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One-step construction of reagentless biosensor based on chitosan-carbon nanotubes-nile blue-horseradish peroxidase biocomposite formed by electrodeposition

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ABSTRACT

A simple and controllable electrodeposition approach was established for one-step construction of novel reagentless biosensors by in situ formation of chitosan-carbon nanotubes-nile blue-horseradish peroxidase (CS-CNTs-NB-HRP) biocomposite film on electrode surface. The mediator effect of NB, conducting performance of CNTs and the biocompatible microenvironment of CS were combined by such one-step non-manual process. NB could interact with CNTs and resulted in good dispersion of CNTs-NB nanocomposites in aqueous solution. Cyclic voltammetry measurements demonstrated that electrons were efficiently shuttled between HRP and the electrode mediated by NB. The developed reagentless 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 reagentless biosensor for the determination of H_2O_2 ranged from 1.0×10^{-6} to 2.4×10^{-4} mol l^{-1} with a detection limit of 1.2×10^{-7} mol l^{-1}. The biosensor exhibited high reproducibility and long-time storage stability. The as-prepared biosensor also showed effective anti-interference capability. The ease of the one-step non-manual technique and the promising feature of the biocomposite could serve as a versatile platform for fabricating electrochemical biosensors.

1. Introduction

Enzyme-based biosensors, which combine the inherent selectivity of enzymatic reactions with the highly efficient electrochemical signal transduction, constitute promising technology in bioanalysis, environmental monitoring and clinical diagnosis due to simplicity, fast response, high sensitivity and selectivity [1,2]. The electrochemical technology based on electron transfer mediator allowed the shuttle of electron from the redox center of enzyme to the surface of working electrode, reduced the operating potential and hopefully avoided the interference from complex samples [3,4]. As compared with solution-phase mediator, immobilization of mediator together with enzyme on an electrode surface using convenient and controllable procedures is of great significance in developing reagentless biosensors for multiple and practical use.

Recently, a series of soluble organic dyes such as methylene blue, nile blue (NB), toluidine blue (Tb) and thionine [3–7] have been proved to be very suitable mediators in horseradish peroxidase (HRP)-based reagentless electrodes due to their high electron transfer efficiency and low cost. It was worth noting that such redox mediators could form stable nanocomposite with carbon nanotubes (CNTs) through π–π electronic and hydrophobic interactions [5,8–10]. The formed nanocomposites showed good dispersion in water, and could thus be conveniently incorporated into the electrode surface for preparing biosensors. It is well known that CNTs have been widely used as predominant materials for preparing modified electrodes in biosensor applications owing to the high accessible surface area, low electrical resistance and high chemical stability. For biosensors constructed by CNTs-organic dye nanocomposites, organic dye could be used as a mediator for electron transfer and CNTs were excellent conductors and matrices for the enzymatic reaction between enzyme and analytical substrate. The combination of such synergistic effects could produce sensitive reagentless biosensor. For example, Liu et al. [5] used Tb, which adsorbed noncovalently on multiwalled carbon nanotubes (MWCNTs), as a mediator for electric communication between HRP and its substrate to prepare regeantless hydrogen peroxide (H_2O_2) biosensor. The fabrication of HRP/Tb-MWCNTs-modified electrode, however, was performed by manual casting. Time-consuming and uncontrollable process might be involved. To make the biosensor reproducible, moreover, special and careful attention must be given for controlling the thickness of the resulting composite film. To easily control the fabrication process, non-manual co-immobilization of enzyme and CNTs-mediator nanocomposites in biocompatible matrix is becoming increasingly important.

