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One-step construction of biosensor based on chitosan–ionic liquid–horseradish peroxidase biocomposite formed by electrodeposition

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ABSTRACT

A simple and controllable electrodeposition approach was established for one-step construction of hydrogen peroxide (H_2O_2) biosensors by in situ formation of chitosan–ionic liquid–horseradish peroxidase (CS–IL–HRP) biocomposite film on electrode surface. A highly porous surface with orderly three-dimensional network was revealed by scanning electron microscopy (SEM) investigation. The biocomposite provided improved conductivity and biocompatible microenvironment. The developed biosensor exhibited a fast amperometric response for the determination of H_2O_2 and 95% of the steady-state current was obtained within 2 s. The linear response of the developed biosensor for the determination of H_2O_2 ranged from 6.0×10^{-7} to 1.6×10^{-4} M with a detection limit of 1.5×10^{-7} M. Performance of the biosensor was evaluated with respect to possible interferences and a good selectivity was revealed. The fabricated biosensor exhibited high reproducibility and long-time storage stability. The ease of the one-step non-manual technique and the promising feature of biocomposite could serve as a versatile platform for the fabrication of electrochemical biosensors.

1. Introduction

The determination of hydrogen peroxide (H_2O_2) is of great importance in clinical, food, pharmaceutical and environmental analyses. Electrochemical biosensor based on horseradish peroxidase (HRP) electrode has been extensively employed for H_2O_2 determination due to the procedural simplicity, intrinsic sensitivity and selectivity (Tangkuaram et al., 2007; Tripathi et al., 2006; Xiong et al., 2007). To improve the performance of the enzyme electrode, effective immobilization of HRP within a biocompatible material by using simple and controllable procedure is of great significance.

Recently, room temperature ionic liquids (ILs) are attracting intensive interest in the area of electrochemistry for their relatively large potential windows, outstanding electrochemical stability, and extremely high ionic conductivity (Xiong et al., 2007; Zhang et al., 2007). In the field of electroanalytical chemistry, the applications of ILs mainly focus on two aspects. One is that ILs are used as the supporting liquid or solid electrolytes (Shen et al., 2005; Xiong et al., 2007; Zhang et al., 2005). Another interesting application is to incorporate ILs into the fabrication of biosensors and bioelectronics. To date, IL-type carbon paste electrodes (Maleki et al., 2006; Sun et al., 2007; Wang et al., 2007a,b,c) or electrodes modified by IL-incorporated composites were well documented. Amongst them, the combination of ILs with conventional matrices, such as, agarose, carbon materials, gelatin, sol–gel-based silica matrices, Nafaion and nano Au particles, gains especially attractive attention for creating unique materials (Li et al., 2006, 2007; Wang et al., 2005, 2007a,b,c; Xiao et al., 2007; Yan et al., 2007; Zhang et al., 2007; Zhao et al., 2004, 2007). It has been proved that the incorporation of ILs can increase sensitivity and allow efficient direct electron transfer of various proteins/enzymes. The combinations of ILs with these conventional matrices, however, were usually achieved by manual casting or dip-coating. Time-consuming and uncontrollable process might be involved. As the thickness of the resulting composite film was difficultly controllable, the biosensor fabrication was easily irreproducible. To easily control the fabrication process, non-manual enzyme immobilization in IL-incorporated biocomposite is becoming increasingly important.

As a biocompatible polymer, chitosan (CS) remains a focus of study in recent years due to its cheapness, hydrophilicity, nontoxicity, excellent film-forming ability and remarkable biocompatibility (Tangkuaram et al., 2007; Wang and Zhang, 2006). Those attractive characteristics have prompted its application as one of the most promising matrix for enzyme immobilization. CS–IL composite materials could find potential applications as electrochemical biosensing platforms. Using hydrophilic IL 1-butyl-3-methyl-imidazolium tetrafluoroborate ($BMIMBF_4$), Lu et al., 2006a,b prepared CS–IL composite materials through manual casting. Direct electron transfer between enzyme and electrode was realized when the CS–IL composite materials were used to entrap

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HRP and hemoglobin (Hb) (Lu et al., 2006a,b). Wang et al. integrated CS with $BMIMBF_4$ and multi-walled carbon nanotubes (MWNTs). The $BMIMBF_4$/MWNTs/CS was manually cast on glassy carbon electrode (GCE) and the modified electrode showed low detection limit and antifouling properties for the determination of β-nicotinamide adenine dinucleotide (NADH) (Wang et al., 2007a,b,c).

