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Direct electron transfer of Horseradish peroxidase on porous structure of screen-printed electrode

Y.J. Teng, S.H. Zuo, M.B. Lan*

Research Center of Analysis & Test and Institute of Advanced Materials, East China University of Science and Technology, No. 130 Meilong Road, Shanghai 200237, PR China

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ABSTRACT

Disposable hydrogen peroxide biosensor was developed based on the direct electron transfer of horseradish peroxidase (HRP) on porous screen-printed carbon electrodes. Conventional screen-printing process was manually performed to fabricate the planar carbon electrodes, which were endowed with porous surfaces especially after anodizing pretreatment. The cyclic voltammetry experiment indicated a pair of stable and well-defined redox peaks with a formal potential of −0.33 V. And the formal potential was pH-dependent, having a slope of −55.2 mV/pH which indicated one electron transfer. The heterogeneous electron transfer rate constant k_s was estimated to be $13.28 \pm 4.80\ s^{-1}$. Additionally, the sensitivity was $143.3\ mA\ M^{-1}\ cm^{-2}$ and the linear range was from 5.98 to 35.36 μM. In conclusion, the present work achieved the direct electron transfer of HRP on screen-printed electrodes without any promoters. The porous structure of screen-printed carbon electrodes facilitated the direct electron transfer between the active sites of HRP and the electrodes due to large amounts of conductive sites available on the surface for contacting with enzyme molecules. Moreover, the proposed biosensor could be mass-produced at low price, promising for commercial application.

1. Introduction

Horseradish peroxidase (HRP), with a molar weight of about 42,000 Da and a diameter of about 5 nm, is a kind of enzyme which contains a heme prosthetic group as its active site. It can oxidize a variety of substrates by one-electron transfer. This behavior has attracted considerable attention to develop the third generation of biosensors which means the electron transfers between the electrode and enzyme directly without any mediators. When HRP catalyzes substrate, such as H_2O_2, the active site form of HRP is from Fe (III) to Fe (II) at the electrode. The direct electron transfer has been achieved by immobilizing HRP in sol–gel films (Di et al., 2005; Yu and Ju, 2002), hydrogel films (Huang and Hu, 2003; Sun et al., 2007), polyelectrolyte films (Kong et al., 2003; Razola et al., 2002), or by using the methods of cross-linking (Qian and Yang, 2006), self-assembly (Gaspar et al., 2001; Xu et al., 2005) and so on.

However, the heterogeneous electron transfer between HRP and the surface of electrode is slow, because the electroactive center of HRP is hard to contact the surface of electrode. In order to increase the signal of direct electron transfer, many papers published by adding different kinds of promoters including Au particles (Xu et al., 2007; Xu et al., 2006b), carbon nanotube (Chen and Dong, 2007; Qian and Yang, 2006; Turdean et al., 2006), semiconductors (Zhang et al., 2004) and so on. But actually, the addition of promoters will induce interference and the complex progress of constructing biosensor. So promoter-free biosensors will be more accurate and applied. This paper uses Nafion as a kind of immobilization material. It is a perfluorinated anionic polyelectrolyte, which has good electrical conductivity. And the Nafion film has high chemical stability, good biocompatibility and the ability to resist interferences from anions and biological macromolecules. Most importantly, it is easy to use which can realize one-step immobilization.

To our knowledge, there are no papers reporting the direct electron transfer of HRP on the screen-printed electrode (SPE). Most papers focus on the glass carbon electrode (Kong et al., 2003), gold electrode (Song et al., 2006), and pyrolytic graphite electrode (Huang and Hu, 2001). However, the high cost and complicated pretreatment limit their commercial application. The technology of screen-printed electrode started from 1960s, which has become more mature after all these years. As SPE can be mass-produced and one-off, it has become preferred electrode material for biosensors. Base on application, this paper shows that the surface of SPE is crude and has porous structure which accelerates the electron's transferring because of the large specific surface area and large amounts of conductive sites.

* Corresponding author. Tel.: +86 21 64253574; fax: +86 21 64252947.
E-mail address: minbolan@ecust.edu.cn (M.B. Lan).

