Biosensors and Bioelectronics 25 (2010) 2244–2248

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Direct electrochemistry of horseradish peroxidase immobilized on the layered calcium carbonate–gold nanoparticles inorganic hybrid composite

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ARTICLE INFO

Article history:
Received 7 January 2010
Received in revised form 1 March 2010
Accepted 2 March 2010
Available online 9 March 2010

Keywords:
$CaCO_3$–AuNPs
Biosensor
HRP
Direct electron transfer
H_2O_2

ABSTRACT

A mediator-free hydrogen peroxide (H_2O_2) biosensor was fabricated based on immobilization of horseradish peroxidase (HRP) on layered calcium carbonate–gold nanoparticles ($CaCO_3$–AuNPs) inorganic hybrid composite. The proposed biosensor showed a strong electrocatalytic activity toward the reduction of H_2O_2, which could be attributed to the favored orientation of HRP in the well-confined surface as well as the high electrical conductivity of the resulting $CaCO_3$–AuNPs inorganic hybrid composite. The hybrid composite was obtained by the adsorption of AuNPs onto the surfaces of layered $CaCO_3$ through electrostatic interaction. The key analytical parameters relative to the biosensor performance such as pH and applied potential were optimized. The developed biosensor also exhibited a fast amperometric response (3 s), a good linear response toward H_2O_2 over a wide range of concentration from 5.0×10^{-7} to 5.2×10^{-3} M, and a low detection limit of 1.0×10^{-7} M. The facile, inexpensive and reliable sensing platform based on layered $CaCO_3$–AuNPs inorganic hybrid composite should hold a huge potential for the fabrication of more other biosensors.

1. Introduction

Direct electron transfer between the redox enzymes and the electrode surface is a goal for which researchers have been pursuing in recent years (Topoglidis et al., 2003; Gooding et al., 2003; Liu et al., 2005a,b) because it can establish a desirable and ideal model for the fundamental study of the redox behavior of the enzymes in biological systems, which may help to elucidate the relationship between their structures and biological functions (Feng et al., 2005). To achieve this propose, much effort has been devoted to designing biocompatible matrix to retain the native structure of redox enzymes (Feng et al., 2005), synthesizing new layered or mesopore structured materials to increase the amount of immobilized redox enzymes (Liu et al., 2005a,b; Cai et al., 2006), or using different nanoparticles to serve as tiny conduction centers to facilitate electron transfer, such as gold nanoparticles (AuNPs) (Cai et al., 2006), titanate nanotubes (Gooding et al., 2003), carbon nanotubes (Liu et al., 2005a,b, 2008), and silica nanoparticles (Luo et al., 2007).

Recently, AuNPs have received much attention for the immobilization of enzymes owing to their good biocompatibility and nano-scaled dimension effect (Daniel and Astruc, 2004). AuNPs can also provide a suitable microenvironment similar to that of enzymes in native systems and allow a favorable orientation of immobilized enzymes for the effective electron transfer, which are essentially important for the achievement of direct electrochemistry of redox enzymes (Feng et al., 2005). AuNPs are often combined with other biocompatible materials such as polymer matrix, sol–gel matrix, and inorganic micro/nanomaterials for enzyme loading. A distinct synergic effect in the electrochemistry of enzyme is often expected compared with a single one. Among them, calcium carbonate ($CaCO_3$) could be deemed as such a good candidate due to its porous surface structure, good biocompatibility, and large surface area (Rosu et al., 1998). Usually, spherical $CaCO_3$ is used in the fabrication of different biosensors (Cai et al., 2006; Wang et al., 2007; Sun et al., 2007), however, layered $CaCO_3$ is rarely reported (Watabe, 1965; Nakahara et al., 1982; Kato et al., 2002). Layered $CaCO_3$ has a high mechanical strength and large surface area, also can provide a friendly environment for enzymes to retain their activity. Natural layered $CaCO_3$, such as nacre of abalone, could be formed via a biomineralization process in the presence of living organisms (Kato et al., 2002), however, the biomineralization process is complex and hard to control. In contrast, in the present paper, a simple method was adopted to prepare layered $CaCO_3$. Furthermore, a novel inorganic hybrid composite material was obtained with the adsorption of AuNPs onto the surface of layered $CaCO_3$ via electrostatic interaction.

