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Direct electrochemistry of horseradish peroxidase on Nafion/[bmim]PF_6/agarose composite film modified glassy carbon electrode

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ABSTRACT

A new strategy to construct electrochemical biosensor for direct electrochemistry of horseradish peroxidase (HRP) on glassy carbon electrode (GCE) based on Nafion, agarose hydrogel and hydrophobic room-temperature ionic liquid (RTIL) 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) composite as sensing platform has been described. [bmim]PF_6 has good conductivity and wide electrochemical windows and agarose can maintain biological activity well. Nafion/[bmim]PF_6/agarose composite combines the advantages of [bmim]PF_6 and agarose. Electrochemical impedance spectroscopy (EIS), ultraviolet visible spectroscopy (UV–vis), fourier transform infrared (FT-IR) spectroscopy and cyclic voltammetry (CV) were used to characterize the composite film, showing that the composite film could be effectively constructed on the GCE surface and greatly enhance the electron transfer between HRP and electrode. The factors influencing the performance of the resulting biosensor were studied in detail. The biosensor responded to H_2O_2 in the linear range from 2×10^{-6} to 1.6×10^{-4} M with a detection limit of 1.2×10^{-7} M (based on the $S/N = 3$). The studied biosensor exhibited good accuracy and high sensitivity. Moreover, the proposed method was economical and efficient.

1. Introduction

Studies of direct electron process between the redox proteins and electrodes has received considerable attention in last few decades [1,2], because it can not only provide us the information to elucidate their metabolic processes in the biological system, but also establish a foundation for constructing the third-generation of electrochemical biosensors and a new kind of bioreactors, in which the redox mediators are no longer needed, and the complications brought about by the mediators are avoided [3,4]. Horseradish peroxidase (HRP) is an important heme-containing enzyme. Because of its known structure and commercial availability, HRP is considered as an ideal model molecule for such study [5]. Unfortunately, because of the deep bury of the electroactive prosthetic groups, the adsorptive denaturation of proteins onto electrodes and the unfavorable orientations at electrodes, HRP generally exhibits a rather slow rate of heterogeneous electron transfer and cannot exhibit electroactivity at conventional electrodes, although their electron transfer is quite fast in biological systems [6,7]. Therefore, it is a challenge to develop matrix to immobilize HRP, which can provide a suitable interface between protein and electrode surface to partly or completely eliminate the denaturation of enzyme molecule on electrode surface and the direct electron transfer rate of protein can be enhanced. Up to now, many materials have been used to modify electrode as a bridge of electron transfer between the redox center of HRP and electrode surface, such as AuNPs [8,9], carbon nanotube [10], dye [11], conducting polymer [12], lipid [13], biopolymer [14], and so on.

Nafion is a perfluorinated sulfonate ionomer which has been widely employed to modify electrodes for a variety of sensor and fuel cell applications [15,16]. Nafion modified electrodes can be easily prepared by solvent casting the polymer directly on the electrode surface and provide several advantages over unmodified electrodes. It has selectivity against anions, which can preconcentrate cations at the electrode surface [17], and acts as a protective coating for the electrode surface. Also, Nafion has excellent properties as an ion conductor, and it provides a biocompatible interface [18].

Room-temperature ionic liquids (ILs), which are composed of ions and are liquid at ambient or even far below ambient temperature, have attracted much attention as novel nonaqueous solvents due to their negligible vapor pressure, extraordinarily high chemical and thermal stability, good conductivity, wide electrochemical windows, no requirement for additional supporting electrolytes, good dissolving capability, etc., and have been widely used in organic synthesis, liquid–liquid extraction, electrochemistry, and inorganic synthesis [19–21]. So far, the application of ILs in electrochemistry has stretched in many areas, such as

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the electrodeposition of metals or semiconductors [22], the electropolymerization of organic compounds [23] and the fabrication of gas sensors and supercapacitors [24].

Agarose is a kind of natural polysaccharide, and has been widely used for immobilization of enzyme [25], antibody [26] and chemical molecule [27] due to its attractive properties of excellent film-forming ability, biocompatibility, non-toxicity, high mechanical strength and cheapness.

To our knowledge, the direct electron transfer of HRP at Nafion/[bmim]PF_6/agarose composite film modified glassy carbon electrode has not yet been reported. In this paper, a novel methodology for construction of HRP-based H_2O_2 biosensor, which combines the merits of Nafion, [bmim]PF_6 and agarose to immobilize HRP, was reported. The direct electron transfer of immobilized HRP and its electrocatalytic response to the reduction of H_2O_2 were studied by cyclic voltammetry. The performance and factor influencing the fabricated biosensor were studied in detail. The experimental results indicated that the proposed biosensor can be directly used for determination of H_2O_2 concentration with an electrochemical method without the aid of an electron mediator. The biosensor exhibited high sensitivity, good repeatability and long-term stability.

