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Direct electrochemistry and electrocatalysis of hemoglobin entrapped in semi-interpenetrating polymer network hydrogel based on polyacrylamide and chitosan

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Abstract

Semi-interpenetrating polymer network (semi-IPN) hydrogel based on polyacrylamide (PAM) and chitosan was prepared to immobilize redox protein hemoglobin (Hb). The Hb–PAM–chitosan hydrogel film obtained has been investigated by scanning electron microscopy (SEM) and UV–VIS spectroscopy. UV–VIS spectroscopy showed that Hb kept its secondary structure similar to its native state in the Hb–PAM–chitosan hydrogel film. Cyclic voltammogram of Hb–PAM–chitosan film-modified glass carbon (GC) electrode showed a pair of well-defined and quasi-reversible redox peaks for Hb Fe(III)/Fe(II), indicating that direct electron transfer between Hb and GC electrode occurred. The electron-transfer rate constant was about 5.51 s^{-1} in pH 7.0 buffers, and the formal potential ($E^{\circ\prime}$) was −0.324 V (vs. SCE). The dependence of $E^{\circ\prime}$ on solution pH indicated that one-proton transfer was coupled to each electron transfer in the direct electron-transfer reaction. Additionally, Hb in the semi-IPN hydrogel film retained its bioactivity and showed excellent electrocatalytic activity toward H_2O_2. The electrocatalytic current values were linear with increasing concentration of H_2O_2 in a wide range of 5–420 μM. The unique semi-IPN hydrogel would have wide potential applications in direct electrochemistry, biosensors and biocatalysis.
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Keywords: Hemoglobin; Direct electrochemistry; Electrocatalysis; Polyacrylamide; Chitosan; Semi-interpenetrating polymer network hydrogel

1. Introduction

Hemoglobin (Hb) is a heme protein that can store and transport oxygen in blood of vertebrate animals. It has a molar mass of approximately 67,000 g mol^{-1}, and contains four polypeptide subunits (two α subunits and two β subunits), each of which has one iron-bearing heme as electron-transfer center. Although Hb does not play a role as an electron carrier in biological systems, it has been shown to possess enzyme-like catalytic activity [1,2]. It can be used as an ideal model molecule for study of electron transfer of heme enzymes due to its commercial availability, moderate cost and a documented structure. But the electron transfer was very slow on conventional electrodes because of protein adsorption and subsequent passivation of the electrodes surface. The other reason for the slow electron transfer is that the electroactive centers of Hb are buried in the polypeptide and inaccessible to the surface of electrodes. Numerous efforts have been made to improve the electron-transfer characteristics by using mediators [3,4].

The direct electron transfer between redox proteins and electrode surface has received widespread attention in recent years [5]. It can be used to establish a foundation for fabricating the new type of biosensors without using mediators [6–8] and can serve as a model to understand the electron-transfer mechanism in biological systems [9,10]. Generally speaking, the direct electrochemistry of proteins was mostly achieved at modified electrodes. Many researches have been reported for entrapment or encapsulation of proteins within biocompatible materials, including natural biomolecules [11], biopolymers [12], hydrogels [13,14], bioceramics [15], and nanomaterials [16,17]. These biocompatible materials can provide favorable microenvironments for the redox proteins to realize direct

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electrochemistry and fabricate excellent biosensors. Hydrogels are cross-linked, three-dimensional hydrophilic polymer networks, which swell but not dissolve when contact with water. Hydrogels possess a degree of flexibility very similar to natural tissue because of their significant water content. Due to their desirable properties, hydrogels have been widely used for immobilizing proteins and enzyme [13,14,18]. However, to the best of our knowledge, no work has been reported on studies of direct electrochemistry and biosensors using semi-interpenetrating polymer network (semi-IPN) hydrogel.

Chitosan is a linear hydrophilic polysaccharide obtained by deacetylation of nature chitin. It is an attractive biocompatible, biodegradable, and nontoxic natural biopolymer that exhibits excellent film-forming ability. Due to its desirable properties, chitosan has been widely used for electrochemical biosensing platforms combined with metal nanoparticles [19], carbon nanotubes [20] and ionic liquids [21]. Polyacrylamide (PAM) is a kind of widely used polymer, which has been successfully used for study of direct electrochemistry [13]. It possesses great affinity toward proteins and other biomolecules. Proteins bound with PAM hydrogel can retain biological activity.

