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Development of hydrogen peroxide biosensor based on in situ covalent immobilization of horseradish peroxidase by one-pot polysaccharide-incorporated sol–gel process

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ABSTRACT

A simple and reliable one-pot approach was established for the development of a novel hydrogen peroxide (H_2O_2) biosensor based on in situ covalent immobilization of horseradish peroxidase (HRP) into biocompatible material through polysaccharide-incorporated sol–gel process. Siloxane with epoxide ring and trimethoxy anchor groups was applied as the bifunctional cross-linker and the inorganic resource for organic–inorganic hybridization. The reactivity between amine groups and epoxy groups allowed the covalent incorporation of HRP and the functional biopolymer, chitosan (CS) into the inorganic polysiloxane network. Some experimental variables, such as mass ratio of siloxane to CS, pH of measuring solution and applied potential for detection were optimized. HRP covalently immobilized in the hybrid matrix possessed high electrocatalytic activity to H_2O_2 and provided a fast amperometric response. The linear response of the as-prepared biosensor for the determination of H_2O_2 ranged from 2.0×10^{-7} to 4.6×10^{-5} mol l^{-1} with a detection limit of 8.1×10^{-8} mol l^{-1}. The apparent Michaelis–Menten constant was determined to be 45.18 μmol l^{-1}. Performance of the biosensor was also evaluated with respect to possible interferences. The fabricated biosensor exhibited high reproducibility and storage stability. The ease of the one-pot covalent immobilization and the biocompatible hybrid matrix serve as a versatile platform for enzyme immobilization and biosensor fabricating.

1. Introduction

The determination of hydrogen peroxide (H_2O_2) is extensively studied in clinical diagnostics, chemical and pharmaceutical industry, environmental control. Amperometric biosensors based on horseradish peroxidase (HRP) have emerged as the most convenient tools for H_2O_2 determination due to the simplicity, high sensitivity and selectivity [1–5]. To improve the performance and long-term stability of the enzyme electrode, effective immobilization of HRP onto the transducer surface through suitable matrix is of great significance.

In recent years, microencapsulation of enzyme in the silica sol–gel matrix has applied as a new platform for the fabrication of biosensors. Typically, those sol–gel matrices possess physical rigidity, chemical inertness, high photochemical biodegradation and negligible swelling in aqueous and organic solutions [2,6–9]. However, silica sol–gel matrices are mainly prepared by using tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS) as precursor. The poor water solubility of such precursors usually involves enzyme immobilization in organic solvents. Therefore, development of enzyme biosensors based on biocompatible silica sol–gel matrix has attracted increasing attention recently. Despite the effects of designing new precursors with improved water solubility and biocompatibility [2,10], silica-based organic–inorganic hybrids are adopted as attractive materials for biosensor fabricating [7,11].

As a biopolymer containing functional groups of –OH and –NH_2, chitosan (CS) remains a focus of study as promising matrix for enzyme immobilization due to the unique characteristics [1,11–15]. Recently, CS-based organic–inorganic hybrid materials have been widely developed as functional biomaterials [2,11,16,17]. The formed organic–inorganic hybrid could overcome the disadvantages of pure CS materials such as unsatisfying mechanical property, swelling, and solubility in acidic conditions. For example, Wang and Zhang developed a new amperometric H_2O_2 biosensor based on sol–gel process of THEOS in the presence of CS. HRP entrapped in the hybrid matrix could retain its native biocatalytic activity and provide a fast amperometric response to H_2O_2 [2]. Despite the formation of hydrogen bonds for preparing CS-based

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hybrid materials, cross-linked organic–inorganic hybridization using epoxy–siloxane is proposed in the pioneering works of Liu and Shirosaki [18–20]. In situ cross-linking of CS and the formation of hybrid using the reactivity between amine groups and epoxy groups have been revealed by a set of investigation such as ^{29}Si NMR, scanning and transmission electronic microscopy. Moreover, the hybrid membrane has been proved to possess high hydrophilicity and good cytocompatibility. Very recently, we have developed a new approach for direct preparation of porous functional matrix based on surface imprinting coating technique and such CS-incorporated sol–gel process [21,22].

