The Analyst

Immobilization of horseradish peroxidase on a self-assembled monolayer modified gold electrode for the detection of hydrogen peroxide

Jian-Jun Sun, Hui-Qun Fang and Hong-Yuan Chen*

Department of Chemistry, State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing, 210093, China

A procedure for developing an enzyme electrode is described, based on the covalent binding of horseradish peroxidase to a self-assembled monolayer on a gold electrode. The resulting electrode exhibits high sensitivity (0.32 Al mol^{-1} cm^{-2}) to hydrogen peroxide in the presence of a mediator (catechol). The current responses of the electrode are related to the concentrations of both substrate and mediator. The enzyme electrode responds well to hydrogen peroxide in the pH range 5.0–8.0. The optimum pH of the solution for the enzyme electrode is 6.0. Voltammetric experiments showed that the linear range of the enzyme electrode to hydrogen peroxide is from 1.0 μM to 1.0 mM and the detection limit is down to 0.5 μM. A decrease in current response is observed as the concentration of hydrogen peroxide increases above 6.0 mM, which might be due to the deactivation of the enzyme electrode at high concentrations of the substrate.

Keywords: *Enzyme electrode; horseradish peroxidase; hydrogen peroxide; self-assembled monolayer; gold electrode*

The detection of hydrogen peroxide is of practical importance in chemical, biological, clinical, environmental and many other fields. Reactive oxygen intermediates may be generated in the lungs during various pathological processes. These intermediates and hydrogen peroxide itself are toxic to cells through their oxidizing effects on proteins, membranes and DNA. Detection of submicromolar concentrations of hydrogen peroxide is vital because these peroxide levels can damage mammalian cells.[1,2]

The conventional methods for the determination of hydrogen peroxide do not satisfy simultaneously the requirements for sensitivity, reliability and operational simplicity. Enzyme modified electrodes have been reported as sensors for hydrogen peroxide.[3–7] Among these, amperometric sensors based on electron transfer between an enzyme and the electrode[5–7] are promising for fabricating sensitive and linearly responding devices. Many approaches can be used for the immobilization of horseradish peroxidase (HRP) on electrodes, such as physical adsorption on pre-treated carbon or graphite electrodes,[8–12] cross-linking with a polymer[13–15] and mixing with graphite oil paste.[16–18]

A new approach for the immobilization of enzyme was recently developed by Willner and Riklin.[19,20] Redox enzymes were organized as a covalently linked protein layer on electrodes by attachment to a functionalized base self-assembled thiol monolayer associated with gold electrode surfaces. Electrical communication between the redox center of the protein and the electrode surface was achieved by the application of diffusional electron mediators or covalent attachment of electron relay units linked to the protein through long and flexible bridge chains. This approach has been applied successfully to develop a glucose biosensor[21] and a bilirubin amperometric biosensor.[22]

In this work, an enzyme electrode was developed by employing immobilized HRP on a self-assembled monolayer (SAM) modified gold electrode coupled with dissolved electron mediators. A rough gold electrode surface was prepared by a procedure of amalgamation (see below), which leads to an obvious increase in the efficient area of the electrode surface. The resulting electrode exhibits high sensitivity to hydrogen peroxide. The effects of the pH of the solution and the concentrations of the diffusing mediator on the current response were studied in detail.

Experimental

Reagents

Horseradish peroxidase (RZ ≈ 3.0, 250 U mg^{-1}) was purchased from Shanghai Biotechnology (Shanghai, China) and cysteamine, glutaraldehyde (25% v/v), hydrogen peroxide (30% w/v) and catechol from Shanghai Chemical Reagents (Shanghai, China).The other reagents were of analytical-reagent grade and were used as received. All solutions were prepared with doubly distilled water.