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As one of the most promising matrix for enzyme immobilization, the biocompatible polymer, chitosan (CS), remains a focus of study in recent years [11,12]. It was now discovered that CNTs could be dispersed in aqueous solution with the help of CS. Such phenomenon makes CS-CNTs composite materials be attractive for potential applications as biosensing platforms by combination biocompatible microenvironment of CS with excellent conductibility of CNTs [5,13]. Due to the possessing of primary amino groups with a p*K*a of about 6.3, CS was a unique pH-shift polymer as its solubility and net charge were pH-dependent. Recently, electrochemical deposition of CS was pioneered by Payne and co-workers and Chen and co-workers [14–17] as a simple non-manual method to obtain composite film with controllable thickness under moderate conditions [18,19]. The electrodeposition 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 CS solubility and hence to the controlled deposition of CS film. Moreover, special biocomposites could be easily achieved through effective incorporation of functional substrates in the process [19,20]. Therefore, electrodeposition method could supply a simple and universal way to construct CS-based biocomposite film containing enzyme, mediator and CNTs on conductive bases for developing biosensors.

This work attempts to disclose a simple and controllable electrodeposition method for one-step construction of novel reagentless biosensors by in-situ formation of CS-CNTs-mediator-enzyme biocomposite film on the surface of electrode. HRP was selected as model enzyme. Nile blue (NB), a phenoxiazine dye, which has one positive charge within one molecule and showed promising properties as a redox mediator in HRP system, was selected as model mediator. The carboxylated CNTs could interact with NB and the resulting CNTs-NB nanocomposites possessed good dispersion. The proposed procedure offered simple and convenient methodology for in-situ incorporation of NB, CNTs 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 as-prepared reagentless biosensor displayed characteristics of fast response, high sensitivity and good stability for the determination of H_2O_2.

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 (98% deacetylation and an average molecular weight of 4.8×10^5 g mol^{-1}, Yuhuan biomedical Corp., China) and MWCNTs (95%, Shenzhen Nanotech. Port. Co., Ltd., China) were used in this study. All other chemicals were of analytical reagent grade and used without further purification. Hydrogen peroxide (H_2O_2) solutions were prepared freshly using a 30% H_2O_2 solution. The 0.1 mol l^{-1} 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 mol l^{-1} 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. Bare gold electrode or modified gold electrode was used as the working electrode. The reference electrode was Ag/AgCl electrode (saturated with KCl) or saturated calomel electrode (SCE), and platinum disk was used as auxiliary electrode, respectively. Scanning electron microscopy (SEM) images were obtained at 5.0 kV on a SIRION (FEI, USA) field emission scanning electron microscope.

2.3. Preparation of CNTs-NB nanocomposite solution

The MWCNTs were purified by refluxing in 3 mol l^{-1} nitric acid for 12 h. The solution was transferred to the polytetrafluoroethylene centrifuge tubes and spun at 2400 × *g* for 2 h. After the supernatant acid was decanted off, the resultant solid was ultrasonicated in concentrated HNO_3 and H_2SO_4 (v/v, 1:3) for 6 h followed by extensive washing and filtrating in double deionized water until the filtrate was neutral. Then the pH was adjusted to 8.0 to achieve net negatively charged carboxylate anions [21]. The negatively charged carboxylated-MWCNTs were centrifuged at 14,000 × *g* for 30 min to remove the supernatant and dried in vacuum at 50 °C. The obtained CNTs (1 mg ml^{-1}) were dispersed in 0.05 mol l^{-1} PBS containing 2 mg ml^{-1} NB with 5 min ultrasonication to obtain the required CNTs-NB nanocomposites.

2.4. Preparation of CS-CNTs-NB-HRP biocomposite film modified electrode

Before each modification, bare gold electrode was successively polished with emery paper and 0.05 μm α-Al_2O_3 slurry. After being ultrasonicated in double deionized water for 5 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 ultrasonicated in double deionized water, the electrode was electrochemically pre-treated by cyclic potential scanning between 1.4 and −0.2 V in 0.1 mol l^{-1} H_2SO_4 until cyclic voltammogram (CV) of clean gold electrode was obtained.