2. Experimental

2.1. Reagents

H_2O_2 (30%) was purchased from Beijing Chemical Reagent Company (Beijing, China). Nafion (perfluorinated ion-exchange resin, 5 wt% solution in a mixture of lower aliphatic alcohols and water) (Sigma, USA) was diluted to 1% with ethanol. 1-Butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) was obtained from Chengjie Chemical Co., Ltd (Shanghai, China) and the 2% of [bmim]PF_6 was prepared by ethanol. Agarose and N, N-dimethyl formamide (DMF) were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). HRP was purchased from Xinjingke Biotechnology Co., Ltd (Beijing, China). All other reagents were of analytical reagent grade and used as received.

2.2. Apparatus

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out at a CHI660 electrochemistry workstation (CH Instrumental Co., USA). The electrochemical cell consisted of a three-electrode system where the modified glass carbon electrode (d = 3 mm) was used as the working electrode, a platinum wire as a counter electrode and a saturated calomel reference electrode (SCE) as the reference electrode. All electrochemical experimental solutions were in nitrogen controlled atmosphere. UV–vis spectroscopy was recorded on a UVmini-1240 spectrophotometer (Shimadzu Co., Japan) and FT-IR spectroscopy was obtained using a Bruker Tensor27 Spectrometer (Germany).

2.3. Preparation of HRP/agarose/[bmim]PF_6/Nafion modified GCE

Prior to modification, a glassy carbon electrode (3 mm in diameter) was polished to a mirror with polish paper and alumina pastes of 0.3 and 0.05 μm (CH Instruments, USA), and cleaned thoroughly in an ultrasonic cleaner with 1:1 nitric acid solution, alcohol, and redistilled water, sequentially.

Agarose hydrogel was firstly prepared by homogeneously mixing 600 ethanol, 50 μL agarose, 10 μL 5 mol L^{-1} NaOH and 60 μL water and kept at 4 °C. DMF was then added into the agarose hydrogel according to different proportions (V/V) to make agarose-DMF solution.

To prepare the HRP/agarose/[bmim]PF_6/Nafion modified GCE, 10.0 μL Nafion, 30.0 μL [bmim]PF_6 and 15.0 μL agarose-DMF were coated on the surface of GCE with a microsyringe successively. The succedent film was not allowed to drop onto the GCE until the former film was dried completely under ambient conditions. In the process of drying, a small bottle was fitted tightly over the electrode so that water evaporated slowly and more uniform films were obtained. At last, 15.0 μL of 1 mg mL^{-1} HRP was pipetted onto the surface of the prepared GCE and spread gently over the entire surface. The film was then dried in air overnight to acquire HRP/agarose/[bmim]PF_6/Nafion film modified GCE. The HRP/agarose/[bmim]PF_6, HRP/agarose/Nafion, HRP/[bmim]PF_6/Nafion film modified electrodes were prepared in the similar way as described above. All resulting electrodes were washed with water and stored at 4 °C when not in use.

3. Results and discussion

3.1. Characterization of HRP/agarose/[bmim]PF_6/Nafion modified GCE

EIS measurements can give information on the impedance changes of the electrode surface during the modification process. The semicircle diameter in the impedance spectrum equals the electron transfer resistance, R_{et}. Fig. 1 shows the results of EIS on Bare GCE (a), Nafion modified GCE (b), [bmim]PF_6/Nafion modified GCE (c), agarose/[bmim]PF_6/Nafion modified GCE (d), HRP/agarose/[bmim]PF_6/Nafion modified GCE (e) in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.1 mol L^{-1} KCl solution (pH 7.0), respectively.

