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Direct electrochemistry and electrocatalysis of hemoglobin on carbon ionic liquid electrode

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ABSTRACT

Hemoglobin in agarose was successfully immobilized on a carbon ionic liquid electrode and the direct electrochemical behavior of hemoglobin was investigated. Room temperature ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was used as the modifier. Ultraviolet–visible spectroscopy, Fourier transform infrared spectroscopy and cyclic voltammetry were used to characterize the hemoglobin on the modified electrode. The results showed that the immobilized hemoglobin retained its bioelectrocatalytic activity. The electrochemistry of hemoglobin provided an opportunity to manufacture a third generation of biosensors. Experimental conditions influencing the biosensor performances such as pH, and potential were optimized and assessed. Under the optimal conditions, hydrogen peroxide was detected in the concentration range from 2×10^{-6} to 1.2×10^{-3} M with a detection limit of 0.2 μM at S/N = 3. The apparent Michaelis–Menten constant was 1.495 mM. The biosensor exhibited some advantages, such as short time respond, high sensitivity, good reproducibility and long-term stability.

1. Introduction

The rapid and accurate detection of hydrogen peroxide (H_2O_2) is of great interest to biochemists because it is not only the product of the reactions catalyzed by many highly selective oxidases but also an essential compound in food, pharmaceutical and environmental analyses [1]. Many methods have been developed for hydrogen peroxide analysis including titrimetry, photometry, chemiluminescence, high performance liquid chromatography and electrochemistry. Among these, amperometric enzyme-based biosensors have received considerable interest because of its high sensitivity, convenience and high selectivity.

For the enzyme-based biosensor biosensors, horseradish peroxidase (HRP) [2,3] is the most commonly used enzyme. But the HRP is not very stable in solution and is expensive. Alternatively, hemoglobin is one of the promising biomolecules that can be used as a bioreceptor element. In contrast to most other proteins, Hb can give long-term stability to the H_2O_2 sensor as compared to other proteins. In addition, Hb is cheaper than the commonly used HRP. Also, as one of the most widely studied proteins, Hb is usually used as model system to study electron transfer properties of proteins and provide the information for constructing biomedical devices, biosensors and bioreactors [4,5]. For this goal, many approaches have been developed for the entrapment of proteins with different modified films. Lim et al. reported a Pd-CNT-based glucose biosensor [6]. Yang et al. fabricated a cholesterol biosensor based on layer-by-layer self-assembled multilayer films of CNTs and platinum nanoparticles [7]. Jitianu et al. prepared the nanocomposites of MWNT and TiO_2 by sol–gel method and used them to immobilize protein [8]. In addition, Liu et al. reported the self-assembly of gold nanoparticles to CNTs using thiol-terminated pyrene as interlinker which can facilitate the direct electron transfer between the protein and the electrode [9]. So far, the immobilizing matrix is still an important factor in achieving the direct electrochemistry of proteins.

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 [10,11]. So far, the application of ILs in electrochemistry has stretched in many areas, such as the electrodeposition of metals or semiconductors [12], the electropolymerization of organic compounds [13] and the fabrication of gas sensors and supercapacitors [14].

Agarose is a kind of natural polysaccharide, and has been widely used for immobilization of enzyme [15], antibody [16] and chemical molecule [17] due to its attractive properties of

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excellent film-forming ability, biocompatibility, non-toxicity, high mechanical strength and cheapness.

The use of solid materials, such as composite electrodes, as detectors for electrochemical analysis is commonly known [18]. A composite electrode results from the combination of two or more materials. Carbon paste electrode is a type of composite electrodes, which is made up of carbon particles and organic liquid [19]. It has the advantages of the diversity of paste modification and the convenience in manipulation, providing a reliable and universal method for immobilization of active species, and has been widely applied in the electroanalytical community due to its low cost, ease of fabrication, high sensitivity for detection and renewable surface [20,21].

In this work, a room temperature ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) was used in the preparation of carbon ionic liquid electrode (CILE) to accelerate the rate of electrons transfer of Hb, and agarose was used as a protein immobilizing material. The methods for construction of the biosensor were very simple and convenient. To the best of our knowledge, this is the first time that the direct electrochemistry of Hb was investigated with this sensor. We also believed that this is the first time that a hydrogen peroxide biosensor was designed based on a Hb-agarose-modified CILE, with the [bmim]PF_6 performing a very important role of enhancing the electrons transfer rate of Hb.

