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Short communication

An amperometric biosensor based on hemoglobin immobilized in poly(ε-caprolactone) film and its application

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Abstract

In this study, poly(ε-caprolactone) (PCL) was synthesized using the ε-caprolactone (CL) monomer ring-opening polymerization. We demonstrated that the hemoglobin (Hb) entrapped in PCL film could retain its original conformation by FT-IR spectra. A pair of well-defined redox peaks with a formal potential ($E^{0\prime}$) of about −0.38 V (vs. SCE) in a pH 7.0 phosphate buffer solution was obtained at the Hb–PCL film modified GC electrode. The dependence of $E^{0'}$ on the pH of the buffer solution indicated that the conversion of heme Fe(III)/Fe(II) was a reaction of one electron coupled to one proton. The apparent heterogeneous electron transfer rate constants (k_s) of Hb confined to PCL was evaluated as $(18.7 \pm 0.8)\,s^{-1}$ according to Laviron's equation. The surface concentration (Γ^*) of the electroactive Hb in the PCL film was estimated to be $(7.27 \pm 0.57) \times 10^{-11}$ mol cm^{-2}. The Hb–PCL film modified electrode was shown to be an excellent amperometric sensor for the detection of hydrogen peroxide. The linear range is from 2 to 30 μM with a detection limit of 6.07×10^{-6} M. The sensor was effectively testified by the determination of the hydrogen peroxide in eyedrops as real samples.

Keywords: Poly(ε-caprolactone); Hemoglobin; Electrochemistry; Hydrogen peroxide; Biosensor

1. Introduction

During the past few years, the direct electron transfer (DET) of protein or enzyme and underlying electrodes had attracted increasing attention (Degani and Heller, 1989; Lotzbeyer et al., 1996; Rusling, 1998; Gorton et al., 1999; Sun et al., 1999; Lai and Bergel, 2002; Varma and Mitra, 2002; Yu et al., 2005; Ding et al., 2007). However, it was generally difficult for the proteins or enzymes with the big and complex structures, the deep burying of the electroactive prosthetic groups and the adsorptive denaturation of proteins onto electrodes and the unfavorable orientations at electrodes (Scheller and Schubert, 1992). To overcome these problems, great efforts had been made to improve the direct electron transfer of protein by incorporating them into various films (Degani and Heller, 1989; Gregg and Heller, 1990; Sun et al., 1999, 2000; Kong et al., 2003; Yan et al., 2005; Yu et al., 2005; Liu et al., 2006; Ding et al., 2007; Zheng et al., 2008), which could provide a favorable microenvironment for protein to facilitate the direct electron exchange between the protein and the underlying electrode.

Hemoglobin (Hb) contains four iron hemes in each subunit acting as the electroactive center, has a molar mass of approximately 67,000 g mol^{-1} (Wade and Castro, 1973), and is a reversible oxygen-carrying and storage protein. Hb had been studied extensively as a model protein for exploring the relationship between the structure and the function of protein by studying its direct electrochemistry (Wang et al., 2005; Yu et al., 2005; Gao and Gao, 2007; Zheng and Zheng, 2007).

Poly(ε-caprolactone) (PCL) is an FDA-approved biodegradable polymer with excellent biocompatibility and flexibility, which has attracted increasing interest in many areas such as biodegradable packaging materials, implantable biomaterials and microparticles for drug delivery (Cho and An, 2006). It has a slow degradation rate (Rezwan et al., 2006) which is good for implantation as biosensor since it could be stable at long-term application in vivo. To our best knowledge, no one has studied the application of PCL in the field of electrochemical biosensors. In the present study, PCL was synthesized using the

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CL monomer ring-opening polymerization. Hb was chosen as a model protein to study the application of PCL in bioelectrochemistry.