Phosphate buffer solutions (PBS) with pH in the range 5.0–8.0 were prepared with 0.05 M KH_2PO_4 and 0.05 M Na_2HPO_4 and buffers of pH 4.0 and 9.0 were prepared with 0.05 M $KH_2C_6H_5O_7$ with adjustment with 0.1 M HCl and 0.05 M $Na_2B_4O_7$ with pH adjustment with 0.1 M NaOH, respectively. The supporting electrolyte was 0.1 M KCl. The test solutions were deaerated with high purity nitrogen for 10 min and kept under a nitrogen atmosphere during the experiments.

Apparatus

Cyclic voltammetric experiments were carried out on a BAS 100B electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN, USA) with a three-electrode system at 25 ± 1 °C. A platinum wire was used as the auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode. A gold disk electrode with a radius of 0.25 mm was made by sealing a gold wire (purity 99.99%) in a glass tube and employed as the working electrode.

Preparation of enzyme electrodes

The gold electrode was polished with sand paper and alumina powder (1.0 μm) successively prior to use. A rough gold electrode was prepared by contacting it with mercury, followed by dissolution of the resulting amalgam layer with concentrated nitric acid. The roughness factor of an electrode represents the ratio of the real area of the electrode surface to the geometric area. During the cycling of a gold electrode in dilute sulfuric acid solution (*e.g.*, 0.5 M) between 0 and 1.6 V *versus* SCE, a specific cathode peak appears at about 0.95 V. The real area of the gold electrode can be calculated from this specific peak area.[23] Roughness factors of 1.2 and 10 were found in this

experiment for smooth and rough gold electrodes, respectively.

The rough gold electrode was soaked in an aqueous cysteamine solution (10 mg ml^{-1}) for 2 h and then rinsed with water thoroughly to remove the non-chemisorbed materials. The resulting electrode was treated for 1 h with a 10% v/v aqueous solution of glutaraldehyde. After rinsing with water, the electrode was immersed in PBS (pH 7.0) containing 10 mg ml^{-1} HRP for 4 h. After rinsing with the buffer, the enzyme electrode was completed and stored in a refrigerator (4 °C) until further use.

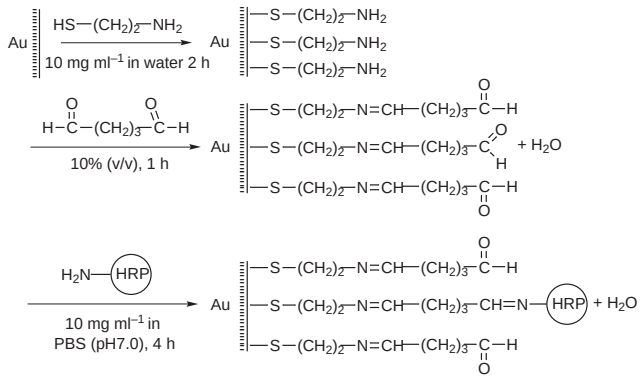
Results and discussion

Scheme 1 shows the reaction steps in the preparation of the HRP modified SAM/Au electrode. After the first step of the electrode modification, the cysteamine monolayers chemisorbed on the gold electrode surface expose an array of amino groups towards the solution. After the second step, the electrode surface was activated with aldehyde groups owing to the second carbonyl group of glutaraldehyde. The formation of bridge structures due to the reaction of both carbonyl groups of glutaraldehyde with two amino groups can be prevented by the application of a high concentration of the bifunctional reagent. After the last step, the amino group of HRP was covalently coupled with the carbonyl group of glutaraldehyde and then an HRP electrode was obtained. This procedure for the preparation of HRP electrode showed good repeatability.

HRP is very different from other enzymes such as glucose oxidase and ureasae. Even though it contains more than 300 amino acid moieties, a few can be used for linking with aldehyde.[24] In addition, as the size of the HRP molecule is much larger than that of an aldehyde group, it might not link with every aldehyde group beneath it. Hence an excess of reactive aldehyde groups remained in the SAM layer (Scheme 1).