2. Experimental

2.1. Reagents

H_2O_2 (30%) was purchased from Beijing Chemical Reagent Company (Beijing, China). 1-Butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF_6) was obtained from Chengjie Chemical Co., Ltd. (Shanghai, China). Agarose and N,N-dimethyl formamide (DMF) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Hemoglobin (Hb) was obtained from Xinjingke Biotechnology Co., Ltd. (Beijing, China). High purity of graphite powder (Shanghai Carbon Plant, Shanghai, China) was used without further treatment. Ultrapure water (18.2 MΩ) was obtained from a Milli-Q water purification system and used throughout. All other reagents were of analytical reagent grade and used as received.

2.2. Apparatus

Cyclic voltammetry and electrochemical impedance spectroscopy were carried out at a CHI660 electrochemistry workstation (CH Instrumental Co., USA). The electrochemical cell consisted of a three-electrode system where the modified carbon paste 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 UVmini-1240 spectrophotometer (Shimadzu Co., Japan) and FT-IR spectroscopy was obtained using Bruker Tensor 27 Spectrometer (Germany).

2.3. Preparation of biosensor

The CILE was prepared as follows: graphite powder and [bmim]PF_6 (4:1, w/w) were mixed in a mortar until a homogeneous paste was obtained. The prepared carbon paste was tightly packed into a PVC tube (internal diameter of 3 mm) and a cope wire was introduced into the other end for electrical contact. Prior to use, the surface of the well-prepared electrode was smoothed with a weighing paper.

For agarose solution, 0.2 g of agarose was dissolved into 100 mL hot water. After being dissolved completely and cooled to the room temperature, certain DMF was added into agarose hydrogel with different ratio (v/v). The prepared solution was kept at 4 °C before used.

To prepare the Hb/agarose modified CILE (Hb/agarose/CILE), firstly, 6.0 μL agarose solution was coated onto the surface of CILE with a microsyringe. When dried at room temperature, a small bottle was fitted tightly over the electrode so that water could be evaporated slowly and a uniform film could be formed. Secondly, 10.0 μL of 10 mg mL^{-1} Hb was pipetted onto the surface of the agarose modified CILE. The process of drying was the same as above. The resulting Hb/agarose/CILE was washed with water and stored at 4 °C when not in use.

3. Results and discussion

3.1. UV–vis spectroscopy

It was important to know whether heme-protein maintained its natural state in composite film. It was well known that position of the Soret absorption band of prosthetic heme group provided information about possible denaturation of heme-proteins [22]. The UV–vis absorption spectroscopy was employed in this experiment for the conformation study of Hb in composite film (Fig. 1). It was obvious that the Soret bands of Hb alone were at about 408 nm, and 411 nm when Hb was immobilized in CILE. When Hb was immobilized in agarose/CILE, the Soret band of Hb was at 409 nm. These indicated that there was no change of Soret band in substance and the native state of Hb was well retained in CILE and agarose/CILE.

3.2. FT-IR spectroscopy

FT-IR was a surface infrared technique ideally suited for the study of the proteins on a solid substrate surface and here was also used to monitor the conformations of Hb in composite film. The shape of the infrared adsorption bands of amide I and amide II provided detailed information on the secondary structure of the polypeptide chain. The amide I band (1700–1600 cm^{-1}) was attributed to the C–O stretching vibrations of peptide linkages in the backbone of protein. The amide II band (1600–1500 cm^{-1}) resulted from a combination of N–H bending and C–N stretching. Fig. 2 shows the FT-IR spectra of Hb (a), Hb/CILE (b), and Hb/agarose/CILE (c). The pure Hb film showed the amide I band at