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Furthermore, this paper established one method to detect H_2O_2 that plays an important part in medicine, chemistry, biology and other fields as it can be used not only as oxidants but also as reductants. For human beings, many diseases including gastrointestinal tract disease, eye disease, heart-lung disease, will be induced if it is taken excessively. So it is necessary to reasonably control its concentration in different fields. Compared with other time-consuming and expensive methods including chemiluminescence (Leite et al., 2007; Yamashiro et al., 2004), fluorescence (Albers et al., 2006; Wang et al., 2006), liquid chromatography (Toyo'oka et al., 2003), electrochemical H_2O_2 biosensor would be a preferential method as it detects more quickly, conveniently and inexpensively. Besides, the H_2O_2 biosensor introduced in this paper has higher sensitivity compared with those introduced in some other papers (Chen and Dong, 2007; Li et al., 2007; Lu et al., 2006) which use promoters. Therefore, it is a convenient method and promising for commercial application as a third-generation biosensor after further development.

2. Experimental

2.1. Materials and reagents

Horseradish peroxidase (HRP, type X, 291 units/mg protein) and Nafion® 117 solution (purum, ~5% in a mixture of aliphatic alcohols and water) were purchased from sigma (USA). Hydrogen peroxide (30% w/v solution) was from Sinopharm Chemical Reagent Co., Ltd. All other chemical reagents used were analytical grade and without further purification. Phosphate buffer saline (PBS, 0.05 M) with various pH values were prepared by mixing with K_2HPO_4 and KH_2PO_4, containing 0.1 M KCl. All the solutions were prepared with doubly distilled water.

2.2. Apparatus and measurements

Electrochemical experiments were carried out at room temperature on a CHI 440 electrochemical station (CHI Instruments Inc., USA). All the electrochemical experiments were performed with a conventional three-electrode system. The Hg/Hg_2Cl_2 (saturated KCl) was used as the reference electrode. The Pt wire acted as the counter electrode and the screen-printed electrode with modified film acted as the working electrode. About 0.05 M PBS (pH 7.0) was used as electrolyte in all experiments. The buffers were purged with highly purified Argon for at least 30 min prior to a series of voltammetric experiments and keeping the argon atmosphere during the experiment. In amperometric experiments, the current-time data were recorded after successive addition of H_2O_2 solution into the buffer. It was carried out by applying a potential of −350 mV on a stirred cell.

The scanning electron microscopic (SEM) image of the SPE surface was recorded by a JSM-6360LV SEM (JEOL Ltd., Japan).

UV–vis absorption spectroscopy was done by a UV-2102 (Unico (Shanghai) Instrument Co., Ltd., USA).

2.3. Preparation of screen-printed electrode

The screen-printed electrodes were fabricated by a TORCH T3244 manualscreen-printer (Beijing Torch Co., Ltd., China). It can once produce an array of twenty electrodes by depositing several layers of inks on PVC substrate. The scheme of steps used in the electrode preparation is shown in our previous work (Zuo et al., 2008). The first step was to force the silver ink (BY2100, Shanghai Baoyin Electronic Materials Co., Ltd., China) to penetrate through the mesh of a screen stencil which acts as conductive layer. Then the first layer cured at 100 °C for 40 min. After drying, the carbon layer (Jelcon CH-10, Jujo Chemical Co., Ltd., Japan) covered on the silver layer by another stencil. This prepared plate also needed a curing step (100 °C for 40 min). And then an insulator layer (Jelcon AC-3G, Jujo Chemical Co., Ltd., Japan) was spread manually over the conductive track, leaving a working area 2 mm × 5 mm and an electrical contact. At last, it was dried at 80 °C for 10 min.

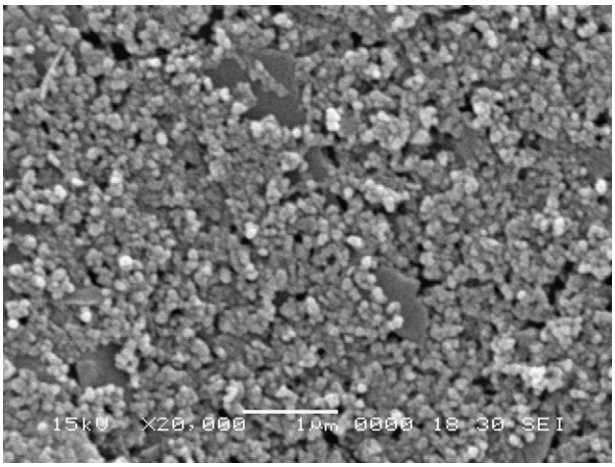

Fig. 1. SEM image of bare screen-printed electrode.