Typically, a homogenous chitosan-carbon nanotubes-nile blue-horseradish peroxidase (CS-CNTs-NB-HRP) mixture was prepared for electrodeposition by mixing 0.5 wt% CS and 1 mg ml^{-1} HRP with the prepared CNTs-NB solution. CS aqueous solution (0.5 wt%, pH 5.0) was prepared according to the previously reported procedure [16,17,19]. Briefly, CS was dissolved in 0.05 mol l^{-1} aqueous HCl solution and the pH of CS solution was adjusted to 5.0 by using a 1.0 mol l^{-1} NaOH solution. Then the CS solution was filtered using a 0.45 μm cellulose filter film. Afterwards, the prepared CNTs-NB solution was added and the solution was ultrasonicated for 5 min. Then HPR was added and the obtained solution was ultrasonicated for 5 min.

A polished and cleaned gold electrode was dipped into the as-prepared CS-CNTs-NB-HRP solution and was polarized as a negative electrode (the cathode). Deposition was performed by applying a voltage of 1.2 V for 3 min. Consequently, H^+ in the solution was reduced to H_2 at the cathode, and pH near the cathode surface gradually increased. CS became insoluble when pH exceeded its p*K*a (about 6.3). As a result, the CS hydrogel incorporated with CNTs, NB and HRP was locally electrodeposited onto the cathode surface. After deposition, the modified electrode (Au/CS-CNTs-NB-HRP) was disconnected from the power supply and removed from the solution. Afterwards, the modified electrode was soaked in 0.1 mol l^{-1} PBS (pH 8.0) for 10 min. Then the CS coated electrode was stored in 0.1 mol l^{-1} PBS (pH 6.5). For comparison with the Au/CS-CNTs-NB-HRP electrodes, Au/CS-NB-HRP electrodes were prepared using the same procedures in CS-NB-HRP solution.

2.5. Electrochemical characterization of the reagentless biosensor

Cyclic voltammetric 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 −0.4 V versus 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 mmol l^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture with the frequencies ranging from 10^4 to 10^{-1} Hz. Saturated calomel electrode was used as the reference electrode for EIS measurement.

3. Results and discussion

3.1. Fabrication of the reagentless biosensor based on one-step electrodeposition

In present investigation, electrodeposition was used for one-step construction of reagentless biosensors by local formation of CS-CNTs-NB-HRP biocomposite film on the surface of electrode. A schematic representation for constructing the reageatless biosensor by one-step formation of CS-CNTs-NB-HRP biocomposite film modified electrode through electrodeposition was illustrated in Fig. 1. It is difficult to disperse CNTs in aqueous water because of their hydrophobic surface. NB is a kind of blue dye and can be easily dissolved in water. As an aromatic compound, NB can easily attach onto CNTs through strong π–π stocking force [8–10]. Such interaction greatly improved the dispersion of CNTs due to the hydrophilicity of the adsorbed NB molecules. The mixed solution became dark green when CNTs-NB nanocomposites formed. The CNTs-NB nanocomposites were stable in aqueous solution for at least 15 days, which highly facilitated the application of CNTs in developing biosensors.

CS is a unique polymer and ideally suited for electrodeposition because its net charge and solubility are pH dependent [14–19]. Moreover, it has been proved that CNTs could be dispersed in aqueous solution with the help of CS [5,13]. As a result, CNTs-NB could mix well with CS aqueous solution to form a homogenous solution. The formed CS-CNTs-NB nanocomposites were dispersed and stable in water. The enzyme HRP, as shown in Fig. 1, was also homogenously dispersed in the CS environment due to the electrostatic interaction between HRP and CS chains. The mechanism for CS deposition at reducing potentials was ascribed to the formation of pH gradient near the cathode surface due to the reduction of water [14–16]. 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 CNTs, NB and HRP was locally electrodeposited onto the cathode surface (Au/CS-CNTs-NB-HRP).

