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Direct electrochemistry and electrocatalysis of hemoglobin in poly-3-hydroxybutyrate membrane

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Abstract

Hemoglobin (Hb) can take direct electron-transfer reactions after being entrapped in poly-3-hydroxybutyrate (PHB) film. A pair of well-defined, quasi-reversible cyclic voltammetric peaks is thus obtained at an Hb–PHB modified pyrolytic graphite electrode. The anodic and cathodic peaks are located at −224 and −284 mV for a pH 5.0 acetate buffer solution. Meanwhile, the peroxidase activity of the protein in the membrane has been greatly enhanced, with the apparent Michaelis-Menten constant calculated to be 1076 μM. According to the direct electron transfer property and enhanced peroxidase activity of Hb in the membrane, a Hb–PHB based hydrogen peroxide biosensor is prepared, with a linear range 6.0×10^{-7} to 8.0×10^{-4} M. The pathway of reductive dehalogenation of trichloroacetic acid is also discussed in detail. The highly reduced form of Hb produced in PHB film can be used to dechlorinate di- and monochloroacetic acid. The catalytic ability of Hb toward the reduction of nitric oxide has been investigated as well. Due to its biodegradability, low cost, chemical inertness, and especially its biocompatibility and non-toxicity, PHB would be a desirable film in the sensor field.

Keywords: Hemoglobin; Poly-3-hydroxybutyrate; Direct electrochemistry; Electrocatalysis

1. Introduction

Direct electrochemistry of proteins or enzymes can provide a good model for mechanistic studies of their electron transfer activity in biological systems. Protein film voltammetry (PFV) (Armstrong et al., 1997) affords a relatively new approach to study the details of electron transfer and the coupled reactions in proteins. Recently, great progress in this field has proved that films modified on electrodes may provide a favorable microenvironment for the proteins to directly exchange electrons with underlying electrodes, and thus afford a new opportunity for the detailed study of the enzyme electrochemistry (Armstrong et al., 1997; Sucheta et al., 1992; Rusling, 1998). Successful approaches have included cast films of proteins with insoluble surfactants (Rusling and Nassar, 1993; Yang and Hu, 1999; Fan et al., 2000b), biological organic substances (Fan et al., 2002; Liu et al., 2004; Shang et al., 2003b), inorganic membranes (Fan et al., 2000c, 2001a,b,c), and films of proteins and polyions grown layer-by-layer (Lvov et al., 1998; Ma et al., 2000; Shang et al., 2003a). Also, protein-containing or enzyme-containing thin films modified on electrode surface have potential applicability in fabricating biosensors, biomedical devices, and enzymatic bioreactors (Turner et al., 1987; Chaplin and Bucke, 1990). Achieving direct electron exchange between redox proteins or enzymes and electrodes simplifies these devices by removing the requirement of chemical mediators, and thus has a great significance in preparing the third generation biosensors (Gorton et al., 1999).

Poly-3-hydroxybutyrate (PHB), a linear polymer of beta-hydroxylate, is produced within bacterial cytoplasm as energy reserve by a range of prokaryotic cells (Beun et al., 2002; Majone et al., 1999; Van Loosdrecht et al., 1997). Due to its good property of biodegradability (Manna and

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Paul, 2000; Murase et al., 2001; Majid et al., 2002; Wang et al., 2002; Reddy et al., 2003; Bonartseva et al., 2003), PHB has been widely used as degradable plastics, and has an extensively application in medicine, membrane technology, and other biotechnology (Van Loosdrecht et al., 1997; Reddy et al., 2003). Besides biodegradability, the distinct advantages of PHB, such as its low cost, chemical inertness, and especially its biocompatibility and non-toxicity, also made it a significant material for the immobilization of biomolecules and even cells. For instance, PHB has been used as a good matrix to regenerate the rat sciatic nerve cells (Hazari et al., 1999). Thus, PHB might be a suitable material for this study.

