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# Studies on direct electron transfer and biocatalytic properties of heme proteins in lecithin film

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**Abstract**

Myoglobin (Mb), hemoglobin (Hb) and horseradish peroxidase (HRP) were incorporated in lecithin (PC) film on glassy carbon (GC) electrode by the method of vesicle-fusion. A pair of well-defined and quasi-reversible cyclic voltammetric peaks was obtained, which reflected the direct electron transfer of heme proteins. UV–Vis and reflectance absorption infrared (RAIR) spectroscopy showed that proteins in PC films remained at their secondary structure similar to their native states. Scanning electron microscopy (SEM) demonstrated the interaction between the proteins and PC would make the morphology of protein-PC films very different from the PC films alone. The immobilized proteins retained their biocatalytic activity to the reduction of NO and hydrogen peroxide, which provide the perspective to be the third generation sensors.


*Keywords:* Myoglobin; Hemoglobin; Horseradish peroxidase; Lecithin; Direct electron transfer

## 1. Introduction

Since 1993, Rusling and Nassar reported the direct electron transfer between electrode and the iron heme protein myoglobin which was incorporated in DDAB [1], the direct electrochemistry of protein has drew a lot of scientists' attention. Rusling reported the direct electrochemistry of proteins, such as hemoglobin, myoglobin and horseradish peroxidase, by incorporating them into the surfactant film, the polymer film and the composite film of surfactant and clay on pyrolytic graphite electrodes [2–12]. Liu improved the electron transfer characteristics of proteins by embedding them into agarose hydrogel film on pyrolytic graphite electrodes [13]. Wang obtained the direct electron transfer of horseradish peroxidase immobilized in DMPC and DDAB films on glassy carbon electrodes, respectively [14,15]. He also reported the direct electrochemistry of hemoglobin at pyrolytic graphite electrodes modified with egg-phosphatidylcholine film and glassy carbon electrodes coated with lipid-protected gold nanoparticles separately [16,17].

Phospholipid vesicles (liposomes) were first described in 1965 by Bangham et al. [18]. It is well known that phospholipids can spontaneously form closed structures when they are hydrated in aqueous solutions. The reason for this is that lipids are amphipathic (both hydrophobic and hydrophilic) in aqueous media, their thermodynamic phase properties and self-assembling characteristics induce entropically driven sequestration of their hydrophobic regions into spherical bilayers, which are referred to as lamellae. Depending on the method of preparation and the lipid used, liposomes can be classed into three sorts [19]: LUVs (large unilamellar vesicles) size range from 0.06 μm, SUVs (small unilamellar vesicles) size range 0.02–0.05 μm and MLVs (multilamellar vesicles) size range 0.1–5.0 μm. They have a number of advantages over other dispersed systems and are one of the best ways to study the properties of biological membranes and simulate the models of biological membranes. The vesicle size is an essential

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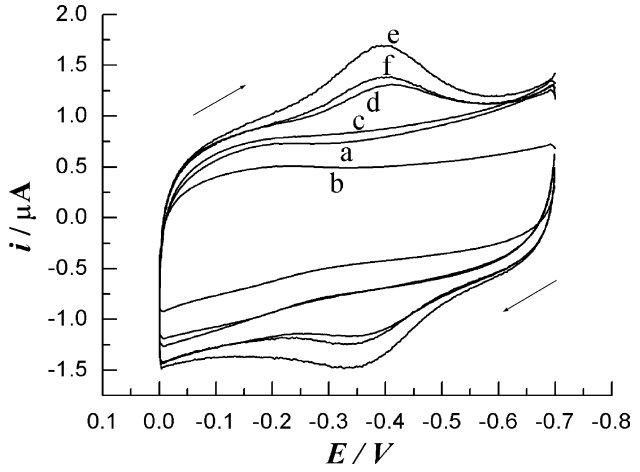


Fig. 1. Cyclic voltammograms at 0.2 V $s^{-1}$ in pH 7.4 buffer for (a) bare GC electrode, (b) Nafion film, (c) Nafion/PC film, (d) Nafion-Hb-PC film, (e) Nafion-Mb-PC film, (f) Nafion-HRP-PC film.

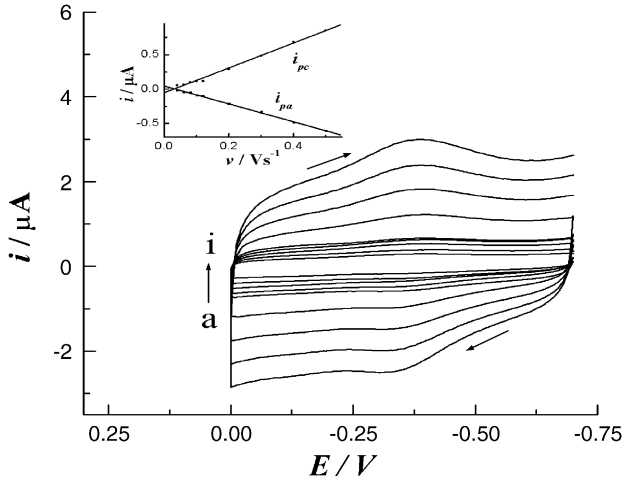


Fig. 2. Cyclic voltammograms of Hb at a Nafion/PC modified GC electrode in a 0.1 M PB solution (pH 7.4) at scan rates of (a) 0.04, (b) 0.06, (c) 0.08, (d) 0.1, (e) 0.12, (f) 0.2, (g) 0.3, (h) 0.4, (i) 0.5 V $s^{-1}$. The inset is a plot of the reduction and oxidation peak current $i$ against the scan rate.

parameter to describe the quality of liposome suspensions. Smaller liposomes, for instant, seem less likely to absorb opsonins because of their higher curvature radii compared with larger ones. Such vesicles, composed of one or more phospholipid bilayer membranes, are similar to the structure of biological membranes so that they can house peptides, proteins, membrane receptors, DNA and so on, which make them have many applications, such as being widely used in pharmacology and pharmacy [20,21].