Electrochemical deposition has been reported recently as a simple method to obtain film with controllable thickness under moderate conditions (Song et al., 2006; Yi et al., 2004). Due to the possessing of primary amino groups, CS was a pH shift polymer with a p*K*a of about 6.3. As a result, the solubility and net charge of CS was pH-dependent. Electrochemical deposition of CS was pioneered by Payne group and Chen group (Wu et al., 2002, 2003, 2005; Xu et al., 2004). The deposition was performed at reducing potentials and H^+ in the solution was reduced to H_2 at the cathode. Using the locally generated H^+ gradient, acidic side chains of CS were titrated, leading to a change in solubility and hence to the controlled deposition of a CS film. Moreover, biocomposites could be conveniently prepared by effective incorporation of functional substrates (Luo et al., 2004, 2005; Zangmeister et al., 2006). Therefore, electrodeposition method could supply a simple and universal way to construct CS-based biocomposite films on conductive bases for biosensors development.

This work attempts to disclose a simple and controllable electrodeposition method for one-step construction of H_2O_2 biosensors by formation of CS–IL–HRP biocomposite film. It offered simple and convenient methodology for in situ incorporation of IL and HRP into three-dimensional structures of CS hydrogel. Such non-manual approach was direct and facile without complicated and time-consuming manual process. Due to the favorable microenvironment and improved conductivity, the H_2O_2 biosensor based on the CS–IL–HRP biocomposite film displayed such characteristics as fast response, high sensitivity and good stability. The preparation methodology and main characteristic features were described and discussed in detail.

2. Experimental

2.1. Reagents

Horseradish peroxidase (HRP, EC 1.11.1.7, 250 U mg^{-1}) was obtained from Shanghai Lizhu Dongfeng Biotechnology Co. Ltd., China. CS with 98% deacetylation and an average molecular weight of 4.8×10^5 g mol^{-1} (Yuhuan Biomedical Corp., China) and 1-butyl-3-methyl-imidazolium tetrafluoroborate ($BMIMBF_4$, Fluka) were used in this study. All other chemicals were of analytical reagent grade and used without further purification. Hydrogen peroxide solutions were prepared freshly using a 30% H_2O_2 solution. The 0.1 M phosphate buffer solutions (PBS) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Then the pH was adjusted with 0.1 M NaOH or H_3PO_4.

2.2. Apparatus and instrumentations

Electrochemical measurements were performed on a CHI 650 electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was used with bare gold electrode or modified gold electrode as the working electrode, Ag/AgCl electrode (saturated with KCl) or saturated calomel electrode (SCE) as the reference electrode, and platinum disk as auxiliary electrode, respectively. Scanning electron microscopy (SEM) images were obtained at 5.0 kV on a SIRION (FEI, USA) field emission SEM. X-ray energy dispersion spectroscopy (EDX) measurements were conducted with a GENENIS 4000 energy dispersive X-ray micro analyzer (EDAX, USA).

2.3. Preparation of the H_2O_2 biosensor

Before modification, bare gold electrode was successively polished with emery paper and 0.05 μm α-Al_2O_3 slurry. After being sonicated in deionized water for 1 min, the electrode was immersed in freshly prepared Piranha solution (30% H_2O_2 and concentrated H_2SO_4, 3:1 (v/v)) for 10 min. After being sonicated in deionized water, the electrode was electrochemically pre-treated by cyclic potential scanning between 1.4 and −0.2 V in 0.1 M H_2SO_4 until cyclic voltammogram of clean gold electrode was obtained.

Typically, a homogenous CS–IL–HRP mixture containing 0.5 wt% CS, 1 mg ml^{-1} HRP, and 1% (v/v) $BMIMBF_4$ was prepared for electrodeposition. CS aqueous solution (0.5 wt%, pH 5.0) was prepared according to the previously reported procedure (Luo et al., 2004, 2005). Briefly, CS was dissolved in 0.05 M HCl aqueous solution and the pH of the CS solution was adjusted to 5.0 by using a 1.0 M NaOH solution. Then the CS solution was filtered using a 0.45-μm cellulose filter film. Afterwards, $BMIMBF_4$ was added to the transparent CS solution. The mixture was stirred for 30 min and placed in an ultrasonic bath for 20 min. Then, HPR was added and the obtained solution was sonicated for 5 min.