In this investigation, a novel H_2O_2 biosensor was developed based on in situ covalent immobilization of HRP by one-pot polysaccharide-incorporated sol–gel process. The siloxane with epoxide ring and trimethoxy anchor groups, γ-glycidoxypropyltrimethoxysiloxane (GPTMS), was applied as a bifunctional cross-linker. The reactivity between amine groups and epoxy groups offered simple and convenient methodology for covalent incorporation of CS and HRP into inorganic polysiloxane network. Such CS-incorporated sol–gel process was easily carried out in aqueous medium without the addition of any organic solvents. Due to the covalent immobilization of HRP, signal loss resulting from the leaching out of enzyme from the electrode could be avoided. The fabricated H_2O_2 biosensor showed promising performance. The preparation methodology and main characteristics were described and discussed in detail.

2. Experimental

2.1. Reagents

Horseradish peroxidase (EC 1.11.1.7, RZ > 3.0, 250 U mg^{-1}) was obtained from Boao Biotechnology Co. Ltd., China. CS with 98% deacetylation and an average molecular weight of 6×10^4 g mol^{-1} (Yuhuan Biomedical Corp., China) and GPTMS (Alfa aesar) 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 (Nanjing Chemical Reagent Factory, China), and the concentration of H_2O_2 solution was determined by titration with $KMnO_4$. The 0.02 mol l^{-1} phosphate buffer solutions (PBS) at various pH values were prepared by first mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Then the pH was adjusted with 0.10 mol l^{-1} NaOH or H_3PO_4. Double distilled water (DDW) was used throughout this work.

2.2. Apparatus and instrumentations

Amperometric and cyclic voltammetric experiments were performed on a CHI 832B electrochemical analyzer and electrochemical impedance spectroscopy (EIS) was performed on a CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was used with bare glass carbon electrode (GCE) or modified GCE as the working electrode, Ag/AgCl electrode (saturated with KCl) or saturated calomel electrode (SCE) as the reference electrode, and platinum wire as auxiliary electrode.

2.3. Preparation of the H_2O_2 biosensor

Prior to use, bare GCE was polished with 1.0, 0.3, 0.05 μm α-Al_2O_3 slurry. The obtained electrode was rinsed with DDW followed by sequential sonicating in acetone, ethanol and DDW. The HRP-modified electrode was prepared by a simple casting method. Briefly, the CS stock solution (2.0 wt%) was firstly prepared by dissolving CS in 0.05 mol l^{-1} acetic acid. After stirring for 1 h, CS/HRP solution was obtained by blending such CS solution with 5 mg ml^{-1} HRP solution at a volume ratio 2:1 (v/v). Using mass ratio of GPTMS to CS of 3:2, GPTMS was then added. The resulting gel (10 μl) was rapidly deposited onto the pre-treated GCE surface and was dried for 10 min at room temperature. Afterwards, the enzyme electrode was thoroughly immersed in 0.02 mol l^{-1} PBS (pH 7.0) to wash out the non-immobilized enzyme from the electrode surface.

2.4. Characterization of the H_2O_2 biosensor

Cyclic voltammetric experiments were carried out in quiescent solutions with the scan rate of 50 mV s^{-1}. In steady-state amperometric experiments, the potential was set at 0.05 V under magnetic stirring. All potentials were reported versus the Ag/AgCl reference electrode. EIS was performed in 1.0 mmol l^{-1} $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) mixture containing 0.1 mol l^{-1} KCl with the frequencies ranging from 10^4 to 10^{-1} Hz. Saturated calomel electrode was used as the reference electrode.

3. Results and discussion

3.1. Fabrication of the H_2O_2 biosensor

In the present investigation, in situ covalent immobilization of HRP via one-pot polysaccharide-manipulated sol–gel process was used for the fabrication of HRP/CS/GPTMS/Au biosensor. Due to the possession of epoxy group and trimethoxy anchor groups, GPTMS acted as both inorganic resource and bifunctional cross-linker. On the one hand, polysiloxane network for the organic–inorganic hybrid formed resulting from self-hydrolysis and self-condensation of GPTMS. On the other hand, the reactivity between amine groups and epoxy groups offered simple and convenient methodology for covalent incorporation of CS and HRP into the inorganic framework [18–22].

The advantages of the strategy for fabricating H_2O_2 biosensor come from the following three aspects. Firstly, the proposed strategy was simple and mild. No addition of organic solvents and other catalysts were involved in CS-manipulated sol–gel process [23,24]. It was different from the microencapsulation of enzyme using sol–gel process of TMOS or TEOS. Secondly, a versatile way for the fabrication of biosensors using covalent incorporation of the enzyme was developed. The reactivity of amine groups on the enzyme molecules appears to be universal [25–29]. The reaction between such amine groups and epoxy groups allowed in situ covalent incorporation of HRP and CS into the inorganic polysiloxane network. Thirdly, the CS/GPTMS hybrid materials have been proved to possess high hydrophilicity [20].