Proteins can be quantitatively entrapped into the aqueous phase of "empty" liposomes, preferably SUVs, by the mixing the solutions of proteins and SUVs in the absence of sonication, detergents or organic solvents. As Mou reported [22,23], by the direct fusion of vesicles onto the mica surface, supported phospholipid membranes can be prepared as staked-up bilayers (multilamellar bilayers). The supported lipid membranes can also be formed on a hydrophobic surface, such as flat hydrophobic alkanethiol surface and dextran modified with lipophilic compounds surface [24–26]. And with the utilization of impedance spectroscopy, FTIR spectroscopy and neutron reflectivity, it demonstrates that the supported lipid membranes can be immobilized by vesicle-fusion without losing their characteristics [27].

In this work, three heme proteins, hemoglobin (Hb), myoglobin (Mb) and horseradish peroxidase (HRP), were mixed with lecithin (PC) SUVs followed by the vesicle-fusion onto the GC electrode hydrophobic surface, and designated as protein-PC films in their general forms. A pair of well-defined and nearly symmetrical redox peaks has been acquired, which indicates the enhanced, reversible electron transfer between heme proteins and the electrode. Wang [15] has reported that HRP can be immobilized in DDAB vesicles and stable films can be formed by the way of casting on glassy carbon electrode. Compared with his work, we obtained more negative formal potential and more symmetrical redox peaks. The values of formal potential ($E^{o\prime}$) for three heme proteins obtained in our work are also more negative than those reports previously [3–6,8,9,17]. Because of the biocompatibility and affinity for protein, the film of lecithin (PC) SUVs may provide a biomembrane-like microenvironment. The method of vesicle-fusion will retain the properties of lipid membrane and the activity of protein. The film also may affect the electrode double layers and will enhance the electron transfer between proteins and the electrode. Furthermore, more sensitive electrocatalytic reductions of NO and $H_2O_2$ were realized at protein-PC

Table 1
Electrochemical parameters of Nafion-protein-PC films

| Films | $E^{o\prime}$ (V vs. SCE) | $\Delta E$ (mV) | $\Gamma$* of electroactive protein ($10^{-11}$ mol $cm^{-2}$) | Electroactive protein (%) |
|---|---|---|---|---|
| Nafion-Hb-PC | −0.381 | 54 | 5.36 | 1.32 |
| Nafion-Mb-PC | −0.377 | 48 | 49.6 | 3.12 |
| Nafion-HRP-PC | −0.384 | 52 | 4.98 | 2.58 |

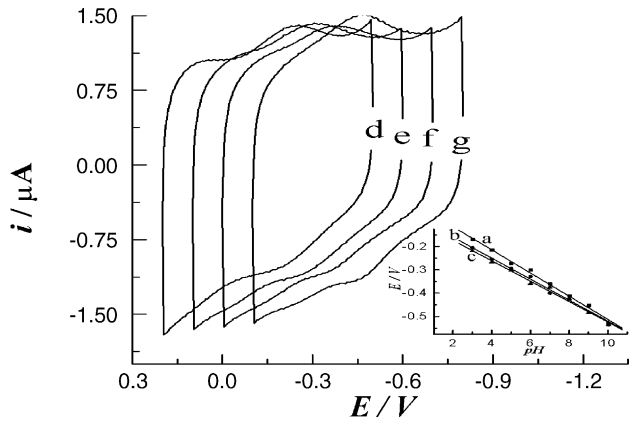


Fig. 3. Cyclic voltammograms of the Nafion-Hb-PC/GC electrode in 0.1 M PBS at pH of (d) 3.0, (e) 5.0, (f) 7.0, (g) 9.0, with the scan rate of 0.2 V $s^{-1}$. The inset shows the relationship between $E^{o\prime}$ of (a) the Nafion-Hb-PC modified electrode (■), (b) the Nafion-Mb-PC modified electrode (●), (c) the Nafion-HRP-PC modified electrode (▲) and the solution pH value.

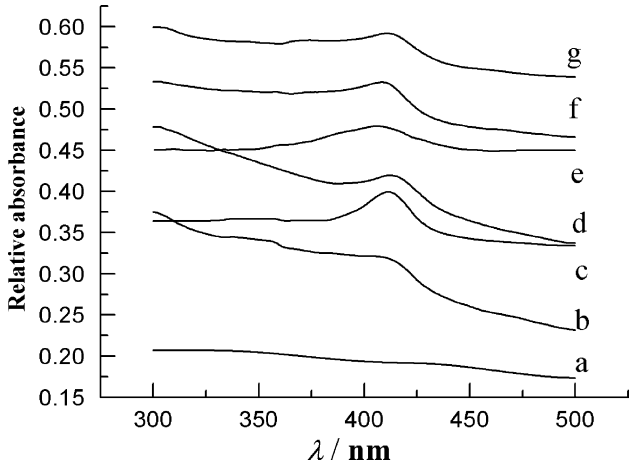


Fig. 4. UV–Vis absorption spectra of dry Nafion/protein-PC films ((b) Hb, (e) Mb, (g) HRP), dry Nafion-protein films ((d) Hb, (c) Mb, (f) HRP) and Nafion-PC film (a). The absorbance coordinate only reflects relative absorbance.

film electrodes, showing the potential application as a foundation of the third generation biosensor or bioreactor.