A polished and cleaned gold electrode was dipped into the as-prepared CS–IL–HRP solution and was polarized as a negative electrode (the cathode). Deposition was performed by applying a voltage of 1.5 V for 3 min. Consequently, H^+ in the solution was reduced to H_2 at the cathode, and the pH near the cathode surface gradually increased. CS becomes insoluble when the pH exceeded its p*K*a (about 6.3). As a result, the CS hydrogel incorporated with IL and HRP was locally electrodeposited onto the cathode surface. After deposition, the modified electrode (Au/CS–IL–HRP) was removed from the solution, rinsed with 0.1 M PBS (pH 8.0), disconnected from the power supply, soaked in 0.1 M PBS (pH 8.0) for 10 min to neutralize CS. Afterwards, the CS coated electrode was stored in 0.1 M PBS (pH 6.5). For comparison with the Au/CS–IL–HRP electrodes, Au/CS–HRP electrodes were prepared using the same procedures in CS–HRP solution.

2.4. Electrochemical characterization of the H_2O_2 biosensor

Cyclic voltammetric (CV) experiments were carried out in quiescent solutions with the scan rate of 100 mV s^{-1}. In steady-state amperometric experiments, the response was obtained at −0.15 V vs. Ag/AgCl and typical steady-state response of the biosensor to successive injection of H_2O_2 was recorded. EIS was performed in 5.0 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) mixture with the frequencies ranging from 10^4 to 10^{-1} Hz. Saturated calomel electrode (SCE) was used as the reference electrode.

3. Results and discussion

3.1. Fabrication of the H_2O_2 biosensor

In present investigation, an electrochemical sensing platform was developed based on the integration of natural polysaccharide CS, IL ($BMIMBF_4$) and enzyme (HRP). Electrodeposition was used for one-step construction of H_2O_2 biosensors by local formation of CS–IL–HRP biocomposite film on the surface of electrode (see Supplementary material). CS is a unique polymer and ideally suited for electrodeposition because its net charge and solubility are pH dependent. As a hydrophilic ionic liquid, $BMIMBF_4$ can mix well with CS aqueous solution to form a homogenous solution. HRP was also homogenously dispersed in the CS–IL environment. The mechanism for CS deposition at reducing potentials was attributed to the formation of pH gradient near the cathode surface due to the