Recently, we studied dopamine (instead of HRP here) covalently bound to a cysteamine SAM on a gold electrode through glutaraldehyde.[25] As dopamine is electrochemically active, the monolayer was checked by an electrochemical method, and its density verified (approximately 1.51 × 10^{-10} mol cm^{-2}).

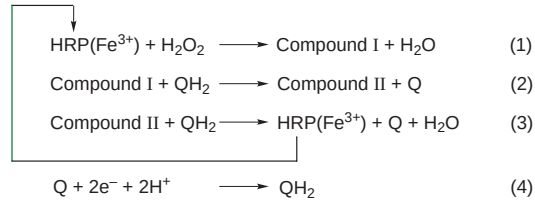
Fig. 1 depicts the cyclic voltammograms (CVs) at the enzyme electrode in PBS (pH 7.0) (a) without and (b, c) with hydrogen peroxide in the presence of mediator. Although a direct electron transfer is possible between an electrode and a peroxidase catalyzing the reduction of hydrogen peroxide,[6,13,14] this is generally a slow process on conventional electrode materials. An appropriate electron donor can mediate the electron transfer between peroxidase and an electrode,[4,5,7,15] and hence such a mediator is expected to improve the performance of a peroxidase- based hydrogen peroxide sensor. It can be seen from Fig.1(a) that the redox of catechol at this electrode



Scheme 1 Reaction steps in the preparation of the HRP electrode.

exhibited quasi-reversible electrochemical behavior. The formal potential, calculated as the average of the potentials of the anodic and cathodic peaks, *i.e.*, $E^{\circ\prime} = (E_{pa} + E_{pc})/2$, was about 150 mV (*versus* SCE) and the peak potential difference, $\Delta E_p = E_{pa} - E_{pc}$, was about 70 mV. The redox of catechol at a bare gold electrode usually exhibited an irreversible electrochemical reaction (not shown). This irreversibility may be due to specific adsorption of some organic molecules, such as catechols or quinones,[26] on the metal electrode surface. The presence of SAM on the gold electrode may prevent catechol molecules from interacting strongly with the electrode surface, and also facilitate the charge transfer between the two.

With addition of hydrogen peroxide to the solution, an increase in cathodic currents was observed in Fig. 1(b) and (c), and the anodic currents were simultaneously reduced. This is a typical enzyme- dependent catalytic process that could be expressed as follows:[27]



Scheme 2

where compounds I (oxidation state +5) and II (oxidation state +4) represent the intermediates in the reactions and QH_2 and Q represent catechol and its oxidized form, respectively. When these reactions proceeded at an electrode surface, compound I was reduced to compound II, and then compound II to the original form of HRP(Fe^{3+}) by a redox mediator QH_2. These approaches result in a reduction current correlated with the concentrations of both peroxide and the mediator in the solution.

Fig. 2 depicts the plot of the current response *versus* the concentration of the mediator in the solution with peroxide concentration fixed. It can be seen that the current rises rapidly at first (up to 0.08 mM) and then more slowly. A leveling off is observed above 0.35 mM. Among mediators for HRP, catechol is one of the best to transfer electrons from electrode surface to compound I or II rapidly.[27,28] Hence, in order to detect submillimolar concentrations of H_2O_2, a millimolar mediator concentration was necessary to overcome the dependence of the response on it and also to achieve a high sensitivity of the electrode. Further, a promising approach was also achieved for the detection of micromolar concentrations of catechol at the enzyme electrode with concentrations of H_2O_2 unchanged.

Fig. 3 illustrates the pH dependence of the current response of the HRP electrode. It is found that the electrode can respond well to H_2O_2 in the pH range 5.0–8.0 and the optimum pH value is around 6.0 (at pH 4.0 and 9.0 the current response decreased sharply). After the treatment of the electrode in the above solutions it became unusable, which might be due to the deactivation of the enzyme immobilized on the electrode surface in such solutions, and the activity of the enzyme could not be restored.

