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Renewable reagentless hydrogen peroxide sensor based on direct electron transfer of horseradish peroxidase immobilized on colloidal gold-modified electrode

Song-Qin Liu and Huang-Xian Ju*

Department of Chemistry, The State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, People's Republic of China

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Abstract

A novel renewable reagentless hydrogen peroxide (H_2O_2) sensor based on the direct electron transfer of horseradish peroxidase (HRP) is proposed. The direct electrochemistry of HRP immobilized on a colloidal gold-modified carbon paste electrode (Au-CPE) was investigated using electrochemical methods. The immobilized HRP displayed a pair of redox peaks in 0.1 M phosphate buffer (PB), pH 7.0, with a formal potential of -0.346 V. The response showed a surface-controlled electrode process with an electron transfer rate constant of $6.04 \pm 0.18\,s^{-1}$ determined in the scan rate range from 120 to 500 mV/s. The biosensor displayed an excellent electrocatalytic response to the reduction of H_2O_2 without the aid of an electron mediator. The sensor surface could be renewed quickly and reproducibly by a simple polish step. The calibration range of H_2O_2 was 0–0.3 mM with linear relation from 0.48 to 50 μM and a detection limit of 0.21 μM at 3σ. The response showed Michaelis–Menten behavior at higher H_2O_2 concentrations. The K_M^{app} value of HRP at HRP-Au-CPE was determined to be 3.69 ± 0.71 mM. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Horseradish peroxidase; Direct electron transfer; Au colloid; Electrocatalysis; Carbon paste electrode; Biosensor

Hydrogen peroxide (H_2O_2) is not only the product of the reactions catalyzed by a large number of highly selective oxidases but also an essential mediator in food, pharmaceutical, and environmental analysis [1,2]. Its determination plays an important role in chemical, biological, clinical, and many other fields. Many techniques, such as titrimetry [3], spectrometry [4], and electrochemistry [5], have been developed for this purpose. Among electrochemical techniques, an amperometric biosensor based on electron transfer between an electrode and immobilized peroxidase, which catalyzes the reduction of hydrogen peroxide, is especially promising. This technique is simple and highly sensitive and can be realized in two ways. The first is based on direct electron transfer between horseradish peroxidase (HRP)[1] and electrodes, producing mediatorless H_2O_2 sensors [6–10]. However, both the sensitivity and the application of these unmediated sensors have been limited due to the slow electron transfer rate and the lack of a simple approach to immobilizing and stabilizing enzymes [11]. Another method uses a mediator, such as ferrocene derivatives [12], hexacyanoferrates [13], or poly(vinylpridine) polymer [14], to shuttle the electron between HRP and the electrode. Although the detection limits of some mediated sensors can reach as low as 10^{-7}–10^{-8} M, it is not convenient to add a mediator to a sample solution, and some mediator molecules will pollute the electrode system or diffuse out the enzyme layer [15]. Thus, it was necessary to develop a highly sensitive unmediated H_2O_2 sensor based on the direct electrochemistry of HRP.

The direct electron transfer of immobilized HRP, with regard to the Fe(III) to Fe(II) conversion, has been reported to be achieved on gold [16,17], carbon black [18], carbon paste [19], spectrographic graphite [6], graphite and Pt [20], and HRP-graphite-epoxy biocomposite [21] by amperometric and cyclic voltammetric [17,21]

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* Corresponding author. Fax: +86-25-3317761.
E-mail address: hxju@jlonline.com (H.-X. Ju).

[1] *Abbreviations used:* CPE, carbon paste electrode; Au-CPE, colloidal gold-modified CPE; HRP, horseradish peroxidase; HRP-Au-CPE, HRP immobilized on Au-CPE; PB, phosphate buffer solution; CV, cyclic voltammetry.

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techniques. The direct electron transfer between enzyme and electrode results from intimate contact with the conducting surface [22]. This contact has been achieved in a silica gel modified with titanium oxide [23], an anionic exchange resin [10], and a DNA film [11]. The metal colloidal film has also been used to construct an interface for direct electron transfer of redox-active protein and retain its bioactivity [8,24]. Colloidal gold, an extensively used metal colloid, has been used to study direct electrochemistry of proteins such as cytochrome *c* [25,26], HRP [8], and hemoglobin [27]. It provides an environment similar to that of redox protein in a native system and allows the protein molecules more freedom in orientation, thus reducing the insulating property of protein shells for direct electron transfer and facilitating the electron transfer through the conducting tunnels of colloidal gold. In previous work, we studied the direct electrochemistry of HRP immobilized on colloidal gold/cysteamine self-assembled monolayers and developed an unmediated H_2O_2 sensor [28]. The stability of this sensor was not sufficient for storage for more than 2 weeks at 4 °C or for determination of high H_2O_2 concentrations due to the deactivation of HRP. An alternative approach, which is more desirable, is to develop a renewable, reproducible surface of immobilized HPR. This objective can be achieved by combining the advantageous features of colloidal gold adsorption and carbon paste technology.