In this paper, hemoglobin (Hb) is incorporated in PHB membrane and is further modified on pyrolytic graphite electrodes. Hb–PHB films can show direct, reversible electrochemistry for heme Fe^{III}/Fe^{II} redox couples. The electrochemical catalytic reductions of hydrogen peroxide (H_2O_2), and trichloroacetic acid (TCA) have been observed, showing the potential applicability of the films as biosensor. Furthermore, the mechanism of the reduction of TCA is discussed in detail. The catalytic activity of the protein towards nitric oxide (NO) has also been studied.

2. Experimental

2.1. Chemicals

Human hemoglobin (M_W = 66,000) and poly-3-hydroxybutyrate (M_W = 3155) were obtained from Sigma. They were all used without further purification. Other chemicals were all of analytical grade. The buffer solutions with different pH values were prepared as follows: pH 3.0 Gly–HCl solution; pH 4.0–5.0 NaAc–HAc solution; pH 6.0–8.0 NaH_2PO_4–Na_2HPO_4 solution; pH 9.0–10.0 Gly–NaOH solution. All solutions were prepared by double distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of > 16 MΩ cm^{-1} and stored in the refrigerator at the temperature of 4 °C.

2.2. Preparation of Hb–PHB film

Pyrolytic graphite (PG) electrode was prepared by putting a PG rod into a glass tube with fixing it by epoxy resin. Electrical contact was made by adhering a copper wire to the rod with the help of Wood alloy.

Prior to coating, the PG electrode was firstly polished on rough and fine sand papers. Then its surface was polished to mirror smoothness with an alumina (particle size of about 0.05 μm)/water slurry on silk. Eventually, the electrode was thoroughly washed by ultrasonicating in both double distilled water and ethanol for about 5 min.

PHB suspension (1 mg mL^{-1}) was prepared by dispersing PHB in double distilled water with ultrasonication for about 45 min. Right before preparing the films, the dispersion was ultrasonicated for another 10 min.

To obtain the best cyclic voltammogram (CV) of protein–PHB films, the experimental conditions for film casting, such as the concentration of Hb, the ratio of Hb/PHB, and the total volume of Hb–PHB dispersion, were optimized. Typically, 10 μL of the dispersion containing 1.2×10^{-5} M Hb and 0.5 mg mL^{-1} PHB was spread evenly onto PG electrodes for preparing Hb–PHB films. A small bottle was fit tightly over the electrode so that water evaporated slowly and more uniform films were formed. Films were then dried overnight in air.

2.3. Apparatus and procedures

Electrochemical experiments were carried out with a Potentiostat/Galvanostat 283 (Princetin Applied Research, USA) and a three-electrode system. A one-compartment glass cell with a modified PG working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode were used for the measurements, with a working volume of 5 mL. All the following potentials reported in this work are versus SCE. Buffer solutions were purged with purified nitrogen, and a nitrogen blanket maintained during scans.

UV–vis absorption spectroscopy was performed on a Model UV-2201 spectrophotometer (Shimidz, Japan). The UV–vis absorption spectra measurements were performed in a 0.2 mg mL^{-1} Hb or mixed solution of 0.2 mg mL^{-1} Hb and 0.1 mg mL^{-1} PHB solution (Hb maintained 0.2 mg mL^{-1} in the test samples).

3. Results and discussion

3.1. Electrochemical behaviors

PHB might provide a desirable membrane environment for Hb to undergo facile electron-transfer reactions. The electrochemical reactions of entrapped Hb have been examined by using cyclic voltammetry (CV) method. Experimental results reveal that Hb can give a pair of well-defined, reversible CV peaks with the formal potential at about −0.25 V versus SCE (Fig. 1b). The peak potential separation is only 60 mV at the scan rate of 200 mV s^{-1}, which indicates a fast heterogeneous electron transfer process. And the peaks are located at the potentials characteristic of the heme Fe^{III}/Fe^{II} redox couples (Rusling and Nassar, 1993; Huang et al., 1996; Ferri et al., 1998). In contrast, no voltammetric peak can be observed for the bare PG electrode or PHB-alone modified PG electrode in the same potential window (Fig. 1a). Thus, it can be reasonably concluded that the redox reactions at the Hb–PHB modified PG electrodes are contributed from the electroactive couples in heme protein.