3.2. Effect of the mass ratio of GPTMS/CS in casting solution on the characteristics of the biosensor

In the present investigation, the electrode was modified with HRP/CS/GPTMS hybrid film. The characteristics of the deposited hybrid film could be controlled by changing the mass ratio of GPTMS to CS in casting solution. The modified electrodes prepared with different ratio of GPTMS/CS were investigated by evaluating the peak current to 15 μmol l^{-1} H_2O_2 in the presence 1.0 mmol l^{-1} $K_4Fe(CN)_6$. The concentration of all CS stock solutions was controlled at 2.0 wt%. Results were shown in Fig. 1. The mass ratios of GPTMS to CS were 1:2, 1:1, 5:4, 3:2, 7:4, 2:1 and 5:2, respectively. As shown, the molar ratios of GPTMS to CS greatly influenced the performance of the biosensor. It was found that too low GPTMS/CS ratio resulted in thin and unstable CS films. With the increase of GPTMS/CS ratio, the thickness of the hybrid films increased and the

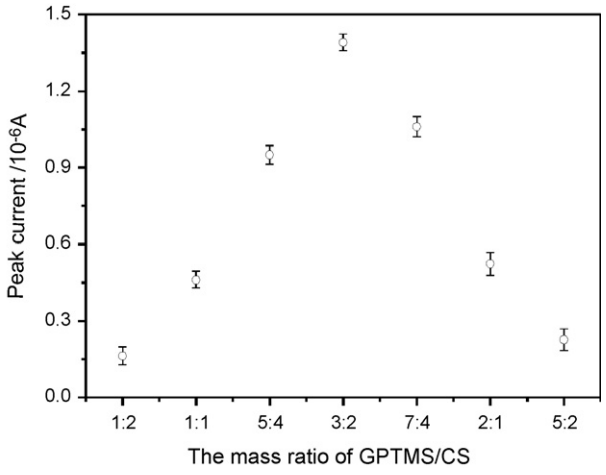

Fig. 1. Effect of the mass ratio of GPTMS/CS on the peak current of the HRP/CS/GPTMS-modified electrode to 15 μmol l^{-1} H_2O_2 in 0.02 mol l^{-1} PBS (pH 7.0) containing 1.0 mmol l^{-1} $K_4Fe(CN)_6$ at the applied potential of 0.05 V versus Ag/AgCl.

attachment of the films to electrode became tight. However, too thick film by high GPTMS/CS ratio would result in large noise and slow response of biosensors [30]. To make stable biosensors with fast and large response, a mass ratio of GPTMS to CS of 3:2 was selected for further investigation.

3.3. Electrochemical characteristics of the developed H_2O_2 biosensor

The electrocatalytic behavior of HRP covalently incorporated in the hybrid material was evaluated by cyclic voltammetry. Since the proposed HRP electrode did not show direct electron transfer between immobilized HRP and GCE, $K_4Fe(CN)_6$ was used as the electron mediator. Cyclic voltammograms (CVs) of the HRP/CS/GPTMS-modified electrode in 0.02 mol l^{-1} PBS (pH 7.0) were given in Fig. 2. When 40 μmol l^{-1} H_2O_2 was introduced, an obvious electrocatalytic response was observed with the increase of reduction current and the decrease of oxidation current. The response process of the biosensor may be expressed as

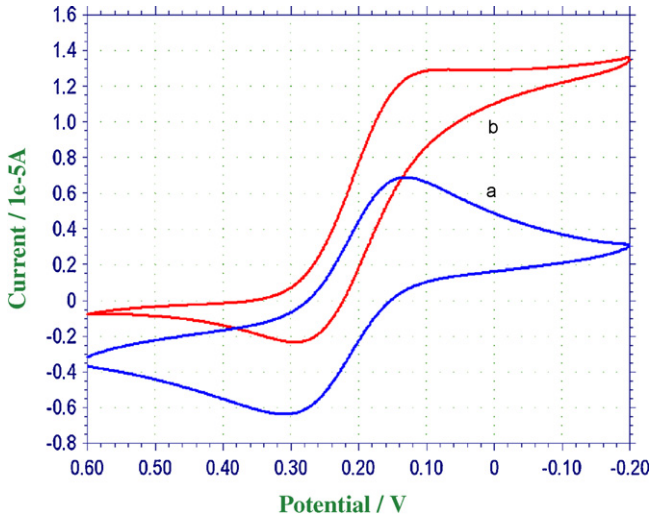

Fig. 2. CVs of the HRP/CS/GPTMS-modified electrode in 0.02 mol l^{-1} PBS (pH 7.0) containing 1.0 mmol l^{-1} $K_4Fe(CN)_6$ at a scan rate of 50 mV s^{-1} (a) without H_2O_2 and (b) with 40 μmol l^{-1} H_2O_2.