It has been recognized that CPEs are very attractive because of their convenient modification by mixing a modifier with the paste [29,30]. The modifier can be in intimate contact and locate in the vicinity of carbon-sensing sites [30,31]. A mediatorless biosensing of H_2O_2 at HRP/CPE has been presented [30]. In this work, we propose a novel approach to developing a renewable, reproducible H_2O_2 sensor. The colloidal gold is used to retain the enzymatic activity and facilitate the direct electron transfer between HRP and carbon-sensing sites. HRP incorporated in the mixture of colloidal gold and carbon paste exhibits a fast electron transfer rate and a high affinity for H_2O_2, which allows quick measurement of H_2O_2 down to 0.21 μM.

Materials and methods

Reagents

HRP (EC 1.11.1.7, $RZ > 3.0$, >250 U/mg) was purchased from Sigma and used as received. $AuCl_3HCl \cdot 4H_2O$ (Au% > 48%) was obtained from Aldrich. Carbon graphite powder (<325 mesh, Johnson Matthey) and paraffin oil (from Fluka) were used to prepare the carbon paste. All other chemicals were of analytical grade and used without further purification. Colloidal gold was prepared by adding 0.5 ml of 1% Na_3-citrate solution to a boiling 50-ml solution of 0.01% $HAuCl_4$ [32]. Prior to use, $HAuCl_4$ and Na_3-citrate aqueous solutions were filtered through a 22-μm microporous membrane filter, and all glassware used in this procedure was cleaned in freshly prepared 3:1 HNO_3:HCl and then rinsed thoroughly in twice-distilled water. The mixture was maintained at the boiling point for 15 min and stirred for another 15 min after removal of the heating source to produce 24-nm-diameter colloidal Au particles. The preparation was stored in a brown glass bottle at 4 °C. Phosphate buffer solutions (0.1 M) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH. All solutions were made up with twice-distilled water.

Electrode preparation

The enzyme-colloidal gold-modified CPEs were prepared according to the literature [30]. Prior to use, the graphite powder was treated at 700 °C for 30 s in a muffle furnace and then cooled to room temperature in a desiccator in the presence of activated silica gel. Graphite powder (100 mg) was mixed thoroughly with 300 μl 24-nm colloidal gold solution. After evaporation of water in a desiccator for 3 h, the colloidal gold-modified carbon powder and 35 mg HRP (or only the modified carbon powder for control) were added into 36 μl paraffin oil. The resulting mixture was stirred (with a glass rod) thoroughly. A portion of the resulting paste was packed into the end of a plastic syringe tube (0.60 mm i.d.) little by little to form an HPR-Au-CPE. Electrical contact to the paste was established by inserting a copper wire down the plastic syringe tube and into the back of mixture. The carbon paste electrode was stored at 4 °C. After the electrode tip was gently rubbed on a fine paper to produce a flat surface, the following experiments were carried out.

Electrochemical measurements

Electrochemical measurements were performed with a BAS-100B electrochemical analyzer connected to a PA-1 preamplifier (Bioanalytical Systems, USA). A three-electrode system comprising a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference, against which all potentials were quoted, and HPR-Au-CPE or Au-CPE as working electrode was used for all electrochemical experiments. The real area of the working electrode was determined to be $1.9 \times 10^{-3}\ cm^2$ from the slope of plot of the anodic peak current of 1.0 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl vs the square root of scan rate. All other experiments were performed at room temperature (26 ± 2 °C) with 0.1 M phosphate buffer (PB) as background electrolyte. All experimental solutions were deoxygenated by bubbling highly pure nitrogen for 15 min and maintained under a nitrogen atmosphere during measurements. Amperometric experiments were carried out by applying a potential step of 0 to −400 mV on a steady cell at 26 ± 2 °C.