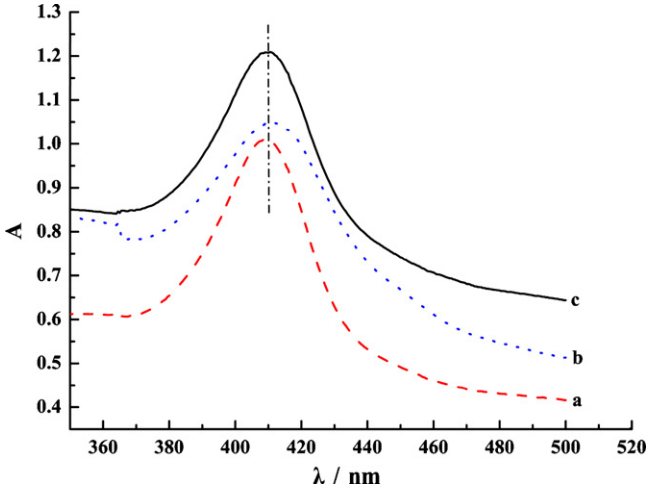

Fig. 1. UV–vis absorption spectra of Hb (a), Hb/CILE (b) and Hb/agarose/CILE (c).

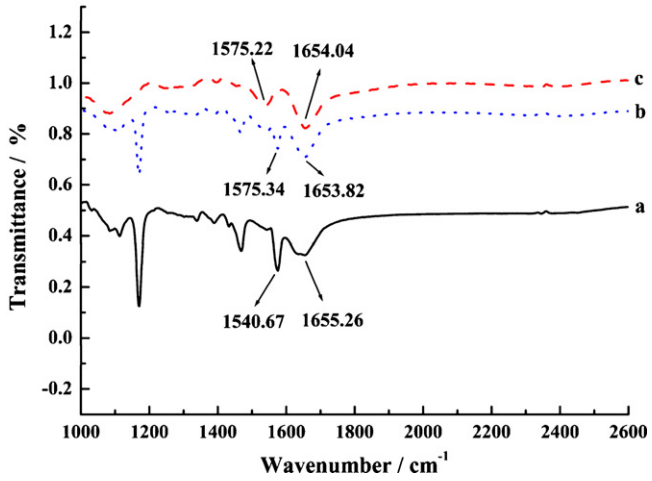

Fig. 2. FT-IR spectra of Hb (a), Hb/CILE (b), and Hb/agarose/CILE (c).

1655.26 cm^{-1} and amide II band at 1540.67 cm^{-1} (Fig. 2a), corresponding to 1653.82 and 1575.34 cm^{-1} of Hb in the CILE (Fig. 2b) as well as 1654.04 and 1575.22 cm^{-1} in the Hb/agarose/CILE (Fig. 2c), which were some slight deviation to the values from those obtained from Hb itself. The results suggested that Hb well retained the essential feature of its secondary structure on the CILE and agarose/CILE.

3.3. Electrochemical behaviors of Hb at the agarose modified CILE

The direct electrochemistry of Hb could be achieved under appropriate conditions, and the bioelectrocatalytic activity of Hb was often applied to construct a third-generation biosensor. The electrochemical behaviors of Hb immobilized at the different films were investigated. Cyclic voltammograms of different modified electrodes in a 0.1 mol L^{-1} N_2 saturated PBS (pH 7.0) are shown in Fig. 3. No voltammetric response was observed on the CILE (a) and agarose/CILE (c), which indicated no redox reaction took place. The cyclic voltammogram of the CILE showed a relative flat curve of charging current. However, the charging current of CILE was obviously bigger than that at the carbon paste electrode which made of graphite powder and paraffin oil (data not shown). The findings were closely related to the replacement of non-conductive organic binder with the conductive ionic liquid (IL). On one hand, the IL had large quantities of caves within its molecule structure which was

