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Thionine Covalently Tethered to Multilayer Horseradish Peroxidase in a Self-Assembled Monolayer as an Electron-Transfer Mediator

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A new approach to construct a reagentless enzyme biosensor is described. Based on multilayer horseradish peroxidase in a self-assembled monolayer configuration, the biosensor was constructed using multilayer thionine covalently tethered to the enzyme as an electron-transfer mediator. The multilayer enzyme and the multilayer mediator were stepwisely synthesized onto an L-cysteine-assembled gold electrode using glutaraldehyde as a bifunctional reagent. The multilayer mediator tethered to the multilayer enzyme could effectively and stably shuttle electrons between the electrode and the multilayer enzyme linked onto the monolayer. The sensitivity of the resulting enzyme biosensor with eight layers of enzyme and three layers of mediator was more than 250 μA cm^{-2} for 1.0 $\times$ 10^{-4} mol/L hydrogen peroxide under optimal conditions, whereas such a modified electrode with one layer of enzyme and one layer of mediator did not yield a detectable response to 1.0 $\times$ 10^{-4} mol/L hydrogen peroxide.

Electrical communication between redox sites of enzyme and electrode is the basis for developing various amperometric biosensor devices.[1] Nevertheless, direct electron transfer is usually not possible with most enzymes because their redox centers are located deep inside the insulated protein shells. Therefore, redox mediators are required in order to establish an electrical connection between these redox centers and the electrode.[2] To fabricate a mediated enzyme biosensor, it would be preferable to immobilize an electron-transfer mediator on the electrode surface.[3] A relatively high mediator concentration is necessary for a high sensitivity of the biosensor, which depends on the mediator concentration as well as the amount of enzyme.[4] However, the electron-shuttling mediator suffers from the inherent drawback that the soluble, or partially soluble, mediating species can diffuse away from the electrode surface into the bulk solution when the biosensor is used continuously or for multiple sample analysis. Hence, immobilization of the mediators plays an important role in the construction of the mediated enzyme biosensors. Our group has used montmorillonite and Nafion gel to prevent the electron-transfer mediator from leaching out of the enzyme electrode.[3,5] Successful mediated enzyme biosensors have been developed with enzymes which have been either chemically modified with electron relays[6] or immobilized in the mediating redox polymers such as polypyrrole,[7] polyloxane,[8] poly(ethylene oxide),[9] and poly(vinylpyridine).[10]

Recently, the self-assembly technique has been used for the fabrication of amperometric enzyme biosensors.[11] Two primary analytical advantages associated with coating an electrode with a self-assembled monolayer (SAM) are as follows: (1) Non-Faradaic background currents are dramatically reduced, largely because the monolayer prevents close approach of solvent and ions to the electrode surface and, therefore, decreases the double-layer capacitance,[12] and reducing background currents is usually desirable in electroanalytical chemistry because it results in improved sensitivity. (2) Self-assembled monolayers made it possible to covalently bind enzyme onto electrode surfaces, and this type of membrane-free amperometric enzyme sensors is of interest because they have, in principle, high amperometric sensitivities and rapid respond time, free from any diffusion problem of substrates or products through the enzyme membrane. On the other hand, the self-assembly techniques provide an elegant strategy in the earlier studies, which allows the preparation of electroactive monolayer, often avoiding excessively laborious organic synthesis work, and this made it possible to bind covalently in situ the redox species. In this way, quinones,[13] viologens,[14] ferrocenes,[15] Ru complexes,[16] and other redox species can be tethered to a self-assembled electrode. However, up to