Results and discussion

Electrochemical properties of HRP-Au-CPE

We have revealed [33] that 24-nm colloidal Au results in a higher catalytic response of HRP to the reduction of H_2O_2. Thus, the small colloidal Au (24 nm) is chosen in this work. The HRP-Au-CPE is characterized by cyclic voltammetry (CV) to observe whether the enzyme retains its electrochemical activity after the entrapping procedure. Fig. 1 shows the cyclic voltammograms of CPE, Au-CPE, HRP–CPE, and HRP-Au-CPE in pH 7.0 PB at 150 mV/s. HRP-Au-CPE exhibits a cathodic peak at −358 mV with the corresponding anodic peak on the reverse scan at −325 mV. No peak was observed at either CPE or Au-CPE, both of which display low background currents. The presence of gold colloid results in a slight decrease in the background current. Obviously, the response of HRP-Au-CPE is attributed to the redox of the electroactive center of HRP on electrode surface. The peak-to-peak separation of 33 mV of HRP-Au-CPE at 150 mV/s is much lower than that of 279 mV reported at a HRP/Au colloid/cysteamine-modified electrode at 20 mV/s [28]. When HRP is mixed with carbon paste without the presence of gold colloid, the HRP–CPE also shows the response of HRP. Thus, carbon microparticle is an important factor for the direct electron transfer of HRP. The response, however, is 2.3 times smaller than that of HRP-Au-CPE, indicating that the adsorption of HRP on colloidal gold surface plays an important role in facilitating the electron exchange between the HRP and the carbon-sensing sites.

From the integration of reduction peak of HRP-Au-CPE at 150 mV/s, the surface coverage of HRP, Γ ($\Gamma = Q/nFA$ and $A = 1.9 \times 10^{-3}\,\mathrm{cm}^2$), is calculated to be $7.5 \times 10^{-11}\,\mathrm{mol/cm}^2$. The coverage is slightly larger than that of a fully packed monolayer of HRP ($5.0 \times 10^{-11}\,\mathrm{mol/cm}^2$) [28] and $5.1 \times 10^{-11}\,\mathrm{mol/cm}^2$ reported in a DNA film [11]. It is obviously less than $7.6 \times 10^{-10}\,\mathrm{mol/cm}^2$ at HRP/Au colloid/cysteamine-modified electrode [28], at which HRP could adsorbed on the gold colloid surface from different orientations of colloidal gold. At HRP-Au-CPE, HRP shows a monolayer coverage.

The effect of scan rate on the response of immobilized HRP is shown in Fig. 2. With increasing scan rate both redox peak currents and peak-to-peak separation increase. The anodic and cathodic peak currents (i_{pa} and i_{pc}) are proportional to the scan rate (inset in Fig. 2); thus the electrode reaction is typical of surface-controlled quasi-reversible process. The formal potential of the Fe^{III}/Fe^{II} redox couple calculated from the average of potentials in the scan rate range from 120 to 500 mV/s is −0.346 (±0.002) V, which is closer to −0.22 V of native HRP in solution [34] than the formal potential of −0.377 V for the HRP entrapped within a solid matrix at a pyrolytic graphite electrode [10], suggesting that most molecules preserve their native structure after being entrapped in the mixture of Au colloid and carbon paste. The difference between the redox potentials of HRP in HRP-Au-CPE and in solution is possibly due to the negative-charged surface of gold colloid. The negative charge around HRP molecules stabilizes the oxi-

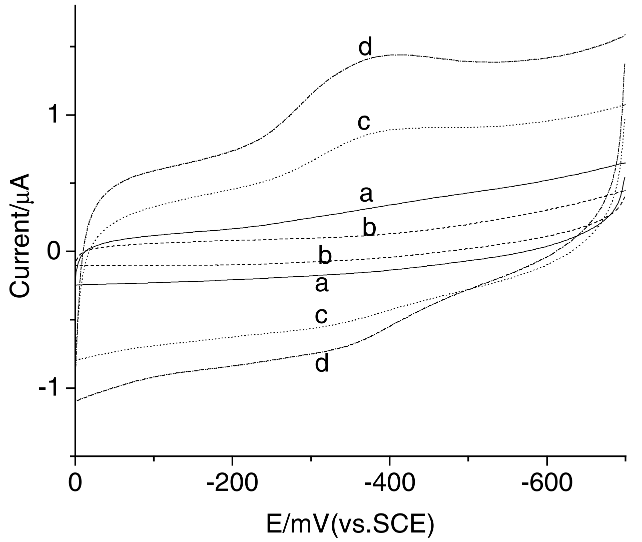

Fig. 1. Cyclic voltammograms of CPE (a, solid line), Au-CPE (b, dash line), HRP–CPE (c, dot line), and HRP-Au-CPE (d, short dash dot line) in pH 7.0 PB at 150 mV/s.