Fig. 4 illustrates the current response of the enzyme electrode to hydrogen peroxide over a wide concentration range from 1.0 μM to 9.0 mM in the presence of 1.0 mM catechol. An excellent linear relationship (r^2 = 0.998) was obtained from 1.0 μM to 1.0 mM (see also the inset), and quasi-linearity prevailed up to 3.0 mM. A maximum current response was reached at about 6.0 mM, and then a decrease in current was observed with further increase in hydrogen peroxide concentration. These results might be due to the formation of an enzymatically inactive form of peroxidase denoted compound

III (oxidation state +6) at high concentrations of peroxide.[29] When this enzyme electrode was dipped once again into the buffer with a concentration of hydrogen peroxide lower than 6.0 mM, the current response could not be restored to the corresponding value. Hence 6.0 mM is the upper limit for the enzyme electrode to respond to hydrogen peroxide reversibly. The detection limit is as low as 0.5 μM. In addition, the sensitivity of the electrode to hydrogen peroxide can be also calculated to be 0.32 A l mol^{-1} cm^{-2}. This value is much higher than that in the literature,[10–15,17,18] *e.g.*, 0.06 A l mol^{-1} cm^{-2} at a pyrolytic graphite electrode with HRP adsorption,[12] 0.073 A l mol^{-1} cm^{-2} at one with HRP within electropolymerized *o*-phenylenediamine[14] and 0.004 A l mol^{-1} cm^{-2} at an HRP-incorporated carbon paste electrode.[17] The amalgamation procedure of the gold electrode leads to substantial enzyme loading on the electrode surface, and hence a large increase in sensitivity. The monolayer immobilization of HRP on the gold electrode surface may assist every HRP molecule to function effectively.

The data used here were all average values from five successive detections. The relative standard deviation was found to be not more than 3.0%.

The HRP electrode was stored in PBS (pH 7.0) at 4 °C when not in use. HRP has been found to be very stable in solution,[27] but the stability of peroxidase electrodes depends on the design of the electrode. In the present case, the activity of the enzyme electrode remained constant during the first 10 d. Two weeks later, the current response to H_2O_2 decreased quickly, and the electrode was completely deactivated after 3 weeks (Fig. 5).

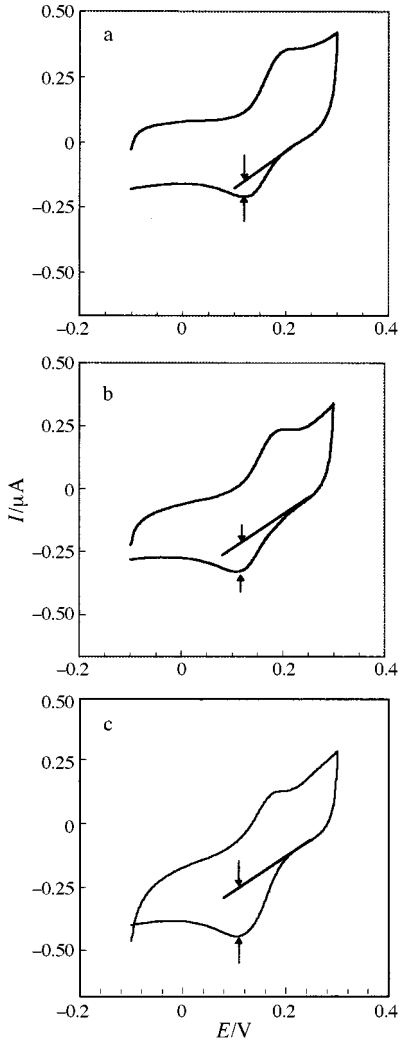


Fig. 1 CVs at the HRP electrode at 50 mV s^{-1} in PBS (pH 7.0) containing hydrogen peroxide at (a) 0 (b) 0.2 and (c) 0.4 mM in the presence of 0.1 mM catechol.