In summary, the advantages of the proposed strategy for one-step fabrication of reagentless biosensor come from the following four aspects. First, the proposed strategy offered simple and convenient methodology for the preparation of CS-CNTs-NB-HRP biocomposite film. Compared with complicated and time-consuming manual process, the non-manual electrodeposition approach was simple and controllable. As a result, the thickness of the resulting biocomposite film was controllable and the biosensor fabrication was reproducible. Second, the fabricated biosensor combined the individual benefits of CNTs, NB and CS, as illustrated in Fig. 1. Due to the presence of mediator and the improved conductivity, the biocomposite film might open up new opportunities in biosensors and solid-state electrochemical devices [5,13]. Third, the entrapped HRP could retain its bioactivity due to favorable microenvironment provided by CS. It was well known that CS was one of the most promising matrix for enzyme immobilization [11,12,22]. Fourth, the immobilization of NB by forming CNTs-NB composite could avoid the diffuse of NB from the film, which would lead to significant signal loss and greatly affect the performance and lifetime of the biosensor.

3.2. Morphology characterization of the CS-CNTs-NB-HRP biocomposite film

The morphology of the CS-CNTs-NB-HRP biocomposite film was examined by SEM observation, as shown in Fig. 2. It was found that the CS-CNTs-NB-HRP biocomposite film exhibited a porous surface with three-dimensional network. Moreover, CNTs were symmetrically dispersed in the CS matrix. Such morphological characteristics might result in high loading of enzyme and fast response to the substrate.

3.3. Electrochemical characteristics of the CS-CNTs-NB-HRP biocomposite film modified electrode

The cyclic voltammograms (CVs) of the biosensor, namely Au/CS-CNTs-NB-HRP electrode, give a pair of well defined redox peaks in PBS (pH 6.5) at scan rate of 100 mV $s\,l^{-1}$, as shown in Fig. 3. The peaks represented characteristics of redox couple of NB. With the addition of H_2O_2, reduction peak current increased significantly while oxidation peak current decreased, indicating that a catalytic reaction occurred on this biosensor. These results demonstrated that the response of the biosensor to H_2O_2 resulted from only the catalytic activity of HRP immobilized in the biocomposite film. NB incorporated in the film could effectively improve the shuttle of electrons between the electrode and the redox center of HRP. The reduction of H_2O_2 catalyzed by HRP and mediated by NB can be described as follows:

$$H_2O_2 + 2H^+ + HRP_{red} \rightarrow HRP_{ox} + 2H_2O$$

$$HRP_{ox} + NB_{red} \rightarrow HRP_{red} + NB_{ox}$$

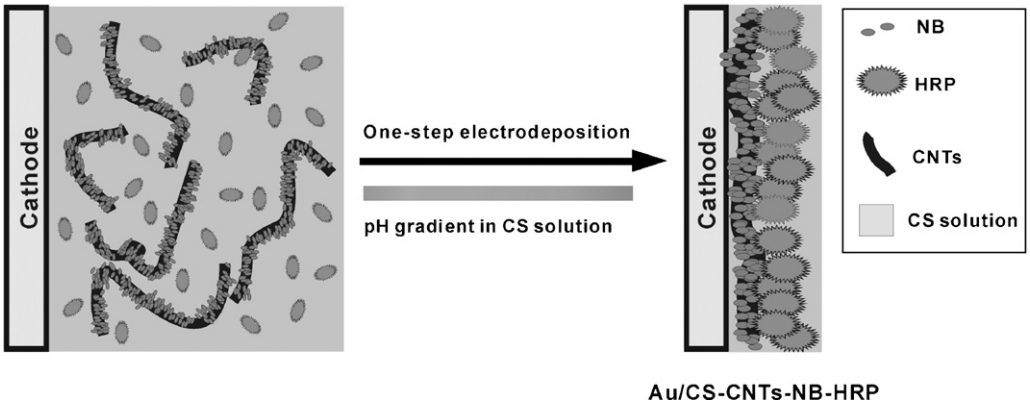

Fig. 1. Schematic representation for construction of the reagentless biosensor by one-step formation of CS-CNTs-NB-HRP biocomposite film modified electrode through electrodepposition.