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doi:10.1016/j.bios.2010.03.006

In order to explore the distinct advantages of current special inorganic hybrid composite in enzyme adsorption and bioactivity, horseradish peroxidase (HRP) was chosen as a model and the direct electron transfer ability of HRP to the electrode surface was investigated. A mediator-free HRP based H_2O_2 biosensor was further constructed by immobilizing HRP on the layered $CaCO_3$–AuNPs inorganic hybrid composite modified Au electrode. Owing to the synergic effect of layered $CaCO_3$ and AuNPs, the prepared biosensor toward H_2O_2 shows good stability, a very wide linear calibration, and a low detection limit. The current fabricated biosensor with the use of layered $CaCO_3$–AuNPs inorganic hybrid composite could be easily extended for more applications in other electrochemical biosensors.

2. Experimental

2.1. Reagents

HRP (EC 1.11.1.7, RZ > 3.0, 250 U mg^{-1}) was obtained from Boao Biotechnology Co. Ltd., China. Hydrogen peroxide (H_2O_2) solutions were prepared freshly using a 30% H_2O_2 solution (Nanjing Chemical Reagent Factory, China), and the concentration of H_2O_2 solutions was determined by titration with $KMnO_4$. Calcium chloride ($CaCl_2$) and sodium carbonate (Na_2CO_3) were purchased from Tianjin Bodi Chemical Co. Ltd., China. $HAuCl_4{\cdot}4H_2O$ was purchased from Sigma. Tween-80 was purchased from Tianjin Kermel Chemical Reagent Co. Ltd. The 0.1 M phosphate buffer solutions (PBS) containing 0.1 M KCl at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.10 M H_3PO_4 or NaOH. Doubly distilled water (DDW) was used throughout this work.

2.2. Apparatus and instrumentations

Amperometric and cyclic voltammetric (CV) experiments were performed with a CHI 832B electrochemical analyzer (Shanghai CH Instrument Company, China). Electrochemical impedance spectroscopy (EIS) was performed on a CHI660C electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was used with bare gold (Au) electrode or modified Au electrode as the working electrode, Ag/AgCl electrode (sat. KCl) as the reference electrode and platinum wire electrode 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 AuNPs and the layered $CaCO_3$ microparticles

All glassware used in the following procedures was cleaned in a bath of freshly prepared solution of HNO_3–HCl (3:1, v/v), rinsed thoroughly in DDW and dried in air. AuNPs were prepared by the conventional citrate reduction of $HAuCl_4$ in aqueous solution according to the literature (Doron et al., 1995). In brief, sodium citrate solution was added to a boiling $HAuCl_4$ solution with a molar ratio of $HAuCl_4$ to sodium citrate of 0.26.

10 mL Tween-80 was dissolved in 20 mL 0.05 M $CaCl_2$ solution under stirring to achieve a homogeneous solution, into which an equal volume of 0.05 M Na_2CO_3 solution was rapidly poured at room temperature under ultrasonication (Li and Li, 2007). 10 s later, the precipitated layered $CaCO_3$ was collected by centrifugation and washed three times with DDW.

2.4. Preparation of the layered $CaCO_3$–AuNPs inorganic hybrid composite

0.5 g layered $CaCO_3$ was dispersed in 50 mL Au colloid solution and sonicated for 2 min. The AuNPs–$CaCO_3$ inorganic hybrid composite was obtained with a color of light purple after centrifugation. The hybrid composite was further washed with DDW and dried (Cai et al., 2006).

2.5. Preparation of the enzyme modified electrode

Au electrode was used as the base electrode for the fabrication of the enzyme biosensor. Before modification, bare Au electrode was immersed in freshly prepared piranha solution (H_2SO_4/H_2O_2, 7:3, v/v) for 30 min. Then, it was carefully polished with 1.0, 0.3, and 0.05 μm α-Al_2O_3 slurry, respectively. The obtained electrode was rinsed with DDW, followed by sonicating in ethanol and DDW alternatively. Finally, the electrode was left to dry at room temperature.