In this article, Hb was entrapped in the semi-IPN hydrogel based on polyacrylamide and chitosan. Chitosan was used as a functional polymer chain to produce this semi-IPN hydrogel, so the affinity and stability of the hydrogel could be improved. Hb entrapped in the semi-IPN hydrogel was characterized by scanning electron microscope (SEM) and UV–VIS spectroscopy. Hb–PAM–chitosan film was cast on a glass carbon (GC) electrode and the direct electrochemistry of Hb was realized and investigated. The electrocatalysis reduction of hydrogen peroxide on Hb–PAM–chitosan film electrode was also studied.

2. Experimental

2.1. Chemicals

Chitosan with deacetylation degree about 90% was purchased from Boao Bio-Technology Company, Shanghai (China). Hemoglobin (from bovine blood), acrylamide (AM) and *N*,*N*′-methylenebisacrylamide (MBA) were purchased from Sigma. They were used as received without further purification. All other chemicals used were analytical grade. Twice-distilled water was used to prepare solutions.

2.2. Preparation of Hb entrapped in PAM–chitosan semi-IPN hydrogel

Chitosan was dissolved into 1% (v/v) acetic acid. The solution was stirred for about 4 h until the solid dissolved entirely and the solid content in solution was 2% (w/v). Hb entrapped in PAM–chitosan semi-IPN hydrogel was prepared based on published procedure with modification [22]: 2.5 ml chitosan solution, 190 mg AM, 10 mg MBA, 4 mg potassium persulfate (KPS), 50 mg Hb and 7.5 ml of 50 mM phosphate buffer solution (PBS), pH 7.0, were put into a 50-ml flask with a nitrogen inlet. The mixture was stirred continuously under a nitrogen atmosphere for 30 min, and then 1 ml 0.16% (w/v) $NaHSO_3$ in phosphate buffer solution was added. The mixture was stirred for another 15 min and stopped. The polymerization progress went on for 3 h under the nitrogen atmosphere.

PAM–chitosan hydrogel and Hb–PAM hydrogel were prepared in the same way as that above just without the addition of Hb or chitosan.

2.3. Construction of film electrode

Prior to coating, GC electrode (diameter 3 mm) was polish with 0.3 μm and 0.05 μm alumina, then ultrasonicated in ethanol and twice-distilled water, respectively. 5 μl Hb–PAM–chitosan hydrogel was cast onto the electrode surface by using a microsyringe to prepare Hb–PAM–chitosan-modified GC electrode. A breaker was covered over the electrode so that water evaporated slowly, and a uniform film could be formed. The Hb–PAM–chitosan film was then dried in air overnight. The PAM–chitosan film and Hb–PAM film-modified GC electrodes were prepared in the same way as described above. Before electrochemical measurements, all the film electrodes were immersed in buffer solution for 1 h, and then washed with twice-distilled water to allow swelling and remove the physically absorbed Hb.

2.4. Electrochemical measurements

All the electrochemical measurements were performed with a CHI 660 electrochemical workstation. A conventional three-electrode system was used with a film-modified or bare GC electrode as working electrode, a platinum wire as counter electrode, and a saturated calomel electrode (SCE) as reference electrode. Buffers were purged with high-purity nitrogen for at least 30 min and a nitrogen environment was then kept over the solutions in the cell. All experiments were done at room temperature (~25 °C).

2.5. SEM and spectroscopic analysis

The morphology of Hb–PAM–chitosan hydrogel and Hb–PAM hydrogel were analyzed by JSM-5600 LV scanning electron microscopy (SEM). Sample films were coated on graphite disks and dried overnight for SEM analysis.

UV–VIS spectroscopy was performed with a UV-2100 spectrophotometer (Lab Tech). Sample films for measurements were prepared by casting Hb solution or Hb–PAM–chitosan hydrogel onto quartz glass slides and drying in air.