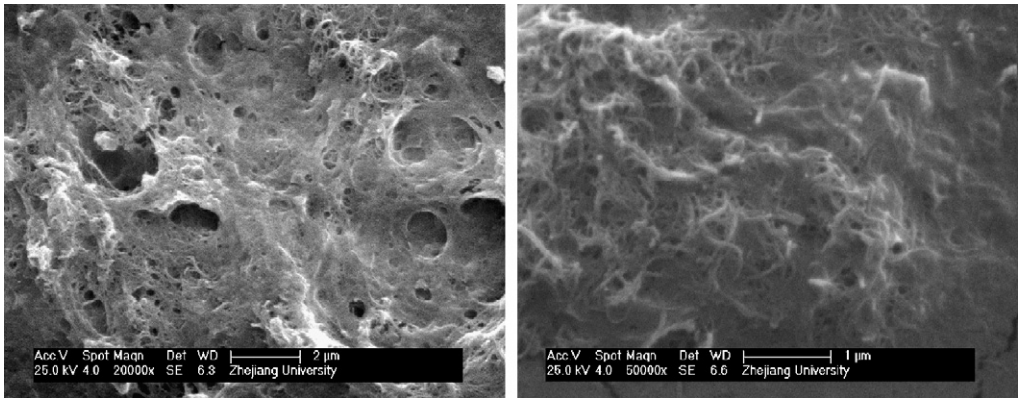

Fig. 2. SEM image of the CS-CNTs-NB-HRP biocomposite film.

$$NB_{ox} + H^+ + 2e \rightarrow NB_{red}$$

where HRP_{ox} and HRP_{red} represent the oxidized and reduced form of HRP, and NB_{ox} and NB_{red} represent the oxidized and reduced form of NB, respectively. In these processes, H_2O_2 in solution first diffused to the biocomposite film on electrode where it is reduced by immobilized HRP_{red}. The immobilized NB_{red} reduced the HRP_{ox} produced in the enzymatic reaction. The NB_{ox} was then electrochemically reduced on the electrode surface [6].

The CVs of the Au/CS-CNTs-NB-HRP electrode in 0.1 mol l^{-1} PBS at different scan rates were shown in Fig. 4. Inset showed the plots of peak current versus the scan rate. A linear relationship of peak current to scan rate was revealed. For the reduction peak current, a slope of 0.327 μA s mV^{-1} and a correlation coefficient of 0.9983 were revealed. For the oxidation peak current, a slope of 0.151 μA s mV^{-1} and a correlation coefficient of 0.9940 were obtained. The results indicated that the electrode reaction initiated by NB was mainly controlled by surface processes.

EIS has been used to characterize the interface properties of the CS-CNTs-NB-HRP biocomposite modified electrodes. Fig. 5 showed the typical results of AC impedance spectra of Au/CS-CNTs-NB-HRP electrode and Au/CS-NB-HRP electrode, respectively. Significant differences in the impedance spectra were observed. The electron transfer resistance (R_{et}) of Au/CS-NB-HRP was esti-