(1) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128−134.
(2) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285.
(3) Lei, C.; Deng, J. *Anal. Chem.* **1996**, *68*, 334−3349.
(4) Bourdillon, C.; Bourgeois, J. P.; Thomas, D. *J. Am. Chem. Soc.* **1980**, *102*, 4231−4235.
(5) Liu, H.; Deng, J. *Anal. Chim. Acta* **1995**, *300*, 65−70.
(6) Heller, A. *J. Phys. Chem.* **1992**, *96*, 3579−3586.
(7) Foulds N. C.; Lowe, C. R. *Anal. Chem.* **1988**, *60*, 2473−2478.
(8) Hale, P. D.; Lee, H.; Okamoto, Y. *Anal. Chem.* **1990**, *62*, 258−263.
(9) Hale, P. D.; Lan, H. L.; Boguslavsky, L. I.; Karan, H. I.; Okamoto, Y.; Skotheim, T. A. *Anal. Chim. Acta* **1991**, *251*, 121−128.
(10) Degani Y.; Heller, A. *J. Am. Chem. Soc.* **1989**, *111*, 2357−2358.
(11) (a) Katz, E.; Schlereth, D. D.; Schmidt, H.-L.; Olsthoorn, A. J. J. *J. Electroanal. Chem.* **1994**, *368*, 165−171. (b) Creager, S. E.; Rowe, G. K. *Anal. Chim. Acta* **1995**, *307*, 277−289. (c) Willner, I.; Lapidot, N.; Riklin, A.; Kasher, R.; Zahavy, E.; Katz, E. *J. Am. Chem. Soc.* **1994**, *116*, 1428. (d) Katz, E.; Lion-Dagan, M.; Willner, I. *J. Electroanal. Chem.* **1995**, *382*, 25. (e) Lotzbeyer, T.; Schuhmann, W.; Katz, E.; Falter, J.; Schmidt, H.-L. *J. Electroanal. Chem.* **1994**, *377*, 291. (f) Willner, I.; Katz, E.; Riklin, A.; Kasher, R. *J. Am. Chem. Soc.* **1992**, *114*, 10965−10966.
(12) Porter, M. D.; Bright, T. B.; Allara, D. L.; Chidsey, C. E. D. *J. Am. Chem. Soc.* **1987**, *109*, 3559−3568.
(13) (a) Katz, E.; Schlereth, D. D.; Schmidt, H. L. *J. Electroanal. Chem.* **1994**, *367*, 59−70. (b) Zhang, L.; Lu, T.; Gokel, G. W.; Kaifer, A. E. *Langmuir* **1993**, *9*, 786.
(14) Katz, E.; Itzhak, N.; Willner, I. *J. Electroanal. Chem.* **1992**, *336*, 357−362.

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now, there have been few reports of combining an enzyme electrode with a redox mediator in the same self-assembled monolayer configuration.[17,18] In addition, the sensitivities of these enzyme electrodes in a self-assembled monolayer configuration are low due to the limited amounts of enzyme attached to the electrode surfaces.[18] To overcome this difficulty, Riklin and Willner developed a glucose- and acetylcholine-sensing multilayer enzyme electrode.[19]

In this work, for the first time, not only was multilayer horseradish peroxidase linked onto the L-cysteine monolayer, but also multilayer thionine was tethered to such a multilayer enzyme. This electrode−enzyme−mediator chain was obtained in situ through covalently linking the amino tails of the L-cysteine monolayer electrode and amino residues of the enzyme and the two amino groups of each thionine molecule successively with glutaraldehyde as a bifunctional reagent. The tethered thionine in this way worked well to speed up electron communication between the multilayer enzyme and the electrode.

EXPERIMENTAL SECTION

Reagents. Horseradish peroxidase (HRP, EC 1.11.7, type VI) was obtained from Sigma. Thionine (TH, not purified before use) was purchased from Fluka. The 25% glutaraldehyde was obtained from Merck. L-Cysteine and hydrogen peroxide (30% w/v solution) were purchased from Shanghai Chemical Reagent Co. (Shanghai, P.R. China). The concentration of more diluted hydrogen peroxide solutions was determined by titration with cerium(IV) to a ferroin end point.[20] All other chemicals were analytical grade reagents. All the solutions were prepared with doubly distilled water.