3. Result and discussion

3.1. Characterization of hydrogel films by SEM and UV–VIS spectroscopy

The morphologies of Hb–PAM–chitosan hydrogel film and Hb–PAM hydrogel film on graphite disks surface were characterized by SEM. As can be seen in Fig. 1B, Hb–PAM–chitosan film exhibited a three-dimensional network porous structure. This would qualify Hb–PAM–chitosan film for good

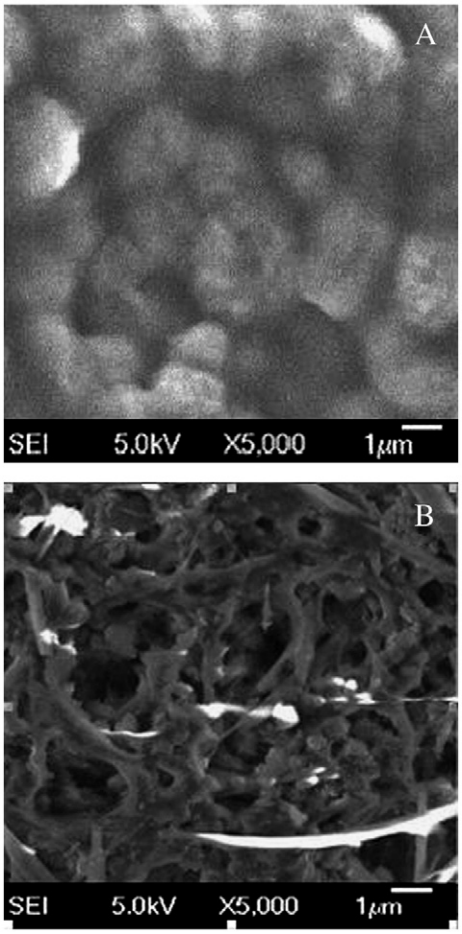

Fig. 1. SEM images of (A) PAM–chitosan and (B) Hb–PAM–chitosan films on graphite disks surface.

conductivity of electron. This means that the semi-IPN hydrogel could be used as an excellent supporting material for electrochemical research. In contrast, the image of Hb–PAM film revealed approximately flat and featureless (Fig. 1A). The compact structure would not benefit the electron transfer.

UV–VIS spectroscopy is a useful means to monitor the possible change of the Soret absorption band in the heme group region. The position of the Soret absorption band of heme can provide information about possible denaturation of heme proteins, especially conformational change in the heme group region [23–25]. As shown in Fig. 2, Hb solution (curve a) had Soret absorption band at 405 nm at pH 7.0. Both dry Hb (curve j) and Hb–PAM–chitosan films (curve i) showed Soret absorption bands at 413 nm, indicating that Hb entrapped in PAM–chitosan film retained nearly the same secondary structure as the native state of Hb in its dry film alone. The effect of pH on the Soret absorption band for Hb–PAM–chitosan film was also investigated. It could be seen that the Hb–PAM–chitosan film absorption spectra in the range of pH 3.0–11.0 exhibited strong heme Soret absorption band at 413 nm (curves c–g), the same as that of dry Hb and Hb–PAM–chitosan films. This result demonstrated that Hb retained its native structure in dry Hb–PAM–chitosan film and in the case that Hb–PAM–chitosan film was immersed in a solution of medium pH range (from 3 to 11). When the pH of solution decreased to lower than 3 or increased to higher than 11, the peak sharp became broader. As shown in curve b and curve h in Fig. 2, the Soret absorption bands almost disappeared at pH 2 and pH 12, suggesting that the conformation of Hb in PAM–chitosan film had been changed in these acidic or alkaline pH environments. When the Hb–PAM–chitosan film was placed in a medium pH range again (e.g., pH 7.0), the disappeared Soret absorption band reappeared at 413 nm again. All these results indicated that the semi-IPN hydrogel based on PAM and chitosan had good biocompatibility for Hb.

The stability of Hb–PAM–chitosan film on quartz glass slide was also studied by UV–VIS spectroscopy. Dry Hb–PAM–chitosan film was immersed in pH 7.0 PBS and UV–VIS absorption spectra were collected for different times. There was no obvious change of absorption spectra in 1 day, indicating that the Hb–PAM–chitosan film was very stable in PBS.

