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Botanical micelle and its application for direct electrochemical biosensor

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ABSTRACT

This paper is concerned about the entrapment of horseradish peroxidase (HRP) within botanical inositol hexakisphosphoric (IP_6) micelles for the preparation of enzyme biosensor. The good affinity of IP_6 micelles with the enzyme provides naturally biocompatible microenvironment for the enzyme immobilization, achieving the direct electron transfer between HRP and electrode surface. The resulting biosensor to H_2O_2 detection exhibits a low detection limit of 0.1 μmol L^{-1} (S/N = 3), a quick response time (3 s), and a long-term stability. The apparent Michaelis–Menten constant is quite tiny about 0.0016 mmol L^{-1}.

1. Introduction

Accurate, rapid, and reagentless determination of hydrogen peroxide (H_2O_2) is very important in fields of food, industry, environmental protection, clinical control and so on (Kulys et al., 1993). Based on its simplicity, high selectivity and intrinsic sensitivity, the electrochemical tracking of biological targets by way of enzyme-based H_2O_2 detection is of special attention (Yi et al., 1999; Lei et al., 2003; Song et al., 2006). Studies on direct electron transfer process between the enzymes and the electrodes cannot only provide dynamic information to elucidate their metabolic processes in the biological system, but also can establish a foundation for constructing the third generation of electrochemical biosensors, in which the redox mediators are no longer needed to avoid the complication of fabrication (Shan et al., 2007). Horseradish peroxidase (HRP), a heme enzyme, is generally used as a representative to investigate the dynamic and thermodynamic mechanism of the enzyme reactions and develop biosensors for the determination of H_2O_2 or related compounds. During the fabrication of biosensors, the strategy employed for immobilizing the active biocatalyst constitutes one of the crucial aspects to be considered because enzymes can be easily inactivated or released from the electrodes if not properly immobilized (Lu et al., 2010). In order to achieve the direct electron transfer (DET) between HRP and electrode, extensive works have been done over the decades. Analysts in this field are always enthusiastic in finding new non-toxic and highly conductive materials for biosensor applications and design of diagnostic kits (Kandimalla et al., 2006). The development of materials science has brought a great momentum to bioelectroanalysis.

Biocompatible nanomaterials seem to be competitive candidates due to their unique advantages such as large surface-to-volume-ratio, high surface reaction activity, high-catalytic activity and strong adsorption ability for enzyme immobilization. Furthermore, biocompatible nanomaterials-based enzyme sensor renders the microenvironment for keeping biological activity and enhancement of the DET between the enzymes active sites and the electrode (Gorton et al., 1999; Jia et al., 2002). It has been reported that micelle-based amplification platforms dramatically enhance the intensity of the electrochemical signal and lead to ultrasensitive bioassays. Immobilizing proteins or enzymes with micelles has become a popular surface derivatization procedure. The reason for this is mostly ascribed to its easy preparation and to increase the loading of electroactive species for signal amplification. As a desire, the modified electrode formed by this platform has the advantages of high organization and uniformity, which could provide a protein immobilization microenvironment and facilitate DET from the electroactive protein to the underlying electrode (Reviejo et al., 1994; Ortiz et al., 1997; Serra et al., 1999). However, so far, the reported micelles are mainly from chemical surfactant agents or detergents.

Inositol hexakisphosphoric (IP_6), a kind of naturally occurring specie as the principal storage form of phosphorus in many plant tissues, especially in brans and seeds, presents unique benefits such as biocompatibility, nontoxicity, and “green” to the environment. These properties constitute favorable conditions for enzyme or protein immobilization (Moraes et al., 2008). IP_6 has six phosphates (see Supplementary Fig. S.1) to react easily with the enzyme. Hence, IP_6 should be used to capture enzyme as a molecular binder instead of employing a polymeric binder (Crea et al., 2008; McKenzie et al., 2002). Quite recently, we found that IP_6 molecules could

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self-assemble into the micelle in sphere structure through some phosphates together inner and some free phosphates remaining on the outside of the sphere under boiling condition (Wang et al., 2009). This unique structure is very similar to the structure of biological membranes, in which the constituent lipids are arranged in the tail-to-tail configuration with the hydrophilic head groups toward outside and proteins are adsorbed onto the surface of or imbedded into the layers (Rusling, 1998; Rusling and Nassar, 1993; Hu et al., 2007). It is an expectation that the free phosphates outer of the micelle shell could conjugate with HRP to fabricate HRP–IP_6 micelle biocomposites. Therefore, botanical IP_6 micelle is expected to offer a promising template for enzyme-based biosensor fabrication because of its satisfying biocompatibility and well-distribution in water for easy spread onto substrate.

