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Direct electrochemistry and bioelectrocatalysis of hemoglobin immobilized on carbon black

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Abstract

It is reported for the first time that hemoglobin (Hb) was immobilized on the surface of carbon black powders modified at the surface of a glassy carbon electrode. The cyclic voltammetric results showed that the immobilized Hb could undergo a direct quasi-reversible electrochemical reaction. Its formal potential, E^0, is −0.330 V in phosphate buffer solution (pH 6.9) at a scan rate of 100 mV/s and is almost independent of the scan rate in the range of 40–200 mV/s. The dependence of E^0, on the pH of the buffer solution indicated that the conversion of Hb–Fe(III)/Hb–Fe(II) is a one-electron-transfer reaction process coupled with one-proton-transfer. The experimental results also demonstrated that the immobilized Hb retained its bioelectrocatalytic activity for the reduction of H_2O_2. Furthermore, the immobilized Hb can be stored at 4 °C for several weeks without any loss of the enzyme activity. Thus, the immobilized Hb may be used as a biocathodic catalyst in biofuel cells.

Keywords: Hemoglobin; Carbon black; Direct electrochemical reaction; Hydrogen peroxide; Biocathode

1. Introduction

The direct electrochemical study of some proteins and enzymes has been paid more and more attention in recent years because of the importance to biological science as well as the potential applications in biosensor and biofuel cells [1–6]. From these study, one can obtain valuable information about the structures of enzymes, mechanisms of electron transfer reactions in biological system and metabolic processes involving redox transformations [7]. If an enzyme immobilized on an electrode surface is capable of direct electron transfer and keeping its bioactivity, it can be used in biosensors without the addition of mediators or promoters onto the electrode surface or into the solution. Unfortunately, it is difficult for an enzyme to carry out a direct electrochemical reaction due to several factors. For example, enzymes would be adsorbed on the electrode surface resulting in the denaturation and thus loss of their electrochemical activities and bioactivities. In addition, usually, the active sites are deeply buried in the molecules, so that promoters or mediators are needed to obtain the electrochemical reactions of proteins or enzymes [6,8,9]. For applications in biosensors, the enzymes should be immobilized on the electrode surface to avoid many complications linked to the solution system. Therefore, it is necessary to find new electrode materials and suitable methods of immobilization of enzymes onto the electrode surface to obtain their direct electrochemical reactions and retain their bioactivities.

Hemoglobin (Hb), an important respiratory protein in red cells, has a molecular weight of approximately 64,500 and consists of four polypeptide chains. Each peptide chain contains one electroactive heme group [10]. Although Hb does not act biologically or physiologically as an electron carrier, it is a desirable mode for function study of the redox behavior of heme proteins in biological systems because of its commercial availability, moderate cost and documented structure. It has been reported that Hb can be used as a substitute of HRP to catalyse the reduction of H_2O_2 since it also has the heme proteins similar to HRP and stable property in the solution [11]. However, it is difficult for Hb to exchange electron with electrode surface

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directly since the redox centers, the heme groups are deeply immersed in the peptide chains [11,12]. In order to obtain the direct electrochemical reaction of Hb, many methods were employed to study the electrochemical reaction of Hb at various modified electrodes [13–22]. For example, silica sol–gel film [16], biomembrane films [15], kieselgubr film [17], nano gold colloid particles [18], the organic solvent [19], CdS nanoparticles [20], mesoporous tungsten oxide [21] and hexagonal mesoporous silica matrix [22] were used to promote the electrochemical reaction of Hb. Wang et al. reported the direct electrochemical reaction of Hb at the glassy carbon electrode coated with lipid-protected gold nanoparticles [23]. More recently, some carbon materials are also extensively used as carriers for immobilization of enzyme. For example, Cai and Chen et al. [24] used the Nafion membrane to immobilize Hb and carbon nanotubes on the surface of the glass carbon electrode and investigated the direct electrochemical reaction of Hb. Jia et al. [25] immobilized Hb onto bamboo-like nitrogen-doping nanotubes and obtained its direct electrochemistry.