2. Experimental

2.1. Materials and chemicals

ε-Caprolactone (CL, 99%) was obtained from Aldrich and dried on molecular sieve for about 12 h before use. Stannous octoate was obtained from Sinopharm Chemical Reagent Co., Ltd., Bovine Hemoglobin (Hb, MW 66,000) was purchased from Sigma and used without additional purification. The hydrogen peroxide (H_2O_2, 30%, w/w) was from Beijing Chemical Company (Beijing, China). The dilute solution of H_2O_2 was prepared daily. Aqueous solutions were prepared with doubly distilled water. All other chemicals and solvents were at analytical grade and used without further purification.

2.2. Preparation of poly(ε-caprolactone)

PCL was synthesized by the ring-opening polymerization of CL with the $Sn(Oct)_2$ as a catalyst. The monomer and the solution of $Sn(Oct)_2$/toluene were added to an ampoule. When the solvent was evaporated under vacuum, the ampoule was sealed and kept at 150 °C for 24 h. After reaction, the products were dissolved in chloroform, precipitated into an excess of methanol, filtered, dried under vacuum until a constant weight.

2.3. Construction of Hb–PCL film

Glassy carbon (GC, 3 mm-diameter), before the use, was firstly polished with emery paper (# 2000), 0.3 and 0.05 μm alumina slurry on a woolen cloth, and then cleaned under bath sonication for 10 min and finally thoroughly rinsed with distilled water.

A total of 5 μL of 5 mg mL^{-1} PCL in *N*,*N*-dimethylformamide was spread evenly onto the freshly abraded GC electrode, and then 5 μL of 20 mg mL^{-1} Hb solution was spread. A small bottle was fitted over the electrode followed by a period of standing in air until dry. Then the electrode (denoted as Hb–PCL/GC electrode, hereafter) was immersed into 0.10 M phosphate buffer.

2.4. Apparatus and characterization

Electrochemical measurements were carried out with a computer-controlled electrochemical analyzer (CHI 650C, CHI, Austin, TX) in a two-compartment and three-electrode cell. The modified GC electrodes were used as working electrode, a platinum spiral wire as counter electrode, and a saturated calomel electrode (SCE) as reference electrode. FT-IR spectra were obtained on a FT-IR PerkinElmer Spectrome 100 spectrometer (PerkinElmer Company, USA) at room temperature.

3. Results and discussion

3.1. Spectroscopic characterization of PCL and Hb–PCL composite films

The formation of PCL was studied with FT-IR spectroscopy as shown in Fig. 1(a), in which the characteristic absorption bands at about 2940 cm^{-1} attributed to the stretching of C–H. The C=O stretching band at 1730 cm^{-1} from the CL was strong and detected clearly in this measurement. The bands at 1190 cm^{-1} were attributed to the C–O stretch (Ha et al., 1999). The amide I band in the region of 1600–1700 cm^{-1} was caused by C=O stretching vibrations of peptide linkages in the backbone of protein. The amide II band in the region 1500–1600 cm^{-1} was caused by the combination of N–H in plane bending and C–N stretching vibrations of the peptide groups (Kauppinen et al., 1981). As shown in Fig. 1(b and c), the amide I band and II band of immobilized Hb on PCL film were observed at 1654 and 1547 cm^{-1}, respectively. They were nearly the same as those obtained for the native Hb, which were appeared at 1653 and 1538 cm^{-1}, respectively. The similarities of the two spectra suggested that Hb retained the essential features of its native secondary structure in Hb–PCL composite film. Moreover, the characteristic absorption band of PCL films was also observed in the FT-IR spectra of Hb–PCL film, as shown in Fig. 1(c). Thus, PCL film may provide a promising matrix for enzyme immobilization and biosensor fabrication because of its satisfying biocompatibility.