In the present work, we constructed a HRP biosensor on glassy carbon electrode (GCE) with the IP_6 micelle film. Herein, two featured points are worth emphasizing. First, botanical IP_6 micelles were utilized as a matrix, exhibiting strong affinity for sufficient HRP immobilization as well as the large nanoscale surface. Second, the stable direct electron transfer of HRP was successfully accomplished and high sensitivity to detection of H_2O_2 was reached.

2. Experimental

2.1. Reagents

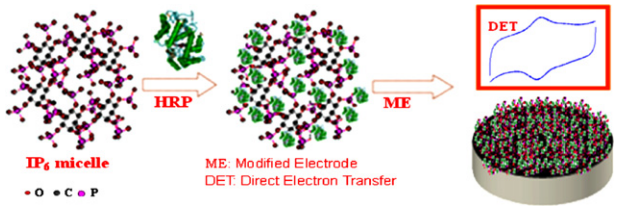
Lyophilized HRP (E.C.1.11.1.7, RZN 3.0, A > 250 U mg^{-1}) was purchased from Shanghai Biochemical Reagents. Dodecasodium of Phytic acid (denoted as $Na_{12}IP_6$) Potassium ferricyanide and Nafion (5% in a mixture of lower aliphatic alcohols and water) were obtained from Sigma. The 0.1 mol L^{-1} phosphate buffer saline (PBS) was prepared with KH_2PO_4, Na_2HPO_4 and KCl. The different pH values were adjusted by adding 0.1 mol L^{-1} H_3PO_4 or NaOH solution. Hydrogen peroxide (30%, w/v solution) was obtained from Shanghai Runjie Chemical Reagent Company. The disinfector was purchased from Shanghai Jinlu Chemical Company. The H_2O_2 solution with different concentrations was freshly prepared for each experiment. Deionized water was used in this experiment throughout. All chemicals were of analytical grade and used without further purification.

2.2. Instrumentations and measurements

A transmission electron microscopy (TEM) image was observed with TEM (JEM-2100, Japan). Field emission scanning electron microscopy (FESEM) (Hitachi S-4800) was used for imaging the topography. Ultraviolet/visible (UV/vis) absorbance spectrum was performed on a UV/vis near-infrared spectrophotometer (UV-8500, Techcomp). Electrochemical measurements were performed with CHI 660D electrochemical workstation (Shanghai CH Instruments, China). A conventional three-electrode system was employed with a modified glassy carbon electrode (Ø = 3 mm) as the working electrode, a platinum flake and a saturated calomel electrode (SCE) as the auxiliary and reference electrodes, respectively. Buffer solutions were purged with highly purified nitrogen for at least 15 min before electrochemical experiments. All glass wares used in the following procedures were cleaned in a bath of freshly prepared solution (1:3 HNO_3/HCl), and thoroughly washed with deionized water.

2.3. Preparation of IP_6 micelles and electrode fabrication

Similar to the previous report (Wang et al., 2010a), IP_6 micelles were prepared with a little modification. 1 mmol L^{-1} $Na_{12}IP_6$ solution was allowed to reflux at 90 °C for 20 min, and then cooled down at room temperature to induce the self-assembly of micelle.



Scheme 1. A schematic fabrication process of Nafion/HRP/IP_6 micelles/GCE.