Compared to bare GCE, after being covered by certain nafion ethanol solution, a substantial increase in the diameter of the semicircle was observed. The reason was that the nafion could act as the inert electron and mass transfer-blocking layer, and it hindered the diffusion of ferricyanide towards the electrode surface. When the nafion modified GCE was further modified with [bmim]PF_6, it was found that the diameter of the Nyquist circle clearly decreased. A [bmim]PF_6-bridged nafion complex might has formed at the electrode/solution interface and [bmim]PF_6 was critical to facilitate the direct electron transfer attributing to its excellent conductivity. After agarose anchored on the modified GCE, the semicircle increased dramatically (curve d) compared to that of the [bmim]PF_6/Nafion modified GCE (curve c), which

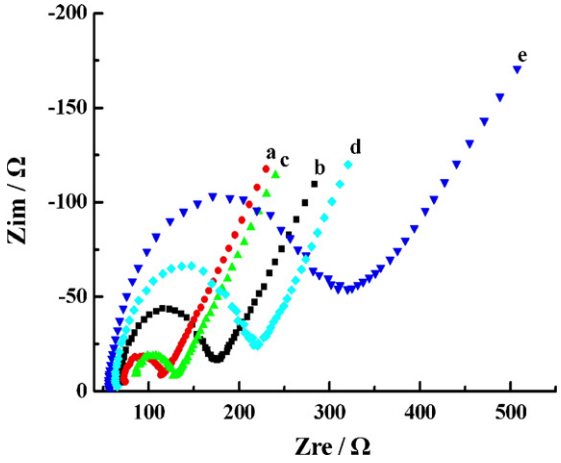

Fig. 1. Nyquist plots of Bare GCE (a), Nafion modified GCE (b), [bmim]PF_6/Nafion modified GCE (c), agarose/[bmim]PF_6/Nafion modified GCE (d), HRP/agarose/[bmim]PF_6/Nafion modified GCE (e) in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ in 0.1 mol L^{-1} KCl solution (pH 7.0), respectively. The frequency range is from 1.0×10^5 to 0.1 Hz at 20 °C, and the perturbation signal is 5 mV.

indicated that agarose layer functioned as barriers that made interfacial charge transfer more difficult. When the HRP molecules were chemisorbed on the modified electrode, it was found that the diameter of the Nquist circle increased again, and it might be caused by the hindrance of the macromolecular structure of HRP to the electron transfer. The above results clearly confirmed the success of the assembly of the electrode.

3.2. Electrochemical behaviors of HRP at the modified GCE

HRP contains heme as a prosthetic group, which is also the active site of protein, and it can catalyze the hydrogen peroxide-dependent one-electron oxidation. The direct electrochemistry of HRP can be achieved under appropriate conditions, and the bioelectrocatalytic activity of HRP is often applied to construct a third-generation biosensor [26]. The electrochemical behaviors of HRP immobilized at the different composite films were investigated. Fig. 2 shows the cyclic voltammograms of HRP/agarose/Nafion (a), HRP/agarose/[bmim]PF_6 (b), HRP/[bmim]PF_6/Nafion (c), HRP/agarose/[bmim]PF_6/Nafion (d) composite films modified GCE in 0.1 mol L^{-1} N_2-saturated phosphate buffer solution (pH 7.0). Almost no cathodic peak was observed at the HRP/agarose/Nafion modified GCE, but one relatively obvious reduction peaks were obtained at the HRP/agarose/[bmim]PF_6 and HRP/[bmim]PF_6/Nafion composite films modified GCE. When the HRP/agarose/[bmim]PF_6/Nafion composite film modified GCE was immersed into 0.1 mol L^{-1} N_2-saturated PBS (pH 7.0), the reduction peak current was obviously increased. The response of HRP/agarose/[bmim]PF_6/Nafion/GCE was attributed to the electroactive center of the immobilized HRP, corresponding to the electrochemical redox reaction. The cathodic peak potential (E_{pc}) was at about −0.35 V. It showed that the agarose/[bmim]PF_6/Nafion composite film formed on the GCE can facilitate the direct electron transfer of HRP.

Fig. 3 displays cathodic peaks for the direct electron transfer behavior of the immobilized HRP on agarose/[bmim]PF_6/Nafion film modified GCE at various scan rates in 0.1 mol L^{-1} PBS (pH 7.0). The cathodic current increased linearly with the scan rate from 20 to 300 mV s^{-1}, as shown in the inset of Fig. 3. For thin layer CV, integration of CV peaks can give the total amount of charge (Q) passed through the electrode for reduction or oxidation of electroactive species in the thin films. Its surface concentration (Γ^*) can be deduced from the following equation: $Q = nFA\Gamma^*$, where n is the number of electron transferred, F is the Faraday constant and A is the electrode area. Integration of CV reduction peaks for HRP/agarose/[bmim]PF_6/Nafion film modified electrode showed that the surface concentration of electroactive HRP (Γ^*) was nearly constant in the range of scan rates from 20 to 300 mV s^{-1}. These results were characteristics of diffusionless, surface-confined electrochemical behavior, in which all electroactive proteins with the heme Fe(III) forms in the films were reduced on the forward cathodic scan, with full conversion of the heme Fe(II) forms back to their oxidized forms on the reversed anodic scan.