Carbon black (CB) is a kind of widely used carbon material due to its good conductivity and large surface area. It consists mainly of elemental carbon as the spherical particles with 30–100 nm in size [26,27]. In the present work, it was reported for the first time that the direct electrochemical reaction of Hb adsorbed on the surface of carbon black with an underlying glassy carbon electrode (noted as Hb–CB/GC electrode). The electrocatalytic reduction of H_2O_2 at the Hb–CB/GC electrode was also investigated.

2. Experimental

2.1. Chemicals and apparatus

Hb (from bovine blood, Sinopharn Chemical Reagent Co, Ltd., Shanghai, China), Nafion solution (5% in methanol with an equivalent molar mass of about 1100, Aldrich Chemicals Co.) and carbon black (specific area of about 550 m^2/g and average pore size of 15 nm, Shanghai Linge Chemical Science and Technology Co., LTD, Shanghai, China) were used as received. Stock solutions of H_2O_2 were freshly diluted from 30% solution. All other chemicals were of analytical grade. All the solutions were prepared with doubly distilled water. The 0.1 M phosphate buffer solutions (PBS, pH 6.9), which were made up from Na_2HPO_4 and NaH_2PO_4, were always employed as supporting electrolyte except that the pH-dependent experiments were carried out in PBS with various pH values.

Electrochemical measurements were performed on a CHI660 electrochemical workstation (CHI, USA) with conventional three-electrode cell. A saturated calomel electrode (SCE) and a platinum electrode were used as the reference and counter electrode, respectively. All potentials were quoted with respect to SCE. All experimental solutions were deoxygenated by bubbling high-purity nitrogen and maintained under nitrogen atmosphere during measurement. The experiments were carried out at room temperature. FTIR spectra of carbon black were recorded with Nicolet 360v FTIR spectrophotometer (Nicolet Instrumental, USA). UV–Vis absorption spectroscopy was performed using a UV–Vis 8453 spectrophotometer (Agilent, USA). XPS experiments were carried out on a RBD upgraded PHI-5000C ESCA system (Perkin Elmer) with Mg Kα radiation ($h\nu = 1253.6$ eV) or Al Kα radiation ($h\nu = 1486.6$ eV).

2.2. Preparation of the Hb–CB/GC electrode

The glassy carbon electrode (3 mm in diameter) was polished to a mirror-like surface with 1.0, 0.3, and 0.05 μm alumina slurry followed by rinsing thoroughly with doubly distilled water. Then the electrode was successively sonicated in nitric acid, acetone and doubly distilled water. Finally, it was dried at room temperature. The Hb–CB/GC electrode was prepared as follows. About 4 mg carbon black powders were dispersed thoroughly in 5 mL 0.1 wt.% cetyltrimethylammonium bromide (CTAB) solution which serves as a dispersant to prevent the CB powders aggregation with the ultrasonication for 30 min. Then 4 mg Hb was added to the suspension and stirred for ten hours at 4 °C. The Hb molecules were adsorbed on the surface of carbon black powders during mixing. Then, 2 μL of the mixture was cast onto the surface of the glassy carbon electrode and dried at ambient temperature. Finally, 1 μL of Nafion (5%) was cast on the surface of the Hb–CB/GC electrode in order to hold the mixture of Hb and carbon black powders on the glassy carbon electrode stably. After the solvent was evaporated, the Hb–CB/GC electrode was obtained. If it is not used immediately, the electrode was stored at 4 °C in a refrigerator. In the above procedure, when Hb was not added, the electrode obtained is the CB/GC electrode.