The GCE was polished by 0.3 and 0.05 μm α-Al_2O_3 powders followed by thoroughly washed with deionized water, and then sonicated in deionized water and ethanol, dried with nitrogen. 5 μL of the IP_6 micelle solution was cast evenly onto the surface of GCE and then dried on the air. After that, 3 μL of the HRP solution was dropped onto the surface of GCE with IP_6 micelle modification and dried in 4 °C about 12 h. It should be mentioned that the loadings of 3 μL HRP (5 mg mL^{-1}, pH 7.4 PBS) onto the micelles modified electrode surface was an optimized one after HRP-loading-dependent cyclic voltammogram experiment (see Supplementary Fig. S.2). And then, the electrode was thoroughly rinsed 3 times with deionized water to remove the physically adsorbed HRP, and the electrode was successively coated with Nafion solution (five times diluted by pH 7.0 PBS). Nafion/HRP/IP_6 micelles/GCE was fabricated, and it was storied at 4 °C in pH 7.0 PBS while not used. A fabrication process is presented in Scheme 1. For comparison, Nafion/HRP/GCE and Nafion/HRP/IP_6/GCE were fabricated using the similar procedures.

3. Results and discussion

3.1. Morphology

The morphology of the IP_6 micelles was observed using a transmission electron microscopy and field emission scanning electron microscopy. The TEM image of IP_6 micelles was taken after dyed the micelles with sodium phosphotungstate. As can be seen from TEM image in Fig. 1a, the average diameter of IP_6 micelles in the well shaped spheres is about 35 nm. FESEM topography (Fig. 1b) of IP_6 micelles onto the pretreated glassy carbon piece was taken after the coating was dried under infrared lamp, displaying nice distribution of micelles at the surface. From Fig. 1c, it can be seen that enzyme molecules (the black dots in IP_6 micelles image) not only adsorb on the biomembrane-like surface of micelle but also imbed into the inner of micelle sphere, which can be potentially exploited to increase the loading of enzyme molecules on the electrode surface and to provide a desirable microenvironment for keeping bioactivity of HRP.

3.2. UV–vis spectroscopic characterization

UV–vis spectroscopy is an effective tool for monitoring the possible change of the Soret absorption band in the heme group region. The band shift may provide information on the tertiary structure of heme proteins, especially that conformational change around the region (George and Hanania, 1953). UV–vis spectra of IP_6 micelles, HRP and HRP–IP_6 micelles composites are presented in Fig. 2. From Fig. 2a, no absorbance of IP_6 micelles is observed. The adsorption peak of HRP is 396 nm (Fig. 2b), while only 1-nm shift is observed in case of the HRP–IP_6 micelles composites (Fig. 2c). It should be mentioned that UV–vis experiment for the HRP–IP_6 micelles composites was conducted after the 5 mg mL^{-1} HRP solution was mixed with 0.001 mol L^{-1} IP_6 micelles solution in volume ratio of 3:5 and then was incubated for 1 h at 4 °C. A tiny shift suggests that the environment of the enzyme is slightly changed and no signif-

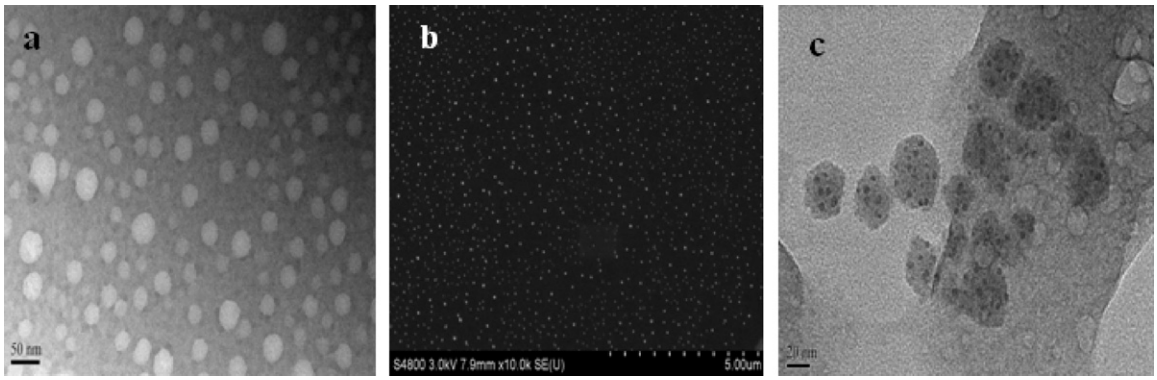


Fig. 1. TEM image of IP_6 micelles in solution (a), FESEM image of IP_6 micelles modified on glassy carbon surface (b) and TEM image of HRP–IP_6 micelles (c).

icant denaturation occurred. We noticed that the Q and CT bands definitely occur at about 500 and 640 nm as the week bands, respectively, in the UV–vis spectrum recorded from pure HRP molecules (Szigeti et al., 2008). Such two broad bands could be clearly observable in UV–vis experimental results of HRP–IP_6 micelles. In other word, the interaction between HRP and IP_6 micelles does not destroy the conformational structure of HRP. Instead, IP_6 micelles may provide a microenvironment for HRP to maintain its native structure and bioactivity.