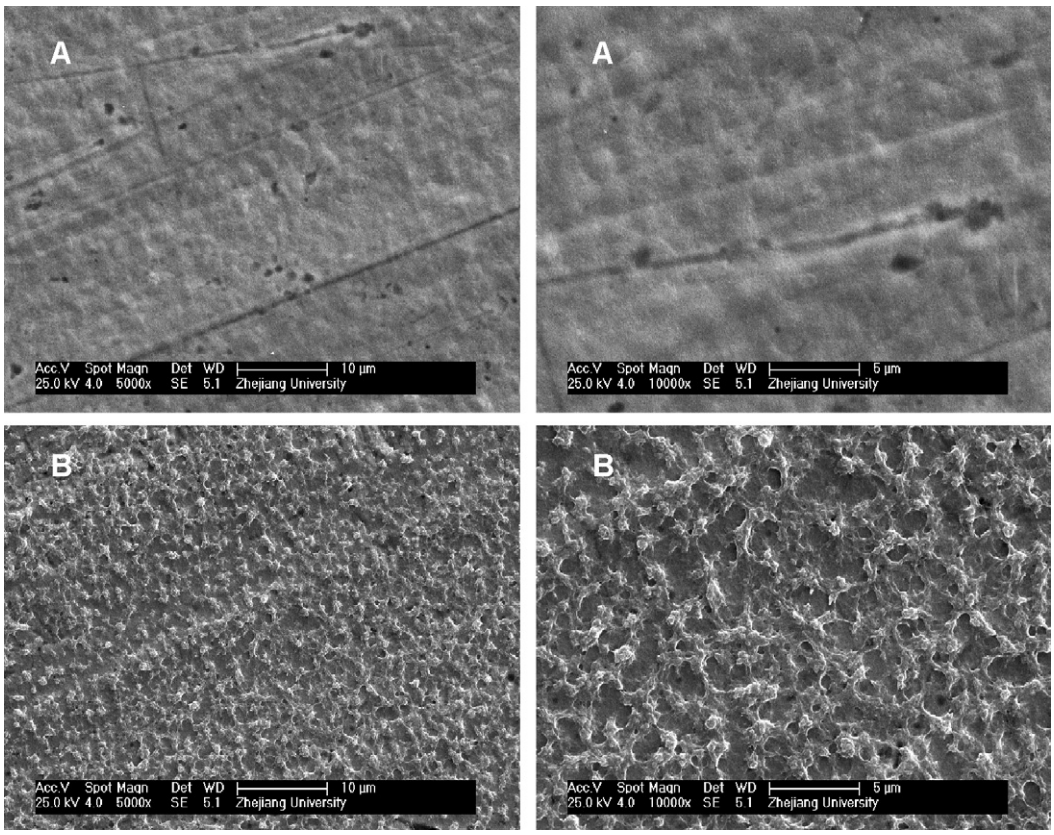

Fig. 1. SEM image of the CS–HRP biocomposite film (A) and the CS–IL–HRP biocomposite film (B).

reduction of water (Wu et al., 2005; Yi et al., 2004). In brief, H^+ in the solution was reduced to H_2 at the cathode, and the pH near the cathode surface gradually increased. When pH exceeded the p*K*a of CS, CS hydrogel incorporated with IL and HRP was locally electrodeposited onto the cathode surface. Consequently, the CS–IL–HRP biocomposite film modified gold electrode (Au/CS–IL–HRP) was obtained.

The advantages of the proposed strategy for one-step fabrication of H_2O_2 biosensor come from the following three aspects. First, the proposed strategy offered simple and convenient methodology for the preparation of CS–IL–HRP biocomposite film. The non-manual electrodeposition approach was simple and controllable. Complicated and time-consuming manual process could be avoided. Moreover, the biosensor fabrication was reproducible and the thickness of the resulting biocomposite film was controllable. Second, the fabricated biosensor combined the individual benefits of IL and CS by in situ incorporation of IL into three-dimensional structure of CS hydrogel. Due to the improved conductivity, CS–IL composite might open up new opportunities for direct electrochemistry, biosensors, biocatalysis and solid-state electrochemical devices (Lu et al., 2006a,b; Wu et al., 2003). Third, the entrapped HRP could retain its bioactivity due to favorable microenvironment provided by CS. It was well known that CS possessed attractive characteristics as immobilization matrix to entrap proteins/enzymes (Tangkuaram et al., 2007; Wang and Zhang, 2006).

3.2. Morphology characterization of the CS–IL–HRP biocomposite film

The morphology of the CS–IL–HRP biocomposite film was examined by SEM observation. For comparison, the CS–HRP biocomposite film prepared in CS–HRP solution was also investigated. Fig. 1 represented the surface morphology of the CS–IL–HRP biocomposite film and the CS–HRP biocomposite film. It was found that the CS–HRP biocomposite film (Fig. 1A) exhibited a slightly rigid surface. The appearance of C, N, and O signals in X-ray EDX firmly confirmed the surface modification (data not shown for brief). For CS–IL–HRP biocomposite film, a totally changed surface was obtained (Fig. 1B). A highly porous surface with orderly three-dimensional network was obtained. Compared with CS–HRP film, the appearance of B and F signals in EDX validate the incorporated of IL into CS–IL–HRP biocomposite film.