3.2. Electrochemical properties

The electrochemical behavior of Hb-modified electrode was studied by cyclic voltammetry. Fig. 3 shows the cyclic voltammograms obtained with Hb–PAM–chitosan- and PAM–chitosan-modified GC electrodes. As shown, no peaks were observed at the PAM–chitosan-modified electrode. When the Hb–PAM–chitosan-modified electrode was immersed into 50 mM PBS, pH 7.0, a pair of stable, well-defined quasi-reversible redox peaks were observed. The formal potential ($E^{\circ\prime}$) calculated by averaging the cathodic and anodic peak potentials was found to be −0.324 V (vs. SCE), characteristic of the Hb heme Fe(III)/Fe(II) redox couple. These results presented strong evidence that direct electron transfer between Hb and GC electrode was achieved. According to Faraday laws

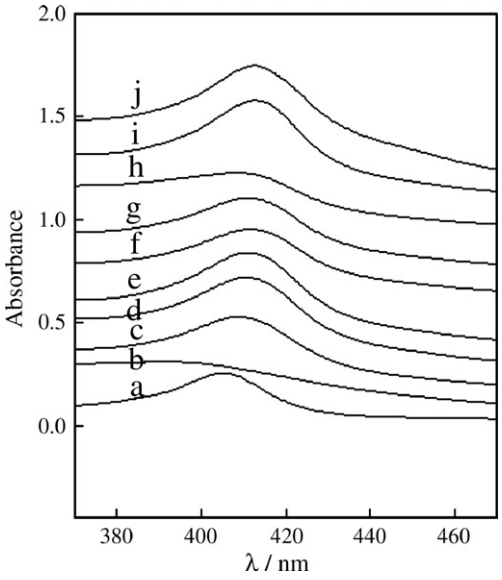

Fig. 2. UV–VIS spectra of (a) Hb solution at pH 7.0, (j) dry Hb film, (i) dry Hb–PAM–chitosan film, and Hb–PAM–chitosan film immersed in different pH buffers: (b) pH 2.0, (c) pH 3.0, (d) pH 5.0, (e) pH 7.0, (f) pH 9.0, (g) pH 11.0, (h) pH 12.0. To demonstrate clearly, the curves were shifted along the *y*-axis.

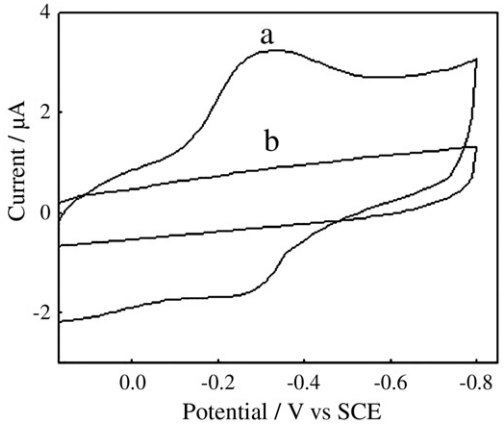

Fig. 3. Cyclic voltammograms of (a) Hb–PAM–chitosan film, (b) PAM–chitosan film-modified GC electrodes in 50 mM pH 7.0 PBS at 0.2 V s^{-1}.

[26], $\Gamma^* = Q/(nFA)$, where Q is the charge involved in the reaction, n is the number of electron transferred, F is the Faraday constant, and A is the effective area of the GC electrode, the surface concentration of electroactive Hb (Γ^*) was estimated to be 5.81×10^{-11} mol cm^{-2}. It was about three times larger than the theoretical monolayer coverage (1.89×10^{-11} mol cm^{-2}) [15]. This showed that several layers of Hb entrapped in the three-dimensional composite hydrogel film participated in the electron-transfer process. The percentage of the electroactive Hb on the electrode surface was about 1.1%, suggesting that only those proteins in the inner layers of the film closed to the electrode and with a suitable orientation can exchange electrons with the electrode surface.

Fig. 4A gives the typical cyclic voltammograms of the Hb–PAM–chitosan-modified GC electrode in pH 7.0 PBS with scan rates from 0.1 to 1.0 V s^{-1}. With the increase of the scan rate, the cathodic and anodic peak currents increased simultaneously. As shown in Fig. 4B, the cathodic peak currents (i_{pc}) increase linearly with scan rate, indicating that the electron transfer of Hb with the GC electrode is a surface-confined electrochemical process. The peak-to-peak separation also increased with the scan rate.