3.3. Electrochemical impedance spectroscopy (EIS) for the modifying process

EIS is particularly useful for providing information on the impedance changes of the electrode surface during the fabrication process. Nyquist plot of the EIS includes a semicircular portion and a linear portion. The semicircle diameter at higher frequencies in the impedance spectrum equals to the electron-transfer resistance. Such resistance controls the electron-transfer kinetics of the redox probe molecule at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process (Foschini et al., 2001; Sundfors et al., 2002). Nyquist plot of a bare GCE (see Supplementary Fig. S.3a) in 1 mmol L^{-1} $[Fe(CN)_6]^{4-/3-}$ solution containing 0.1 mol L^{-1} KCl exhibits a small semicircle but an almost straight line, which implies the characteristic of a diffusion limiting step of the electrochemical process. The EIS semicircle increases, after modification of IP_6 micelle composites (see Supplementary Fig. S.3b), indicating that IP_6 micelles as barriers make interfacial charge transfer more difficult. Comparing with the IP_6 micelles/GCE, an obvious increase in the interfacial resistance is observed at the HRP/IP_6 micelles/GCE (see Supplementary Fig. S.3c), marking the successful immobilization of HRP at the electrode. As modified with Nafion, the EIS of Nafion/HRP/IP_6 micelles/GCE (see Supplementary Fig. S.3d) shows a higher interfacial resistance. The EIS results demonstrate that HRP molecules are effectively immobilized on the GCE by the linkage of IP_6 micelles and Nafion should enhance the robust of modified electrode.

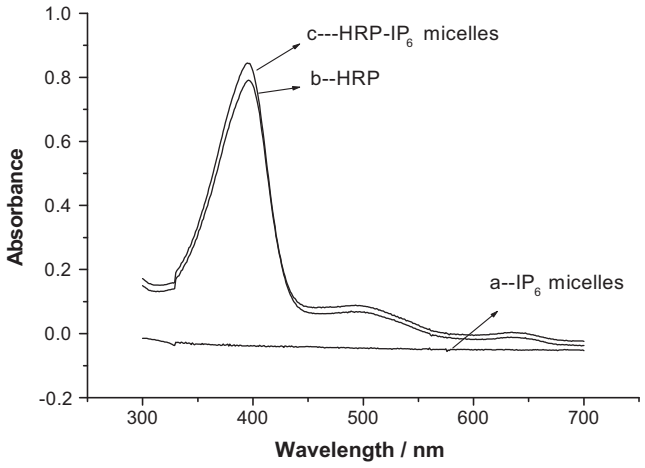


Fig. 2. UV–vis absorption spectra of IP_6 micelles (a), HRP (b) and HRP–IP_6 micelles composites (c).

3.4. Direct electron transfer of Nafion/HRP/IP_6 micelles/GCE

The DET between the HRP and the underlying GCE can be readily achieved via a high biocompatible composite system based on IP_6 micelles. Fig. 3 shows the typical cyclic voltammograms (CVs) of the bare GCE, Nafion/HRP/GCE, Nafion/HRP/IP_6/GCE and Nafion/HRP/IP_6 micelles/GCE. A pair of high, stable and nearly reversible redox peaks of HRP in pH 7.0 PBS for the HRP (Fe^{III})/HRP (Fe^{II}) redox couple transformation is observed at the Nafion/HRP/IP_6 micelles/GCE (Fig. 3d), which is ascribed to the electron transfer between the HRP and the underlying electrode. No peak is observed at bare GCE (curve a) and Nafion/HRP/GCE (curve b). There are relatively smaller peaks observed for HRP in the IP_6 molecules modified film (curve c), comparing to that in the presence of IP_6 micelles. Therefore, IP_6 micelles are the key factor to enhance the direct electron transfer of HRP. The redox peaks