3.3. Electrochemical characteristics of the developed H_2O_2 biosensor

The electrochemical activity of the $Fe(CN)_6^{3-/4-}$ redox couple at the CS–IL–HRP biocomposite modified electrode (Au/CS–IL–HRP) was probed using cyclic voltammetry. Fig. 2 compared the CV responses at Au/CS–IL–HRP and Au/CS–HRP in 5 mM $Fe(CN)_6^{3-/4-}$ at a scan rate of 100 mV s^{-1}. A well-defined cyclic voltammogram was observed at Au/CS–HRP. The peak current obtained at Au/CS–IL–HRP increased obviously and the peak-to-peak separation decreased. This revealed that the electron transfer between $Fe(CN)_6^{3-/4-}$ and electrode could be easily performed with good reversibility associated with good sensitivity. CV responses of Au/CS–IL–HRP electrode in 5 mM $Fe(CN)_6^{3-/4-}$ with scan rates from 10 to 300 mV s^{-1} was also investigated. There was good linear relationship between peak currents and the square root of scan rates, which showed a typical diffusion-controlled electrochemical behavior.

EIS has been used to characterize the interface properties of the CS–IL–HRP biocomposite modified electrodes. Fig. 3 showed the typical results of AC impedance spectra of Au/CS–IL–HRP

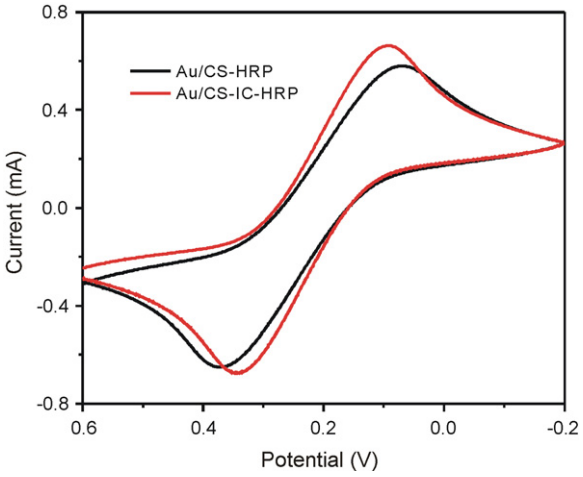

Fig. 2. Cyclic voltammograms of the Au/CS–IL–HRP and Au/CS–HRP modified electrode in 0.1 M PBS (pH 6.5) containing 5.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ at a scan rate of 100 mV s^{-1}.

electrode and Au/CS–HRP electrode, respectively. Significant differences in the impedance spectra were observed. The electron transfer resistance (R_{et}) of Au/CS–HRP was estimated to be 425 Ω. For Au/CS–IL–HRP, the value of R_{et} was found to be 821 Ω, implying that CS–IL–HRP biocomposite film facilitated the electron transfer of the electrochemical probe.

3.4. Influence of pH and applied potential on biosensor response

The conditions of amperometric determination of H_2O_2 were optimized. The dependence of the biosensor response on the pH of the measurement solution was investigated. The pH of the solution ranged from 5.5 to 8.0. The current response reached the maximum at pH 6.5 (see Supplementary material). Therefore, the suitable pH with the maximum activity of the immobilized HRP was at pH 6.5, which was in agreement with that reported for HRP entrapped in CS matrix (Tangkuaram et al., 2007).

Further studies were performed to investigate the dependence of the biosensor response on the applied potential. Amperometric responses of the proposed biosensor to H_2O_2 at different potentials were examined. Results showed that applied potential possessed significant effect on the response of the biosensor (see Supplementary material). Little current was seen at 0.05 V, but current remarkably increased at −0.10 V. The current response arrived at a maximum value at −0.15 V. As a result, the operating potential of −0.15 was selected for further investigation.

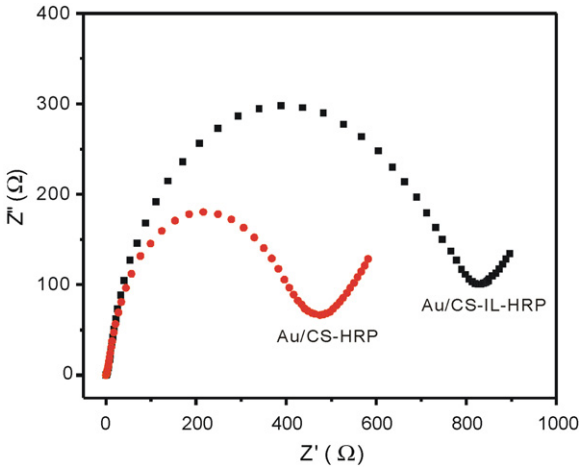

Fig. 3. Electrochemical impedance spectroscopy for Au/CS–IL–HRP and Au/CS–HRP in a solution of 5.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ with SCE as the reference electrode.