The kinetic parameters α and k_s were estimated using the model of Laviron [27]:

$$\log(k_s\ /s^{-1}) = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\left[\left(\frac{RT}{nF\upsilon}\right)/s\right] - \frac{\alpha(1-\alpha)nF\Delta E_p}{2.3RT}$$

where α is the transfer coefficient, k_s is the heterogeneous electron-transfer rate constant, ΔE_p is the peak-to-peak separation, n is the number of the number of electrons transferred in the rate-determining reaction, R is the gas constant, T is the absolute temperature and υ is the scan rate. A graph of ΔE_p versus the logarithm of the scan rate yielded a straight line. Based on this, the values of $\alpha = 0.65\pm0.08$ and $k_s = 5.51\pm0.30$ s^{-1} were calculated.

The influence of buffers pH on cyclic voltammograms of Hb–PAM–chitosan-modified electrode was also studied. Both

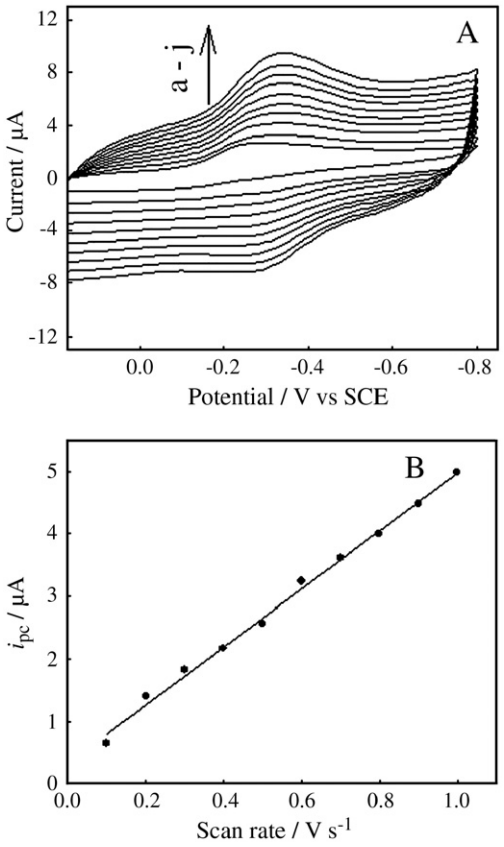

Fig. 4. (A) Cyclic voltammograms of Hb–PAM–chitosan film-modified GC electrodes in 50 mM pH 7.0 PBS over a range of scanning rates (a–j: 0.1–1.0 V s^{-1}). (B) Plot of cathodic peak currents vs. scan rates.

reduction and oxidation peaks for Hb–PAM–chitosan film shifted negatively as pH increased. It can be seen from Fig. 5 that the formal potential $E^{\circ\prime}$ of the heme Fe(III)/Fe(II) redox couple for the Hb–PAM–chitosan-modified electrode had a linear relationship with pH from 3.0 to 11.0. The slope of $E^{\circ\prime}$ versus pH was −49.6 mV pH^{-1}, which was approximately close to the theoretical value of −59.0 mV pH^{-1} at 25 °C for a reversible one-proton- and one-electron-transfer reaction [28,29]. This result indicated that the electron transfer between

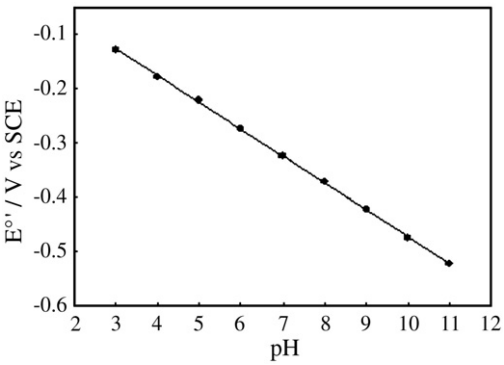

Fig. 5. Influence of pH on the formal potential for Hb–PAM–chitosan film-modified GC electrode at 0.2 V s^{-1}.

Hb in Hb–PAM–chitosan film and the electrode was coupled by proton transportation. The reaction could be expressed as:

$$HbFe(III) + e^- + H^+ \rightleftharpoons HbFe(II)$$

3.3. Electrocatalytic properties

Many literatures reported that Hb has good electrocatalytic activity to hydrogen peroxide, nitrite, tricholoroacetic acid, oxygen, etc. [12,14,17,24,26]. Taking H_2O_2 as an example, the electrocatalytic properties of Hb–PAM–chitosan-modified GC electrode were studied. When H_2O_2 was added to the PBS, the voltammograms of Hb–PAM–chitosan-modified GC electrode showed a significant increase in the reduction peak with the disappearance of the oxidation peak (Fig. 6A), suggesting that an electrocatalytic reduction of H_2O_2 occurred. The reduction peak current i_{pc} increased with the concentration of H_2O_2. No peaks were observed at the PAM–chitosan-modified GC electrode in the potential range of 0.2 to −0.8 V in the presence of H_2O_2. The exact mechanism of catalytic reduction of H_2O_2 by Hb–PAM–chitosan-modified GC electrode is not yet clear. According to He et al. and Arnao et al. [12,30], heme can react with H_2O_2 to form a first intermediate of compound I, which has catalytic activity to H_2O_2. Thus the simplified mechanism for the electrochemical catalytic reaction of H_2O_2 by Hb–PAM–chitosan-modified GC electrode may be expressed as the following schemes:

$$HbFe(III) + H_2O_2 \rightarrow compoundI + H_2O$$

$$compoundI + H_2O_2 \rightarrow HbFe(III) + O_2$$

$$HbFe(III) + e^- + H^+ \rightleftharpoons HbFe(II) \text{ at electrode}$$

$$HbFe(II) + O_2 \rightarrow HbFe(II){-}O_2 \text{ fast}$$

$$HbFe(II){-}O_2 + 2e^- + 2H^+ \rightarrow HbFe(II) + H_2O_2 \text{ at electrode}$$

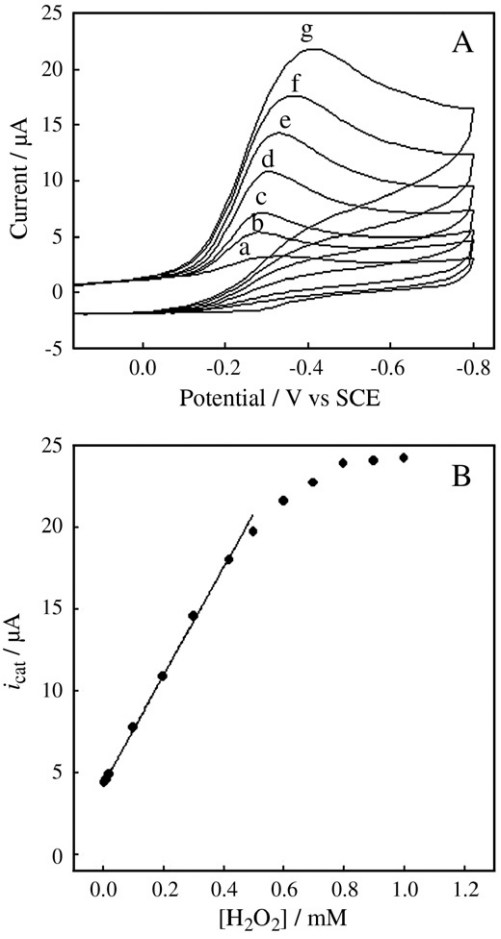

Fig. 6. (A) Cyclic voltammograms at 0.2 V s^{-1} of Hb–PAM–chitosan film-modified electrode in the presence of (a) 0, (b) 0.005, (c) 0.02, (d) 0.1, (e) 0.2, (f) 0.3, (g) 0.5 mM H_2O_2. (B) Plot of the electrocatalytic current vs. H_2O_2 concentration.

Table 1
Electrocatalytic performance of some Hb films toward H_2O_2

Films	Linear range (μM)	Detection limit (μM)	K_m (μM)	Reference
Hb–PAM–chitosan	5–420	1.6	330	Present work
Hb–PAM	15–180	8.0	1200	Present work
Hb–gelatine	50–1200	3.4	na [a]	[32]
Hb–silica sol–gel	5–700	na [a]	898	[33]
Hb–kieselgubr	5–300	2.1	975	[34]
Hb–chit-Aus [b]	740–13000	6.4	1400	[35]

[a] na, not available.
[b] chit-Aus, chitosan-stabilized gold nanoparticles.