The CV reduction and oxidation peaks currents for immobilized Hb are found to increase linearly with potential scan

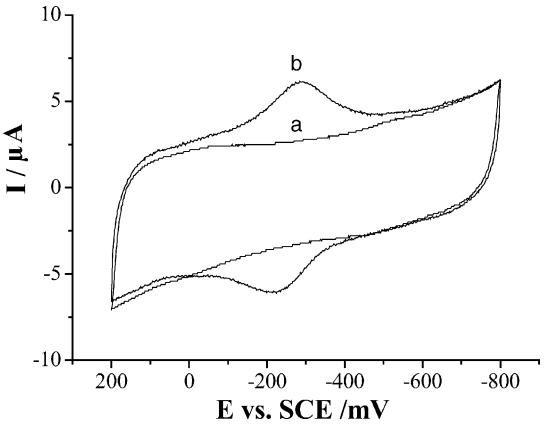

Fig. 1. Cyclic voltammograms at 200 mV s^{-1} in acetate buffer (pH 5.0) for (a) PHB film, (b) Hb–PHB film.

rates from 0.03 to 1 V s^{-1}. The linear regression equations are: $y = 1.13524 + 0.03348x$, $r = 0.999$ (cathodic peak), and $y = -1.07782 - 0.03366x$, $r = 0.999$ (anodic peak). Integration of reduction peaks at different scan rates gave nearly constant charge (Q) values. All these are characteristic of diffusionless, thin-layer electrochemical behavior, that is, nearly all electroactive proteins in the films are reduced on the forward cathodic scan, with full conversion of the reduced proteins back to their oxidized forms on the reversed anodic scan (Murry, 1984).

On the other hand, linear relationship between the peak potential E_p and $\ln v$ can be obtained for both the cathodic and anodic peaks, and the slope of the two straight lines are very similar. Fig. 2 shows that in the range from 400 to 900 mV s^{-1}, the cathodic peak potential E_p is linear to $\ln v$ with a linear regression equation of $y = -0.35166 - 0.04775x$, $r = 0.995$. According to Laviron's equation (Laviron, 1974):

$$E_p = E^{0'} + \frac{RT}{\alpha n F} - \frac{RT}{\alpha n F} \ln v$$

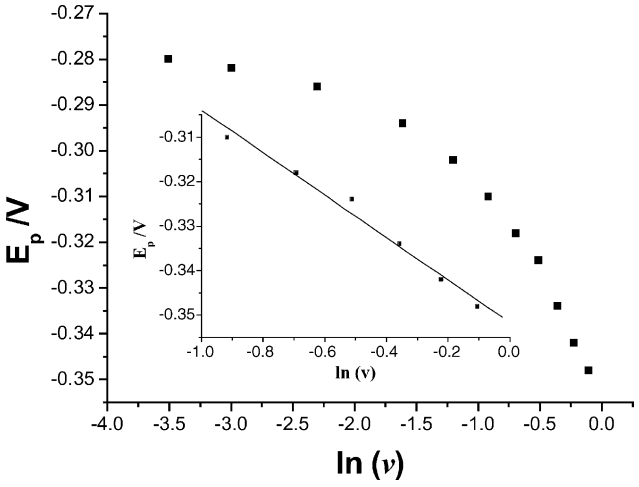

Fig. 2. The value of E_p vs. $\ln v$ and its linear fitting at scan rate from 400 to 900 mV s^{-1} (inset).