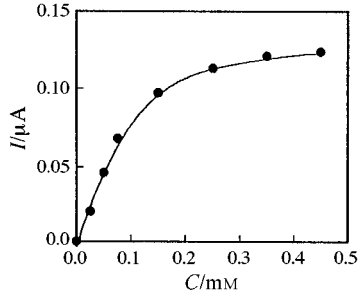


Fig. 2 Plot of the cathodic peak currents of CVs at 50 mV s^{-1} *versus* concentration of catechol in PBS (pH 7.0) containing 0.1 mM hydrogen peroxide.

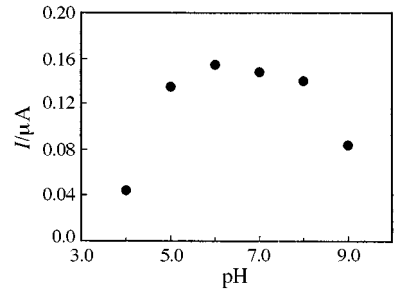


Fig. 3 pH dependence of the HRP electrode in buffer containing 0.1 mM catechol and 0.2 mM hydrogen peroxide.

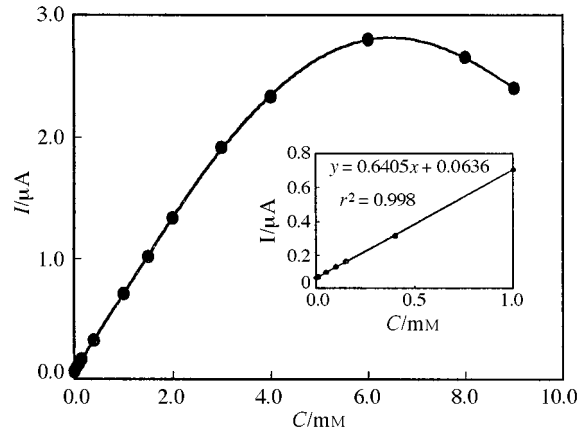


Fig. 4 Plots of the cathodic peak current of CVs at 50 mV s^{-1} at the HRP electrode *versus* concentration of hydrogen peroxide in PBS (pH 7.0) in the presence of 1.0 mM catechol.

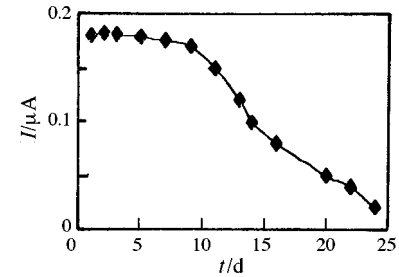


Fig. 5 Time course of the HRP electrode for detection of hydrogen peroxide (0.2 mM). Other conditions as in Fig. 4.

Owing to the specific characterization of the enzyme with respect to its substrates, few substances can interfere with the response of this HRP electrode to H_2O_2 except the other peroxides (*e.g.*, cumene hydroperoxide and butan-2-one peroxide) and mediators (*e.g.*, hydroquinone and dopamine). The presence of such interfering substances should be avoided.

Conclusions

An enzyme electrode based on the covalent binding of HRP to a SAM modified gold electrode was developed that is simple in design and more sensitive than most other electrodes. The procedure is promising for immobilizing HRP-linked antigens or antibodies on a gold electrode for applications in electrochemical immunoassay. Work on this aspect is in progress.

Financial support from the National Natural Science Foundation of China and the Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Science, is gratefully acknowledged.