Preparation of the Thionine-Modified Self-Assembled Monolayer Electrode (TH/SAME). Gold electrodes were prepared by sealing polycrystalline gold wires (1.08 mm diameter, area = 0.0092 cm^2) in epoxy. Electrodes were polished with alumina and chamois leather, sonicated in distilled water, and etched for 10 min in a 1:3:4 (v/v/v) solution of concentrated HNO_3−concentrated HCl−H_2O prior to coating. This polishing−etching protocol has been shown to yield surfaces which are excellent substrates on which to form self-assembled monolayers (SAMs).[21] The etched electrodes were thoroughly rinsed with water prior to modification. The cleaned electrodes were immersed for 12 h at room temperature in a 0.02 M L-cysteine aqueous solution (deoxygenated by bubbling nitrogen). The resulting monolayer-modified gold electrodes were rinsed with water, and thus the self-assembled monolayer electrodes (SAMEs) were obtained. SAMEs were treated with a 7% (v/v) aqueous solution of glutaraldehyde for 20 min and then rinsed with water. Such a treated SAME was incubated in 1.0×10^{-3} mol/L thionine aqueous solution for 20 min and then was rinsed with water. Such a two-step procedure using the reaction with glutaraldehyde and the thionine solution was used to assemble the thionine-modified self-assembled monolayer electrode (TH/SAME) with the desired number of TH layers. Similarly, horseradish peroxidase-linked self-assembled monolayer electrode (HRP/SAME) and the thionine-tethered HRP/SAME (TH/HRP/SAME) electrode could be assembled. All resulting electrodes were washed with water and stored in a refrigerator (4 °C) when not in use.

Apparatus. Cyclic voltammetry and amperometric measurement were done with a three-electrode system comprising TH/SAME, HRP/SAME, or TH/HRP/SAME as a working electrode, a saturated calomel reference electrode, and a platinum wire auxiliary electrode. The electrodes were connected to a cyclic voltammetry apparatus (Scientific Equipment Co., Fudan University, P.R. China) in line with a type 3086 *x*−*y* recorder (Yokowa Hokushin Electric, Tokyo, Japan). All electrochemical experiments were carried out in 5 mL of 0.1 mol/L phosphate buffer (pH 6.8, apart from the pH parameter experiments) containing 0.1 mol/L KCl in a thermostated and stirred electrochemical cell at 25.0 ± 0.2 °C. All experimental solutions were thoroughly deoxygenated by bubbling nitrogen through the solution for at least 5 min.

RESULTS AND DISCUSSION

Preparation of the Modified Self-Assembled Electrodes. The reaction of glutaraldehyde with a primary amino group by the formation of the Schiff-base bond has been studied and applied in covalently binding compounds and enzyme-containing amino groups.[11a,22] In our experimental procedure, the gold electrode surface was functionalized with primary amino groups by chemisorption of L-cysteine as reported.[23] Next, the amino tails of SAMs formed in this way reacted with the bifunctional reagent glutaraldehyde and then reacted the thionine containing two primary amino groups. The electrode modification process of TH/SAME, HRP/SAME, and TH/HRP/SAME is illustrated in Scheme 1.

Electrochemical Characteristics of TH/SAME. The cyclic voltammograms of TH/SAME in 0.1 mol/L phosphate buffer are shown in Figure 1. The linked TH exhibited an electrochemically quasi-reversible redox process. Estimated from the integration of the cathodic or anodic peak, the surface density of TH/SAME with one, two, three layers of TH corresponded to 7.4×10^{-12}, 15.6×10^{-12}, and 21.2×10^{-12} mol/cm^2, respectively.

TH/SAME with one layer of TH displayed the cathodic peak potential $E_{pc} = -0.16$ V and the anodic peak potential $E_{pa} = -0.11$ V at a scanning rate of 40 mV/s (see Figure la). The formal potential ($E^{o\prime}$) of the thionine layers was −0.135 V (vs SCE); this value was slightly negative compared to that of free thionine ($E^{o\prime} = -0.12$ V). The ΔE_p value (the anodic to cathodic peak separations) produced by TH/SAME was 50 mV, indicating that there were fast charge transfers through SAM as well as charge transfers from SAM to the electrode. The more TH layer the TH/SAME was linked to, the larger the ΔE_p value TH/SAME showed,