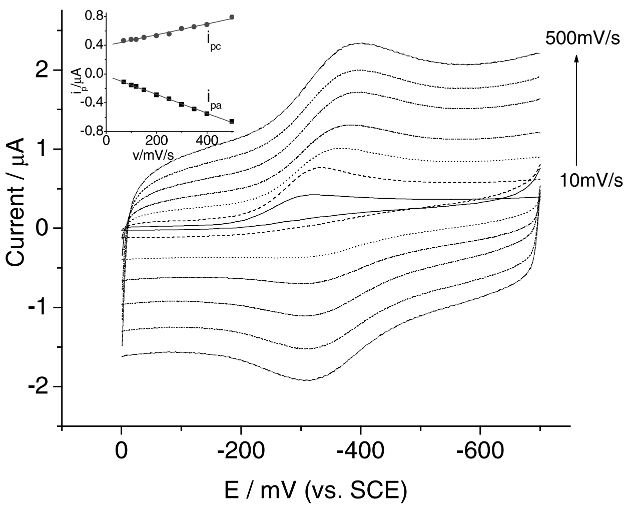

Fig. 2. Cyclic voltammograms of HRP-Au-CPE in pH 7.0 PB at 10, 40, 120, 200, 300, 400, and 500 mV/s (from lowest to highest peak currents). (Inset) Plot of peak current vs scan rate.

dized form of HRP, resulting in the shift of redox potentials of HRP to more negative values.

The small peak-to-peak separation indicates a fast electron transfer rate and a uniform distribution of HRP in HRP-Au-CPE. While the large peak-to-peak separation reported in [28] was ascribable to the various orientations of immobilized HRP molecules. Supposing the charge transfer coefficient, α, is between 0.3 and 0.7, the electron transfer rates k_s can be estimated with the formula $k_s = mnFv/RT$ when the peak-to-peak separation is less than 200 mV [35], where m is a parameter related to the peak-to-peak separation. The peak-to-peak separations of 28, 44, 59, 73, and 88 mV at 120, 200, 300, 400, and 500 mV/s respectively produce an average k_s value of $6.04 \pm 0.18\,\mathrm{s}^{-1}$. The peak-to-peak separations at scan rates less than 500 mV/s are less than that of HRP in TBBMPC matrix [10], indicating a faster electron transfer rate. The k_s value is also much larger than $1.13\,\mathrm{s}^{-1}$ of HRP immobilized on DNA film [11]. The increase in electron transfer rate results from the strong interaction between HRP molecules and colloidal gold particles. The small colloidal gold particles can function as electron-conduction pathways between the prosthetic groups of HRP and carbon-sensing sites and therefore facilitate the electron transfer process.

Effect of solution pH on direct electron transfer of HRP

The effect of solution pH on the response of HRP-Au-CPE is shown in Fig. 3. Obviously, an optimal pH range occurs between 6.5 and 7.0 with a maximum peak current at pH 7.0. The same result was also observed for soluble HRP [36], indicating that the Au-CP matrix does not alter the optimal pH value for electron transfer of immobilized HRP. The decrease of HRP response at pH 8.0 is possibly due to the decreases of proton concentration and bioactivity of immobilized HRP.

Influence of Au colloid content for preparation of HRP-Au-CPE

Fig. 4 shows the relation between the peak current of HRP response and the volume of gold colloid solution mixed with per milligram carbon powder for preparation of HRP-Au-CPE. The cathodic peak current increases gradually with increasing volume of Au colloid solution, indicating that the colloidal Au particles facilitate the electron transfer between HRP and carbon-sensing sites. After the volume is more than 3 μl, the HRP response decreases with the increase in Au colloid content. This phenomenon is attributed to the increase in the resistance and double-layer capacitance of the electrode due to the decrease of ratio of carbon-sensing sites in the paste. A maximum response occurs at a gold colloid volume of 3 μl per milligram carbon powder.