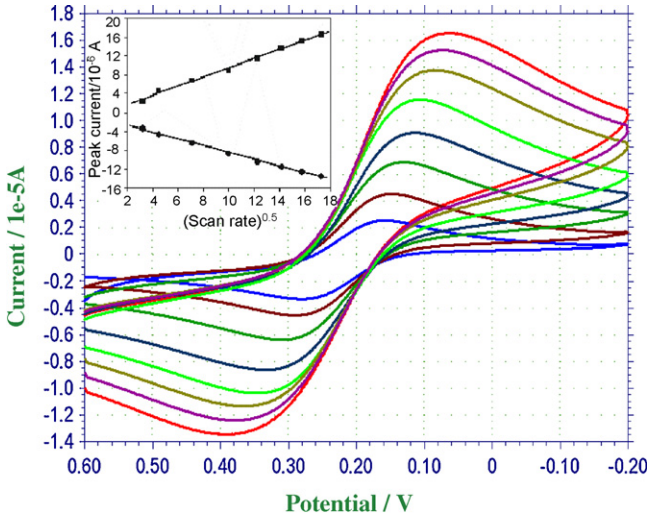

Fig. 3. Typical CVs of the HRP/CS/GPTMS-modified electrode in 0.02 mol l^{-1} PBS (pH 7.0) containing 1.0 mmol l^{-1} $K_4Fe(CN)_6$ under different scan rates from 10 to 300 mV s^{-1}. Inset showed the plots of peak current versus the square root of the scan rate.

follows [31,32]:

$$H_2O_2 + 2Fe(CN)_6^{4-} + 2H^+ \xrightarrow{HRP} 2H_2O + 2Fe(CN)_6^{3-}$$

In the presence of immobilized HRP, $K_4Fe(CN)_6$ was oxidized to $K_3Fe(CN)_6$ and $K_3Fe(CN)_6$ was subsequently reduced at the surface of the electrode.

The CVs of the developed biosensor to various scan rates were also investigated. As shown in Fig. 3, well-characterized redox peaks were observed when scan rate ranged from 10 to 300 mV s^{-1}. In addition, it was found from inset that the peak currents were proportional to the square roots of the scan rates, which showed a typical diffusion-controlled electrochemical behavior.

EIS has been used to characterize the interface properties of the modified electrodes. Fig. 4 showed the typical results of AC impedance spectra of the bare GCE (curve a), CS/GPTMS/GCE (curve b), and HRP/CS/GPTMS/GCE (curve c). The electron transfer resistance (R_{et}) of a bare GCE was estimated to be 1172 Ω. After CS/GPTMS hybrid film formed on the surface of GCE, the

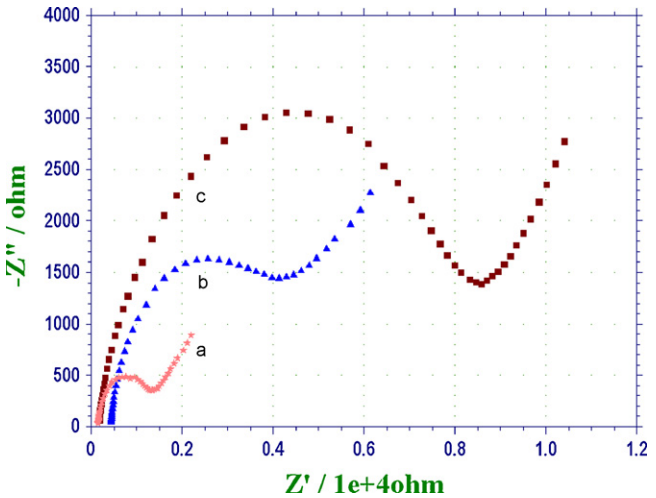

Fig. 4. EIS for (a) bare GCE, (b) CS/GPTMS/GCE, and (c) HRP/CS/GPTMS/GCE in a solution of 0.1 mol l^{-1} KCl containing 1.0 mmol l^{-1} $Fe(CN)_6^{3-}$ and 1.0 mmol l^{-1} $Fe(CN)_6^{4-}$ with SCE as the reference electrode.

value of R_{et} was found to be 3703 Ω, implying CS/GPTMS hybrid film that obstructs the electron transfer of the electrochemical probe. When HRP was incorporated into the hybrid material, the R_{et} of HRP/CS/GPTMS/GCE electrode was calculated and equalled to 8409 Ω. The increase of R_{et} resulted from the hindered pathway of electron transfer by HRP. The results demonstrated that HRP was successfully immobilized into the CS/GPTMS hybrid film.