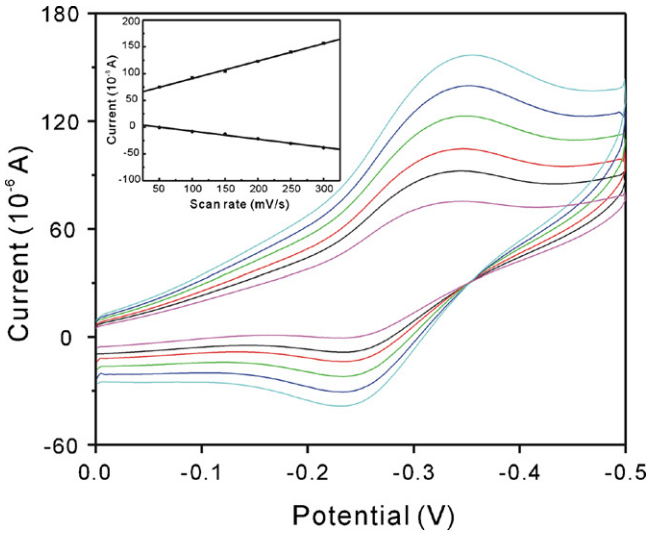

Fig. 4. Typical cyclic voltammograms of the CS-CNTs-NB-HRP biocomposite film modified electrode in 0.1 mol l^{-1} PBS (pH 6.5) at different scan rates from 50 to 300 mV s^{-1}. Inset showed the plots of peak current versus the scan rate.

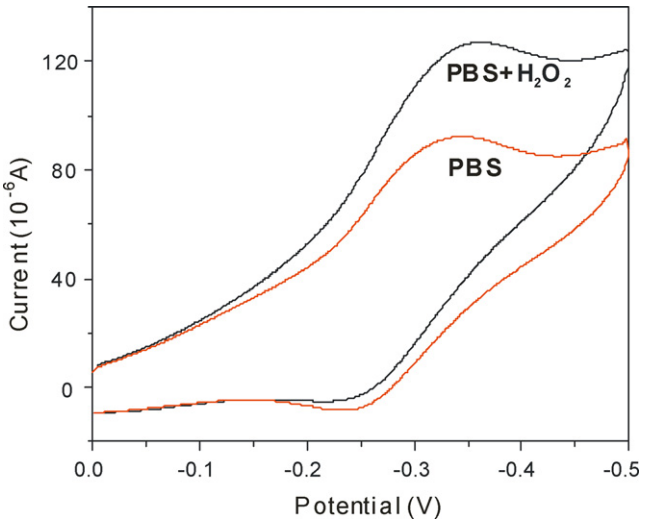

Fig. 3. Cyclic voltammograms of the CS-CNTs-NB-HRP biocomposite film modified electrode in 0.1 mol l^{-1} PBS (pH 6.5) at a scan rate of 100 mV s^{-1} without H_2O_2 and with 1×10^{-4} mol l^{-1} H_2O_2.

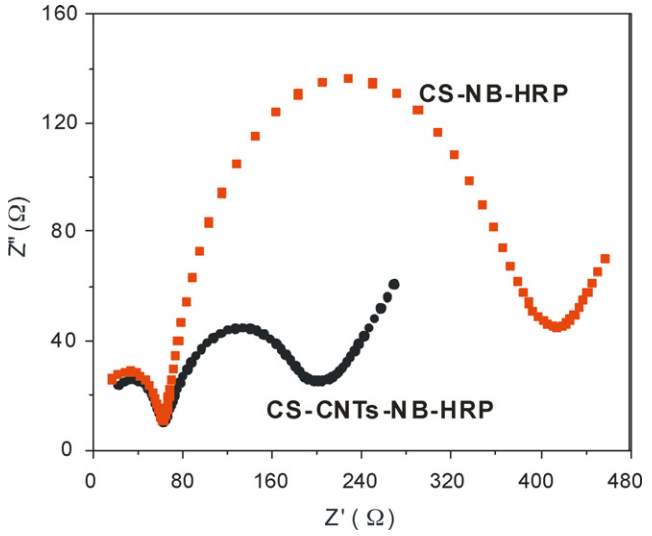

Fig. 5. Electrochemical impedance spectroscopy for Au/CS-CNTs-NB-HRP and Au/CS-NB-HRP in a solution of 5.0 mmol l^{-1} $K_4Fe(CN)_6/K_3Fe(CN)_6$ with SCE as the reference electrode.