3. Results and discussion

The procedure of immobilization of Hb on the surface of CB does not alter the integral structure of Hb or damage Hb leading to a denatured state. This conclusion can be verified by the FTIR spectrum. Fig. 1c is the FTIR spectrum of carbon black. The peaks at 1632 and 1377 cm^{-1} are due to stretching vibrations of C═O and C–OH, respectively. The results indicated that the oxygen-contained groups were present on the surface of CB powders. Fig. 1a is the FTIR spectrum of free Hb. The amide I band of Hb, which is caused by C═O stretching vibrations of peptide linkages, appears at 1656 cm^{-1}. The signal at 1542 cm^{-1} indicates the characteristic of amide II, which results from a combination of N–H in plane bending and C–N stretching vibrations of the peptide groups . Amide I (1700–1600 cm^{-1}) and amide II (1620–1500 cm^{-1}) can provide the detailed information on the secondary structure of the polypeptide chain. Fig. 1b is the FTIR spectrum of Hb immobilized on the surface of CB. Obviously, the amide I and II bands in Fig. 1b had similar shapes to that of free Hb (Fig. 1a) except that the bands shifted to 1640 and 1562 cm^{-1}, respectively. The results indicate the Hb was not denatured after it was immobilized on the surface of carbon black powers.

The position of Soret absorption band of heme may provide information of the conformational structure of heme proteins, especially that of the conformational change in the heme group region . UV–Vis spectroscopy is a useful tool for conformational

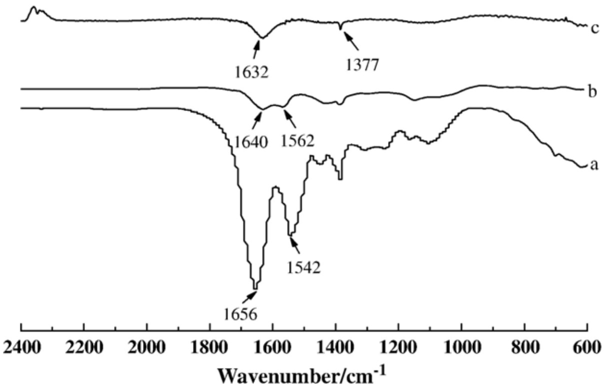

Fig. 1. The spectra of (a) free Hb, (b) Hb immobilized on the surface of carbon black and (c) carbon black powders.

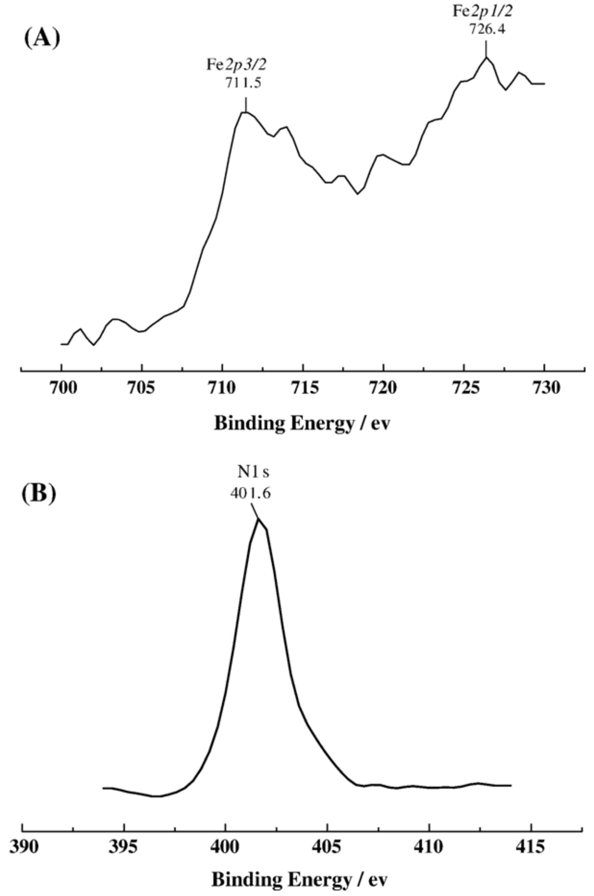

Fig. 3. The XPS spectra of Hb immobilized on the surface of carbon black in (A) Fe*2p* and (B) N*1s* regions.

study of heme proteins. Fig. 2 shows the UV–Vis spectra of Hb (Fig. 2a) and Hb/CB composite (Fig. 2b) in PBS (PH 6.9), respectively. The Soret band of Hb/CB appears at 408 nm (Fig. 2b), same as that of native Hb in phosphate buffer (Fig. 2a). This indicates that secondary structure of Hb immobilized on the surface of CB is not destroyed and Hb retains its biological activity.