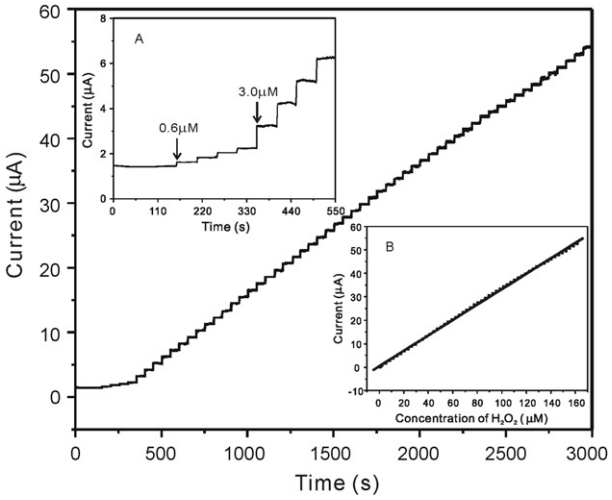

Fig. 4. Typical amperometric response of the fabricated biosensor to successive addition of H_2O_2 in a stirred 0.1 M PBS (pH 6.5) containing 30 μM hydroquinone. Applied potential was −0.15 V vs. Ag/AgCl. (A) Amperometric response of 0.6 μM (155–355 s) and 3.0 μM (from 355 s) H_2O_2 and (B) calibration curve between the current and the concentration of H_2O_2.

3.5. Amperometric response of the developed H_2O_2 biosensor

Amperometric response of the Au/CS–IL–HRP electrode to successive addition of H_2O_2 in 0.1 M PBS (pH 6.5) containing 30 μM hydroquinone was given in Fig. 4. The biosensor responded rapidly when H_2O_2 was added to the stirring PBS and 95% of the steady-state current could be obtained within 2 s (Fig. 4A). Such rapid response could attribute to the presence of IL and fast diffusion of substrate in the porous and open three-dimensional network of CS–IL–HRP biocomposite film (Lu et al., 2006a,b; Wang and Zhang, 2006). In addition, well-defined current proportional to the H_2O_2 concentration was observed. The inset (Fig. 4B) displayed calibration curve of the amperometric response of the biosensor to the concentration of H_2O_2. The linear range of the developed biosensor for the determination of H_2O_2 was found to be 6.0×10^{-7}–1.6×10^{-4} M with a slope of 328.4 μA mM^{-1} and a correlation coefficient of 0.9996 ($n = 57$). The detection limit of 1.5×10^{-7} M was estimated at a signal-to-noise ratio of 3.

To demonstrate the performance of the developed H_2O_2 biosensor, a comparison of the response time, linear range, detection limit and long-time storage stability obtained by several CS-based HRP electrodes for H_2O_2 detection was made in Table 1. HRP electrodes based on incorporation CS with IL, carbon nanotubes, nano Au particles and organically modified sol–gels were mainly compared. The detection limit obtained with the present method was lower than those obtained by HRP immobilization in CS–GD (Miao and Tan, 2000), CS–IC (Lu et al., 2006b), CS–Tb–MWCNT (Liu et al., 2008), CS–MWCNT (Qian and Yang, 2006), CS–nano Au (Lei et al., 2003, 2004; Tangkuaram et al., 2007), CS–THEOS sol–gel (Wang and Zhang, 2006) and FBCS (Garcia et al., 2007) systems, but higher than that obtained using HRP entrapping in CS–APDMOS sol–gel system (Wang et al., 2003). The response obtained with the present method was fast and only HRP electrode based on CS–APDMOS sol–gel system provided similar response rate (Wang et al., 2003).