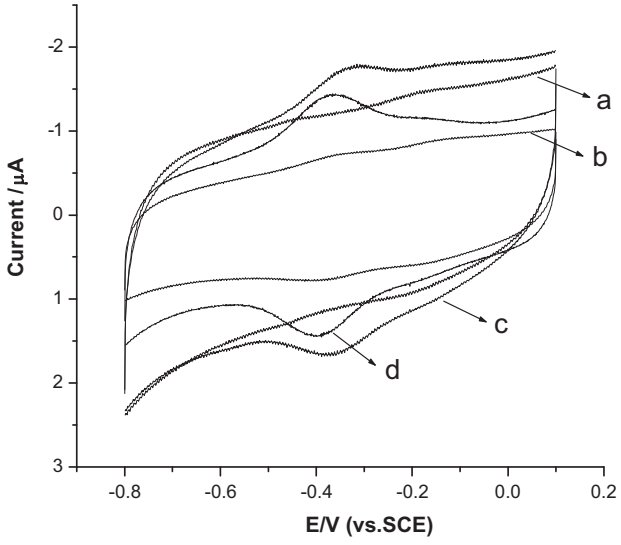


Fig. 3. Cyclic voltammograms of bare GCE (a), Nafion/HRP/GCE (b), Nafion/HRP/IP_6/GCE (c), Nafion/HRP/IP_6 micelles/GCE (d) in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate at 300 mV/s.

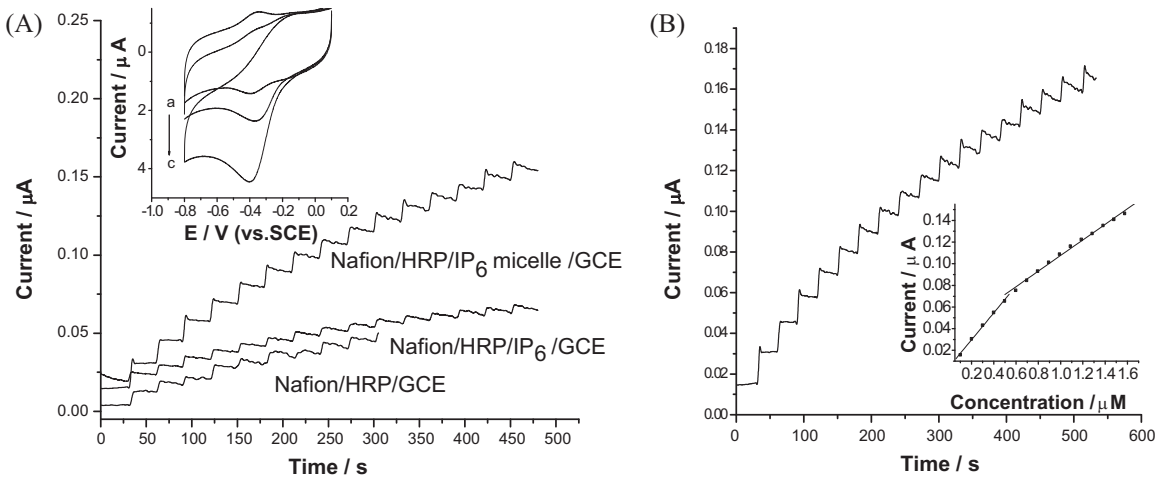


Fig. 4. (A) Amperometric responses of the Nafion/HRP/IP_6 micelles/GCE, Nafion/HRP/IP_6/GCE and Nafion/HRP/GCE at −0.24 V to successive addition of 10 μL 0.1 mmol L^{-1} H_2O_2 in a 10 mL PBS (pH 7.0) under stirring. Inset: CVs of the Nafion/HRP/IP_6 micelles/GCE electrode in the absence (a) and presence of 1×10^{-5} mol L^{-1} (b) 5×10^{-4} mol L^{-1} (c) H_2O_2 in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate at 300 mV s^{-1}. (B) The chronoamperometric curve of Nafion/HRP/IP_6 micelles/GCE obtained through successive additions of H_2O_2 into the 10 mL 0.1 mol L^{-1} PBS. Inset: plots of the linear calibration curves.