## 2. Materials and methods

### 2.1. Reagents

Pig hemoglobin (MW 68000), horse heart myoglobin (MW 17800) and horseradish peroxidase (MW 175000) were purchased from Sigma and used without further purification. Lecithin (soybean) was acquired from Shanghai Boao Biotechnology Ltd. Nafion (5%) was obtained from Sigma. $H_2O_2$ was the product of Beijing Chemical Reagent Factory (Beijing, China). The saturated NO solution (the concentration is 1.8 mmol/L at 20 °C) was produced by adding 2 mol/L $H_2SO_4$ slowly to saturated solution of $NaNO_2$ with the aid of a dropping funnel, then was passed through washing bottle containing 2 mol/L NaOH twice and water once to remove possible $NO_2$ and other impurities, and then passed into 20 ml 0.1 mol/L phosphate buffer solution (PBS) for 30 min, which kept in glass flask fitted with a rubber septum to be used. All apparatus were degassed meticulously with $N_2$ for 30 min to exclude $O_2$. All other chemicals were of analytical grade

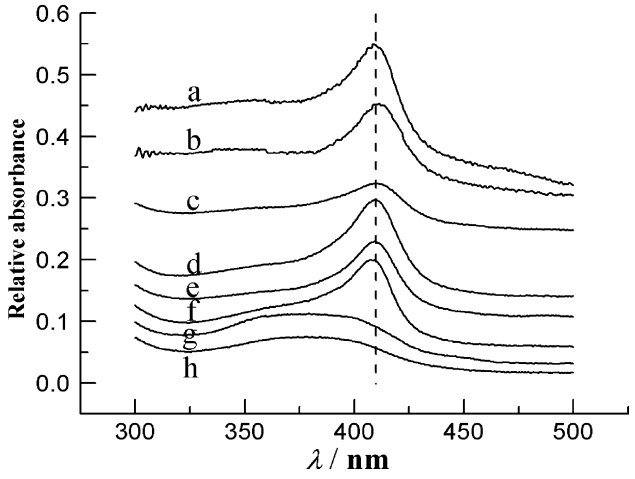


Fig. 5. UV–Vis absorption spectra of (a) dry Nafion-Mb-PC film, (b) dry Nafion-Mb film and Nafion-Mb-PC films in different pH PB solutions: (c) pH 10, (d) pH 9, (e) pH 7.4, (f) pH 5, (g) pH 4, (h) pH 3. The absorbance coordinate only reflects relative absorbance.

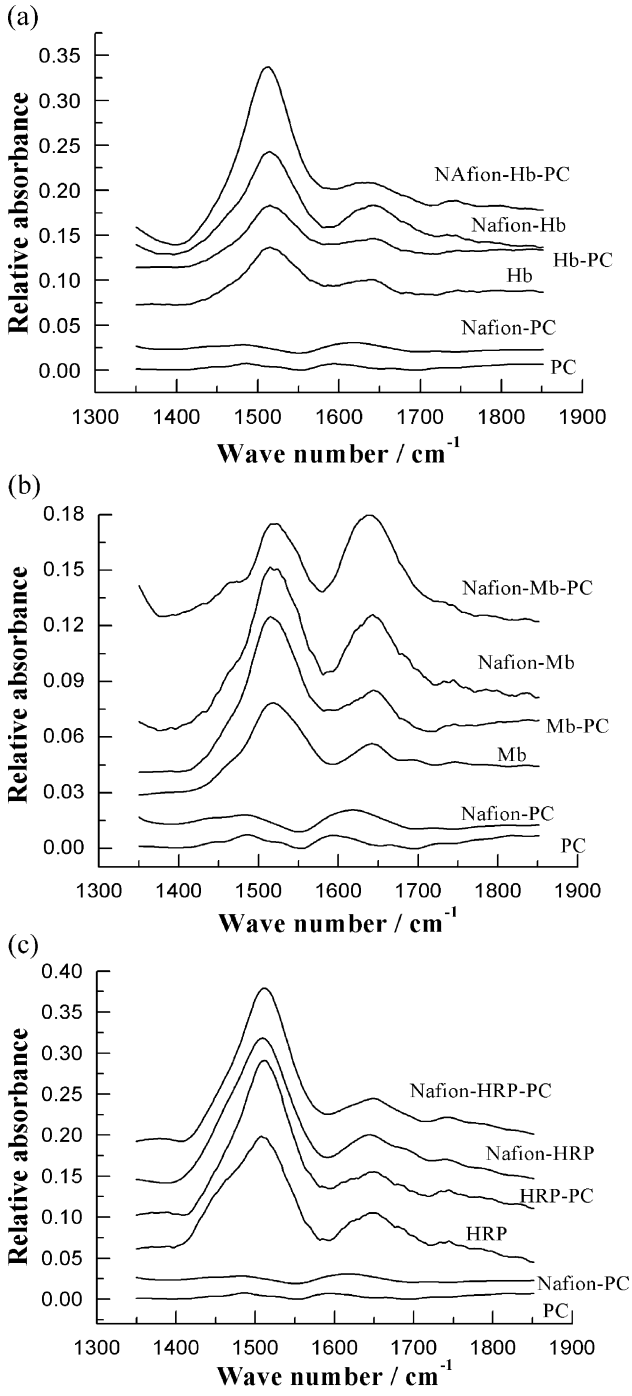


Fig. 6. Reflectance absorbance infrared (RAIR) spectra of (a) Hb and Hb-PC films, (b) Mb and Mb-PC films, (c) HRP and HRP-PC films. The absorbance coordinate only reflects relative value.

and used as obtained. All solutions were prepared with twice-distilled water.