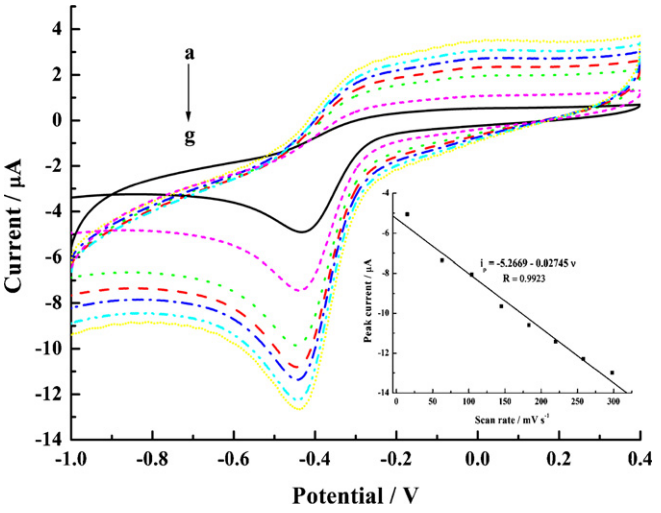

Fig. 3. Cyclic voltammograms of HRP/agarose/[bmim]PF_6/Nafion modified GCE in 0.1 mol L^{-1} PBS (pH 7.0) at different scan rates (from inner to outer) of 20, 60, 140, 180, 220, 260, 300 mV s^{-1}. Inset: plot of cathodic current vs. scan rate.

3.3. Optimization of experimental conditions

Nafion has weak electrical conductivity, too much Nafion would embarrass the electron transfer between the HRP and electrode, and this would decrease the peak current. Fig. 4 shows the effect of the volume of Nafion used to prepare HRP/agarose/[bmim]PF_6/Nafion modified GCE. The cathodic current could achieve the biggest when 5.0 μL of Nafion was used, and it would decrease if went on increasing the volume of Nafion. So 5.0 μL of Nafion was chosen to modify GCE.

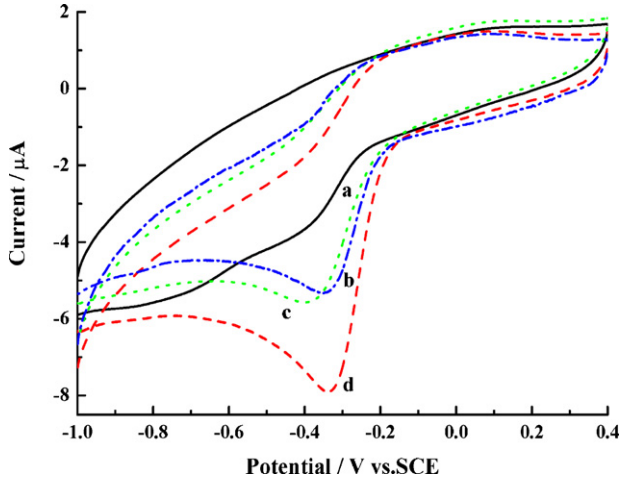

Fig. 2. Cyclic voltammograms of (a) HRP/agarose/Nafion, (b) HRP/agarose/[bmim]PF_6, and (c) HRP/[bmim]PF_6/Nafion, (d) HRP/agarose/[bmim]PF_6/Nafion modified GCE in 0.1 mol L^{-1} PBS (pH 7.0), scan rate = 100 mV s^{-1}.

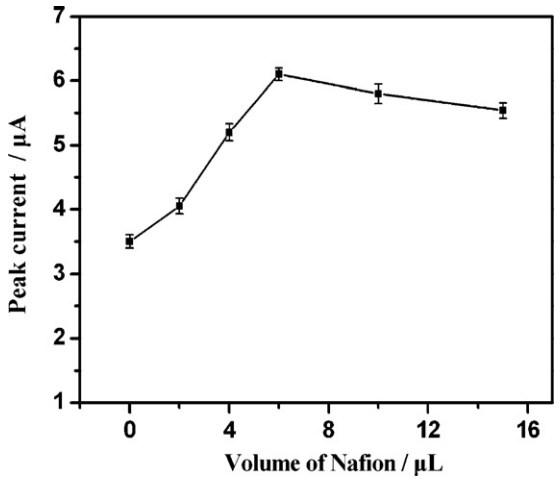

Fig. 4. The influence of the amounts of 1% Nafion on the response of the biosensor in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate = 100 mV s^{-1}.