References

1 Jolliet, P., *Crit. Care Med.*, 1994, **22**, 157.
2 Test, S. T., and Weiss, S. J., *J. Biol. Chem.*, 1984, **259**, 399.
3 Aizawa, M., Karube, I., and Suzuki, S., *Anal. Chim. Acta*, 1974, **69**, 431.
4 Hahn, Y., and Olson, C., *Anal. Chem.*, 1979, **51**, 444.
5 Frew, J. E., Harmer, M. A., Hill, H. A. O., and Libor, S. I., *J. Electroanal. Chem.*, 1986, **201**, 1.
6 Armstrong, F. A., and Lanon, A. M., *J. Am. Chem. Soc.*, 1987, **109**, 7211.
7 Tatsuma, T., Okawa, Y., and Watanabe, T., *Anal. Chem.*, 1989, **61**, 2352.
8 Kulys, J., and Schmid, R. D., *Bioelectrochem. Bioenerg.*, 1990, **24**, 305.
9 Dominguez Sanchez, P., Tunon Blanco, P., Fernandez Alvarez, J. M., Smyth, M. R., and O'Kennedy, R., *Electroanalysis*, 1990, **2**, 303.
10 Jonsson, G., and Gorton, L., *Electroanalysis*, 1989, **1**, 465.
11 Vidal, J. C., Yague, M. A., and Castillo, J. R., *Sens. Actuators B*, 1994, **21**, 135.
12 Wollenberger, U., Bogdanovskaya, V., Bobrin, S., Scheller, F., and Tarasevich, M., *Anal. Lett.*, 1990, **23**, 1795.
13 Tatsma, T., Gondaira, M., and Watanabe, T., *Anal. Chem.*, 1992, **64**, 1183.
14 Deng, Q., and Dong, S., *J. Electroanal. Chem.*, 1994, **377**, 191.
15 Liu, H., Zhang, X., Wei, J., Wu, X., Qi, D., Liu, Y., Dai, M., Yu, T., and Deng, J., *Anal. Chim. Acta*, 1996, **329**, 97.
16 Dominguez-Sanchez, P., Miranda Ordieres, A. J., Costa Garcia, A., and Tunon Blanco, P., *Electroanalysis*, 1991, **3**, 281.
17 Wollenberger, U., Wang, J., Ozsoz, M., Gonzalez-Romero, E., and Scheller, F., *Bioelectrochem. Bioenerg.*, 1991, **26**, 287.
18 Luo, W., Liu, H., Deng, H., Sun, K., Zhao, C., Qi, D., and Deng, J., *Anal. Lett.*, 1997, **30**, 205.
19 Willner, I., and Riklin, A., *Anal. Chem.*, 1994, **66**, 1535.
20 Riklin, A., and Willner, I., *Anal. Chem.*, 1995, **67**, 4118.
21 Willner, I., Riklin, A., Shoham, B., Rivenson, D., and Katz, E., *Adv. Mater.*, 1993, **5**, 912.
22 Shoham, B., Migron, Y., Riklin, A., Willner, I., and Tartakovsky, B., *Biosen. Bioelectron.*,1994, **10,** 341.
23 Woods, R., in *Electroanalytical Chemistry*, ed. Bard, A. J., Marcel Dekker, New York, 1980, vol. 9, p. 125.
24 Zhu, M. H., and Shi, W. Z., *Modern Analytical Chemistry*, High Education Press of China, Beijing, 1991, p. 181 (in Chinese).
25 Sun, J.-J., Xue, J.-J., Fang, H.-Q., and Chen, H.-Y., *Bioelectroanal. Bioenerg.*, 1997, **44**, 45.
26 White, J. H., Soriaga, M. P., and Hubbard, A. T., *J. Electroanal. Chem.*, 1985, **185**, 331.
27 Ruzgas, T., Csoregi, E., Emneus, J., Gorton, L., and Maryo-Varga, G., *Anal. Chim. Acta*, 1996, **330**, 123.
28 Pantano, P., Morton, T. H., and Kuhr, W. G., *J. Am. Chem. Soc.*, 1991, **113**, 1832.
29 Aderian, S. A., and Lambeir, A.-M., *Eur. J. Biochem.*, 1989, **186**, 571.

Paper 7/05841A
Received August 11, 1997
Accepted January 2, 1998