3.2. Direct electron transfer of Hb–PCL film modified GC electrode

Fig. 2A shows the typical cyclic voltammograms (CVs) of the Hb–PCL/GC (solid line), Hb/GC (dot line) and PCL/GC (dashed line) modified electrode in 0.10 M a phosphate buffer solution of pH 7.0 at scan rate of 100 mV s^{-1}, respectively. As shown in CVs (Fig. 2A), a couple of stable and well-defined redox peaks were observed at the Hb–PCL/GC (solid line). Its anodic peak potential and cathodic peak potential were located at −0.34 and −0.43 V versus SCE, respectively. The formal potential was

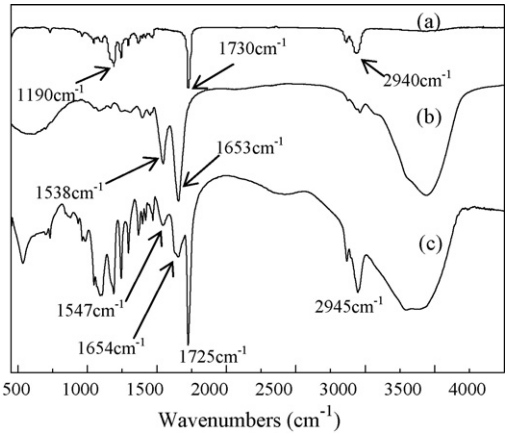

Fig. 1. FT-IR spectra of PCL (a), Hb (b) and Hb–PCL (c).

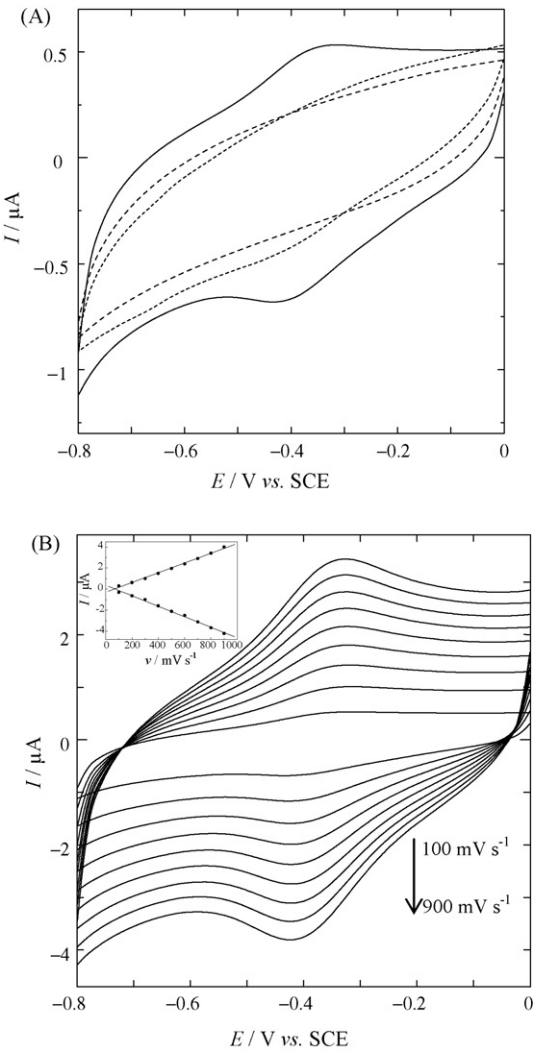

Fig. 2. (A) Cyclic voltammograms (CVs) obtained at Hb–PCL/GC (solid line), Hb/GC (dot line) and PCL/GC (dashed line) electrodes at 100 mV s^{-1} and (B) CVs obtained at Hb–PCL/GC electrode at 100, 200, 300, 400, 500, 600, 700, 800, and 900 mV s^{-1} (from inner to outer) in 0.10 M N_2-saturated phosphate buffer. Inset in (B): a plot of cathodic and anodic peak currents against potential scan rate.