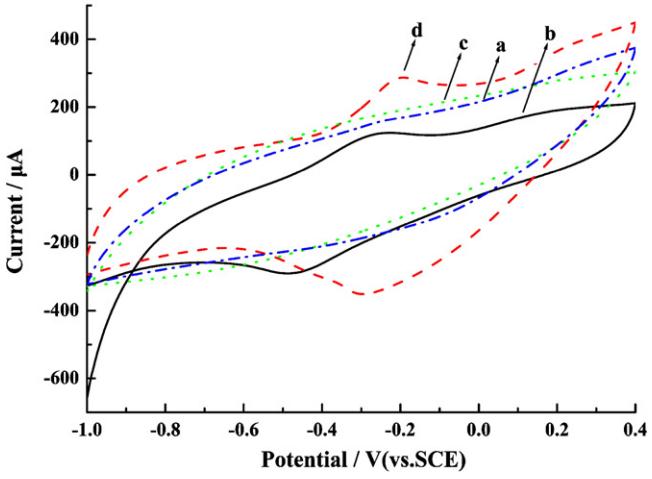

Fig. 3. Cyclic voltammograms of CILE (a), Hb/CILE (b), agarose-DMF/CILE (c) and Hb/agarose-DMF/CILE (d) in 0.1 mol L^{-1} PBS (pH 7.0), scan rate = 100 mV s^{-1}.

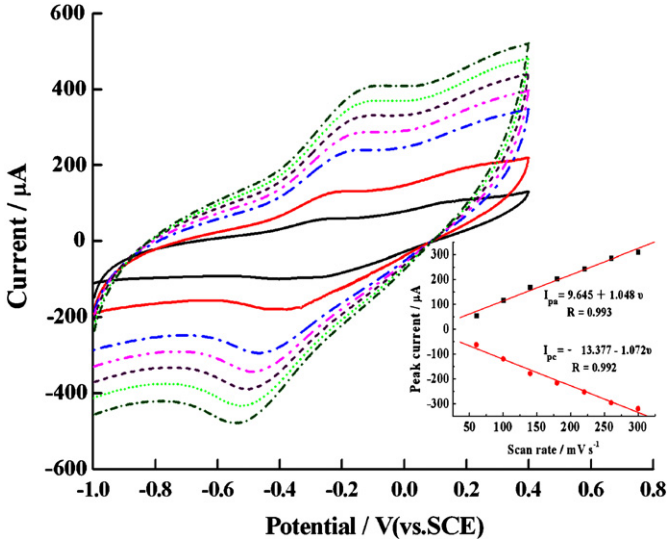

Fig. 4. Cyclic voltammograms of Hb/agarose/CILE in 0.1 mol L^{-1} PBS (pH 7.0) at different scan rates of 60, 100, 140, 180, 220, 260, 300 mV s^{-1} (from inner to outer). Inset: plots of cathodic and anodic peak current with the scan rate.

facile to hold more charges. On the other hand, its good conductive performance facilitated fast transportation and thus reduced surface diffusion capacitance. Therefore, the IL played an important role in promoting electrochemical performance of CILE. While on the Hb/CILE (b), there was a pair of very small redox peaks. However, to the Hb/agarose/CILE (d), a pair of more apparent anodic and cathodic peaks appeared which locate at about −0.2 and −0.3 V (vs. SCE), respectively. The improved electrochemical responses could be attributed to the direct electron transfer of Hb with the help of CILE and agarose which greatly facilitated the electron transfer, corresponding to the electrochemical redox reaction between HbFe(III) and HbFe(II).

Fig. 4 shows the cyclic voltammograms of Hb/agarose/CILE at different scan rates from 60 to 300 mV s^{-1} in PBS (pH 7.0). It could be seen that the redox peak currents increase and the redox peak potentials shift simultaneously with the increase of scan rates, and the peak-to-peak separation become greater, indicating the more irreversible of the electrode process. The redox peak currents had almost the same values in the selected range of scan rates, which indicated that all the electroactive ferric hemoglobin HbFe(III) was reduced to ferrous hemoglobin HbFe(II) on the forward scan and the HbFe(II) was reoxidized to HbFe(III) on the reverse scan. The redox peak currents increased linearly with the scan rate from 60 to 300 mV s^{-1}, as shown in the insert of Fig. 4. The relationships of the redox peak currents (I_p) with scan rate (υ) were further investigated with two well-defined straight lines got as I_{pa} (μA) = 9.645 + 1.048υ (mV s^{-1}) (R = 0.993) and I_{pc} (μA) = 13.377 − 1.072υ (mV s^{-1}) (R = 0.992), respectively. The experimental results proved that this redox process was a typical diffussionless surface-controlled electrode process [23].