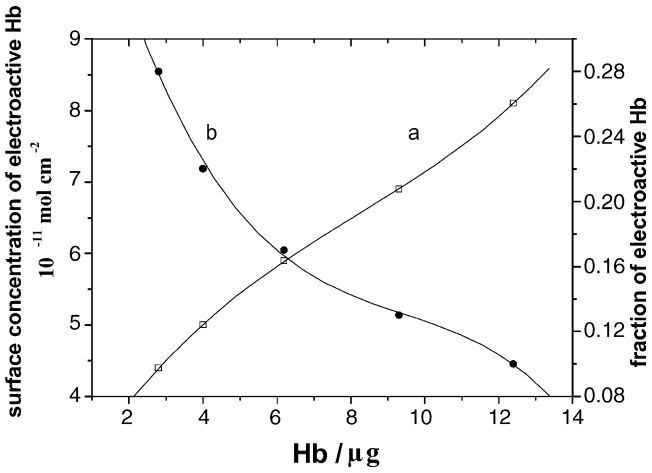

Fig. 3. Surface concentration (a) and fractions of electroactive Hb (b) for Hb–PHB films with different amounts of Hb with a constant ratio of Hb/PHB.

where α is the charge transfer coefficient and n is the number of electrons, αn is calculated as 0.54. Given $0.3 < \alpha < 0.7$ in general, we conclude that $n = 1$ and $\alpha = 0.54$. From the width of the peak at mid height at low scan rate, we can also obtain $n = 1$. So, the redox reaction of Hb is a single electron transfer process. Given α in Laviron's equation being 0.54, the apparent heterogeneous electron transfer rate constant (k_s) is figured out to be 10.33 s^{-1} (Laviron, 1979).

We have also investigated the influence of film thickness on the fraction of electroactive protein. Various amounts of Hb–PHB solutions with the same Hb/PHB ratio were deposited on PG electrodes, and CVs have been run to obtain the values of Q and Γ^* (the average surface concentration of electroactive proteins in the films) Results show that with increasing film thickness, the total surface concentration of electroactive Hb in the films increased, while the fraction of electroactive Hb decreased (Fig. 3), suggesting that only Hb in the inner layers closest to the electrode surface can exchange electrons with the electrode.

Experimental results have also revealed that the protein–PHB modified PG electrodes are very stable. No significant decrease of the peak currents is observed after at least 10 days storage at 4 °C.

3.2. Influence of pH on voltammetry

Nearly reversible voltammograms can be observed for all the pH range tested from 3.0 to 10.0, with stable and well-defined peaks. And the same CV can be obtained if the protein–PHB modified electrode is transferred from one solution with different pH value to its original solution. So the pH-induced variations in the peak shape and potentials in voltammograms are reversible. Meanwhile, with an increase of the pH value, both cathodic and anodic CV peaks shift negatively. The formal potentials ($E^{0'}$) were found to be linearly proportional to pH value in the range of 3.0–10.0, with linear regression equation of $y = -26.46429 - 44.42857x$, r

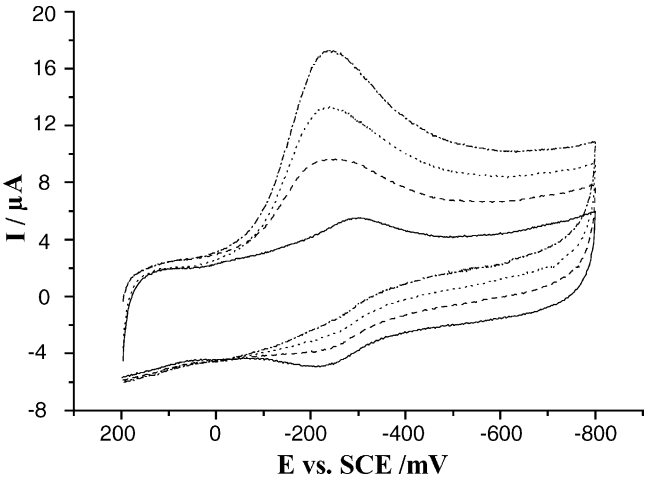

Fig. 4. Cyclic voltammograms of Hb–PHB/PG electrode at 200 mV s^{-1} in pH 5.0 buffers with the H_2O_2 concentration of 0, 1×10^{-4}, 2×10^{-4}, 3×10^{-4} M (from inner to outer).