The assembly of 4-aminothiophenol (ATP) monolayer was performed by direct immersion of the clean bare Au electrode into ethanol solution containing 0.5 mM ATP for 8 h. After that, the obtained electrode was washed carefully with 0.1 M PBS (pH 7.0), and then 10 μL of layered $CaCO_3$–AuNPs hybrid composite suspension was deposited on the surface of ATP modified Au electrode. 4 h later, the modified electrode was washed thoroughly with PBS (pH 7.0). Subsequently, the resulting electrode was incubated in 3 mg/mL HRP for 8 h to allow HRP molecules loading onto the surface of the layered AuNPs–$CaCO_3$ modified electrode, and the enzyme electrode was denoted as HRP/$CaCO_3$–AuNPs/ATP/Au. All the resulted electrodes were washed with PBS (pH 7.0) and stored at 4 °C when not in use.

2.6. Characterization of the developed biosensor

CV experiments were carried out in quiescent solutions at a scan rate of 50 mV s^{-1}. Amperometric experiments were carried out in a stirred system by applying a potential of −0.2 V to the working electrode. Typical steady-state response of the biosensor to successive injection of H_2O_2 was recorded after a steady-state current had been achieved. EIS experiments were performed in 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture containing 0.1 M KCl with the frequency ranging from 10^4 to 10^{-1} Hz.

3. Results and discussion

3.1. Immobilization of HRP on layered $CaCO_3$–AuNPs inorganic hybrid composite

In present investigation, a mediator-free H_2O_2 biosensor was fabricated based on immobilization of HRP on layered $CaCO_3$–AuNPs inorganic hybrid composite modified Au electrode. Schematic illustration of the fabrication process is given in Fig. 1. With the isoelectric point (IEP) at pH 8.5 (Volodkin et al., 2004), the layered $CaCO_3$ were positively charged at pH 7.0, while AuNPs were negatively charged at the same pH. As a result, AuNPs were adsorbed on the surface of layered $CaCO_3$ through electrostatic interaction (Cai et al., 2006).

Self-assembling technique has been proved as a promising method for the construction of interface with specific functionality and controllability (Song et al., 2008). The ATP was firstly self-assembled on the bare Au electrode surface via sulfur–Au interaction (Jiao et al., 2005; Zhang et al., 2003). The exterior amine group on the monolayer could be further used to attach layered $CaCO_3$–AuNPs composite on the electrode surface through Au–NH_2 interaction (Ferapontova, 2004). Then, the HRP, its IEP of 8.8, could

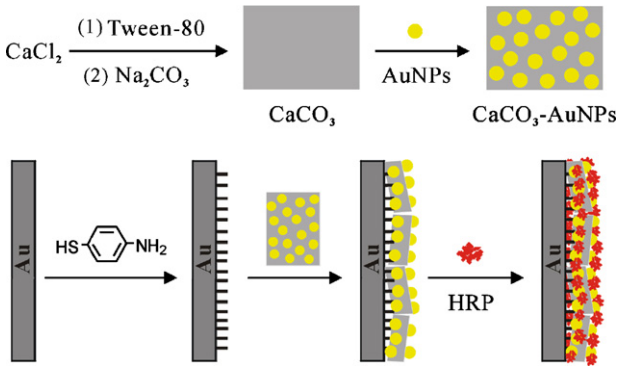

Fig. 1. Schematic representation for the fabrication of the enzyme electrode based on the layered $CaCO_3$–AuNPs inorganic hybrid composite.

be electrostatically adsorbed onto the AuNPs surface at pH 7.0. Simultaneously, the amine group of adsorbed HRP could play a stabilization role for the AuNPs (Cai et al., 2006).