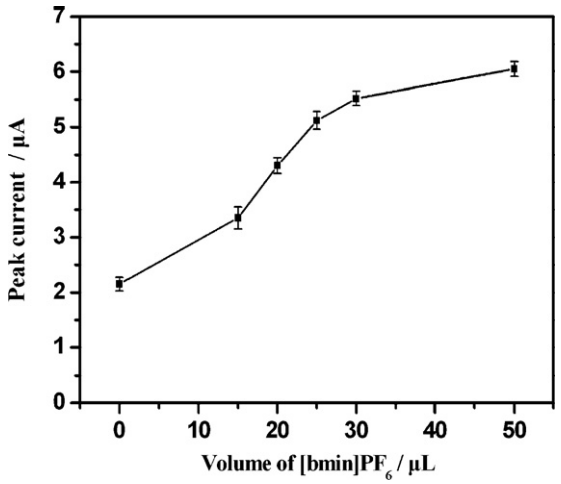

Fig. 5. The influence of the amounts of 2% [bmim]PF_6 on the response of the biosensor in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate = 100 mV s^{-1}.

Fig. 5 shows the effect of the volume of [bmim]PF_6 used to prepare the modified electrode. Because [bmim]PF_6 has good conductivity, a dramatic increase in the cathodic current was obtained when the volume of [bmim]PF_6 increased from 0 to 30.0 μL. However, the cathodic current had almost no change when further increased the volume of [bmim]PF_6. As a result, 30.0 μL of 2% [bmim]PF_6 suspension was therefore used for the fabrication of HRP/agarose/[bmim]PF_6/Nafion modified GCE.

The report of Pang and co-workers [28] has showed that DMF has important influence on the electrochemical behavior of protein in the membrane of agarose hydrogel. With the join of DMF, the environment of HRP was more hydrophobic and a gelatification process of agarose hydrogel was enhanced. This will help to increase the peak current. Herein, the influence of the proportion of agarose to DMF (V/V) was also investigated. Fig. 6 shows that the reduction peak current was very weak when no DMF was contained in the agarose hydrogel. The cathodic current increased quickly with the change of agarose:DMF from 2:1 to 4:1 (V/V). However, the cathodic current decreased if further increased the proportion, and it was found that the protein would precipitate from the hydrogel solution. So the proportion of agarose to DMF (V/V) = 4:1 was used to fabricate the modified electrode.

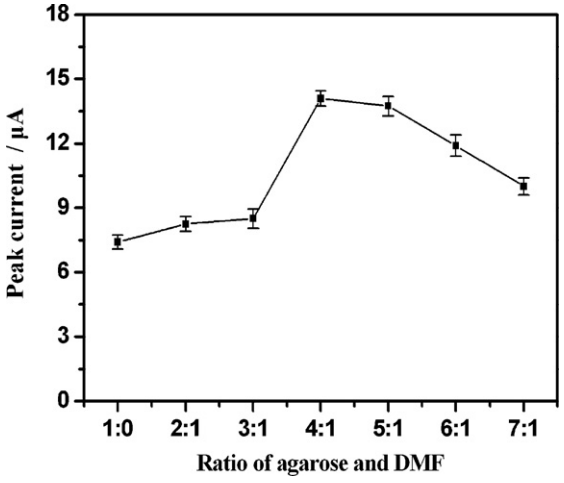

Fig. 6. The influence of the proportion of agarose to DMF on the response of the biosensor in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate = 100 mV s^{-1}.

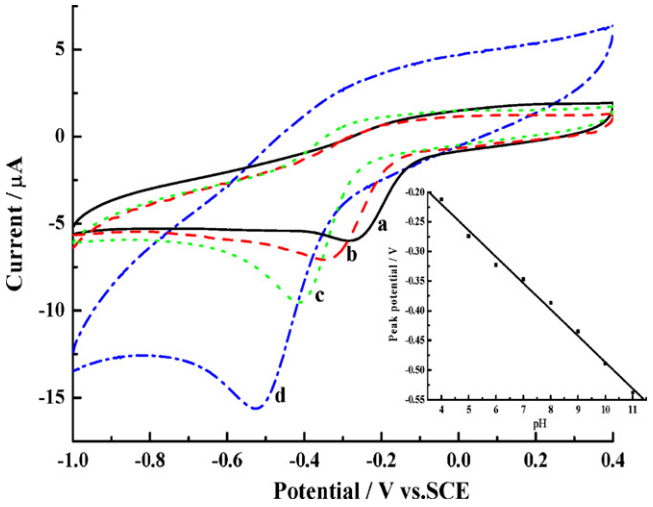

Fig. 7. Cyclic voltammograms of HRP/agarose/[bmim]PF_6/Nafion modified GCE at different pHs. Scan rate = 100 mV s^{-1}. Inset: plot of E_p vs. pH.