of IP_6 micelle-based HRP electrode with anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) were located at −0.389 and −0.366 V, respectively. And the formal potential ($E^{0\prime} = (E_{pa} + E_{pc})/2$) at −0.378 V is obtained in Fig. 3d. It agrees with those of other heme-containing proteins including horseradish peroxidase (Lu et al., 2006), hemoglobin (Xu et al., 2008), and myoglobin (Ma et al., 2000). The peak-to-peak separation of 23 mV at a scan rate of 300 mV s^{-1} indicates that HRP undergoes a quasi-reversible redox progress and a quick electron transfer process in film of IP_6 micelles. Herein, the IP_6 micelles provide a more favorably biocompatible environment for electron transfer of HRP with underlying GCE than that of IP_6 molecules binding as well as the decrease of background current. Additionally, Nafion is helpful in protecting the biocompatibility of the enzyme and preventing it from leakage. The average surface concentration of active HRP (Γ^*) is estimated to be 17.2×10^{-11} mol cm^{-2} by the equation of $\Gamma^* = Q/nFA$, suggesting that HRP in multilayer take part in the electrochemical reaction due to IP_6 micelles immobilizing more HRP, considering that the theoretical value for monolayer coverage of HRP on the electrode is calculated to be 2×10^{-11} mol cm^{-2}.

The influence of scan rate on the response of the modified electrode in PBS was investigated. Nafion/HRP/IP_6 micelles/GCE displays a well-defined peak shape at different scan rates and shows nearly equal height of reduction and oxidation peaks at the same scan rate (see Supplementary Fig. S.4A) and no change of their positions with the increasing of scan rate. Both anodic (i_{pa}) and cathodic (i_{pc}) peak currents vary linearly with the scan rates in the range from 100 to 600 mV s^{-1} (as shown in Fig. S.4B), indicating a surface-confined electrode process (Zhao et al., 2006a).

The apparent heterogeneous electron transfer rate constant (k_s) for Nafion/HRP/IP_6 micelles/GCE was estimated by cyclic voltammetry with Laviron's method (Laviron, 1979) of the following equations:

$$E_{pc} = E^{0\prime} - \frac{2.3RT}{\alpha nF}\log \upsilon$$

$$E_{pa} = E^{0\prime} - \frac{2.3RT}{(1-\alpha)nF}\log \upsilon$$

$$\log k_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nF\upsilon} - \frac{(1-\alpha)\alpha F\Delta E_p}{2.3RT}$$

where E_{pc} and E_{pa} are, respectively, the peak potentials of cathode and anode, $E^{0\prime}$ is the apparent potential of the electrode reaction, α is the charge transfer coefficient, R is the gas constant, T is the absolute temperature, F is the Faraday constant, n is the number of electrons transferred and υ is the scan rate. From the equations, α was estimated as 0.61 and assuming one electron transferred, k_s of HRP was calculated to be about 5.6 s^{-1}, which was higher than the previously reported enzyme-based H_2O_2 biosensors (Mohammadi et al., 2009; Zhao et al., 2008; Xu et al., 1998).

The pH dependence of the electrochemical behavior of Nafion/HRP/IP_6 micelles/GCE in the electrolyte solution was further investigated. Negative shifts in both E_{pa} and E_{pc} potentials are observed with the increase of pH value (see Supplementary Fig. S.5A). Moreover, $E^{0\prime}$ potentials exhibit linear relationship with the pH values in the range of pH from 4.0 to 9.0 (see Supplementary Fig. S.5B). The slopes of −45.84 mV/pH, for a reversible one-electron transfer coupled by single-proton transportation, which is smaller than the theoretical value of −57.6 mV/pH at 18 °C. It is suggested that the groups near the heme iron made an impact on redox potential. Thus, the protonation of distal or proximal histidines, or the protonation of the water molecule coordinated to the central iron might be responsible for the pH dependence of $E^{0\prime}$ (Yamazaki et al., 1978; Liu et al., 2004a,b). It should be mentioned that, all changes in CV peak potentials and currents with pH were quasi-reversible between pH 4.0 and 9.0 and the same CV response could be obtained if the electrode was transferred from a solution with a different pH to its original solution.