(15) (a) Hickman, J. J.; Ofer, D.; Laibins, P. E.; Whitesides, G. M.; Wrighton, M. S. *Science* **1991**, *252*, 688. (b) Hickman, J. J.; Ofer, D.; Zou, C.; Wrighton, M. S.; Laibinis, P. E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 1128−1132. (c) Chidsey, C. E. D.; Bertozzi, C. R.; Putvinski, T. M.; Musjce, A. B. *J. Am. Chem. Soc.* **1990**, *112*, 4301−4306. (d) Creager, S. E.; Rowe, G. K. *J. Electroanal. Chem.* **1994**, *370*, 203−211.

(16) (a) Finklea, H. O.; Ravenscroft, M. S.; Sinder, D. A. *Langmuir* **1993**, *9*, 223. (b) Finklea, H. O.; Hanshew, D. D. *J. Am. Chem. Soc.* **1992**, *114*, 3173−3181.

(17) Willer, I.; Helg-Shabtai, V.; Katz, E.; Tao, G. *J. Am. Chem. Soc.* **1996**, *118*, 10321−10322.

(18) Willner, I.; Riklin, A. *Anal. Chem.* **1994**, *66*, 1535−1539.

(19) (a) Riklin, A.; Willner, I. *Anal. Chem.* **1995**, *67*, 4118−4126. (b) Willner, I.; Lion-Dagan, M.; Marx-Tibbon, S.; Katz, E. *J. Am. Chem. Soc.* **1995**, *117*, 6581−6592.

(20) Hurdis, E. C.; Romeyn, H., Jr. *Anal. Chem.* **1954**, *26*, 320.

(21) Creager, S. E.; Hockett, L. A.; Rowe, G. K. *Langmuir* **1992**, *8*, 854.

(22) Kajiya, Y.; Okamoto, T.; Yoneyama, H. *Chem. Lett.* **1993**, 2107−2110.

(23) Katz, E.; Solov'ev A. A. *J. Electroanal. Chem.* **1990**, *291*, 171−186.

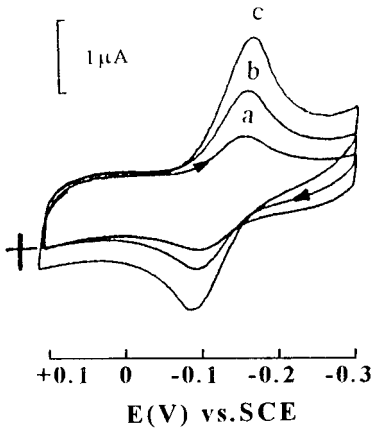
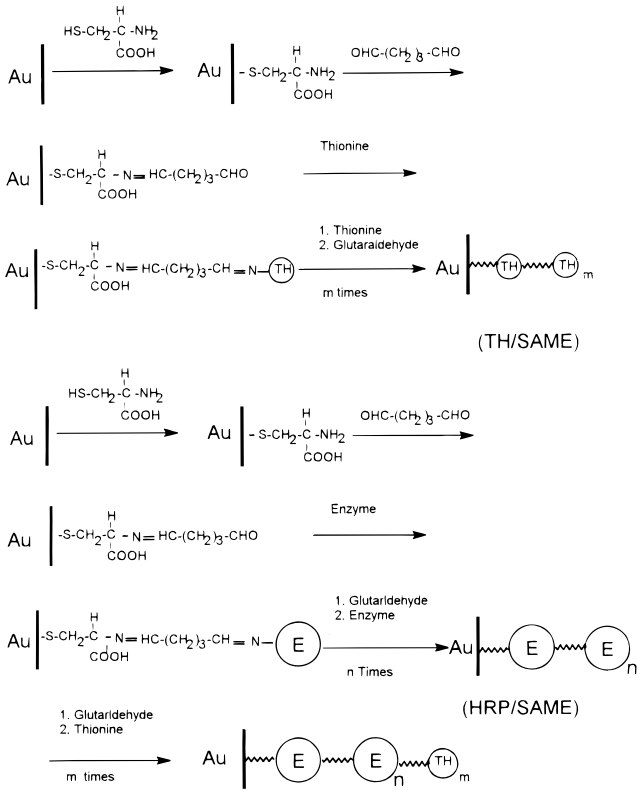


Figure 1. Cyclic voltammograms of TH/SAME with (a) one layer of TH, (b) two layers of TH, and (c) three layers of TH at a scan rate of 40 mV/s.