The electrocatalytic current (i_{cat}) linearly increased with increasing concentration of H_2O_2 in the beginning and thereafter began to level off (Fig. 6B), indicating that the relationship between i_{cat} and the concentration of H_2O_2 showed a Michaelis–Menten response. Here the i_{cat} value was defined as the difference between i_{pc} in the presence of H_2O_2 and i_{pc} in the absence of H_2O_2 for the Hb–PAM–chitosan-modified GC electrode. The i_{cat} values were linear with increasing concentration of H_2O_2 in the range of 5–420 μM for the Hb–PAM–chitosan-modified GC electrode. The apparent Michaelis–Menten constant (K_m) can be obtained from the electrochemical

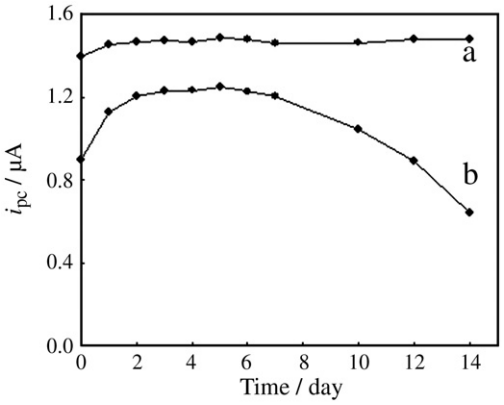

Fig. 7. Changes of cathodic peak currents of (a) Hb–PAM–chitosan- and (b) Hb–PAM-modified GC electrodes at 0.2 V s^{-1} during the storage in pH 7.0 PBS.

version of the Linweaver–Burk equation [31]. The K_m value for the Hb–PAM–chitosan-modified GC electrode was found to be 330 μM. Identical experiments were performed using the Hb–PAM-modified GC electrode, and their analytical performances were collected in Table 1. For comparison, other reported protein films were also listed in Table 1. In contrast with Hb–PAM and other films reported so far, the Hb–PAM–chitosan film presented excellent analytical performance in the determination of H_2O_2 for a wider linear range. The smaller K_m value meant that Hb entrapped in PAM–chitosan semi-IPN hydrogel exhibited a higher affinity for H_2O_2.

3.4. Stability of the Hb–PAM–chitosan-modified GC electrode

Compared with the Hb–PAM-modified GC electrode, the stability of Hb–PAM–chitosan-modified GC electrode was tested by cyclic voltammetry. In the stability study, The Hb–PAM–chitosan- and Hb–PAM-modified GC electrodes were stored in PBS at 4 °C, and cyclic voltammograms were performed periodically. The changes of cathodic peak currents of Hb–PAM–chitosan- and Hb–PAM-modified GC electrodes were shown in Fig. 7. As shown, for Hb–PAM–chitosan-modified GC electrode, the cathodic peak currents almost kept constant during 2 weeks. In contrast, the cathodic peak currents of Hb–PAM-modified GC electrode fast increased during the first 2 days. This result was similar to that obtained by Sun et al. [13]. It was attributed to the influence of water on the microenvironment of the films in which Hb resided. But different from the excellent stability of Hb–PAM film on the pyrolytic graphite electrode in the research of Sun et al. [13], Hb–PAM film on the GC electrode showed much less stability. When the time for storage of Hb–PAM-modified GC electrode in PBS was more than 1 week, the cathodic peak currents decreased rapidly. Then, the cathodic peak was almost disappeared after 2 weeks of storage. All these results indicated that semi-IPN hydrogel based on PAM and chitosan provided more excellent microenvironment for Hb than PAM hydrogel. And Hb entrapped in PAM–chitosan semi-IPN hydrogel could exhibit better stability. The excellent stability of Hb–PAM–chitosan-modified GC electrode might attribute to both the good biocompatibility of the composite hydrogel based on PAM and chitosan and the strong interaction between Hb and PAM–chitosan.

4. Conclusion

In this paper, a new type of semi-IPN hydrogel based on polyacrylamide and chitosan was utilized for the immobilization of Hb. The UV–VIS spectra measurement showed that the semi-IPN hydrogel did not change the original secondary structure of Hb. Furthermore, the direct electron transfer of Hb was achieved when Hb–PAM–chitosan film was cast onto the surface of GC electrode. The semi-IPN structure of the hydrogel film provided a favorable microenvironment around Hb to retain the enzymatic bioactivity and native structure of Hb. The immobilized Hb showed good bioelectrocatalytic activity toward H_2O_2. It could be anticipated that the unique semi-IPN hydrogel would have widely potential applications in direct electrochemistry, biosensors and biocatalysis, as it can provide a new promising electrochemical sensing platform for redox proteins and enzymes.

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