3.4. Influence of pH and applied potential on biosensor response

The dependence of the biosensor response on pH of the measurement solution was investigated. The pH of the solution ranged from 6.0 to 8.0. The current response increased from pH 6.0 and reached the maximum at pH 7.0. Then, the current response decreased from pH 7.0 to 8.0. Therefore, the suitable pH with the maximum activity of the immobilized HRP was at pH 7.0, which was in agreement with that reported for soluble HRP [2]. The covalent immobilization of HRP and the GPTMS/CS hybrid matrix did not alter the optimal pH value for the catalytic behavior of HRP.

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. Little current was seen at 0.20 V, but current was highly increased at 0.10 V. The current response arrived at a maximum value at 0.05 V and a flat step was observed from −0.05 to −0.15 V. As a result, the operating potential of 0.05 V was determined to give a good sensitivity for further study.

3.5. Amperometric response of the developed H_2O_2 biosensor

Amperometric response of the HRP/CS/GPTMS/GCE electrode to successive addition of H_2O_2 in 0.02 mol l^{-1} PBS (pH 7.0) containing 1.0 mmol l^{-1} $K_4Fe(CN)_6$ was given in Fig. 5. The concentration of H_2O_2 in the solution was also indicated in Fig. 5. When H_2O_2 was added to the stirring PBS, the biosensor responded rapidly and 95% of the steady-state current could be obtained within 5 s (Fig. 5a). Such a rapid response could attribute to the fast diffusion of the substrate in the porous and open frameworks of sol–gel hybrid film. Moreover, well-defined current proportional to H_2O_2 concentration has been observed. Fig. 5b displayed calibration curve of the amperometric response of the biosensor to the concentration of H_2O_2. Inset showed the linear part of the calibration curve. The linear range of the proposed biosensor for the determination of H_2O_2 was found to be 2.0×10^{-7} to 4.6×10^{-5} mol l^{-1} with a slope of 84.3 μA ml mol^{-1} and a correlation coefficient of 0.9991 ($n = 26$). The detection limit of 8.1×10^{-8} mol l^{-1} was estimated at a signal-to-noise ratio of 3.

When H_2O_2 concentration was larger than 4.6×10^{-5} mol l^{-1}, the current response showed a level-off tendency and the curve tended to maximum at 8.8×10^{-5} mol l^{-1} H_2O_2, demonstrating the typical Michaelis–Menten kinetic characteristic of enzyme-based electrode.

The apparent Michaelis–Menten constant (K_M^{app}) can give an indication of the enzyme–substrate kinetics. It could be calculated from the electrochemical version of the Lineweaver–Burk equation [33]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}C}$$

where I_{ss} was the steady-state response current after the addition of substrate, I_{max} was the maximum current measured under saturated substrate conditions, C referred to the bulk concentration of the substrate. In our work, K_M^{app} was estimated to be 45.18 μmol l^{-1}. This value was much smaller than HRP entrapped in THEOS–CS gel and TMOS sol–gel [2,34], indicating that the present electrode exhibits a higher affinity for H_2O_2. The covalent immobilization of HRP for the fabrication of as-prepared electrode appears to be beneficial to improve biosensor's performance.