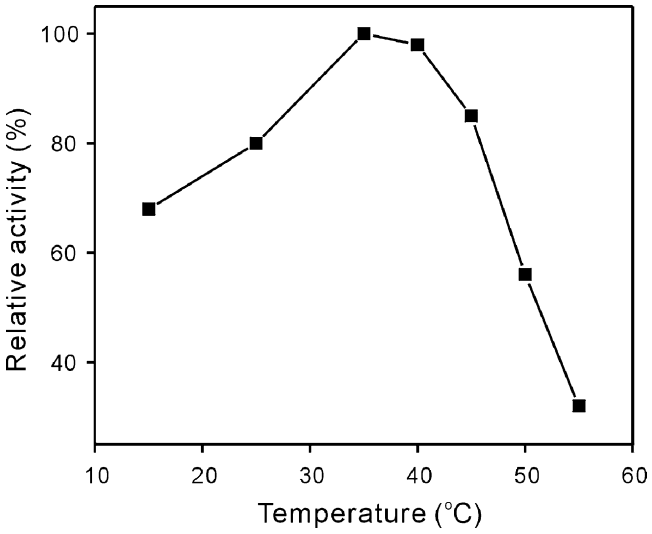

Fig. 6. Effect of temperature on the response of the biosensor. The maximum response was set as 100%.

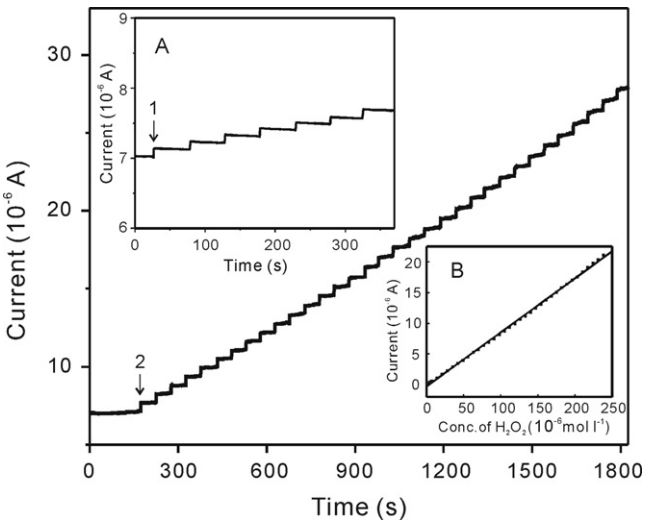

Fig. 7. Typical amperometric response of the fabricated biosensor to successive addition of (1) 1 μmol l^{-1} H_2O_2 and (2) 7 μmol l^{-1} H_2O_2 in a stirred 0.1 mol l^{-1} PBS (pH 6.5). (B) Calibration curve between the current and the concentration of H_2O_2.

mated to be 334 Ω. For Au/CS-CNTs-NB-HRP, the value of R_{et} was found to be 125 Ω, implying that the incorporation of CNTs greatly facilitated the electron transfer of the electrochemical probe.

3.4. Influence of pH, applied potential and temperature 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. A range of pH values between 5.0 and 8.0 was studied. The current response reached the maximum at pH 6.5. Therefore, the suitable pH with the maximum performance of the biosensor was set at pH 6.5, which was in agreement with that reported for HRP entrapped in CS matrix [22].

Studies to investigate the dependence of the biosensor response on the applied potential were also performed. The amperometric response of the CS-CNTs-NB-HRP biocomposite film modified electrode to 30 μmol l^{-1} H_2O_2 was investigated over the potential range of −0.25 to −0.5 V. The response increased sharply from −0.25 to −0.4 V, similar to the trend of cyclic voltammetric response. Thus, −0.4 V was selected as the applied potential for detection of H_2O_2.

The current responses at different temperatures were determined from 25 to 55 °C. Results were given in Fig. 6. At relative low temperature, the response increased with the temperature and reached a maximum value at 35 °C. The result was similar with that of native HRP and confirmed the fact that the immobilization of HRP on the biocompatible CS film caused no deformations of its structure [23,24]. When the temperature was higher than 45 °C, the current decreased rapidly. The phenomenon might be ascribed to the denaturation of the enzyme.