Table 1
Comparison of the developed H_2O_2 biosensor with other HRP-based biosensors

HRP electrode	Response time (s)	Linear range (μM)	Detection limit (μM)	Stability	Reference
Au/CS–IC	2	0.6–160	0.15	92%/30d	This work
CPE/CS–GD	10	47–2000	3.0	85%/30d	Miao and Tan (2000)
GCE/CS–IC	–	0.75–135	0.25	95%/30d	Li et al. (2006)
GE/CS–Tb–MWCNT	8	10–400	1.7	80%/24d	Liu et al. (2008)
GCE/CS-MWCNT	–	16.7–740	10.3	–	Qian and Yang (2006)
GCE/CS–nano Au	≤10	6.1–1800	6.1	70%/30d	Lei et al. (2004)
CPE/CS–nano Au	15	12.2–2430	6.3	60%/30d	Lei et al. (2003)
SPCE/CS–nano Au	–	10–11300	0.65	95%/30d	Tangkuaram et al. (2007)
GCE/CS–THEOS	5	1.0–250	0.4	90%/30d	Wang and Zhang (2006)
Au/CS–APDMOS	2	0.005–0.1	0.002	75%/60d	Wang et al. (2003)
GCD/FBCS	20	35–2000	15	94%/14d	Garcia et al. (2007)

CPE, carbon paste electrode; GD, glutaraldehyde; GE, graphite electrode; Tb, toluidine blue; MWCNT, multiwalled carbon nanotubes; SPCE, screen printed carbon electrode; THEOS, tetrakis(2-hydroxyethyl) orthosilicates; TMOS, tetramethoxysilane; APDMOS, 3-aoryloxypropyl dimethoxymethylsilane; GCD, glassy carbon disk; FBCS, ferrocene branched chitosan.

3.6. Reproducibility and stability of the developed H_2O_2 biosensor

The repeatability of the same electrode in the measurements was obtained by recording the response for 75 μM H_2O_2 through successive experiments. The relative standard deviation (RSD) was 2.6%. The current response was not lowered after the biosensor was continuously used for 30 times. To evaluate electrode-to-electrode reproducibility, six electrodes were prepared under the same conditions independently. Their response in presence of 75 μM H_2O_2 was investigated. The results revealed a RSD of 3.0%. The good reproducibility can be ascribed to the non-manual approach with simplicity, controllability and reproducibility. The long-time storage stability of the fabricated biosensor was examined by intermittent measuring the current response to H_2O_2 standard solution every 3 days in 30-day storage. The electrode was immersed in PBS (pH 6.5) at 4 °C in a refrigerator when not used. The catalytic current response could maintain about 92% of its original response after the biosensor was stored for 30 days. The long-time storage stability was higher than those obtained by HRP immobilization in CS–GD (Miao and Tan, 2000), CS–Tb–MWCNT (Liu et al., 2008), CS–nano Au (Lei et al., 2003, 2004) and CS–THEOS sol–gel (Wang and Zhang, 2006) systems, but lower than those obtained with GCE/CS–IC–HRP (Lu et al., 2006b) and SPCE/CS–nano Au–HRP (Tangkuaram et al., 2007). The good storage stability was ascribed to the excellent biocompatibility of the CS–IL–HRP biocomposite, which can provide a favorable microenvironment for the entrapped enzyme (Lu et al., 2006a,b).

3.7. Selectivity of the developed H_2O_2 biosensor

The selectivity of the developed H_2O_2 biosensor was evaluated by studying the effect of substances that might interfere with the determination of H_2O_2. The current obtained for each potential interfering substance (80 μM) in the presence of H_2O_2 (40 μM) was compared with that obtained in H_2O_2 solution. Glucose, sucrose, ethanol, citric acid, acetic acid and ascorbic acid did not cause any observable interference. The results indicated that the developed H_2O_2 biosensor possessed high sensitivity.

4. Conclusion

The successful preparation of the H_2O_2 biosensor demonstrated the efficiency of one-step electrodeposition of CS–IL–HRP biocomposite on electrode surface. Such non-manual approach offered direct and facile methodology with controllability and reproducibility. The obtained CS–IL–HRP biocomposite film possessed a porous morphology with orderly three-dimensional network. Due to the favorable microenvironment and improved conductivity, the enzyme entrapped in such biocomposite possessed high electrocatalytic activity and fast amperometric response to H_2O_2. The ease of the one-step electrodeposition and the biocompatible matrix endowed the electrode with high reproducibility and storage stability. The promising performance of the developed biosensor makes this methodological study and application attractive as excellent biosensing platform.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.03.023.

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