The dependence of the electrochemical behavior of the biosensor on the pH of the working solution was studied at various pH values ranged from 4.0 to 10.0. It was found that an increase of pH in solution led to a negative shift in potential of both cathodic and anodic CV peaks for Hb/agarose/CILE. The cathodic potential (E_{pc}) had a linear relationship with the linear regression equation of E_{pc} = 0.1064 − 0.068 pH (R = 0.996) for Hb, it showed that the reaction was accompanied by the transfer of protons. The slope value was a little bigger than the theoretical value of 59 mV pH^{-1} at 20 °C for a single-proton coupled, reversible one-electron transfer [24]. This might be because of the influence of protonation states of trans-ligands to the heme iron and amino acids around the heme,

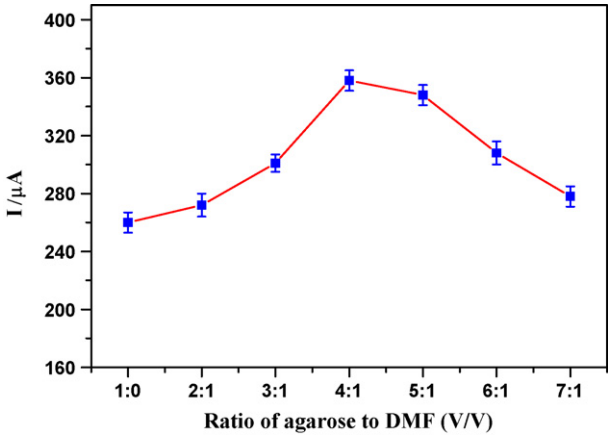

Fig. 5. The influence of Hb/agarose/CILE containing different ratio of agarose to DMF (v/v). (a) without DMF, (b) 2:1, (c) 3:1, (d) 4:1, (e) 5:1, (f) 6:1, (g) 7:1, and (h) 8:1.

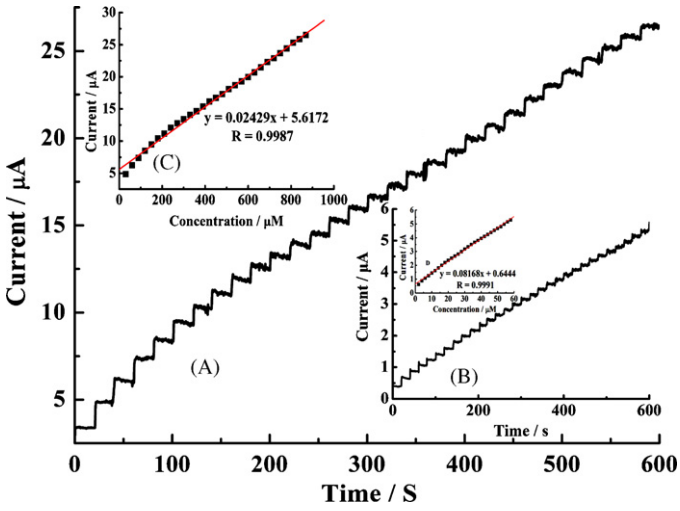

Fig. 6. Amperometric response of Hb/agarose/CILE to H_2O_2, conditions: −300 mV constant potential, pH 7.0; (A) successive addition of 30 μM and (B) 2 μM (inset: the calibration curve of the biosensor).

or the protonation of the water molecule coordinated to the central iron [25].

3.4. Optimum conditions of preparation of the biosensor

The optimization of the condition to prepare the ion liquid modified electrode was studied. The mass ratio of graphite powder to ion liquid including 7:1, 6:1, 5:1, 4:1, 3:1 and 2:1 were investigated, respectively. When [bmim]PF_6 was insufficient, the graphite powder could not be mixed well-proportioned and the mixture of [bmim]PF_6 and graphite powder was easy to brush off from the electrode. However, the mechanical strength of the carbon paste would be too weak and the carbon paste was difficult to shape if too much [bmim]PF_6 was employed. Considering the above problems, the mass ratio of 4:1 was used.