Scheme 1. Organization of Multilayer TH/SAME, HRP/SAME, and TH/HRP/SAME



indicating slower charge transfer displayed by TH/SAME (see Figure l).

The cathodic (or anodic) peak currents of TH/SAME with three layers of TH were linearly related to the scan rates, as expected for a redox species linked to SAM. The plot of ΔE_p as a function of scan rates is shown in Figure 2. A small ΔE_p ($\leq$50 mV) was obtained at a slow potential scan rate ($v \leq 20$ mV s^{-1}). ΔE_p was nearly independent of the potential scan rate from 40 to 100 mV/s ($\Delta E_p \approx 50$ mV). When the potential scan rate was higher than 100 mV/s, ΔE_p increased dramatically and was proportional to log v (see Figure 2). The formal potential, $E^{\circ\prime}$, calculated as the average of E_{pa} and E_{pc}, $E^{\circ\prime} = 0.5\ (E_{pa} + E_{pc})$, was almost unchanged when the peaks were symmetrically shifted

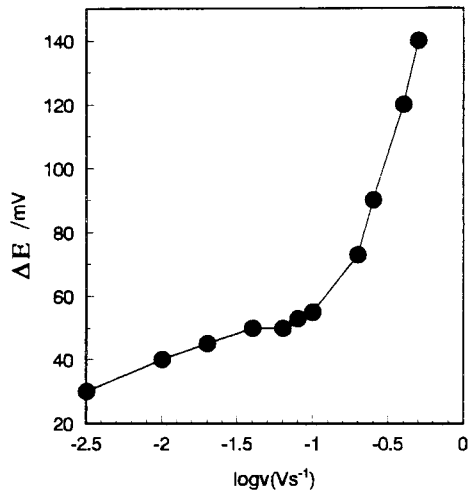


Figure 2. ΔE_p of TH/SAME with three layers of TH as a function of scan rates. H_2O_2 in 0.1 mol/L phosphate buffer (pH 6.8) containing 4 $\times$ 10^{-5} mol/L TH.

in the positive and negative directions with increasing potential scan rate: $\delta E_{pa}/\delta(\log v) \approx \delta E_{pc}/\delta(\log v) \approx 100$ mV. Thus, it could be assumed that the anodic and cathodic transfer coefficients α_a and α_c are both about 0.5. The electron-transfer rate constant from the electrode to TH was determined by Laviron's method.[24] The derived value of the apparent electron-transfer rate constant for the heterogeneous electron transfer process corresponded to 1.7 s^{-1}.

The redox peak potentials of the linked TH in this way were pH dependent, and the slope of the plot of the cathodic peak potential as a function of pH (5.5−7.5) corresponded to 30 mV/pH unit, suggesting that the reduction process of TH involved two electrons and one proton at a neutral pH.[25]

Response of the TH/HRP/SAME to Hydrogen Peroxide. From Figure 3a, TH/HRP/SAME exhibited quasi-reversible redox waves in the absence of hydrogen peroxide, which was similar to that of TH/SAME (see Figure 1). When hydrogen peroxide was added to the electrochemical cell, the cyclic voltammograms changed dramatically, with an increase of reduction current and an almost complete disappearance of oxidation current (see Figure 3b), showing that a bioelectrocatatylic reaction of hydrogen peroxide occurred at TH/HRP/SAME. Indeed, the TH tethered to HRP/SAME facilitated electron communication between the protein redox site and the electrode, as presented in Scheme 2. The response mechanism of TH/HRP/SAME to hydrogen peroxide is summarized as follows:

$$H_2O_2 + \text{HRP(red)} \rightarrow H_2O + \text{HRP(ox)}$$

Then, the oxidized HRP oxidized LTH to TH$^+$:

$$\text{HRP(ox)} + \text{LTH} \rightarrow \text{HRP(red)} + \text{TH}^+$$

(24) Laviron, E. *J. Electroanal. Chem.* **1979**, *101*, 19−28.
(25) Clavilier, J.; Svetlicić, V.; Zutic, V. *J. Electroanal. Chem.* **1995**, *386*, 157−163.