### 2.2. Preparation of PC SUVs

Small unilamellar vesicles (SUVs) were prepared by the method described elsewhere [28]. 10 mg lecithin was dissolved in 5 mL chloroform in a 100 mL round bottom flask. The solvent was removed using a rotary evaporator under reduced pressure to form a thin film on the walls of the flask. The film was dried under high vacuum for 30 min. Then it was hydrated with PBS (pH 7), incubated above the bilayer phase transition temperature of the phospholipid employed, and vortexed intermittently about 30 min. Finally the solution was sonicated with a high power supersonic wave generator in a water bath heated above the lipid's transition temperature until the solution became clear (usually 2 h).

### 2.3. Preparation of the samples

A GC electrode was first polished with 0.05 μm alumina slurry, then sonicated in nitric acid (1:1), ethanol and twice-distilled water in turn.

Proteins (8 mg/mL) in pH 6.8 PBS was mixed with PC SUVs in 1:1 (V/V) ratio. Then 5 μL of the mixture was applied to the freshly cleared GC electrode surface. With the action of vesicle-fusion, the films were formed and became dried at ambient temperature over night. Finally, 1 μL Nafion was cast and used as a binder to hold the protein-PC films on the electrode surface stably, and the solvent was allowed to evaporate before use. The final electrodes are taken as Nafion/protein-PC/GC electrodes.

### 2.4. Measurements

Cyclic voltammetry (CV) was performed with a CHI 660B electrochemical workstation (CH Instruments, Shanghai, China) in a conventional three-electrode cell. A Nafion/protein-PC/GC electrode was used as the working electrode, and a saturated calomel electrode (SCE) and a platinum electrode were used as the reference electrode and the counter electrode, separately. Prior to each experiment, the buffer solutions were purged with high-purity nitrogen for at least 30 min and a nitrogen environment was then kept over the solution in the cell. All experiments were carried out at room temperature.

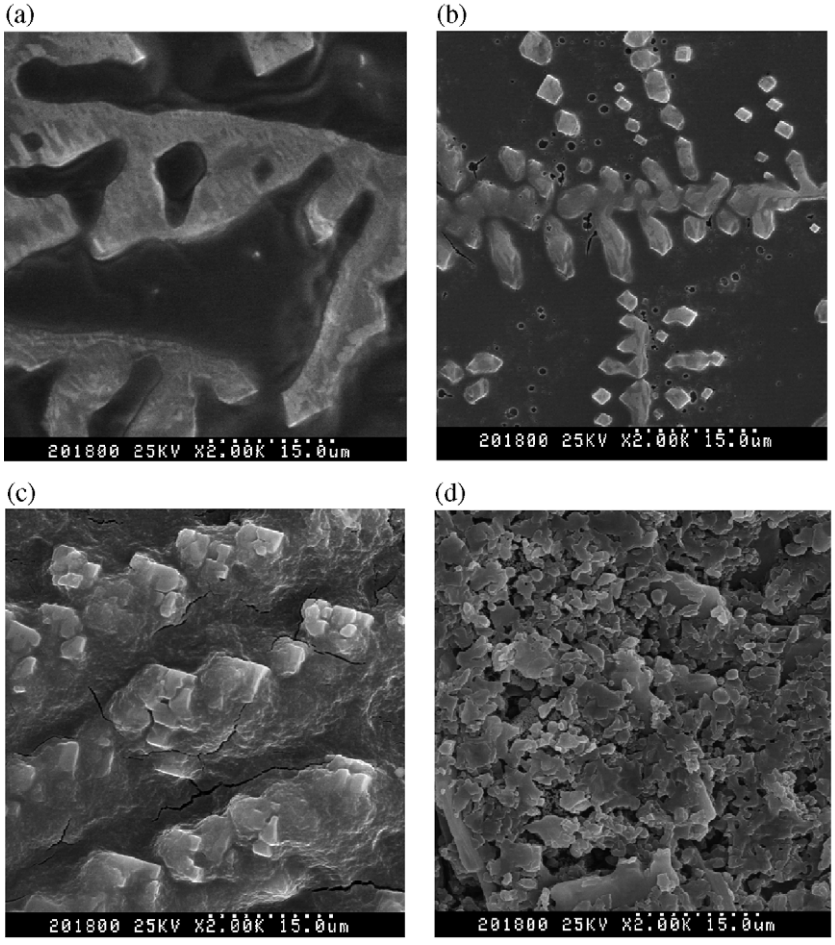


Fig. 7. SEM views with the same magnification: (a) PC film; (b) Mb-PC film; (c) Hb-PC film; (d) HRP-PC film.

UV–Vis spectroscopy was recorded on a UV–Vis Spectrophotometer TU-1901 (Beijing Purkinje General Instrument, China), using twice-distilled water as the background. The samples for spectroscopy were prepared by depositing protein-PC solutions onto glass slides with the same concentration as for GC electrodes described above and were dried in air overnight.

Reflectance absorption infrared (RAIR) spectroscopy was collected by using Magna-IR Spectrometer 500 (Nicolet Instrument Corporation, USA). Scanning electron microscopy (SEM) was done with an X-650 scanning electron microanalyzer (Hitachi).