3.4. Effect of pH

An increasing of pH value leaded to a negative shift in potential for cathodic peaks of HRP/agarose/[bmim]PF_6/Nafion modified GCE (Fig. 7). Well-defined cathodic peaks were obtained for HRP in the pH range of 4.0–11.0. In this pH range, the cathodic potential (E_p) had a linear relationship with linear regression equation of $E_p = -0.0423 - 0.0514$ pH ($R = 0.9963$) for HRP (inset of Fig. 7), suggesting that the reaction was accompanied by the transfer of protons. The slope value was smaller than the theoretical value of 57.6 mV pH^{-1} at 18 °C for a single-proton coupled, reversible one-electron transfer [29]. The reason might be the influence of the protonation states of trans ligands to the heme iron and amino acids around the heme, or the protonation of the water molecule coordinated to the central iron [30].

3.5. UV–vis spectroscopy

It is important to know whether heme-protein maintain its natural state in agarose/[bmim]PF_6/Nafion composite film. It is well known that position of the Soret absorption band of prosthetic heme group provides information about possible denaturation of heme-proteins [31]. Fig. 8 shows the UV–vis spectra of HRP dry film (a) and HRP in agarose/Nafion (b), agarose/[bmim]PF_6 (c), [bmim]PF_6/Nafion (d), agarose/[bmim]PF_6/Nafion (e) films on optical glass slides, respectively. It was obvious that HRP in agarose/Nafion, agarose/[bmim]PF_6, [bmim]PF_6/Nafion and agarose/[bmim]PF_6/Nafion film all kept nearly the same Soret band as dry HRP film, especially in the film of agarose/[bmim]PF_6/Nafion, indicating that HRP in the composite film well maintained its natural state due to its provision of hydrophilic environment for heme-protein.

3.6. FT-IR spectroscopy

FT-IR spectroscopy can provide some useful information on the structure and conformation of protein molecules. So it was employed to investigate the existing state of the immobilized HRP. The position of the infrared adsorption bands of amide I and amide II provides detailed information on the secondary structure of the polypeptide chain. The amide I band (1700–1600 cm^{-1}) is attributed to the C–O stretching vibrations of peptide linkages in the backbone of protein. The amide II band (1620–1500 cm^{-1}) results from a combination of N–H

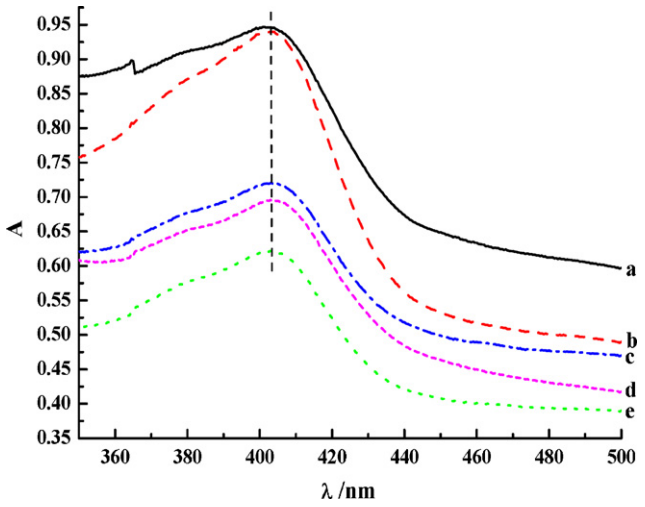

Fig. 8. UV–vis absorption spectra of (a) HRP; (b) HRP-Nafion/agarose; (c) HRP/agarose/[bmim]PF_6; (d) HRP/[bmim]PF_6/Nafion; (e) HRP/agarose/[bmim]PF_6/Nafion films on optical plates.