2.4. Electrode modification

The HRP-Nafion-SPE electrode was prepared according to the following procedure. Before modification, short pre-anodization periods of SPE, which could increase the surface functionalities and roughness and remove off surface contamination, are suggested to increase the heterogeneous electron transfer. According to the research, which has discussed the pretreated conditions of screen-printed electrodes (Wang et al., 1996), the present SPEs were pre-anodized in 0.05 M PBS (pH 7.4) on 1.7 V for 3 min, then cleared and allowed to dry at room temperature. 100 μl HRP solution (3 mg/ml) was mixed with 10 μl Nafion. Then 3 μl mixture was dropped on the pretreated SPE and allowed to dry under ambient conditions overnight. The modified electrodes were rinsed with doubly distilled water for twice or thrice to get rid of the non-firmly adsorbed HRP. Before electrochemical test, it should be immersed into 0.05 M pH 7.0 PBS for 30 min. The obtained HRP-Nafion-SPE electrodes were stored in 0.05 M PBS (pH 7.0) at 4 °C in a refrigerator when not in use. And the Nafion-SPE electrode followed the procession of fabricating the HRP-Nafion-SPE electrode.

3. Results and discussion

3.1. Characteristic of SPE, HRP-Nafion-SPE film

Fig. 1 shows that the surface of screen-printed electrode covers plenty of carbon particles which have several hundred nanometers to form the porous structure. The diameter of HRP is about 5 nm, which is much smaller compared with the size of pore. So it is inferred that HRP can insert into the pore freely. At the same time, the coverage of HRP on the surface of electrode is increased and the carbon particles also provide many conductive sites, resulting that more H_2O_2 would be catalyzed by HRP.

Fig. 2 is the UV–vis spectrum of 2 μl HRP (3 mg/ml) after (a) and before (b) mixing with 20 μl Nafion on ITO electrode. The position of Soret absorption band of heme may provide information about possible denaturation of heme proteins (Theorell and Ehrenberg, 1951). The HRP, embedded in the Nafion film (curve a), has the

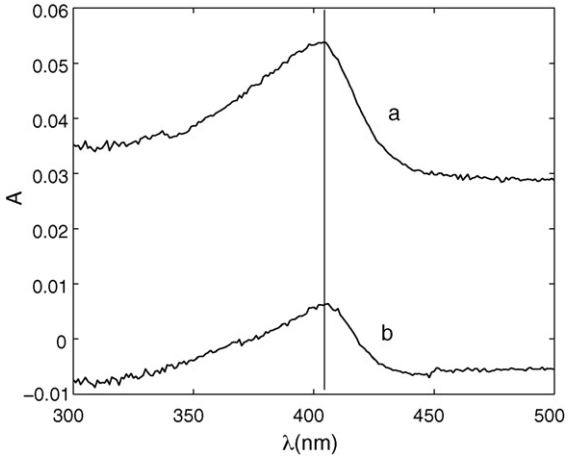

Fig. 2. UV–vis spectrum after (a) and before (b) mixing with Nafion to immobilize HRP on ITO electrode.

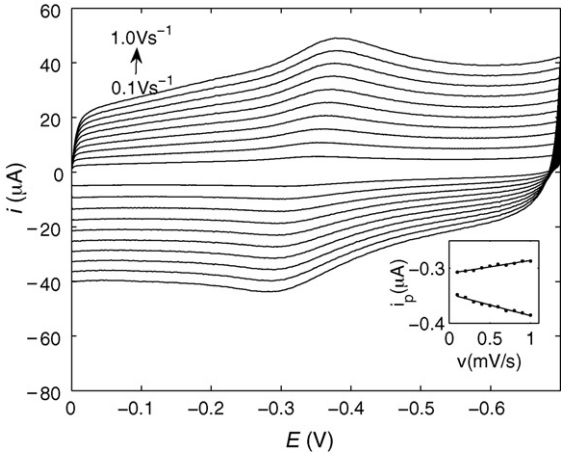

Fig. 4. Cyclic voltammograms of HRP-Nafion-SPE electrode in 0.05 M, pH 7.0, PBS at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 V s^{-1}. Inset: plot of the cathodic (i_{pc}) and anodic (i_{pa}) peak currents versus the scan rates.

characteristic absorption band at the position of 408 nm, same as the native HRP (curve b) in PBS solution. It proves that the addition of Nafion keeps original structure of HRP and does not change the activity of HRP.