3.5. Electrochemical response to H_2O_2

A noticeable electrochemical transformation of H_2O_2 is observed by cyclic voltammetry in Fig. 4A. As shown in inset of Fig. 4A for Nafion/HRP/IP_6 micelles/GCE in 0.1 mol L^{-1} PBS, the cathodic peak current increases with increasing H_2O_2 concentration, accompanied by a decrease or disappearance of the oxidation peak. It indicates that HRP–IP_6 micelles composites exhibit excellent electrocatalytic activity toward H_2O_2. The reaction mechanism (Zhang et al., 2007; Liu and Hu, 2005) of the H_2O_2 biosensor is briefly summarized as below:

$$HRP(Fe^{II}) + H_2O_2 \rightarrow HRP(Fe^{III}) + H_2O$$

$$HRP(Fe^{III}) + e \rightarrow HRP(Fe^{II})$$

Taking the sensitivity and selectivity into consideration, the applied potential of −0.24 V was chosen for the amperometric determination of H_2O_2 in this work (see Supplementary Fig. S.6). Amperometric responses of various modified electrodes to H_2O_2

Table 1
Comparison of the performance of different H_2O_2 biosensors.

Modified electrode	DET	Response time	Detection limit (μmol L^{-1})	K_M^{app} (mmol L^{-1})	K_s (s^{-1})	Reference
Nafion/HRP/IP_6 micelles/GCE	Yes	3 s	0.1	0.0016	5.6	This work
HRP/GNPs/IP_6/GNPs/IP_6/Au	No	3 s	3.3	0.078	–	Wang et al. (2010a,b)
HRP/NiO NPs/GC	Yes	–	123	–	0.75	Mohammadi et al. (2009)
Clay–HRP–Clay/AuCS–GCE	Yes	–	9.0	23.15	2.95	Zhao et al. (2008)
$\{IP_6/Mb\}_{10}$/PG	Yes	–	–	–	44.1	Yang et al. (2007)
Hb/(Fe_3O_4/chitosan–IP_6)$_4$/GCE	Yes	–	–	–	–	Zhao et al. (2006a,b)
HRP–MB/MWNT/GCE	Yes	30 s	1.0	0.12	–	Xu et al. (2003)
HRP/Nafion–methylene-green/GCE	Yes	20 s	0.1	2.1	–	Wang and Dong (2000)
HRP/Thionine/GCE	Yes	20 s	0.1	1.6	0.097	Xu et al. (1998)

DET is the direct electron transfer. K_M^{app} is the apparent Michaelis–Menten constant. K_s is apparent heterogeneous electron transfer rate constant.

were investigated by successively adding H_2O_2 to a continuously stirred PBS solution of pH 7.0 in Fig. 4A. The Nafion/HRP/IP_6 micelles/GCE shows the largest response to H_2O_2, which should result from a high activity of HRP entrapped by IP_6 micelle composite matrix. As comparison, for Nafion/HRP/IP_6/GCE and Nafion/HRP/GCE, the response currents to the addition of H_2O_2 are relatively faint.

Obviously, in Fig. 4B, the electrocatalytic currents of the Nafion/HRP/IP_6 micelles/GCE biosensor increase with successive additions of 10 μL 0.1 mmol L^{-1} H_2O_2 into the 10 mL PBS and achieve 95% of steady-current within 3 s, indicating a fast electrocatalytic response. The current response and the concentration of H_2O_2 have two linear relationships within the concentration range from 0.1 to 0.5 μmol L^{-1} with a correlation coefficient of 0.998 (n = 5) and from 0.6 to 1.6 μmol L^{-1} with a correlation coefficient of 0.997 (n = 11) (inset of Fig. 4B). The detection limit of 0.1 μmol L^{-1} is estimated at signal-to-noise ration of 3 (S/N = 3), which is much lower than some reported electrochemical biosensors, 4.5 μmol L^{-1} for Hb/Au/ITO electrode (Zhang and Oyama, 2004), and 1.8 μmol L^{-1} for PVB/HRP–SA/Au electrode (Liu et al., 2009).