Electrocatalysis of HRP immobilized in HRP-Au-CPE to reduction of H_2O_2

Upon addition of H_2O_2 to the electrochemical cell comprising an HRP-Au-CPE, the reduction current of cyclic voltammogram for the direct electron transfer of HRP increases dramatically (Figs. 5c–e), while no obvious change is observed at Au-CPE (Figs. 5a and b). The changes in redox currents of HRP display an obvious electrocatalytic behavior of immobilized HRP to the reduction of H_2O_2. Furthermore, the reduction peak current increases with increasing H_2O_2 concentration. The electrocatalytic process can be expressed as follows [8]:

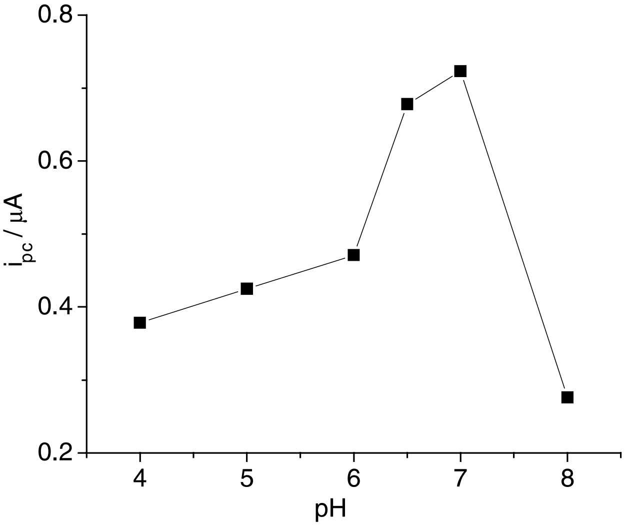

Fig. 3. Effect of pH on cathodic peak current of HRP-Au-CPE in 0.1 M PB at 150 mV/s.

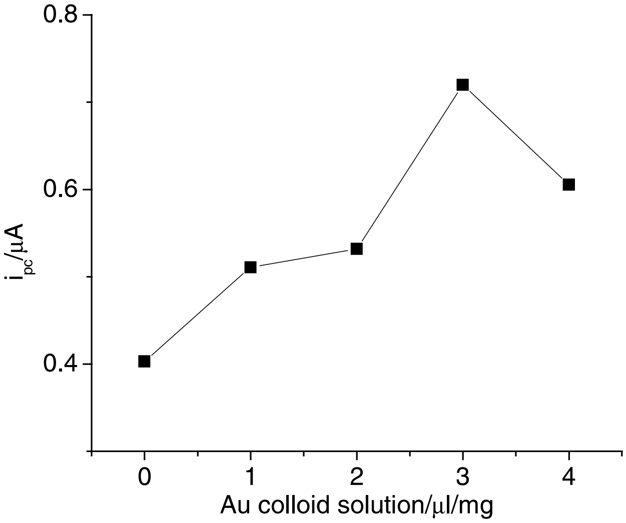

Fig. 4. Effect of the content of colloidal gold for preparation of sensor.

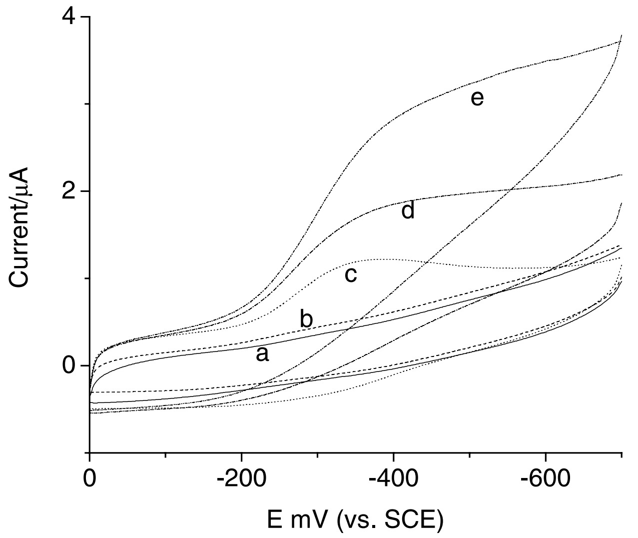

Fig. 5. Cyclic voltammograms of Au-CPE (a and b) and HRP-Au-CPE (c, d, and e) in pH 7.0 PB in the absence of H_2O_2 (a and c) and in the presence of 0.24 (d) and 0.48 mM H_2O_2 (b and e) at 150 mV/s.

$$H_2O_2 + HRP_{red} \rightarrow HRP_{ox} + H_2O \quad (1)$$

$$HRP_{ox} + 2e^- \rightarrow HRP_{red} \quad (2)$$

In the presence of H_2O_2, HRP is efficiently converted to its oxidized form, HRP_{ox} (reaction (1)). Consequently, HRP_{ox} is reduced at the electrode surface by the direct electron transfer (reaction (2)).