## 3. Results and discussion

### *3.1. Direct electrochemistry of heme proteins in Nafion/PC film*

The cyclic voltammetry (CV) was used to investigate the electrochemical properties of the Nafion/protein-PC films modified electrodes. Fig. 1 shows the cyclic voltammograms of the bare GC electrode, the GC electrode covered with Nafion, the GC electrode covered with Nafion/PC film and the GC electrode modified with Nafion/protein-PC films in protein-free 0.1M PBS (pH7.4). There is no redox peaks at the bare GC electrode (curve a) obviously. The electrodes covered with Nafion (curve b) and Nafion/PC film (curve c) show the same electrochemical behaviors. A pair of well-defined and nearly symmetrical redox peaks is observed at the Nafion/protein-PC electrodes (curves d, e, f). This illuminates that the redox peaks in curves d, e, f attribute to the electrochemical reaction of proteins immobilized in the Nafion/PC films. The cyclic voltammograms of proteins only immobilized on a bare GC electrode surface using Nafion was also performed (not shown). Compared with the cyclic voltammograms of Nafion/protein-PC/GC electrodes, the electrochemical reaction of proteins at a bare GC electrode had little contribution to the observed redox peaks. The anodic and cathodic peak potentials are located at about $-0.35$ V and $-0.40$ V, for those proteins, respectively. The formal potentials ($E^{\circ\prime}$), defined as the average of the anodic and cathodic peak potentials, are about $-0.38$ V. A pair of peaks was the characteristic of heme Fe(III)/Fe(II) redox couple for the heme proteins [1]. The electrochemical parameters of the three Nafion/protein-PC film electrodes obtained from CV are listed in Table 1. The ratio of the cathodic current over the anodic one was close to one, which suggested that proteins underwent a quasi-reversible redox process at the Nafion/PC modified electrode.

Fig. 2 displays an overlap of CVs of hemoglobin at scan rates ($v$) from 0.04 V $s^{-1}$ to 0.5 V $s^{-1}$. The reduction and oxidation peak currents exhibit a linear relationship with the scan rate (shown in the inset with $r=0.998$). At the same time, $Q$ (the charge consumed in coulombs) obtained from integrating the anodic or cathodic peak area in cyclic voltammograms under the background correction was invariable in substance. In addition, the $\log I_{pc}-\log v$ and $\log I_{pa}-\log v$ plots demonstrated linearly and the ratio of the slopes ($S_{Ipc}/S_{Ipa}$) was about one. All these results are the

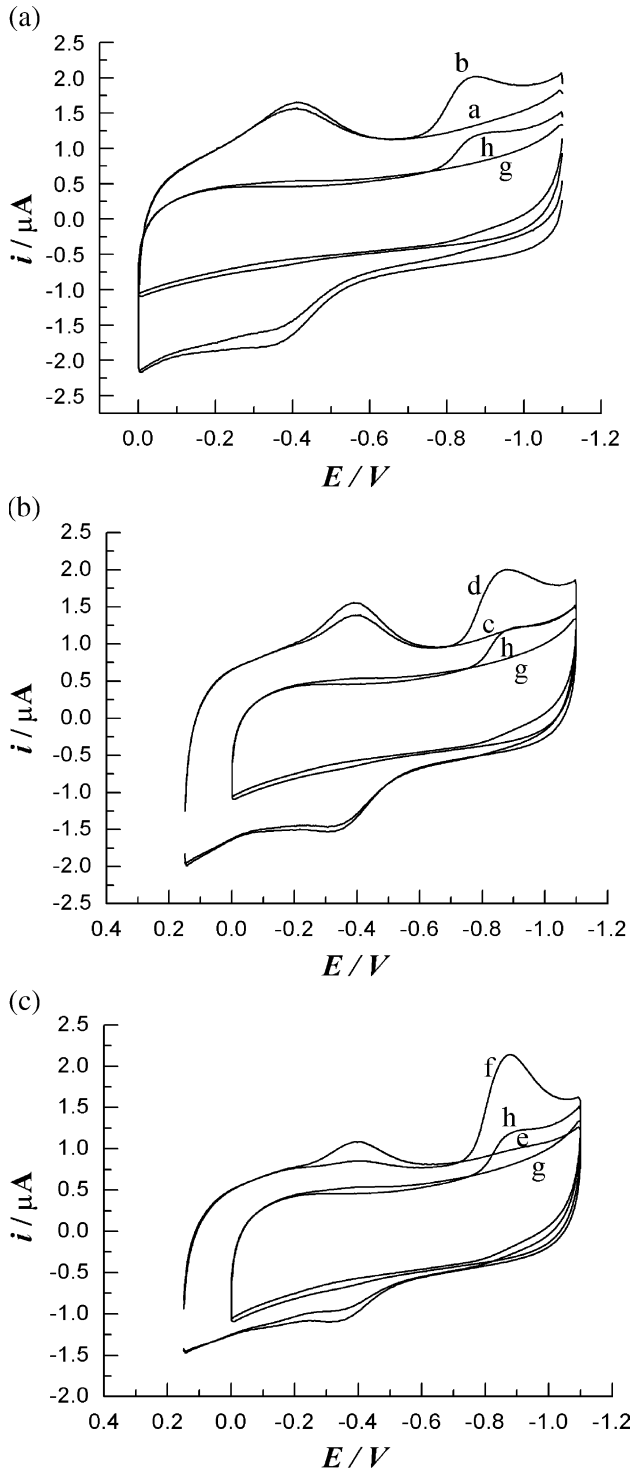


Fig. 8. Cyclic voltammograms at 0.2 V $s^{-1}$ in pH 7.4 buffer for (a) Nafion-Hb-PC film containing no NO (a) and containing 9 μM NO (b); (b) Nafion-Mb-PC film containing no NO (c) and containing 9 μM NO (d); (c) Nafion-HRP-PC film containing no NO (e) and containing 9 μM NO (f) as well as for Nafion/PC film containing no NO (g) and containing 9 μM NO (h).