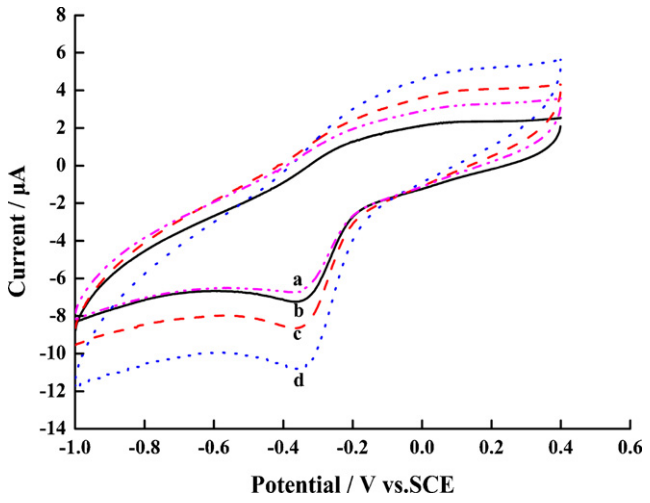

Fig. 10. Cyclic voltammograms of HRP/agarose/[bmim]PF_6/Nafion modified GCE in 0.1 mol L^{-1} PBS (pH 7.0) in the presence of 5×10^{-6} (a), 8×10^{-6} (b), 2×10^{-5} (c) and 5×10^{-5} mol L^{-1} H_2O_2 (d). Scan rate = 100 mV s^{-1}.

bending and C–N stretching. Fig. 9 shows the FT-IR spectra of HRP (a), HRP/agarose/[bmim]PF_6 (b), HRP/agarose/Nafion (c), HRP/[bmim]PF_6/Nafion (d), HRP/agarose/[bmim]PF_6/Nafion (e) composite films. The positions of the amide I (1572.67 cm^{-1}) and amide II bands (1656.20 cm^{-1}) of HRP/agarose/[bmim]PF_6/Nafion film deviated slightly from those obtained from pure HRP (1538.66 and 1653.54 cm^{-1}), respectively. The present data indicated that the major portion of HRP essentially retains its native structure in the HRP/agarose/[bmim]PF_6/Nafion film.

3.7. Electrocatalytic response of hydrogen peroxide at the modified GCE

In view of its simplicity, low-cost and real time detection, electrochemical techniques have been considered ideal for the determination of H_2O_2. Although H_2O_2 is electroactive, the potentials needed are prone to be interfered and therefore an enzymatic route is often preferred.

The HRP in agarose/[bmim]PF_6/Nafion film showed good catalytic activity towards hydrogen peroxide. Fig. 10 shows the bioelectrocatalytic behavior of the enzyme electrode towards different concentrations of H_2O_2 in 0.1 mol L^{-1} PBS (pH 7.0). Compared with the system with no H_2O_2 present, an obvious increase in the CV reduction peak at about −0.36 V was observed when H_2O_2 was added. The reduction peak current increased with the concentration of H_2O_2 enhanced. While at higher H_2O_2 concentration, a response plateau was observed. This was also observed and discussed in detail in previous publications [32,33]. The apparent Michaelis–Menten constant (K_m), which gives an indication of the enzyme-substrate kinetics, can be obtained from the Lineweaver–Burk equation [34]: ${i_{ss}}^{-1} = {i_{max}}^{-1} + K_m \cdot {i_{max}}^{-1} \cdot c^{-1}$, i_{ss} is the steady-state current after the addition of substrate, c is the bulk concentration of substrate, and i_{max} is the maximum current measured. The K_m in this work was found to be 233 μmol L^{-1}, implying that the HRP/agarose/[bmim]PF_6/Nafion modified glassy carbon electrode exhibited high affinity for H_2O_2. As is well known, the smaller K_m shows the higher catalytic ability. Therefore, it clearly showed that the peroxidase activity of entrapped HRP was greatly enhanced. These results indicated that the catalytic current was mainly due to the direct electron transfer between HRP and the electrode. The presence of agarose/[bmim]PF_6/Nafion provided well microenvironment to keep HRP bioactivity and assisted the electron transfer between the protein and the bulk electrode surface.

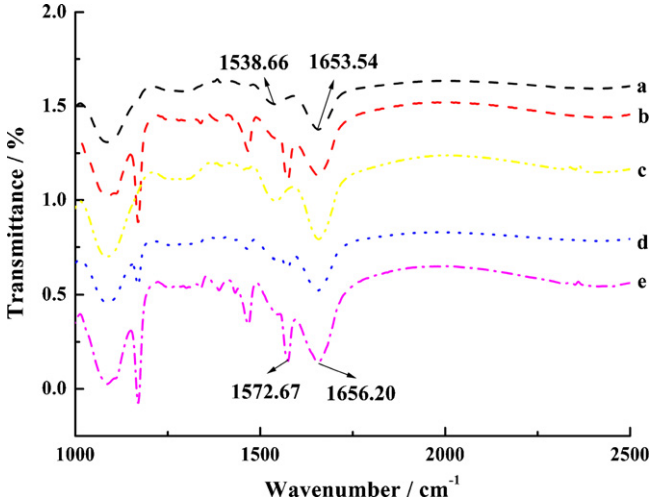

Fig. 9. FT-IR spectroscopy for (a) HRP; (b) HRP/agarose/[bmim]PF_6; (c) HRP/agarose/Nafion; (d) HRP/[bmim]PF_6/Nafion; (e) HRP/agarose/[bmim]PF_6/Nafion films.