In order to confirm the existence of Hb on the surface of carbon black powders, XPS experiments were carried out. Fig. 3 shows the XPS spectra of Hb immobilized on the surface carbon black in the Fe*2p* and N*1s* regions. It can be observed that the peaks Fe$2p_{3/2}$ and Fe$2p_{1/2}$ of Fe (III) are located at 711.5 and 726.1 eV, respectively, (Fig. 3(A)) and the characteristic peak of N*1s* of N is at 401.6 eV (Fig. 3(B)). Since both Fe and N are from Hb, the results indicate the existence of Hb on the surface of carbon black powers.

Fig. 4 shows the cyclic voltammograms of the CB/GC electrode, Hb–CB/GC electrode and Hb–CTAB/GC electrode in 0.1 M PBS (pH 6.9) at a scan rate of 100 mV/s. No redox peaks were observed in the cyclic voltammogram of the CB/GC electrode (Fig. 4a), indicating that both carbon black and CTAB do not undergo the electrochemical reactions in the potential range studied. However, a pair of well-defined and nearly symmetrical redox peaks was obtained at the Hb–CB/GC electrode (Fig. 4b), illustrating that the redox peaks were due to the electrochemical reaction of Hb. In the cyclic voltammogram of the Hb–CTAB/GC electrode (Fig. 4c), no redox peaks were observed. The above results demonstrated that the direct electrochemical reaction of Hb is not promoted by surfactant CTAB or Nafion, but is promoted by carbon black due to the presence of the oxygen-contained groups and many active sites on the surface [24,28].

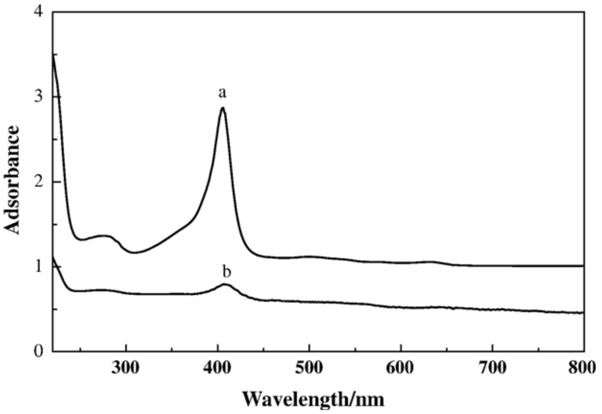

Fig. 2. UV–Vis spectra of (a) Hb and (b) Hb/CB composite in 0.1 M PBS (PH 6.9).

It was observed from Fig. 4b that the anodic and cathodic peaks of Hb are located at −0.295 and −0.361 V (*vs.* SCE),

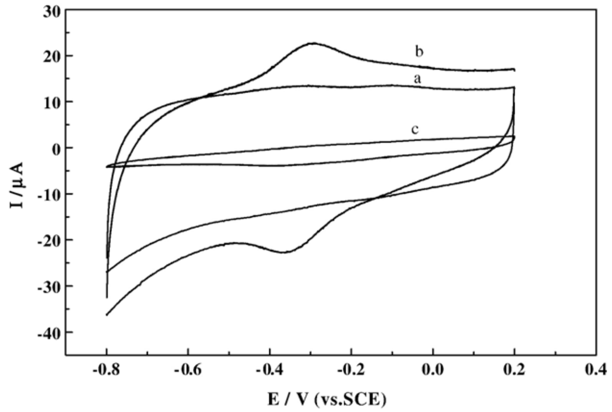

Fig. 4. The cyclic voltammograms of: (a) the CB/GC electrode, (b) the Hb–CB/GC electrode, (c) the Hb–CTAB/GC electrode in the phosphate buffer solution (pH 6.9) at the scan rate of 100 mv/s.