3.2. Cyclic voltammetric behavior of HRP-Nafion-SPE

The insert plot in Fig. 3 shows the original cyclic voltammograms of different electrodes in 0.05 M pH 7.0 PBS with scan rate 0.1 V s^{-1}. The Nafion-SPE (a) has no peak which represents it is electroinactive in the potential window. Whereas, the HRP-Nafion-SPE electrode (b) is electroactive. In Fig. 3, the background-corrected cyclic voltammogram of HRP-Nafion-SPE electrode, which is obtained by the Nafion-SPE (a) serving as background, shows a great peak corresponding to the reduction of HRP. At the same time, a corresponding oxidation peak is observed at the HRP-Nafion-SPE electrode. The reduction peak and oxidation peak are observed at −350 and −310 mV, respectively. Besides, the formal potential ($(E^{\circ\prime}) = (E_{pa} + E_{pc})/2$) is −330 mV, which is similar to the $E^{\circ\prime}$, obtained for native HRP in solution (−270 mV versus NHS at pH 7.0) (Harbury, 1957).

Thus the direct electron transfer performed between the electrode and the immobilized HRP without any mediators. It is obviously attributed to the redox of the electroactive center of the immobilized HRP.

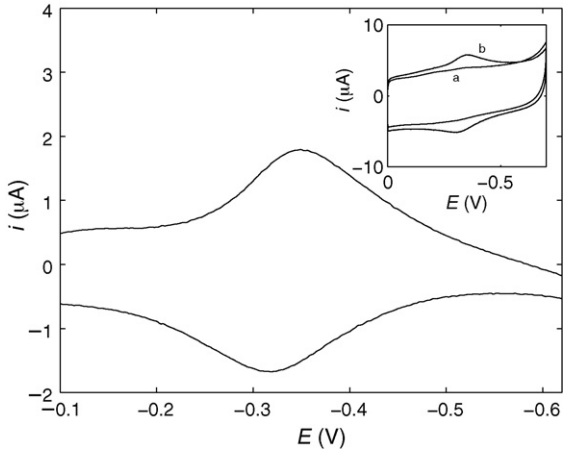

Fig. 3. Background-corrected cyclic voltammogram of HRP-Nafion-SPE in 0.05 M, pH 7.0, PBS with scan rate 0.1 V s^{-1}. Inset: cyclic voltammograms of Nafion-SPE (a) and HRP-Nafion-SPE (b).

The cyclic voltammograms of HRP-Nafion-SPE electrode in 0.05 M pH 7.0 PBS from 0.1 to 1.0 V s^{-1} was shown in Fig. 4. With the increase of the scan rates, the redox peak currents increased linearly. And the anodic and cathodic peak potentials of HRP shifted in positive and negative directions, respectively, indicating a surface-controlled and quasi-reversible process. The small peak-to-peak separation values (ΔE_p) illustrate a fast electron transfer rate. The electron transfer rate constant (k_s) is estimated to be $13.28 \pm 4.80\ s^{-1}$ using the following equation:

$$k_s = \frac{mnFv}{RT} \quad (1)$$

when the $n\Delta E_p$ is less than 200 mV (Laviron, 1979), where m is a parameter related to the peak-to-peak separation, T is the temperature, n is the number of electrons, and v is the scan rate. This value is larger than the corresponding values observed for HRP immobilized on DNA ($3.27 \pm 0.91\ s^{-1}$) (Wang et al., 2007), gold nanoparticles ($7.8\ s^{-1}$) (Di et al., 2005), Nafion-cysteine ($1.2\ s^{-1}$) (Hong et al., 2007). It indicates that a fast electron transfer rate resulted from the strong interaction between HRP molecules and screen-printed electrode. As the screen-printed electrode is covered with many carbon particles acted as conductive sites. And the porous structure is beneficial for the active site of HRP to close the conductive site. In quantity, there are more enzymes immobilizing on the surface of electrode compared with other plane electrodes. All the reasons promote the ability of direct electron transfer.