−0.38 V versus SCE which was consistent with the reported values for the Fe(III)/Fe(II) redox center of the heme group in the Hb (Nassar et al., 1997; Zhang et al., 2002), suggesting that the Hb molecules entrapped in the PCL film retained their native structure. However, such redox process was unable to achieve at Hb-coated GC electrode (without modification with PCL) under the same condition (dot line), suggesting that it was difficult to realize the direct electron transfer of the Hb at the bare GC electrode, which coincided with the previous reports (Yu et al., 2005; Gao and Gao, 2007). Moreover, no redox peaks were observed at the PCL/GC electrode (dashed line). Hence, it could be concluded that PCL membrane was quite effective to facilitate the direct electron transfer between Hb and GC electrode. The reason might be that the PCL was a synthetically biodegradable polymer with good biocompatibility and could provide a favorable micro-environment for immobilized protein.

To further investigate the characteristics of Hb–PCL/GC electrode surface, we also systematically studied the dependence of the peak currents and the peak potentials on the scan rate. Fig. 2B shows the cyclic voltammograms of the Hb–PCL/GC electrode in 0.1 M PBS (pH 7.0) at different scan rates. The cathodic and anodic peak potentials exhibited a small shift and the increase of peak-to-peak separation with the increase of the scan rate, as well as the increase of cathodic and anodic peak currents (I_p) of the Hb could be observed. As shown in the inset of Fig. 2B, the cathodic and anodic peak currents increased linearly with the scan rate in the examined range of 100–900 mV s^{-1}. All these results demonstrated a quasi-reversible surface-confined feature of the electron transfer between Hb and electrode. When the peak-to-peak separation (ΔE) larger than 200 mV could be obtained experimentally, the apparent heterogeneous electron transfer rate constants (k_s) would be easily calculated with the help of Laviron's equations (Laviron, 1979). The k_s of Hb confined within PCL was evaluated as $18.7 \pm 0.8\ s^{-1}$, which was larger than that in previous reports (Wang et al., 2005; Zhao et al., 2005a; Qi et al., 2006). Therefore, we concluded that in our study the use of biodegradable PCL enhanced the electron transfer rate between the immobilized Hb and GC electrode.

From the integration of the Fe^{III} reduction peaks of Hb–PCL/GC at different scan rates, the surface concentration (Γ^*) of the electroactive Hb in the PCL film was estimated to be $(7.27 \pm 0.57) \times 10^{-11}$ mol cm^{-2} by Faraday's law, $Q = nFA\Gamma^*$ at the scan rate of 100–500 mV s^{-1}. Here F is the Faraday's constant, Q could be obtained by integrating the cathodic peak of Hb, n and A stand for the number of electron transferred and the geometric area of the electrode surface, respectively. This value was close to the theoretical monolayer coverage of the Hb on the surface of the GC electrode.

The effect of the pH value on electrochemical behavior of Hb in the PCL membrane had been also examined. The increase of the pH value (from 5.0 to 9.0) in the solution led to a negative shift of potential of both reduction and oxidation peaks. Moreover, E_{pa}, E_{pc}, and $E^{0'}$ was found to exhibit linear relationships with pH value in the range of 5.0–9.0, with a linear regression equation of $E_{pc} = -11.9\text{–}43.3\text{pH}$, $R = 0.999$; $E_{pa} = 10.1\text{–}52.8\text{pH}$, $R = 0.997$; $E^{0'} = -0.9\text{–}48.1\text{pH}$, $R = 0.999$, respectively. These slope values were close to the theoretical value of -58 mV pH^{-1} at 20 °C for the reaction of one electron coupled one proton (Bond, 1980).