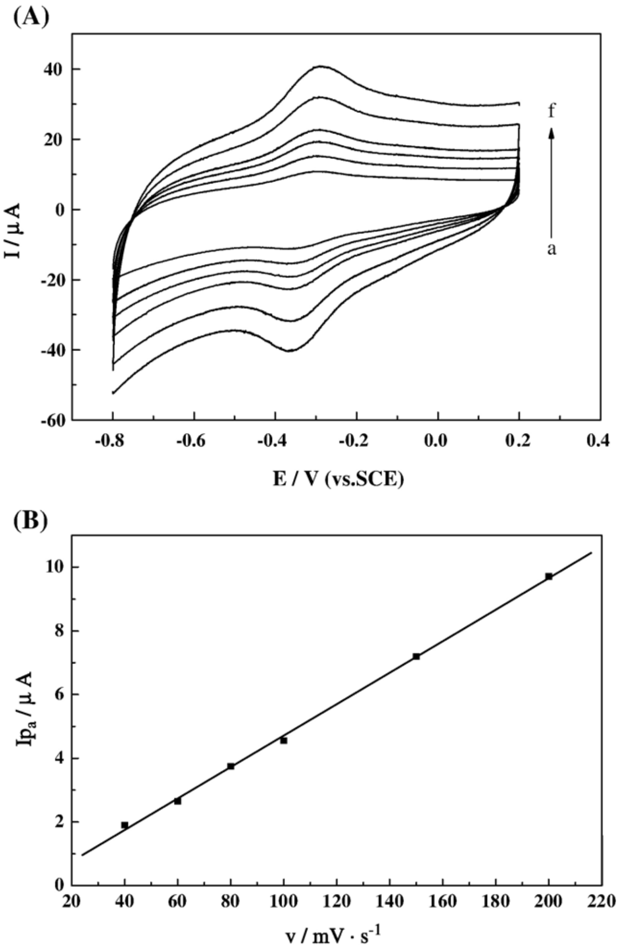

Fig. 5. (A) The cyclic voltammograms of the Hb–CB/GC electrode in 0.1 M PBS (pH 6.9) at the scan rate of (a)40, (b)60, (c)80, (d)100, (e)150 and (f) 200 mV/s. (B) The plot of anodic peak current *vs.* scan rate from Fig. 5 (A).