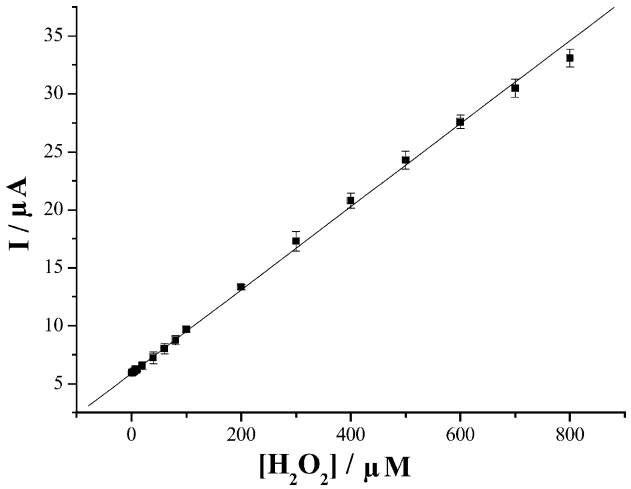

Fig. 5. Linear calibration plot of the catalytic peak current to the H_2O_2 concentration. Others are same as Fig. 4.

= 0.996. The slope value is close to the theoretical value of −57.6 mV pH^{-1} at 18 °C for a reversible one-proton coupled single-electron transfer (Meites, 1965; Bond, 1980), which can be represented as

$$\text{heme Fe}^{\text{III}} + \text{H}^+ + \text{e}^- = \text{heme Fe}^{\text{II}}$$

3.3. Catalytic ability

The electrocatalytic behaviors of Hb incorporated in PHB film toward various substrates have been characterized. The catalytic activity toward H_2O_2 is first examined. As is well known, Hb has some intrinsic peroxidase activities because of the close structural similarity to peroxidases (Matsui et al., 1999). Experimental results reveal that the peroxidase activity of Hb is greatly enhanced in the PHB films. When H_2O_2 is added to a pH 5.0 buffer, an increase in reduction peak for Hb Fe^{III} at about −0.24 V is observed, accompanied by the disappearance of the oxidation peak for heme Fe^{II} (Fig. 4), which is characteristic of an electrochemically catalytic reaction (Bard and Faulkner, 1980). No corresponding electrochemical signal can be observed at either bare PG electrodes or PHB (free of protein) film electrodes in the same H_2O_2 solutions in this potential range. Therefore, the catalytic process comes from the specific enzymatic catalysis between Hb and H_2O_2, which indicates a large decrease in activation energy for the reduction of H_2O_2 in the presence of Hb.

Since in a large range of pH (3.0–10.0) the reductive peak current changes slightly, and reaches a maximum at pH 5.0, we have used solution of pH 5.0 for the detection of H_2O_2. A linear dependence between the catalytic peak current and the concentration of H_2O_2 is obtained in the range 6.0×10^{-7} to 8.0×10^{-4} M H_2O_2 (Fig. 5). The linear regression equation is $y = 5.95528 + 0.03541x$, with a correlation coefficient of 0.999. Its detection limit is 2.0×10^{-7} M with a sensitivity of 0.03541 μA μM^{-1} H_2O_2. Six independent determinations at a H_2O_2 concentration of 50 μM show a relative standard deviation (R.S.D.) of 3.3%, which displays nice reproducibility of these measurements. The curve levels off at a concentration of 6 mM. It is consistent with an enzyme-like catalytic process of the substrate.

The apparent Michaelis-Menten constant (K_m^{app}), which gives an indication of the enzyme-substrate kinetics, can be calculated by Lineweaver-Burk equation (Li et al., 1996)

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max} c}$$

where I_{ss} is the steady current after the addition of substrate (with the current derived from heme proteins subtracted), c is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated substrate condition. The K_m^{app} of Hb–PHB films is, thus, calculated to be 1076 μM. It is well known that the smaller K_m^{app} represents the higher catalytic ability. The value of K_m^{app} for Hb in this work is smaller than 1300 μM which is obtained from an HRP-Au-SPCE (Xu et al., 2003). Therefore, it clearly shows that the peroxidase activity of entrapped Hb is greatly enhanced, which is comparable to or even higher than that of the native peroxidase, HRP.