3.3. An amperometric H_2O_2 biosensor

Fig. 3 shows the amperometric responses of the Hb–PCL films modified GC electrode, using the chronoamperometric technique upon the successive additions of H_2O_2 to 0.10 M pH 7.0 PBS at an applied potential of −0.40 V. Obviously, the steady-state response current increased as the H_2O_2 concentration increased. The linear range of this biosensor to H_2O_2 concentration was 2–30 μM which could be described by a linear regression equation of ΔI (nA) = 34.7 + 13.0C (μM), (R = 0.987) (inset of Fig. 3). The detection limit of the biosensor was 6.07 μM at a signal to noise ratio of 3. When the

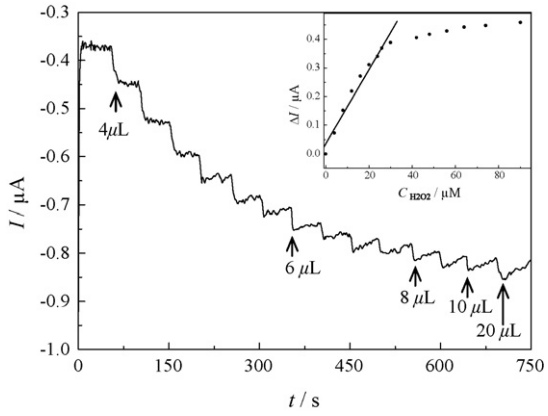

Fig. 3. Amperometric responses of Hb–PCL/GC electrode in stirred solutions to the successive additions of H_2O_2. Applied potential, −0.40 V vs. SCE; supporting electrolyte, 0.10 M pH 7.0 PBS. Insets: calibration curve of electrocatalytic current vs. concentrations of H_2O_2.

concentration of H_2O_2 was higher than 30 μM, a plateau was observed, showing a characteristic of the Michaelis–Menten kinetic mechanism. According to the Lineweaver–Burk form of the Michaelis–Menten equation (Kamin and Wilson, 1980):

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

where I_{ss} was the steady-state current after the addition of a substrate and obtained from amperometric experiments, I_{max} was the maximum current under saturated substrate conditions, c was the concentration of the substrate, and K_m^{app} was the Michaelis–Menten constant. The K_m^{app} value calculated from inset of Fig. 3 was 36.76 μM, which was markedly smaller than that reported previously (Zhao et al., 2005b; Li et al., 2006; Gao and Gao, 2007; Shan et al., 2007). This result showed that the immobilized Hb in PCL film retained its bioactivity and possessed a high biological affinity to H_2O_2.

When the H_2O_2 biosensor had been stored in a 0.10 M PBS for 2 weeks at 4 °C, the biosensor retained over 90% response of its initial sensitivity to the reduction of H_2O_2, demonstrating its good long-term stability. The reproducibility of the biosensor was examined in 20 μM H_2O_2, and the relative standard deviation was 3.54% for five successive assays. In addition, to further demonstrate the practicality of the proposed method, we also investigated the concentration of H_2O_2 in eyedrops as real samples. The real sample was firstly diluted with PBS (pH 7.0) before the detection, in order to make the concentration of H_2O_2 locating in the detection limit range. The concentration of H_2O_2 in real samples was estimated to be 0.249 mM by using the linear equation and the dilution ratio, and the relative standard deviation was 4.78% for five successive assays. This value was consistent with that obtained by the conventional permanganate index method (0.241 mM), which indicated that the present method was reliable and effective in applications. These results also suggested that the PCL film might be useful for the development of new electrochemical biosensor.

4. Conclusions

From the results given above, it was demonstrated that PCL film could provide favorable micro-environment for Hb. The Hb entrapped in PCL film retained its original conformation by FT-IR spectra, and the direct electron transfer between the immobilized redox proteins in PCL film and the electrode were easily performed. The formal potential $E^{0'}$ of the heme Fe(III)/Fe(II) redox couple in Hb varied linearly with increase of pH from 5.0 to 9.0 with a slope of 48.1 mV. The hydrogen peroxide biosensor based on Hb immobilized in PCL film showed a fast amperometric response, very high sensitivity, good reproducibility and stability. Moreover, this proposed method had been applied to the determination of H_2O_2 in real samples. These properties were believed to be very useful for the future development of application of biodegradable polymer in the new bioelectronic nanodevices, e.g., electrochemical biosensor and enzyme-based biofuel cells.

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