Fig. 6 illustrates the chronoamperometric response of HRP-Au-CPE with successive additions of H_2O_2 to 0.1 M PB, pH 7.0. Upon a potential step to the sensor in an unstirred system the reduction current decreases steeply to reach a stable value. The sensor achieves 95% of the steady-state current in 100 ms. The response rate is much faster than those reported for other sensors [10,11,28,37]. The electrode with a direct electron transfer rate constant of $1.13\,s^{-1}$ for immobilized HRP achieves 95% of the steady-state current within 4 s [11]. Thus, the fast response is attributed to the fast electron transfer between HRP and carbon-sensing sites.

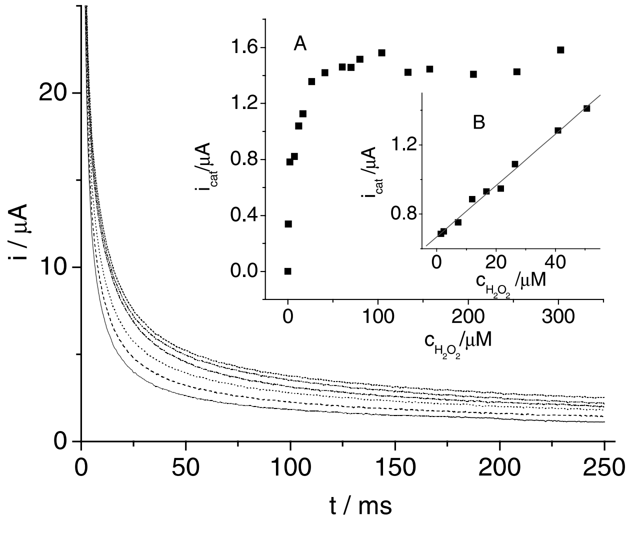

Fig. 6. Chronoamperometric responses of H_2O_2 sensor in unstirred pH 7.0 PB containing 0, 0.48, 0.96, 1.44, 1.92, and 2.40 μM H_2O_2 (from bottom to top). (Inset) Plot of catalytic current vs H_2O_2 concentration (A) and linear calibration curve (B).

With increasing H_2O_2 concentration the amperometric response of the enzyme electrode increases. Inset A in Fig. 6 shows the calibration curve of the enzyme electrode under the optimized experimental conditions. The calibration range of H_2O_2 is 0–300 μM. The linear response range of the sensor to H_2O_2 concentration is from 0.48 to 50 μM with a correlation coefficient of 0.995 ($n = 9$) and a detection limit of 0.21 μM at a signal-to-noise ratio of 3.

When the concentration of H_2O_2 is higher than 50 μM, a platform is observed, showing a characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_M^{app}), a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants, can be obtained from the electrochemical version of the Lineweaver–Burk equation [38]. The K_M^{app} value for HRP-Au-CPE is determined to be 3.69 ± 0.71 mM. This value is lower than 5.5 mM for membrane-entrapped HRP [10] and 11 mM for HRP in solution and slightly higher than 2.3 mM for the HRP/Au colloid self-assembled monolayer electrode [28]. These results indicate that HRP molecules entrapped in the mixture of Au colloid and carbon paste are of a higher affinity than those of soluble HRP and reported in [10].

Stability and renewal of H_2O_2 sensor

When the enzyme electrode was not in use, it was stored in PB at 4 °C. No obvious decrease in the response to H_2O_2 was observed after 3 weeks of storage. After a 40-day storage period, the sensor retained 90% of its initial current response. The stability is much better than that based on a colloidal gold/cysteamine self-assembled monolayer [28]. Thus colloidal gold mixed in carbon paste is very efficient at retaining the activity of HRP and preventing it from leaking out of the sensor.

After the electrode is used in a solution containing H_2O_2 at higher than 10 mM, the response of the sensor decreases irreversibly due to the denaturation of immobilized HRP [39]. The surface with denatured HRP can be renewed by a gently rubbing on a fine paper. The current response of the renewed surface is examined at a H_2O_2 concentration of 2.4 μM. The relative standard deviation is 4.6% for six successive renewals. Thus the method is rapid, easy, and, more importantly, reproducible for removing surface-denatured HRP film. The fabrication reproducibility of six electrodes, made independently, shows an acceptable reproducibility with a relative standard deviation of 5.2% for the current determined at 2.4 μM H_2O_2.

Acknowledgments

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