The influence of the ratio of agarose to DMF (v/v) has also been studied (Fig. 5). The peak currents increased quickly while enhancing the ratio of agarose to DMF from 2:1 to 4:1 (v/v). However, the smaller of the peak currents were obtained when the ratio of agarose to DMF was further increased. As mentioned above, the volume ratio 4:1 of agarose to DMF could act as excellent reagent besides as a binder, which benefits the accumulation of Hb to the electrode. However, the too low and too high volume ratio will go against the electron transfer and mass transfer. Withal, the background currents also increased with the proportion of agarose to DMF (v/v) enhancement. When the proportion was about 4:1 (v/v), the signal-to-background ratio was the highest. So, the ratio of agarose to DMF was fixed at 4:1 (v/v) in this work.

3.5. Optimum conditions of the biosensor for detecting H_2O_2

The catalytic properties of the sensor towards H_2O_2 were studied. When 1.0×10^{-4} M H_2O_2 was added into the electrolyte solution, an obvious increase of the cathodic peak current and the ultimate disappearance of the anodic peak current were observed. However, no similar cathodic peak corresponding to the reduction of H_2O_2 could be observed at CILE and agarose modified CILE electrode under the same condition, so it could be concluded that Hb immobilized on agarose modified CILE showed good catalytic activity toward hydrogen peroxide. Also, this obvious increase in the cathodic current indicated that the Hb kept its natural structure after immobilizing onto the agarose modified CILE.

The pH value was one of the most important factors for working with biosensors that depend on the response of the electrode to H_2O_2. Thus, the effect of pH on the sensor response in the presence of 1.0×10^{-4} mol/L H_2O_2 was investigated between 4.5 and 9.0 in 0.1 M PBS by cyclic voltammetry. Experimental results revealed that the current response increased from pH 4.5 to 7.0 and attained its maximum level at pH 7.0, and decreased from pH 7.0 to 9.0. The reason might be that a strong acidic or alkaline solution would decrease the bioactivity of Hb, leading to an obvious decrease in the response current. Therefore, pH 7.0 was chosen to be used for the working media solution to determine H_2O_2 of this sensor.

The influence of applied potential on the current response of the biosensor in the presence of 1.0×10^{-4} M H_2O_2 was also investigated in the range from 0 to −500 mV. Experimental results revealed that the current response increased sharply from 0 to −300 mV, and reach the maximum at −300 mV, then slightly decreased at more negative potentials than −300 mV. Therefore, a constant potential of −300 mV was adopted to get the maximum sensitivity.

3.6. Amperometric detection of H_2O_2 at Hb/agarose/CILE

The amperometric response to H_2O_2 at the Hb/agarose/CILE was investigated by successively adding H_2O_2 to a continuous stirring PBS solution under the optimized conditions. Fig. 6 shows the steady-state amperometric response of the biosensor to the successive addition of aliquot H_2O_2 into the PBS (pH 7.0) with an operating potential of −300 mV. When 30 μM H_2O_2 was added, the background current was changed and the reduction current rose steeply to reach the maximum value. This sensor could achieve more than 95% of the steady-state current within 3 s, indicating a fast amperometric response to H_2O_2 reduction. Also, the increasing H_2O_2 concentration resulted in an increase of the amperometric response. Fig. 6 (inset) shows the calibration plot obtained for the Hb/agarose/CILE. Under the optimized experimental parameters, the sensor showed a linear response within the range of 2×10^{-6} to 1.2×10^{-3} M of H_2O_2. The calibration plot over the concentration range 2–58 μM (29 points) had a slope of 81.68 nA μM^{-1} and a correlation coefficient of 0.999. The detection limit was determined to be 2×10^{-7} M (based on the S/N = 3). The performance of the proposed biosensor was compared with other biosensors. The results are shown in Table 1.

The apparent Michaelis–Menten constant K_m^{app}, which gives an indication of the enzyme–substrate kinetics, can be obtained from the Lineweaver–Burk equation: $1/I_{ss} = 1/I_{max} + K_m^{app}/I_{max} \cdot 1/C$, where I_{ss} is the steady-state current after the addition of substrate, C the bulk concentration of the substrate and I_{max} is the maximum

Table 1
The performances of the various types of modified electrodes.