3.3. Effect of solution pH on direct electron transfer of immobilized HRP

Fig. 5 shows the direct electrochemistry of immobilized HRP with a strong dependence on solution pH. An increase of solution pH caused a negative shift in both cathodic and anodic peak potentials, which was reversible by testing from pH 5.0 to pH 8.0 and then back. The inset plot shows the formal potential had linear relationship with pH, presenting a slope of −55.2 mV/pH that approximated to the theoretical value of −57.6 mV/pH (Meites, 1965) at 18 °C for a single proton transfer, coupled with reversible single electron transfer which illustrated one proton participating in the electron transfer process (Bond, 1980). Thus for HRP immobilized on the screen-printed electrode, the electrode reaction is a single electron

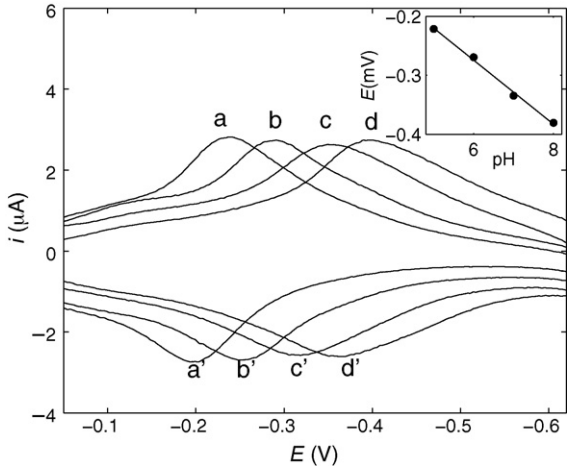

Fig. 5. Background-corrected cyclic voltammograms of HRP-Nafion-SPE electrode in 0.05 M PBS with different pH (a) 5.0 (b) 6.0 (c) 7.0 and (d) 8.0. Inset: plot of the formal potential versus the pH.

transfer process which can be expressed in the following equation:

$$HRP(Fe^{3+}) \rightarrow HRP(Fe^{2+}) \quad (2)$$

3.4. Electrocatalysis of immobilized HRP to reduction of H_2O_2

HRP is heme-protein which can catalyze the reductive reaction of H_2O_2. The principle of catalyzing H_2O_2 was presented by the following (Ferri et al., 1998):

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow CompoundI(Fe^{4+} = O, P^{\bullet+}) + H_2O \quad (3)$$

$$CompoundI(Fe^{4+} = O, P^{\bullet+}) + e^- + H^+ \rightarrow CompoundII(HRP(Fe^{4+} = O)) \quad (4)$$

$$CompoundII(HRP(Fe^{4+} = O)) + e^- + H^+ \rightarrow HRP(Fe^{3+}) + H_2O \quad (5)$$

From the above equations, there are several states in the bioelectrocatalytic cycles of HRP. It firstly forms a powerful enzymatic oxidizing agent known as compoundI(HRP(Fe^{4+} = O, $P^{\bullet+}$)), which is a two-equivalent oxidized form containing an oxyferryl heme and a porphyrin π cation radical. CompoundI is catalytically active and can abstract one electron from the substrate to form a second intermediate, compoundII(HRP(Fe^{4+} = O)), which is subsequently reduced back to the resting state of the native enzyme, HRP (Fe^{3+}) by accepting an additional electron from the substrate. According to Ferris', HRP (Fe^{3+}) can also be further reduced to HRP (Fe^{2+}) at lower potential on the electrode.

In order to investigate the catalytic ability of HRP-Nafion-SPE electrode, the amperometric *i–t* had been used to detect the reduction reaction of compoundI to HRP (via compoundII) at the surface of electrode. The amperometry performed at the different constant potential, such as −150, −200, −250, −300, −350, −400, and −450 mV. The response current to 12 μM H_2O_2 increased with the potential until −350, −350, −400, and −450 mV give the similar value (result not shown). As the other electroactive species normally are present at lower potential. And the modified electrodes are unstable at lower potential. In order to reduce interference, obtain sensitive response current and keep stable of modified electrode, −350 mV will be the best choice. Fig. 6(A) is standard amperometric *i–t* curve, after successively adding 60 μl 2 mM H_2O_2 in 20 ml 0.05 M pH 7.0 PBS at the working potential of −350 mV, the amperometric response of H_2O_2 is observed and the response value of every addition is similar. The linear range is 5.98–35.36 μM with a correlation coefficient of 0.9967 (n = 9) as shown in Fig. 6(B). The sensitivity is 143.3 mA M^{-1} cm^{-2} and the detection limit is 0.48 μM with the signal to noise ratio of three. The coefficient of variation (CV) of the sensor response to 24 μM H_2O_2 was 4.71% for six successive measurements.