According to the Lineweaver–Burk form of the Michaelis–Menten equation (Kamin and Wilson, 1980): $1/I_{ss} = K_M^{app}/I_{max}C + 1/I_{max}$. Here I_{ss} is the steady-state current after the addition of substrate, which can be obtained from amperometric experiments. I_{max} is the maximum current. C is the concentration of the substrate, and the apparent Michaelis–Menten constant (K_M^{app}), is the apparent Michaelis–Menten constant and a characteristic parameter of the enzyme-substrate kinetics. The value of K_M^{app} is estimated to be 0.0016 mmol L^{-1}, which is obtained by the analysis of slope and intercept of the reciprocals of the steady-state currents versus concentrations. Clearly, K_M^{app} of the Nafion/HRP/IP_6 micelles/GCE is in a relatively good position in Table 1, compared to some reports regarding H_2O_2 biosensors such as HRP/GNPs/IP_6/GNPs/IP_6/Au with K_M^{app} of 0.078 mmol L^{-1} (Wang et al., 2010b), HRP–MB/MWNT/GCE with K_M^{app} of 0.12 mmol L^{-1} (Xu et al., 2003), HRP/Nafion–methylene-green/GCE with K_M^{app} of 2.1 mmol L^{-1} (Wang and Dong, 2000), and HRP/Thionine/GCE with K_M^{app} of 1.6 mmol L^{-1} (Xu et al., 1998). These observations show that the resulting biosensor in the present work possesses a higher affinity to H_2O_2.

3.6. Stability and reproducibility

The stability of the Nafion/HRP/IP_6 micelles/GCE was examined. No band relative to HRP absorption peaks normally at 390, 500, and 640 nm in UV–vis absorption spectrum (see Supplementary Fig. S.7) recorded from a solution taken out of 0.1 mol L^{-1} PBS in which Nafion/HRP/IP_6 micelles/GCE has been immersed for one week can be visible. Therefore, HRP leaching from the Nafion/HRP/IP_6 micelles/GCE seems not to happen after removal of infirmly attached HRP from biosensor by careful rinse with deionized water. It means that the IP_6 micelles modified electrode is suitable for immobilization of sufficient enzyme and preventing HRP molecules from leakage should be attributed to IP_6 micelles capturing the HRP molecules as well as the Nafion is helpful in the protection of enzymes at the electrode.

No obvious change of CV curves presenting DET could be observed even 40 continuous cyclic scans were repeated in the potential range from −0.8 to 0.1 V with a scan rate of 100 mV s^{-1} (see Supplementary Fig. S.8). A freshly synthesized Nafion/HRP/IP_6 micelles/GCE was conducted successive detections within 20 operation days and its amperometric response to 0.1 μmol L^{-1} H_2O_2 solution remained 92% of the initial current signal. In all, the stability of the HRP at the electrode surface over the period of operation is accepted.

The reproducibility was estimated by determining the amperometric responses to 0.1 μmol L^{-1} H_2O_2. Three H_2O_2 biosensors fabricated independently with the same protocol, showed an acceptable reproducibility with the R.S.D. of 6.4% for the amperometric responses to H_2O_2 (see Supplementary Fig. S.9). The result demonstrates that this biosensor has good repeatability.

3.7. H_2O_2 determination in disinfector sample

Nafion/HRP/IP_6 micelles/GCE was applied to detect H_2O_2 concentration in commercial disinfector. The sample was diluted 1000-fold with PBS (pH 7.0). The average concentration of H_2O_2 in sample was determined to be 0.851 mol L^{-1} by the prepared biosensor for four assays (see Supplementary Table S.1). Using the potassium permanganate titration method, H_2O_2 concentration in sample was measured to be 0.868 mol L^{-1}. The relative standard deviation (R.S.D. %) of results obtained by two methods was 1.96%, indicating that it was feasible to apply the developed biosensor to determine H_2O_2 in medicine sample.

4. Conclusions

HRP can be effectively immobilized in IP_6 micelle spheres with good biocompatibility, high surface activity, high organization and uniformity. The Nafion/HRP/IP_6 micelles/GCE realizes the DET between the HRP and the electrode. The biosensor possesses a low apparent Michaelis–Menten constant (0.0016 mmol L^{-1}) and a low detection limit (0.1 μmol L^{-1}), fast response (3 s) and operational convenience of fabrication. This IP_6 micelles-based protocol may be extended to provide a promising platform for the development of other biosensors.

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Appendix A. Supplementary data

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