3.2. Characterization of the layered $CaCO_3$–AuNPs inorganic hybrid composite

SEM is a powerful tool to characterize the morphologies of different composites. Fig. 2 shows the SEM images of the layered $CaCO_3$ microparticles before (Fig. 2a) and after loading AuNPs (Fig. 2b). The layered $CaCO_3$ microparticles were simply prepared by the reaction of $CaCl_2$ with Na_2CO_3 solution in the presence of Tween-80. The Tween-80 was used to control the shape and size of $CaCO_3$ nanoparticles to some extent (Watabe, 1965; Nakahara et al., 1982). It was obvious to see that the prepared $CaCO_3$ microparticles possessed large surface area and a uniformly multilayered structure. After the adsorption of AuNPs (Fig. 2b), AuNPs were seen to be uniformly distributed on the plane surface of the layered $CaCO_3$ microparticles and some of them intercalated into the interior layers.

3.3. Electrochemical characteristics of the developed enzyme biosensor

CV was employed to evaluate the performance of the fabricated biosensor during stepwise modification. CVs of differently modified electrodes in 0.1 M PBS (pH 7.0) at a scan rate of 50 mV s^{-1} are presented in Fig. 3. No redox peaks were observed on the bare Au electrode (curve a), ATP/Au (curve b) and $CaCO_3$–AuNPs/ATP/Au (curve c). In comparison, a pair of well-defined redox peaks, corresponding to heme Fe(Ox) and Fe(Red) redox couples in HRP, was obtained on HRP/$CaCO_3$–AuNPs/ATP/Au (curve d) with the peak

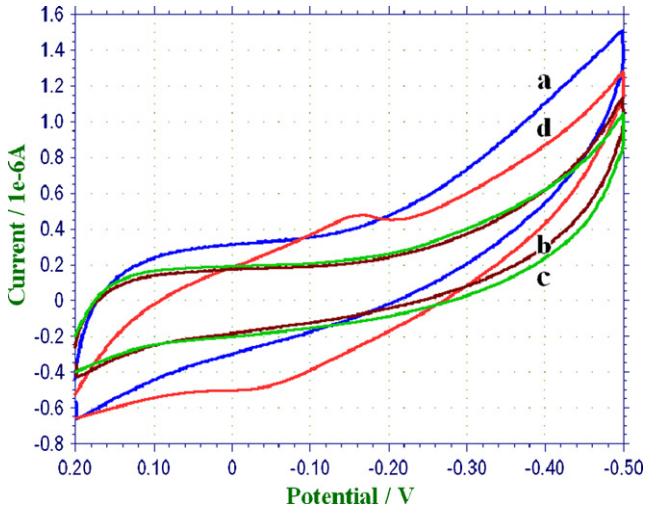

Fig. 3. CVs of (a) the bare Au electrode, (b) ATP/Au, (c) $CaCO_3$–AuNPs/ATP/Au, and (d) HRP/$CaCO_3$–AuNPs/ATP/Au in 0.1 M N_2-saturated PBS (pH 7.0) at a scan rate of 50 mV s^{-1}.

potential located at about −0.05 and −0.15 V, respectively. The formal potential, the average value of the anodic and the cathodic peaks potentials was −0.1 V, which is more positive than that obtained by Xu et al. (2006), implying the easier electron transfer on the HRP/$CaCO_3$–AuNPs/ATP/Au. The favored orientation of the HRP molecules and the high electrical conductivity of $CaCO_3$–AuNPs inorganic hybrid composite may contribute to the direct electron transfer reaction between HRP and the electrode surface. Furthermore, the layered $CaCO_3$ microparticles and AuNPs may play a synergic effect for the direct electron transfer of assembled HRP (Cai et al., 2006).

