode in N_2-saturated PBS is shown in Fig. 7. Cyclic voltammograms were obtained at hemoglobin-outer shell membrane/glassy carbon electrode before and after injection of aliquots of concentrated H_2O_2 solution into 10 mmol L^{-1} PBS. In the absence of H_2O_2, a pair of redox peak of hemoglobin is observed in curve a. As shown in curves b and c, when H_2O_2 was added to the PBS, a significant increase in the reduction peak current was observed accompanying the decrease of oxidation peak current, demonstrating a typical electrocatalytic reduction process of H_2O_2. With the increasing concentration of H_2O_2, the cathodic peak current increases simultaneously (curve c). No electrochemical reduction peak was observed when the cyclic voltammetric scan

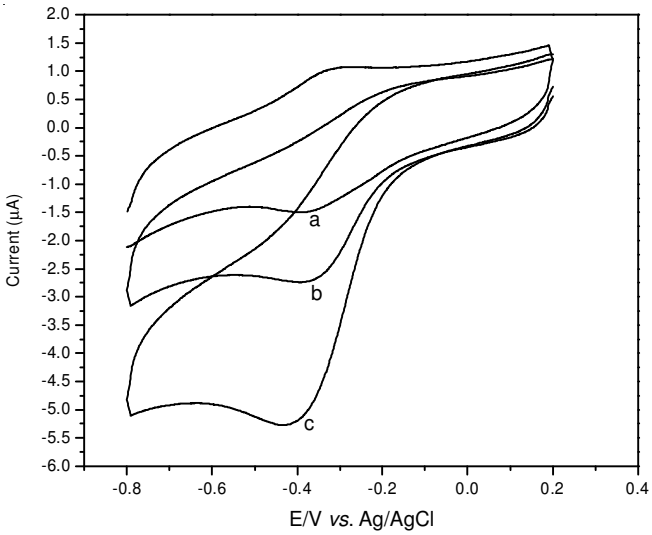

Fig. 7. Cyclic votammograms of hemoglobin-outer shell membrane/glassy carbon electrode in PBS containing (a) 0, (b) 0.1, (c) 0.3 mmol L^{-1} H_2O_2. Scan rate: 100 mV s^{-1}

was performed at bare glassy carbon electrode or outer shell membrane/glassy carbon electrode under the same conditions. Thus, we can conclude that the catalytic reduction of H_2O_2 is due to hemoglobin.

To further investigate the bioactivity of immobilized hemoglobin, the electrocatalytic reduction of H_2O_2 at hemoglobin-outer shell membrane/glassy carbon electrode is studied by amperometry in stirring N_2-saturated PBS. The typical time dependence of the response current of the enzyme electrode on successive step changes of H_2O_2 is demonstrated in Fig. 8a. When an aliquot of concentrated H_2O_2 solution is added into the stirring buffer solution, the reduction current rises steeply to reach a stable value. The calibration curve is shown in Fig. 8b. A clearly defined reduction current proportional to the concentration of H_2O_2 was observed. The linear range of H_2O_2 concentration is between 4×10^{-5} and 2.4×10^{-4} mol L^{-1} with a correlation coefficient of 0.997.

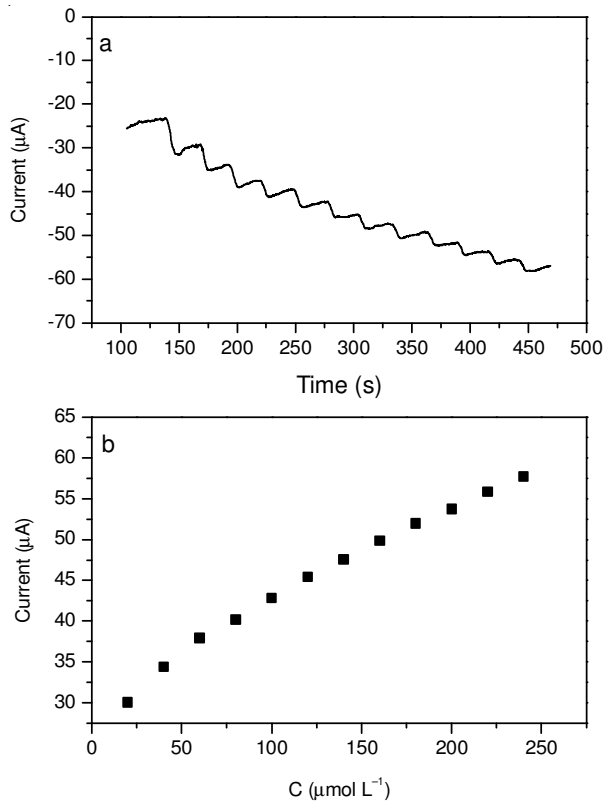

Fig. 8. (a) Typical current-time response of the sensor with the increasing concentration of H_2O_2 in 20 μmol L^{-1} steps in PBS. Potential: -100 mV. (b) Calibration curve of the biosensor

Conclusion

In this paper, eggshell membrane was modified on the surface of glassy carbon electrode. Electrochemical and electrocatalytic behaviour of immobilized hemoglobin was studied on membrane modified electrode. A pair of well-defined redox peaks of hemoglobin was obtained at the outer shell membrane modified electrode. Hemoglobin in outer shell membrane can retain its biological activity to exhibit catalytic ability for electrochemical reduction of O_2 and H_2O_2. Eggshell membrane as natural biomaterial with many advantages such as biocompatibility, easy maintenance of enzyme, simplicity of construction and good stability can be applied to incorporate more other proteins and enzymes. It is expected that eggshell membrane easy immobilizing protein with the retention of activity can achieve more potential applications in biosensors and biocatalysis.

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