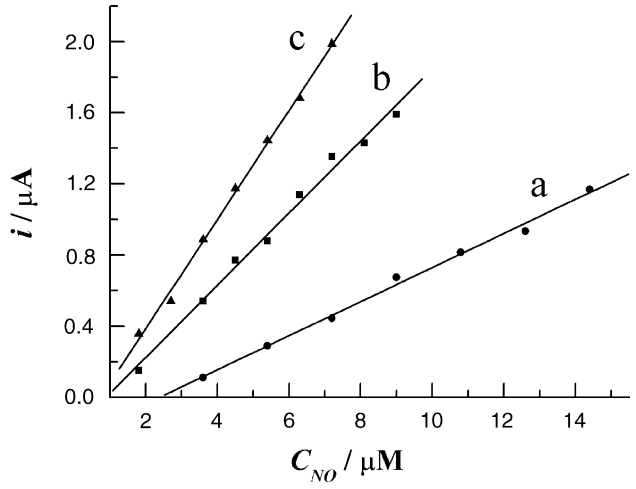


Fig. 9. Plot of the peak currents against the concentration of NO in 0.1M buffer (pH 7.4) at the scan rate of 0.2 V $s^{-1}$ for (a) Nafion-Hb-PC film, (b) Nafion-Mb-PC film and (c) Nafion-HRP-PC film.

characteristic of surface-controlled electrochemical reaction, not diffusion-controlled electrochemical one [1,10]. In this process, all electroactive ferric proteins (protein-Fe(III)), which were produced by the oxidation of ferrous proteins (protein-Fe(II)) on the anodic scan, could be reduced to protein-Fe(II) on the cathodic scan. The average surface concentration of electroactive proteins in the films, $\Gamma^*$ (in mol/$cm^2$), could be calculated by using the Faraday's law and is listed in Table 1. Compared with the total amount of proteins modified on the electrode, the fractions of electroactive proteins are different for different protein, ranging from 1.32% to 3.12%.

The peak potentials of Nafion/protein-PC films are dependent on the solution pH linearly, as Fig. 3 depicts. As can be seen, within the pH range from 3.0 to 10.0, the peak potentials of Nafion/protein-PC films shift to the negative direction with a slope of −0.050 V $pH^{-1}$ for Hb (a, inset), −0.045 V $pH^{-1}$ for Mb (b, inset) and −0.044 V $pH^{-1}$ for HRP (c, inset). All these slope values are smaller than the theoretical value of −0.059 V $pH^{-1}$ for a single-proton coupled, reversible one-electron transfer [10]. The reason for this is as yet unclear. However, the linear relationship between the peak potential and the solution pH suggests that the electron transfer of the proteins is accompanied by proton transfer [13].

### 3.2. UV–Vis spectroscopy of the films

The position of the sensitive Soret absorption band of heme prosthetic group for heme proteins may provide additional information about possible denaturation of the proteins, especially that of conformational change in the heme group region [29,30]. Fig. 4 gives the Soret band positions of the dry Nafion/protein films (d, c, f), dry Nafion/protein-PC films (b, e, g) and dry Nafion/PC film (a), which were cast on crystal glass slides. The dry Nafion/PC film shows no position of Soret absorption band. While the dry Nafion/protein-PC films give almost the same peak positions of Soret band as those of the dry Nafion/protein films alone. It indicates that the proteins embedded in Nafion/PC films have a secondary structure similar to the native state of the proteins.

The position of Soret absorption band depended on the solution pH when the Nafion/protein-PC films were immerged into buffer solutions with a different pH value [3,10]. For example, as Fig. 5 describes, at pH from 5.0 to 9.0, the Soret band of Nafion/Mb-PC film appears at 412 nm (d–f) as same as that of dry Nafion/Mb (b) and dry Nafion/Mb-PC (a) films. It suggests that in the medium pH range, Mb basically retains its native structure in the Nafion/PC film. But when pH was changed towards more acidic or alkaline direction, the peak position of Soret absorption band changed or even distorted. For instance, at pH 10 (c), the Soret band of Nafion/Mb-PC film falls, and at pH 3 (h) and 4 (g), the Soret band becomes much broader and smaller. These phenomena indicate the denaturation of Mb in the low or high pH environment. Nafion/Hb-PC film and Nafion/HRP-PC film showed similar UV–Vis spectroscopic behaviors.

### 3.3. Reflectance Absorption Infrared (RAIR) spectroscopy

Reflectance absorption infrared spectroscopy can also provide more information on the secondary structure of polypeptide chain in detail and be used to detect the conformation change of the proteins [31,32]. The shapes of amide I and amide II infrared absorbance bands drew our attention. The amide I band at 1700–1600 $cm^{-1}$ is caused by the C=O stretching vibrations of the peptide linkage. The amide II band at 1600–1500 $cm^{-1}$ results from a combination of N–H in-plane bending and C–N stretching of the peptide groups. Fig. 6 shows the RAIR spectra of Hb and Hb-PC films (Fig. 6(a)), Mb and Mb-PC films (Fig. 6(b)) as well as HRP and HRP-PC films (Fig. 6(c)). Obviously, the RAIR spectra of Nafion/protein-PC films have very similar sharps and the positions of amide I and amide II bands to those of protein films alone, which also suggests that proteins immobilized in Nafion/PC film do not denature.