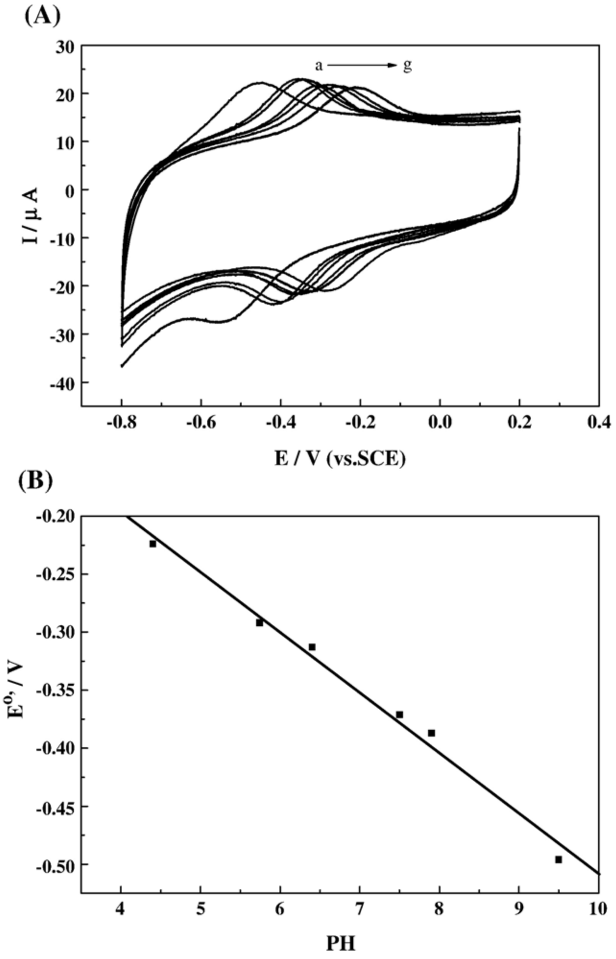

Fig. 6. (A) The cyclic voltammograms of the Hb–CB/GC electrode in 0.1 M PBS at pH: (a) 9.5, (b) 7.9, (c) 7.5, (d) 6.9, (e) 6.4, (f) 5.7 and (g) 4.4. Scan rate: 100 mV/s. (B) The plot E^0, of Hb *vs.* pH of the solution from Fig. 6(A).

respectively. The separation of peak potentials, ΔE_p, is 66 mV. The cathodic current (Ipc) is almost equal to the anodic peak current (Ipa). Thus, it can be concluded that Hb immobilized on the surface of the carbon black powders can undergo a direct quasi-reversible electrochemical reaction. Its formal potential, E^0, is −0.330 V, which is similar to those previously reported in Refs [29,30]. Thus, this suggests that Hb immobilized on carbon black is not denatured.

The cyclic voltammograms of the Hb–CB/GC electrode at various scan rates were measured as shown in Fig. 5(A). It was found that the anodic and cathodic peak currents are linearly proportional to a scan rate in the range of 40–200 mV/s (Fig. 5(B)), suggesting that the electrochemical reaction is surface-controlled as expected for immobilized systems [31,32]. According to the Laviron equation [33], from the dependence of peak-to-peak separation (ΔE_p) on the various scan rates, the electron transfer rate constant k_s value was estimated to be 1.02 s^{-1}, which is larger than those obtained for Hb immobilized on Au–colloid–cysteamine-modified gold electrode (0.49 s^{-1}) [34], Hb modified CNT powder microelectrodes electrodes (0.062 s^{-1}) [35], indicating a reasonably fast electron transfer between the immobilized Hb and the underlying electrode. The surface concentration (Γ) of Hb was estimated from integration of the cathodic peaks in Fig. 5(A) according to Faraday's law ($Q = nFA\Gamma$) to be 3.55×10^{-9} mol/cm^2.

In most cases, protein redox behavior is often significantly dependent on the solution pH. Fig. 6(A) shows strong dependence of the CVs of Hb–CB/GC electrode on solution pH. It was found that an increase in the solution pH leads to a negative shift of the potentials for both anodic and cathodic peaks, E^0, shifts linearly

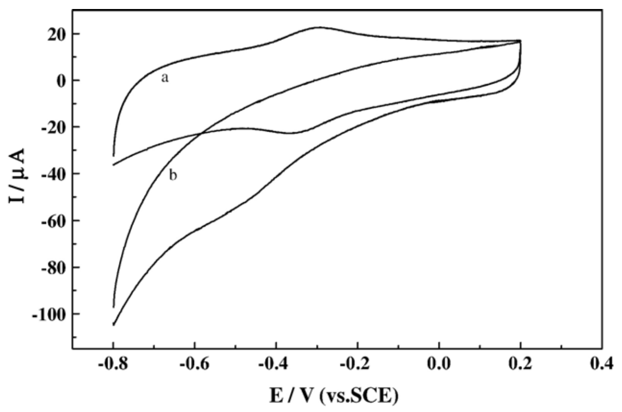

Fig. 7. The cyclic voltammograms of the Hb–CB/GC electrode in 0.1 M PBS (pH 6.9) in (a) the absence and (b) presence of 5 mM H_2O_2. Scan rate is 100 mV/s.