3.5. Amperometric response of the developed H_2O_2 biosensor

Amperometric measurements were performed in a stirred 0.1 mol l^{-1} PBS (pH 6.5) at an applied potential of −0.4 V. Fig. 7 showed the typical current–time responses at CS-CNTs-NB-HRP biocompatible film modified electrode for successive addition of H_2O_2. A sharp increase of current was observed after each addition of H_2O_2, and the response reached 95% of the steady-state value within 2 s. Such rapid response could attribute to the presence of CNTs-NB and fast diffusion of substrate in the porous network of CS-CNTs-NB-HRP biocomposite film. Moreover, well-defined current proportional to the H_2O_2 concentration was observed. The inset (Fig. 7B) displayed calibration curve between the amperometric response of the biosensor and the concentration of H_2O_2. The linear range of the developed biosensor for the determination of H_2O_2 was found to be 1.0×10^{-6} to 2.4×10^{-4} mol l^{-1} with a slope of 87.9 μA ml mol^{-1} and a correlation coefficient of 0.9990 (n = 40). The detection limit of 1.2×10^{-7} mol l^{-1} was estimated at a signal-to-noise ratio of 3.

3.6. Reproducibility and stability of the developed H_2O_2 biosensor

To prove the precision and practicability of the proposed method, the reproducibility and storage stability of the biosensor were examined. The relative standard deviation (RSD) of the biosensor was 2.8% for twelve successive assays at the H_2O_2 concentration of 30 μmol l^{-1}. To evaluate electrode-to-electrode reproducibility, six electrodes were prepared under the same conditions independently. Their response in presence of 30 μmol l^{-1} H_2O_2 was investigated. The results revealed a RSD of 3.5%. When the electrode was kept by suspending it in above 0.1 mol l^{-1} PBS (pH 6.5) at 4 °C in a refrigerator, 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 current response decreased 10% of its initial value after 30 days, showing a long lifetime.

3.7. Selectivity of the developed H_2O_2 biosensor

The potential interference of some biological substance was investigated. The current in an assay solution containing both 30 μmol l^{-1} H_2O_2 and a 0.3 mmol l^{-1} interfering substance was first obtained. The current in an assay solution containing only 30 μmol l^{-1} H_2O_2 was then obtained. The interference degree from interfering substances can be evaluated by comparison the above two currents. Glucose, ascorbic acid, uric acid and L-cysteine were investigated. These results indicated that above four tested reagents would not cause observable interference for the determination of H_2O_2. The results indicated that the developed H_2O_2 biosensor possessed high sensitivity.

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 (%)
15.00	20.00	34.56	97.8
50.00	30.00	80.71	102.4
100.00	50.00	151.47	102.9

3.8. H_2O_2 determination in real samples

To demonstrate the practical usage of the as-prepared H_2O_2 biosensor, recovery experiments of three real samples were performed by standard addition method. As listed in Table 1, the recovery rate was in the range 97.8%–102.9%. These results indicate that the biosensor can be directly used to determine H_2O_2 in real samples. The phenomenon might be ascribed to the high stability and selectivity of the biosensor.

4. Conclusion

The one-step electrodeposition of CS-CNTs-NB-HRP biocomposite on electrode surface is shown to be a highly efficient method for the development of new type of reagentless biosensors with sensitivity, stability and reproducibility. The obtained CS-CNTs-NB-HRP biocomposite film possessed unique characteristics for the improvement of the electrochemical performance. Due to the favorable microenvironment and the improved conductivity, enzyme entrapped in such biocomposite possessed high electrocatalytic activity and fast amperometric response to H_2O_2. Such non-manual approach offered direct and facile methodology with controllability and reproducibility. The ease of the one-step electrodeposition and the biocompatible matrix endowed the electrode with high reproducibility and storage stability. Both the unique one-step construction method and the promising performance of the developed biosensor enable the construction of an attractive biosensing platform.

Acknowledgements

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