The electrocatalytic reduction of H_2O_2 at the HRP/$CaCO_3$–AuNPs/ATP/Au is shown in Fig. 4. With the addition of 0.5 mM H_2O_2 into 0.1 M PBS (pH 7.0), it was observed that the reduction peak current increased, while the oxidation peak current decreased (Fig. 4b), showing a typical electrocatalytic reaction behavior of HRP toward H_2O_2. The electrocatalytic mechanism could be expressed as follows:

$$HRP(Red) + H_2O_2 \rightarrow HRP(Ox) + H_2O$$

$$HRP(Ox) + 2e^- + 2H^+ \rightarrow HRP(Red) + H_2O$$

$$\text{Net reaction}: H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$$

EIS could provide information on the impedance changes of the electrode surface during stepwise modification. Fig. 5 shows

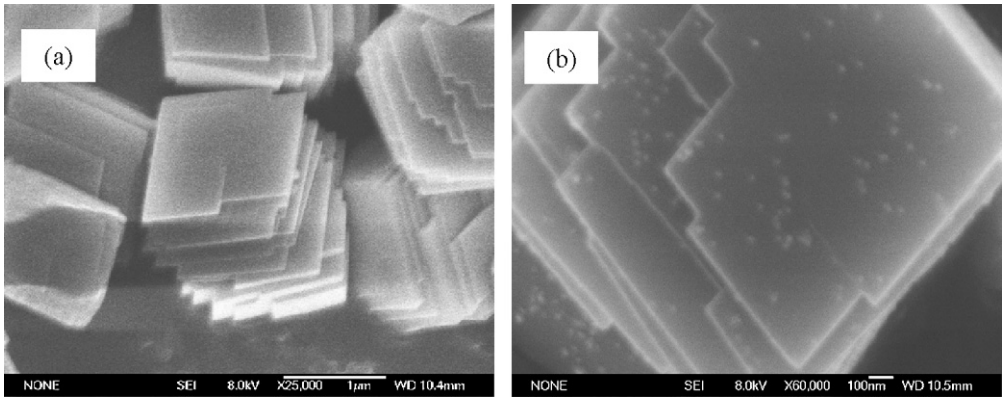

Fig. 2. SEM images of the layered $CaCO_3$ microparticles before (a) and after (b) loading AuNPs.

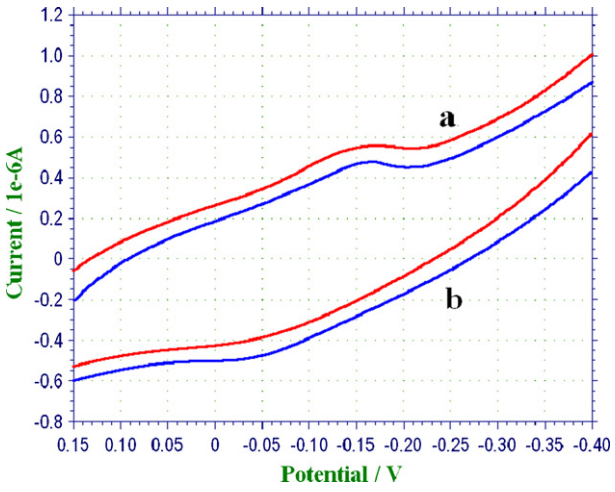

Fig. 4. CVs of the HRP/$CaCO_3$–AuNPs/ATP/Au (a) with 0.5 mM H_2O_2 and (b) without H_2O_2 in 0.1 M N_2-saturated PBS (pH 7.0) at a scan rate of 50 mV s^{-1}.

the Nyquist plots obtained on (a) bare Au electrode, (b) ATP/Au, (c) $CaCO_3$–AuNPs/ATP/Au and (d) HRP/$CaCO_3$–AuNPs/ATP/Au in a solution of 0.1 M KCl containing 1.0 mM $Fe(CN)_6^{3-}$ and 1.0 mM $Fe(CN)_6^{4-}$. After the bare Au electrode was modified by ATP (Fig. 5b), the resulting amino-functioned surface showed a higher interfacial electron transfer resistance (R_{et}), indicating that ATP could obstruct the electron transfer. Decreased R_{et} value was observed when layered $CaCO_3$–AuNPs inorganic hybrid composite was assembled on the ATP-modified electrode (Fig. 5c), indicating that the layered $CaCO_3$–AuNPs inorganic hybrid composite could enhance the electron transfer. The R_{et} of HRP/$CaCO_3$–AuNPs/ATP/Au (Fig. 5d) was found larger than that of $CaCO_3$–AuNPs/ATP/Au, indicating the successful immobilization of HRP onto $CaCO_3$–AuNPs composite.