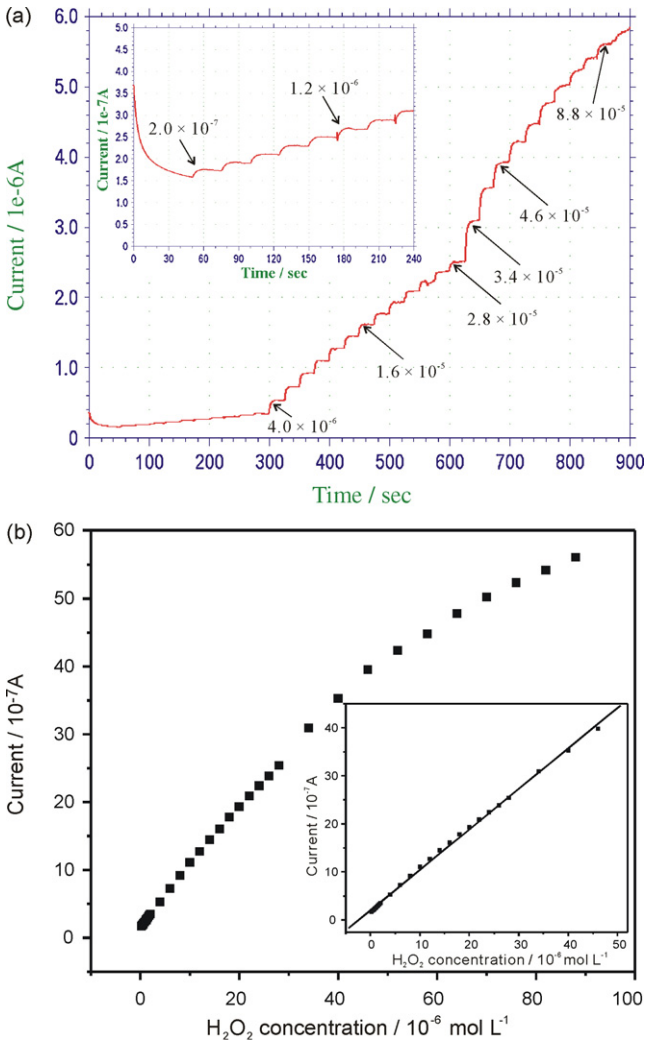

Fig. 5. (a) Typical amperometric response of the fabricated biosensor to successive addition of H_2O_2 into stirring 0.02 mol l^{-1} PBS (pH 7.0) containing 1.0 mmol l^{-1} $K_4Fe(CN)_6$. Applied potential was 0.05 V versus Ag/AgCl and (b) calibration curve between the current and the concentration of H_2O_2. Inset showed the linear part of the calibration curve.

3.6. Reproducibility and stability of the developed H_2O_2 biosensor

The reproducibility and its storage stability of the developed biosensor in a drying state at 4 °C were investigated. Results showed that the current response to 15 μmol l^{-1} H_2O_2 was not lowered after the biosensor was tested continuously for 30 times. To evaluate the electrode-to-electrode reproducibility, six biosensors were prepared under the same conditions independently. The R.S.D. (3.1%) obtained with the present method indicated an acceptable electrode-to-electrode reproducibility. The storage stability of the fabricated biosensor was examined by intermittently measuring the current response to H_2O_2 standard solution every 3 days in the period of 2 months. The catalytic current response could maintain about 81% of its original response in 2 months. The good

Table 1
H_2O_2 concentration in real samples tested by the developed H_2O_2 biosensor

$C_{Original}$ ($\mu mol\,l^{-1}$)	C_{Added} ($\mu mol\,l^{-1}$)	C_{Found} ($\mu mol\,l^{-1}$)	Recovery (%)
1.000	1.500	2.469	97.9
8.000	6.000	14.110	101.8
20.000	5.000	25.276	105.5

reproducibility and storage stability were ascribed to the covalent immobilization of HRP in CS/GPTMS hybrid films which provided a biocompatible microenvironment [20,21,35].

3.7. Selectivity and recovery experiments 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 ($0.10\,mmol\,l^{-1}$) in the presence of H_2O_2 ($30.0\,\mu mol\,l^{-1}$) was compared. Glucose, sucrose, ethanol, acetic acid, citric acid and cystine did not cause any observable interference. Only ascorbic acid interfered significantly. Ascorbic acid can reduce the hexacyanoferrate(III) produced in the peroxidase reaction and thus interfered the determination of H_2O_2.

In order to demonstrate the analytical applicability of developed H_2O_2 biosensor, recovery experiments of three real H_2O_2 samples were performed by standard addition method. As listed in Table 1, the recovery rate was in the range 97.9–105.5%. It can be seen that the results in Table 1 were satisfactory.

4. Conclusion

The successful preparation of the H_2O_2 biosensor demonstrated the efficiency of in situ covalent immobilization of enzyme using polysaccharide-incorporated sol–gel process. Such one-pot process offered simple and convenient methodology and could be easily carried out in aqueous medium without the addition of any organic solvents. The enzyme covalently immobilized in such biocompatible hybrid matrix possessed high electrocatalytic activity and fast amperometric response to H_2O_2. The ease of the one-pot covalent immobilization 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 in enzyme immobilization and fabrication of biosensors.

Acknowledgements

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