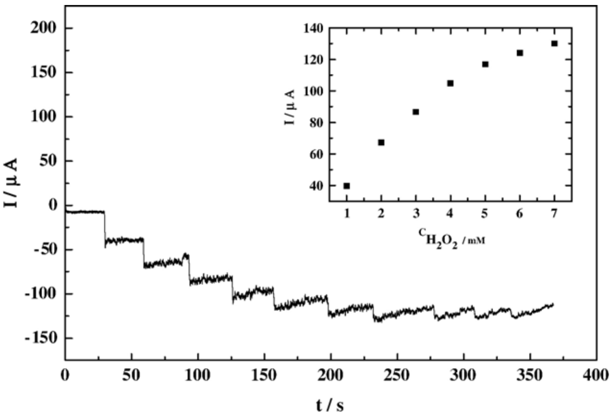

Fig. 8. The chronoamperometric curve of the Hb–CB/GC electrode in 0.1 M PBS (pH6.9). Fixed potential: −0.5 V, Addition of H_2O_2: 1 mM H_2O_2 after every 20–25 s. Inset: the plot of the current for the reduction of H_2O_2 *vs.* the concentration of H_2O_2.

to the negative direction with increasing pH with a slope of −53.0 mV/pH (Fig. 6(B)), which is close to the theoretical value of −57.6 mV/pH at 18 °C for the electrochemical reaction with one electron and one proton. Therefore, the reaction could be represented as follows:

$$\text{Hb heme Fe(III)} + \text{H}^+ + \text{e}^- \rightarrow \text{Hb heme Fe(II)}$$

Fig. 7 shows the cyclic voltammograms of the Hb–CB/GC electrode in 0.1 M PBS (pH 6.9) in the absence and presence of 5 mM H_2O_2. In the absence of H_2O_2, a pair of redox peaks of Hb was observed (Fig. 7a), which is the same as Fig. 4b. In the presence of H_2O_2, a cathodic peak for the reduction of H_2O_2 was observed (Fig. 7b). Such cathodic peak was not observed at the bare GC or CB/GC electrode in the presence of H_2O_2. These results indicated that the immobilized Hb retained its bioelectrocatalytic activity for the reduction of H_2O_2 and was not denatured.

Fig. 8 displays the chronoamperometric curve of the Hb–CB/GC electrode in 0.1 M PBS (pH 6.9) with H_2O_2, whose concentration is changed by adding 1 mM H_2O_2 after every 20–25 s. It can be observed that the electrocatalytic current of the Hb–CB/GC electrode increases with each addition of H_2O_2. The current reaches its maximum value within 6 s after each addition of H_2O_2, suggesting that the Hb–CB/GC electrode can respond rapidly to the change of the concentration of H_2O_2.

The stability of the electrocatalytic activity of Hb–CB/GC electrode for the reduction of H_2O_2 was also checked. After the Hb–CB/GC electrode was stored at 4 °C for several weeks, the electrocatalytic activity of the Hb–CB/GC electrode for the reduction of H_2O_2 were almost unchanged. These results indicate that the Hb–CB/GC electrode has a good stability toward the electrocatalytic reduction of H_2O_2.

4. Conclusions

From the above results, it can be concluded that carbon black can promote the direct electrochemical reaction of Hb and thus Hb immobilized on the surface of carbon black powders could undergo a direct quasi-reversible electrochemical reaction. The immobilized Hb is not denatured and retains the bioelectrocatalytic activity for the reduction of H_2O_2 and its bioelectrocatalytic activity is stable. After the Hb–CB/GC electrode was stored at 4 °C for several weeks, the electrocatalytic activity of the Hb–CB/GC electrode for the reduction of H_2O_2 were almost unchanged. Therefore, the Hb–CB/GC electrode can be used as a biocathode in biofuel cells. The method presented here can be easily extended to immobilize and obtain the direct electrochemistry of other enzymes or proteins.

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