Table 2
Relationship between catalytic currents and NO concentrations

| Modified electrode | $C_{NO}$ (μM) | Linear regression equations | Correlation coefficients | Number of measurements |
|---|---|---|---|---|
| Nafion-Hb-PC | 3.6–14.4 | $I$/μA=−0.23+0.096$C$/μM | 0.997 | 7 |
| Nafion-Mb-PC | 1.8–9.0 | $I$/μA=−0.18+0.203$C$/μM | 0.995 | 8 |
| Nafion-HRP-PC | 1.8–7.2 | $I$/μA=−0.23+0.307$C$/μM | 0.999 | 7 |

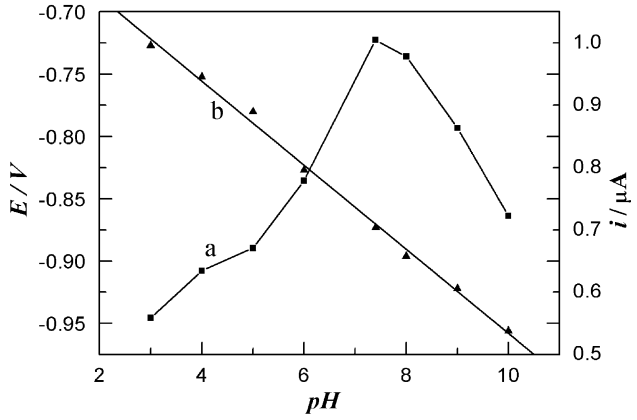


Fig. 10. Plots of (a) current (■) and (b) potential (▲) of Nafion-Mb-PC films vs. pH.

### 3.4. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to probe and compare the morphology of PC and protein-PC films [33]. The top view of a PC film on a GC block displays a cross-linked needle-like crystal structure (Fig. 7(a)). While the top view of polished GC electrode revealed relatively flat and featureless at the same magnification (not shown). Compared with PC film, the protein-PC films appear much different morphologies. The Mb-PC film shows a crystal structure (Fig. 7(b)). And the Hb-PC film (Fig. 7(c)) demonstrates a similar structure but every crystal unit is much thicker and larger. The HRP-PC film (Fig. 7(d)) illustrates a quite different view from the Hb-PC film and Mb-PC film. It seems no any crystalline structure but a conglomerated one.

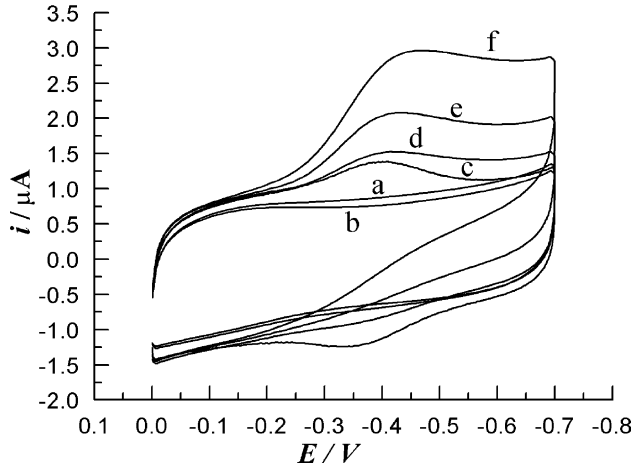


Fig. 11. Cyclic voltammograms at 0.2 V $s^{-1}$ in pH 7.4 buffer for (a) Nafion/PC film in buffer containing no $H_2O_2$, (b) Nafion/PC film in buffer containing 11.34 μM $H_2O_2$, (c) Nafion-HRP-PC film in buffer containing no $H_2O_2$, (d) Nafion-HRP-PC film in buffer containing 11.34 μM $H_2O_2$, (e) Nafion-HRP-PC film in buffer containing 23.94 μM $H_2O_2$, (f) Nafion-HRP-PC film in buffer containing 36.54 μM $H_2O_2$.

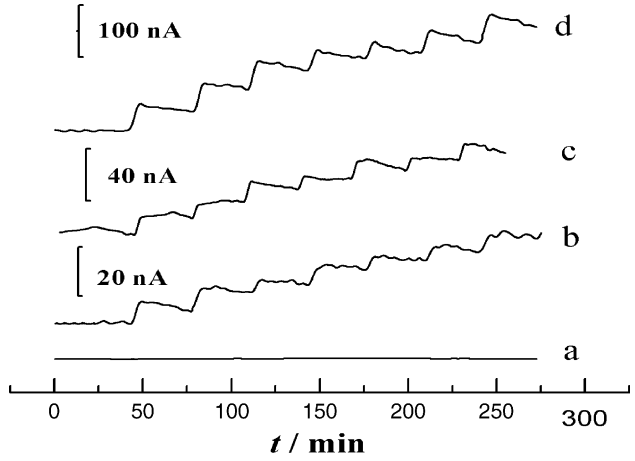


Fig. 12. Amperometric responses at various modified electrodes: (a) Nafion/PC film, (b) Nafion-Hb-PC film, (c) Nafion-Mb-PC film, (d) Nafion-HRP-PC film, at constant potential of −0.39 V in 5 mL buffer (pH 7.4) with injecting 0.252 μM $H_2O_2$ every 30 s.