3.4. Optimization of experimental conditions

Peroxidases are pH-dependent enzymes and present their maximum activities at different pH values (Abelskov et al., 1997). The dependence of the biosensor response on pH of the measurement solution was investigated. A range of pH values between 5.0 and 9.0 was studied (see supplementary material). The redox current increased from pH 5.0 and reached a maximum at pH 7.0. Then, the

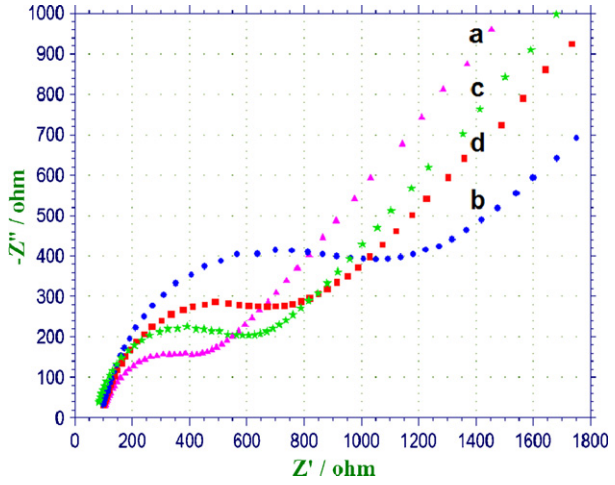

Fig. 5. EIS for (a) the bare Au electrode, (b) ATP/Au, (c) $CaCO_3$–AuNPs/ATP/Au, and (d) HRP/$CaCO_3$–AuNPs/ATP/Au in a solution of 0.1 M KCl containing 1.0 mM $Fe(CN)_6^{3-}$ and 1.0 mM $Fe(CN)_6^{4-}$.

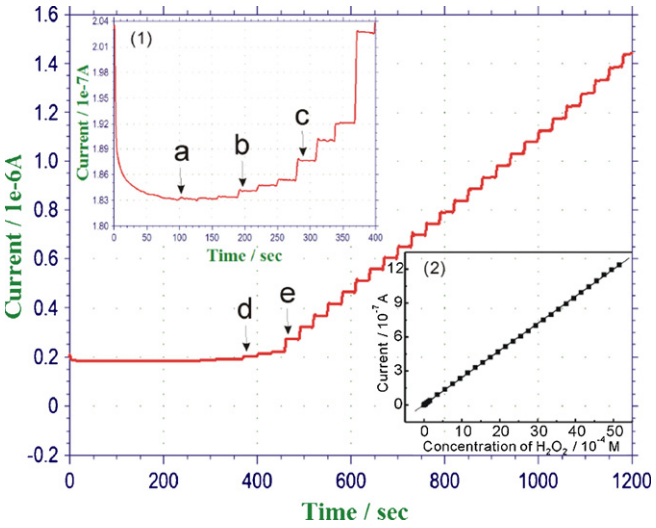

Fig. 6. Typical amperometric response of the HRP/$CaCO_3$–AuNPs/ATP/Au to successive addition of H_2O_2 (a) 0.5 μM, (b) 2.0 μM, (c) 8.0 μM, (d) 40 μM and (e) 200 μM into stirring 0.1 M N_2-saturated PBS (pH 7.0). Inset showed the (1) amperometric response to H_2O_2 in 400 s and the (2) linear part of the calibration curve between the current and the concentration of H_2O_2.

current response was found to be decreased from pH 7.0 to 9.0. In acidic medium, the $CaCO_3$–AuNPs inorganic hybrid composite suffered from dissolution and the hydrogen bonding network around the heme group was destroyed, resulting in the release of the heme itself from the protein cavity, with concomitant enzyme inactivation (Chattopadhyay and Mazumdar, 2000). When pH is above 7.0, the response current decreased, which is probably due to the partial denaturation of HRP. Taking the response and the lifetime of the biosensor into consideration, we selected the suitable pH with the maximum response of the HRP/$CaCO_3$–AuNPs/ATP/Au at pH 7.0.