Michaelis–Menten constant (K_m) is the concentration when the velocity reached the half-maximum velocity. It is the characteristic constant which weighs the affinity between enzyme and substrate. According to the Lineweaver–Burk equation (Kamin and Wilson, 1980):

$$\frac{1}{i_{ss}} = \left(\frac{K_m}{i_{max}}\right)\left(\frac{1}{C}\right) + \frac{1}{i_{max}} \quad (6)$$

where i_{ss} is the steady-state current after addition of substrate, C is the substrate concentration in the bulk solution and i_{max} is the maximum current measured under saturated substrate conditions. When the value of K_m is smaller, the affinity between the enzyme and substrate will be stronger. The K_m value of HRP-Nafion-SPE electrode was calculated to be 4.5 ± 0.2 μM, which is lower than the corresponding values observed for HRP immobilized on gold electrode (1.1 mM, Di et al., 2005; 8.01 mM, Tong et al., 2007; 2.3 mM, Yi et al., 2000), glass carbon electrode (0.57 mM, Xu et al., 2006a; 1.89 ± 0.21 mM, Yu and Ju, 2002; 7.5 μM, Lu et al., 2006) by combining with different promoters. It can be inferred that the affinity of enzyme and substrate increases on the screen-printed electrode.

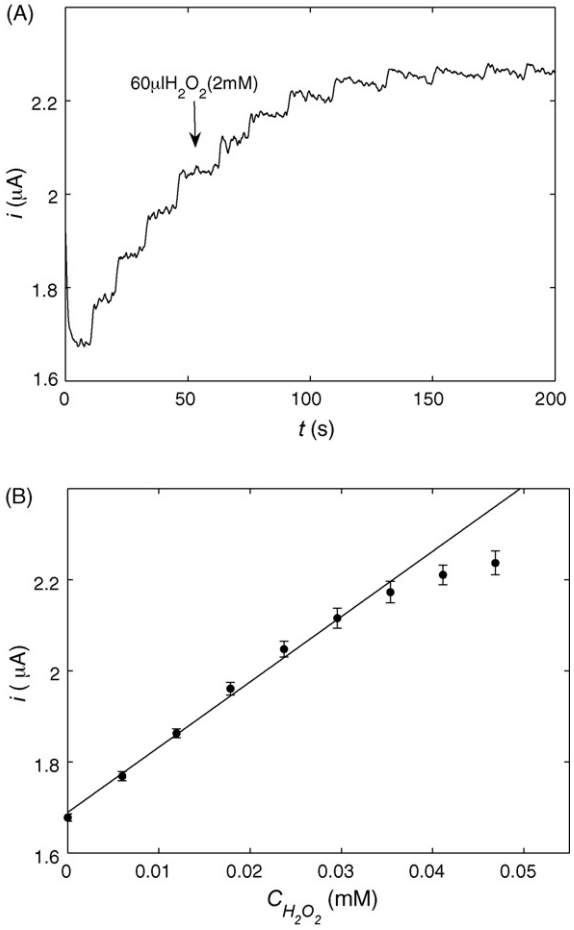

Fig. 6. (A) Amperometric responses of HRP-Nafion-SPE at −350 mV upon successive additions of 60 μl 2 mM H_2O_2 to 20 ml pH 7.0 PBS. (B) Calibration curve of the biosensor for H_2O_2.

3.5. Stability and reproducibility of the H_2O_2 sensor

To examine the stability of HRP-Nafion-SPE electrodes, cyclic voltammetric sweeps over potential range from −0.7 to 0 V at 0.1 V s^{-1} for 30 cycles. But the current values retained which illustrates the present modified electrode is stable. The signal decreased 4.7% after stored in 0.05 M pH 7.0 PBS in a refrigerator at 4 °C for 10 days. The reproducibility between bare SPEs were assessed in 2 mM $[Fe(CN)_6]^{3-}$ in 0.5 M KCl solution with a relative standard deviation (R.S.D.) of 3.5% (n = 15). After modification, the reproducibility of the HRP-Nafion-SPE electrodes was examined by successively testing in 12 μM H_2O_2 solution. The R.S.D. of current response is 7.5% (n = 5). It is believed that the reproducibility of SPEs, including the modification process of enzyme electrode, could be improved after mechanized production.

4. Conclusion

The present paper demonstrates that the direct electron transfer of HRP can be performed on the porous surface of screen-printed electrode without any mediators or promoters. The modified electrode system acts as an efficient and stable amperometric biosensor. In absence of mediators, the direct electrochemistry of HRP offers an opportunity to build up a biosensor for H_2O_2. At the same time, the results are good compared with the biosensors adding promoters. As the screen-printed electrodes could be mass-produced at low price, the proposed biosensors for H_2O_2 could be more accessible to commercial application.

Acknowledgements

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