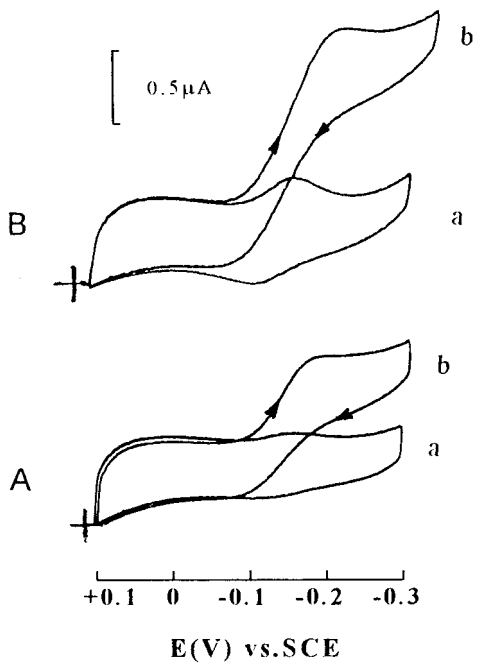
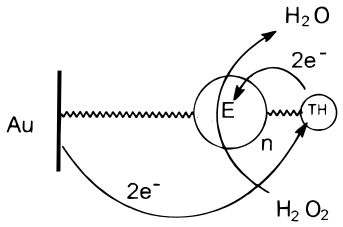


Figure 3. Cyclic voltammograms of TH/HRP/SAME with five layers of HRP and one layer of TH (A) or two layers of TH (B) in the absence (a) and presence (b) of 1×10^{-4} mol/L H_2O_2 at a scan rate of 40 mV/s.

Scheme 2. Electrical Communication of the HRP-Assembled Multilayer and the Electrode Surface Using TH Tethered to Protein



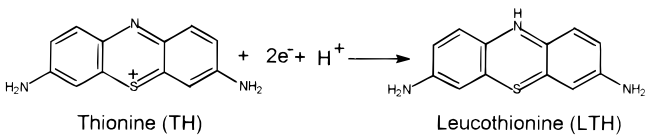
(this overall rereduction of HRP(ox) included two separate steps.[26]

$$\mathrm{HRP(I) + LTH \rightarrow HRP(II) + TH^{\bullet}} \quad (1)$$

$$\mathrm{HRP(II) + TH^{\bullet} \rightarrow HRP(red) + TH^{+}} \quad (2)$$

where one electron was donated at a time, HRP(I) was HRP(ox), and $TH^{\bullet}$ represented the free radical formed during the reaction.) Then, TH^+ was reduced to LTH_2 at TH/HRP/SAME, resulting in the cathodic catalytic current:

$$\mathrm{TH^{+} + H^{+} + 2e^{-} \rightarrow LTH_2}$$



The effect of the number of HRP layers on TH/HRP/SAME was investigated by control experiments. When one layer of TH was tethered to HRP/SAME, the response of TH/HRP/SAME with one, three, and five layers of enzyme to hydrogen peroxide was investigated respectively. To 1×10^{-4} mol/L hydrogen peroxide, such a TH/HRP/SAME with three layers of HRP yielded a lower amperometric response than that with five layers of HRP, whereas such a TH/HRP/SAME with one layer of enzyme did not yield a detectable cathodic current. This result indicated that the sensitivity of TH/HRP/SAME was tuned by the number of enzyme layers. We see that, by increasing the number of HRP layers, the electrocatalyzed current was enhanced. The response of TH/HRP/SAME with eight layers of HRP to hydrogen peroxide was examined, and it was found that the electrocatalyzed current was higher than that of the five layers one.