The effect of applied potential on the biosensor performance was also investigated from −0.35 to −0.10 V (see supplementary material). The current increased rapidly with the applied potential shifting from −0.10 to −0.20 V. At a potential more negative than −0.20 V, a slight decrease in the current was observed due to the irreversible reduction of the ferric iron of HRP in the active site. The current approached a maximum value at −0.20 V, so the potential of −0.20 V was chosen for further measurements.

3.5. Amperometric response of the developed H_2O_2 biosensor

To further investigate the bioactivity of immobilized HRP, amperometric response of the HRP/$CaCO_3$–AuNPs/ATP/Au to successive addition of H_2O_2 in 0.1 M PBS (pH 7.0) is given in Fig. 6. 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 3 s (Fig. 6(1)). Such a fast response can be attributed to the biocompatibility and high electronic conductivity of $CaCO_3$–AuNPs hybrid composite. Moreover, well-defined current proportional to H_2O_2 concentration has been observed. Under the optimized experimental conditions, the proposed biosensor showed a very wide linearly increased amperometric response to H_2O_2 ranging from 5.0×10^{-7} to 5.2×10^{-3} M with a relative coefficient 0.9999 ($n = 37$, inset (2) in Fig. 6). This linear range of four orders of magnitude was much wider than the ranges for H_2O_2 sensor based on direct electrochemistry of HRP (Cai et al., 2006; Xu et al., 2006; Zhao et al., 2008; Kafi et al., 2008; Wang et al., 2009). The detection limit of 1.0×10^{-7} M at a signal-to-noise ratio of 3 was also much lower than those of 1.0 μM based on AuNPs–$CaCO_3$ hybrid composite modified GCE (Cai et al., 2006), 10.3 μM at HRP cross-linked MWCNT/chitosan film (Qian and Yang, 2006),

4.0 μM at HRP coimmobilized in the gelatine matrix and cross-linked with formaldehyde (Yao et al., 2005) and 12.89 μM based on the direct electrochemistry of HRP immobilized in sol–gel-derived ceramic–carbon nanotube nanomaterial film (Chen and Dong, 2007).

3.6. Stability and reproducibility of the HRP modified electrode

For HRP/$CaCO_3$–AuNPs/ATP/Au, the relative standard deviation (R.S.D.) determined by 11 successive assays of 1.0 mM H_2O_2 was 2.7%. The fabrication reproducibility was investigated by preparing six biosensors independently using the same procedure and a R.S.D. of 3.1% was obtained. When the modified electrode was stored at 4 °C for 30-day, it retained more than 96.4% of its initial sensitivity to the reduction of H_2O_2, which may be attributed to the unique layered structure and excellent biocompatibility of $CaCO_3$–AuNPs inorganic hybrid composite to preserve HRP from suffering from loss and denaturation.

4. Conclusions

A mediator-free H_2O_2 biosensor was fabricated based on immobilization of HRP on layered $CaCO_3$–AuNPs inorganic hybrid composite. Direct electrochemistry of HRP and its electro-catalytic activity for the reduction of H_2O_2 were obtained due to the unique structure, excellent biocompatibility and high electrical conductivity of the layered $CaCO_3$–AuNPs inorganic hybrid composite. The proposed biosensor possessed a wide linear range and a sensitive response toward H_2O_2 detection. At the same time, the adopted construction method was simple, which not only could be fit for HRP but also could be extended for more other bioactive macromolecules. The layered $CaCO_3$–AuNPs inorganic hybrid composite also provides a promising platform for the development of more other biosensors.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 Program, 2007CB936000), the National Natural Science Funds for Distinguished Young Scholar (No. 20725518), the Major Program of National Natural Science Foundation of China (No. 90713019), the National Natural Science Foundation of China (Nos. 20875058, 20775039), Science and Technology Development Programs of Shandong Province of China (No. 2008GG30003012), the Natural Science Foundation of Shandong Province of China (No. ZR2009BM031), and the Open Foundation of State Key Laboratory of Electroanalytical Chemistry (2009004).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.03.006.

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