Modified electrode	Linear range (μM)	LOD (μM)	Stability[a] (percentage/days)	Reference
HRP in nano-Au/carbon ceramic electrode	12.2–1100	6.1	75%/35	[26]
Hb in nano-Au/ITO	10–7000	4.5	75%/40	[27]
Hb in egg phosphatidylcholine/pyrolytic graphite electrode	10–100	3.90	90%/14	[28]
Hb in silica sol–gel/carbon paste electrode	1–280	0.86	93%/21	[29]
Hb in carbon nanotube powder microelectrodes electrodes	210–900	9.00	Not reported	[30]
Hb in thiolated-viologen/Au electrode	10–125	0.25	90%/30	[31]
Hb in CHT/nano-$CaCO_3$/GCE	37–830	8.30	Not reported	[32]
Hb in agarose/CILE	2–1200	0.20	95%/20	This work

[a] The biosensor retained percentage of its initial response after a storage period.

Table 2
Measurement of H_2O_2 level in disinfecting preparation.

Sample	Proposed biosensor ($g\,L^{-1}$)	RSD (%)	$KMnO_4$ titration method ($g\,L^{-1}$)	RSD (%)
1	30.12	4.2	30.25	3.3
2	32.34	3.6	32.47	3.2
3	35.12	3.8	35.45	2.1
4	31.65	4.2	31.50	2.2
5	33.25	2.5	33.10	2.5

current measured under saturated substrate conditions. K_m^{app} can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus H_2O_2 concentration. The K_m^{app} value for the electrocatalytic activity of Hb/agarose/CILE to H_2O_2 was determined to be 1.495 mM, which implied that the present electrode exhibited a higher affinity for H_2O_2. As was well known the smaller K_m^{app} showed the higher catalytic ability. The low value of K_m^{app} indicated that Hb immobilized in Hb/agarose film retained its bioactivity and had a high biological affinity to H_2O_2.

3.7. Real sample analysis and selectivity against interferences

The real analytical applicability of the proposed biosensor was examined by the determination of H_2O_2 level in regent H_2O_2. The results obtained by the biosensor were compared with those determined by the classical $KMnO_4$ titration method (Table 2).

The effect of some substances that interfered with the response of the proposed biosensor was also evaluated. The interfering substance was added to the PBS containing 0.1 mM H_2O_2. The results showed that 0.2 mM of glucose, acetic acid, ethanol, L-cysteine, L-tyrosine and citric acid have almost no influence on the current response of H_2O_2 (signal change below 5%).

3.8. Repeatability and stability of the Hb/agarose/CILE

To examine the repeatability and stability of the proposed method, the direct electrochemistry of Hb/agarose/CILE was investigated by CV. The current responses decreased less than 5% over 200 cycles at a scan rate of 100 mV s^{-1} from −1.0 to 0.4 V. The modified electrode was stored in 0.1 mol L^{-1} PBS (pH 7.0) for about 20 days. The peak current of a 5 μM H_2O_2 solution was recorded using the same method every two days. The results showed that the current response was almost unchanged (data changed $\leq$ 5%). After 20 days, 95% of its initial current response was obtained, indicating the composite film was enough efficient for Hb bioactivity retention and Hb molecule leakage prevention. When a 5 μM H_2O_2 solution was determined with six modified electrodes made independently, the relative standard deviations for amperometric response current were 4.38%. It indicated that the protein electrode had an acceptable reproducibility.

4. Conclusions

A room temperature ionic liquid modified carbon paste electrode was constructed based on the substitute of paraffin with l-butyl-3-methyl imidazolium hexafluorophate ([bmim]PF_6) to mix with certain graphite power. The electrochemical response could be greatly improved with the high conductivity of RTIL and the modified CILE showed good mechanic property and stability. Besides this, the usage of agarose could not only increase the activity and stability of Hb because of the good biocompatibility of its film, but also could provide a join in the electrode reaction of hemeproteins owing to its hydrogen ion. The Hb incorporated in the composite film can catalyze the reduction of hydrogen peroxide and therefore a high-powered third-generation reagentless H_2O_2 biosensor was constructed along with good stability and repeatability.

Acknowledgments

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