### 3.5. Catalytic reactivity

The electrochemical catalytic behavior of Nafion/protein-PC films towards NO was tested by CV. As Fig. 8 shows, for Nafion/Hb-PC film ((a), curves a, b), Nafion/Mb-PC film ((b), curves c, d) and Nafion/HRP-PC film ((c), curves e, f), with the addition of NO in a pH 7.4 buffer, a new reduction peak at about −0.846V is observed, and the further addition of NO causes an increase of the peak current (not shown). Meanwhile, the reduction and oxidation peak currents of the heme Fe(III)/Fe(II) redox couple for proteins decrease. For Nafion/PC film (curves g, h), there is also a new reduction peak appeared at −0.896V, negative than those of Nafion/protein-PC films, and the peak current is smaller. This indicates that the Nafion/

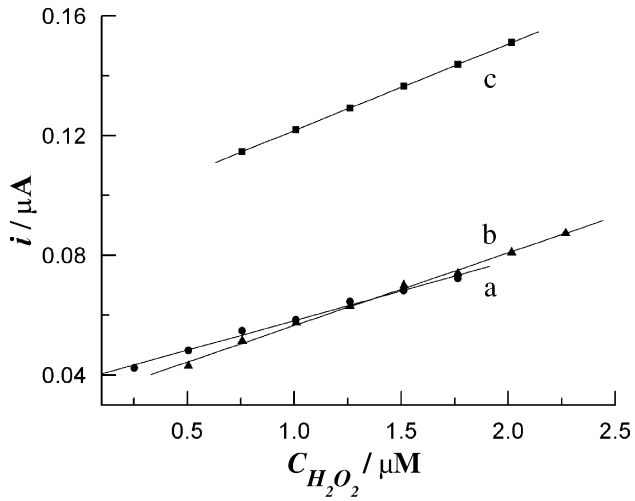


Fig. 13. Calibration curves of (a) Nafion-Hb-PC film, (b) Nafion-Mb-PC film and (c) Nafion-HRP-PC film. Experimental conditions are same as those of Fig. 12.

Table 3
Relationship between currents and $H_2O_2$ concentrations

| Modified electrode | Concentration of $H_2O_2$ (μM) | Linear regression equations | Correlation coefficients | Number of measurements |
|---|---|---|---|---|
| Nafion-Hb-PC | 0.252–1.764 | $I$/μA=−0.038+0.020$C$/μM | 0.996 | 7 |
| Nafion-Mb-PC | 0.504–2.268 | $I$/μA=−0.032+0.024$C$/μM | 0.998 | 8 |
| Nafion-HRP-PC | 0.756–2.016 | $I$/μA=−0.093+0.029$C$/μM | 0.999 | 6 |

protein-PC films are sensitive to NO. The reduction current of NO has a linear relationship with the concentration of NO for all the films as shown in Fig. 9. The data of the relationship between catalytic currents and NO concentrations are listed in Table 2. The dependence of the reduction peak current and the peak potential for NO on the solution pH was also examined. Taking example for Nafion/Mb-PC film (Fig. 10), in the range of 3.0–10.0 of pH, the peak potential shifts negatively with an increase in pH, and shows a linear dependence on the solution pH with a slope of −0.034V/pH (curve b). This indicates that the reduction of NO catalyzed by Mb is a reaction with one proton transferring [34]. The reduction peak current of NO also has relations with the pH of buffer. In the neutral or alkalescent buffer, biggish peak current can be obtained (curve a). From the same experiments on Nafion/Hb-PC film and Nafion/HRP-PC film, we can get similar results.

The electrochemical catalytic characters of hydrogen peroxide at Nafion/protein-PC films were also examined by CV. For instance (Fig. 11), when $H_2O_2$ is added to the pH 7.4 buffer, comparing with the Nafion/HRP-PC film in the absence of $H_2O_2$ (curve c), an increase of the reduction peak at about −0.41 V is observed obviously accompanied by the decrease of the oxidation peak (curve d). The reduction peak current increases with the addition of $H_2O_2$ in buffer (curves e, f). While for Nafion/PC film, there is no difference between the absence (curve a) and the presence (curve b) of $H_2O_2$. It means that the direct reduction of $H_2O_2$ at blank Nafion/PC film cannot be obtained. The catalytic reduction peak current has a linear relation with $H_2O_2$ concentration, which can be used to determine the concentration of $H_2O_2$ in any samples. For Nafion/Hb-PC film and Nafion/Mb-PC film, we can get the same characters. The electrocatalytic reduction of $H_2O_2$ at Nafion/protein-PC films was also studied by amperometry, which is one of the most widely applied techniques for biosensors. As shown in Fig. 12, the currents produced by the electrocatalytic reaction at Nafion/protein-PC modified electrodes get stepped growth at a constant potential of −0.39 V with the increase of $H_2O_2$ concentration. In comparison with the Nafion/protein-PC films (curves b, c, d), a Nafion/PC film (curve a) modified electrode is tested by amperometry and no current is obtained with the addition of $H_2O_2$. Among the three proteins, HRP appears the most sensitive to $H_2O_2$. The calibration curves of the Nafion/protein-PC films are shown in Fig. 13, and the linear relationship between the currents and $H_2O_2$ concentrations are listed in Table 3.

## 4. Conclusions

Heme proteins incorporated in lecithin SUVs film, using Nafion as a binder, obtained direct, stable and quasi-reversible CV responses on GC electrode. The formal potentials ($E^{o\prime}$) are more negative than others reported. The Nafion/protein-PC films have good electrocatalytic property and sensitivity to NO and $H_2O_2$. Based on the direct electron transfer of the heme proteins, it may supply an application perspective as a new type of biosensors.

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