Electrocatalytic reduction of TCA by the Hb in PHB films has also been tested. When TCA is added to a pH 5.0 buffer, the Hb Fe^{III} reduction peak of Hb–PHB film electrodes at about −0.28 V increased in height (Fig. 6A), accompanied by decrease of Hb Fe^{II} oxidation peak. The Hb Fe^{III} peak current is linearly proportional to the concentration of TCA. These are characteristic of an electrochemically catalytic reaction (Bard and Faulkner, 1980). However, no corresponding electrochemical signal can be observed in the same potential window employing either a bare PG electrode or a PHB-alone-modified PG electrode in the same TCA solution. Therefore, the catalytic process comes from the specific enzymatic catalytic reaction between Hb and

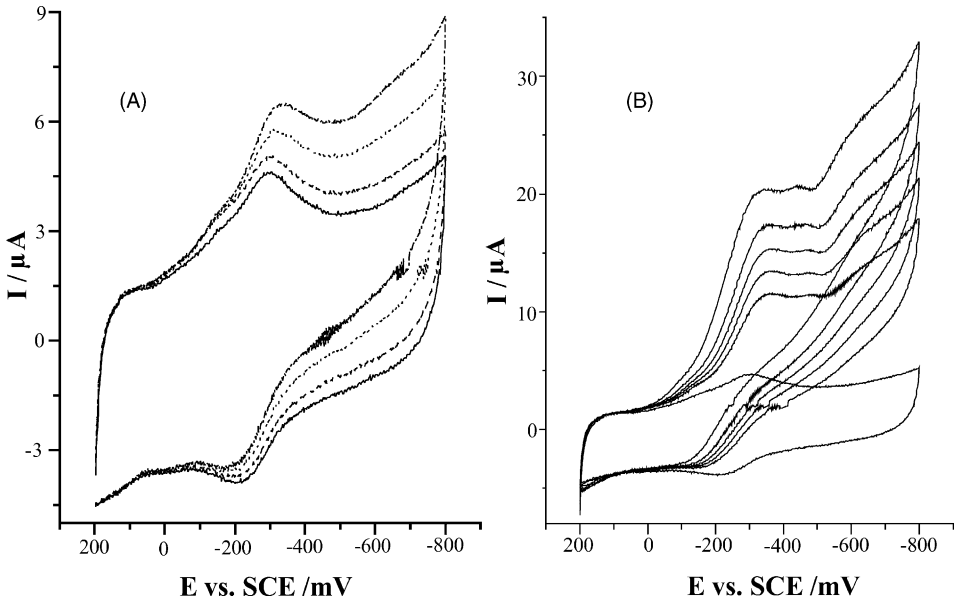

Fig. 6. (A) Cyclic voltammograms of Hb–PHB/PG electrode at 200 mV s^{-1} in pH 5.0 buffers with the TCA concentration of 0, 1×10^{-3}, 4×10^{-3}, 8×10^{-3} M (from inner to outer). (B) Cyclic voltammograms of Hb–PHB/PG electrode at 200 mV s^{-1} in pH 5.0 buffers with the TCA concentration of 0, 4×10^{-2}, 6×10^{-2}, 8×10^{-2}, 1.0×10^{-1}, 1.2×10^{-1} M (from inner to outer).

TCA, and the activation energy for the reduction of TCA has also been largely decreased in the presence of Hb.

It should be mentioned that when the concentration of TCA is larger than 0.04 M, a new reduction peak located at −0.45 V is observed, and the peak current increased with the concentration of TCA (Fig. 6B). In the previous work, using spectroelectrochemistry method, Nassar and Rusling found a highly reduced form of myoglobin (Mb) which was produced in DDAB films (Nassar et al., 1995). This highly reduced Mb, possibly Mb Fe^{I}, was suggested to be an active reductant during the dechlorination of tetra- and trichloroethylenes (Nassar et al., 1995). Here, with Hb–PHB films, we have found a new reduction peak in the solution with high concentration of TCA, and tentatively assign it to the highly reduced form of Hb (Hb Fe^{I}). This active reductant might dechlorinate di- and monochloroacetic acid after the dechlorination of TCA by Hb Fe^{II}. Therefore, from these observations such a series of processes can be proposed: reduction of Hb Fe^{III} (from ferric to ferrous heme), reduction of TCA with the help of heme, reduction of Hb Fe^{II} (from Fe^{II} to Fe^{I}), reduction of di- and monochloroacetic acid with the help of Hb Fe^{I}, and re-oxidation of Hb Fe^{I}. The following equations may illustrate these processes:

$$\text{Hb Fe}^{\text{III}} + \text{e} \rightarrow \text{Hb Fe}^{\text{II}} \quad (1)$$

$$2\text{Hb Fe}^{\text{II}} + \text{Cl}_3\text{CCOOH} + \text{H}^+ \rightarrow \text{Cl}_2\text{CHCOOH} + 2\text{Hb Fe}^{\text{III}} + \text{Cl}^- \quad (2)$$

$$\text{Hb Fe}^{\text{II}} + \text{e} \rightarrow \text{Hb Fe}^{\text{I}} \quad (3)$$

$$2\text{Hb Fe}^{\text{I}} + \text{Cl}_2\text{CHCOOH} + \text{H}^+ \rightarrow \text{ClCH}_2\text{COOH} + 2\text{Hb Fe}^{\text{II}} + \text{Cl}^- \quad (4)$$

$$2\text{Hb Fe}^{\text{I}} + \text{ClCH}_2\text{COOH} + \text{H}^+ \rightarrow \text{CH}_3\text{COOH} + 2\text{Hb Fe}^{\text{II}} + \text{Cl}^- \quad (5)$$

It should be noticed that no obvious oxidation peak is found corresponding to re-oxidation of Hb Fe^{I}. This is because of the electrochemically catalytic reasons mentioned above. There may also exist such a condition that Hb Fe^{I} donors two electrons to dechlorinate di- and monochloroacetic acid. Since the identity of Hb Fe^{I} is not yet clear, further researches are required.

The catalytic reduction of NO has been studied as well. However, no significant phenomenon of catalytic reduction of NO can be observed. The reduction peak current for Hb Fe^{III} in Hb–PHB films increase slightly when NO is present, and only a small catalytic reduction peak is found at about −0.7 V, when the concentration of NO is larger than 2.0×10^{-6} M. It seems that NO has not reacted with Hb in a feasible way. We ascribe this to the special transport properties of PHB films and the adsorption competition between NO and nitrous oxide (N_2O). PHB has a low barrier character against small gas molecules such as oxygen, nitrogen and NO (Miguel and Iruin, 1999). Therefore, NO can permeate PHB films easily to react with Hb in the inner layers. After the reduction of NO by Hb heme, N–N coupled gases N_2O and other products are generated (Fan et al., 2000a). It is reported that PHB can be catalogued as a good barrier material against larger gas molecules, carbon dioxide for instance (Miguel and Iruin, 1999). N_2O has nearly the same or even larger dimension than

carbon dioxide, so we suggest that N_2O cannot be transported out of PHB films. The accumulation of reductive product (N_2O) inhibits the process of positive reaction. Moreover, NO and N_2O have comparable affinities to bind to the same sites on hemoglobin (Sampath et al., 2001). This further interferes reactions between NO and Hb. Thus, no obvious catalytic reductive signal of NO can be observed in Hb–PHB films.

4. Conclusions

Hemoglobin incorporated in biocompatible natural PHB films modified on PG electrodes demonstrated direct, stable and nearly reversible CV responses. Hb retained its native state in the PHB films. Good electrocatalytic property and stability of Hb–PHB films may provide an application perspective for the films as a new type of biosensors based on direct and mediator-free electrochemistry of hemoglobin. The Hb–PHB films were catalytically active for the reduction of a range of organic and inorganic reactants, such as hydrogen peroxide, trichloroacetic acid, etc. The films provide a biomembrane-like environment for their biologically relevant reactions. Furthermore, a reduced form of Hb Fe^{II} can be produced in the films.

Acknowledgements

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