The relationship between the reduction peak current and the concentration of H_2O_2 was studied under the optimized conditions. The reduction peak current (i_p) was proportional to the concentration of H_2O_2 over the range from 2×10^{-6} to 1.6×10^{-4} mol L^{-1}, obeying the following equation: $i = 3.2479 + 5.8594c$ ($R = 0.9993$, i in μA, c in mol L^{-1}). The detection limit was 1.2×10^{-7} mol L^{-1} (based on the $S/N = 3$). The high sensitivity of the sensor reflected the high efficiency of the immobilization strategy in which the nature of immobilized HRP in the agarose/[bmim]PF_6/Nafion films was essentially identical with that of native HRP.

The H_2O_2 detection performances of the proposed biosensor were compared with those of HRP biosensors based other matrices and the results were shown in Table 1. It can be seen that the HPR/agarose/[bmim]PF_6/Nafion/GCE offered reasonable linear range for H_2O_2 detection and the detection limit was lower than that of some reported literatures. These indicated that the proposed biosensor was an excellent platform for the detection of H_2O_2.

Table 1
Comparison of performances of proposal biosensor for H_2O_2 detection with those of other biosensors based on differential matrices.

Electrode	Applied potential (V)	Linear range (μM)	Limit of detection (μM)	Reference
HRP–AuNPs–SF/GCE	−0.60	10–1800	5.0	[35]
HRP–ZnO–GNPs–Nafion/GC	−0.30	15–1100	9.0	[36]
HRP/APTMS/colloidal Au/ITO	−0.25	20–8000	8.0	[37]
HRP/nanogold/CCPE	−0.30	12.2–2430	6.3	[38]
HRP-MB/MWNT/GCE	−0.30	4–2000	1.0	[10]
HRP/agarose/[bmim]PF_6/Nafion/GCE	−0.35	2–140	0.12	This paper

GNPS: gold nanoparticles; APTMS: (3-aminopropyl)trimethoxysilane; CCPE: chitosan-entrapped carbon paste electrode; MB: methylene blue; MWNT: multiwall carbon nanotube; SPCE: screen-printed carbon electrode.

3.8. Stability and repeatability of the modified GCE

The stability of the HRP enzyme electrode was studied. The direct electrochemistry of HRP modified electrode could retain constant current values upon continuous cyclic sweep over the potential range from −1.0 to 0.4 V at 100 mV s^{-1}. When the modified electrode was stored in 0.1 mol L^{-1} PBS (pH 7.0) for about 2 weeks, it could retain >96% of its initial current response.

The fabrication reproducibility of the HRP enzyme electrode was also investigated. The fabrication of 10 HRP/agarose/[bmim]PF_6/Nafion modified GCE, made independently, showed an acceptable reproducibility with a relative standard deviation (R.S.D.) of 4.62% for the current determined at a H_2O_2 concentration of 1×10^{-5} mol L^{-1}. Also, the biosensor showed a good reproducibility for the determination of H_2O_2 in its linear range. The concentration of 1×10^{-5} mol L^{-1} H_2O_2 was measured consecutively for 10 times and the R.S.D. of 3.6% was obtained.

4. Conclusions

In this paper, we successfully immobilized HRP on the agarose/[bmim]PF_6/Nafion composite film modified glass carbon electrode by layer-to-layer casting method, which provided a biocompatible microenvironment for the HRP, and it gave the HRP molecules more freedom in orientation and thus increased the possibility that the cofactor of the enzyme approached the metal particle surface, reduced the insulating property of the protein shell for the direct electron transfer reaction. UV–vis and FT-IR spectroscopy indicated that HRP retained its secondary structure in the film. The modified electrode showed an excellent electrocatalytic activity towards the reduction of H_2O_2 along with good stability and repeatability. Based on the composite film, a high-powered third-generation reagentless biosensor could be constructed for the determination of H_2O_2.

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