For TH/HRP/SAME with five layers of HRP, the response of this electrode with one and two layers of TH to 1×10^{-4} mol/L hydrogen peroxide was 38 and 109 μA cm^{-2} respectively (see Figure 3A,B). This showed that the electrocatalytic current was enhanced with increasing number of layers of tethered TH, but the enhancement of current by increasing layers of TH is different from that obtained by increasing the number of layers of HRP. When the number of inner enzyme layers is constant, only three layers of TH can make the electrocatalytic current reach a plateau value.

Although the electrocatalytic current enhanced with increasing the number of enzyme and mediator layers, this does not mean that the number of HRP layers has no limits. When HRP/SAME is immersed in the glutaraldehyde solution for too long a time or too many times, HRP might be denatured, which would lead to low sensitivity and short lifetime of TH/HRP/SAME. With eight layers of HRP and three layers of TH, the electrocatalytic current developed in the electrochemical cell to hydrogen peroxide was examined. The sensitivity of TH/HRP/SAME was more than 250 μA cm^{-2} for 1×10^{-4} mol/L hydrogen peroxide.

The effect of pH on the hydrogen peroxide sensor response has two main aspects: one was that pH affected the activity of HRP, and the other was that pH affected the peak potentials of TH (as discussed above) Therefore, both the oxidation and reduction reactions of HRP-catalyzed cycle were influenced by the pH of the working buffer. The effect of pH was studied at different pH values in 0.1 mol/L phosphate buffer (see Figure 4a). It can be seen that the optimal response was obtained at pH 6.5−6.8, which was critical for the proper peak potentials of the tethered TH to catalyze the reduction of oxidized HRP. This pH value also guaranteed the high activity of HRP for both the oxidation and reduction reactions of the catalyzed cycle. In this work, the buffer solution of 0.1 mol/L phosphate buffer (pH 6.8) was used throughout this paper.

The potential dependence of the steady-state current is shown in Figure 4b. Electroreduction of hydrogen peroxide was observed already at approximately −0.09 V, and the steady-state current increased with the applied potential, decreasing from −0.09 to −0.16 V. It is preferable to control the lower working potential to avoid or decrease the interference caused by some electroactive species at TH/HRP/SAME. Figure 5 shows the cathodic currents of TH/HRP/SAME at different hydrogen peroxide concentrations at an applied potential of −0.18 V. A

(26) (a) Maehly, A. C. *Methods Enzymol.* **1995**, *2*, 801. (b) Durliat, H.; Courteix, A.; Comtat. M. *Bioelectrochem. Bioenerg.* **1989**, *22*, 197−209. (c) Danner, D. T.; Brignac, P. J., Jr.; Arceneaux, D.; Patel, V. *Arch. Biochem. Biophys.* **1973**, *156*, 759.

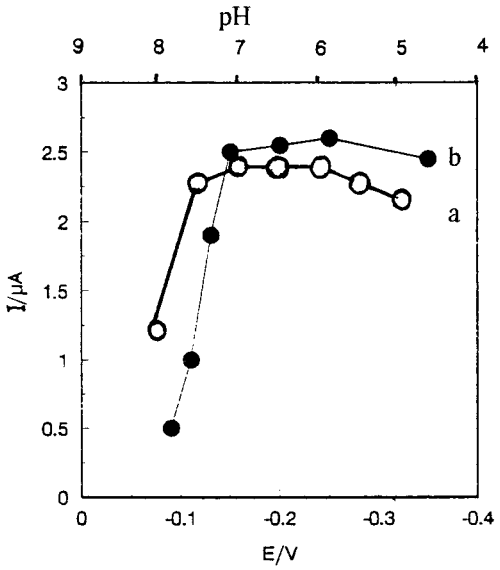


Figure 4. Effect of pH (a) and applied potential (b) on TH/HRP/SAME with eight layers of HRP and three layers of TH when [H_2O_2] was 1×10^{-4} mol/L.

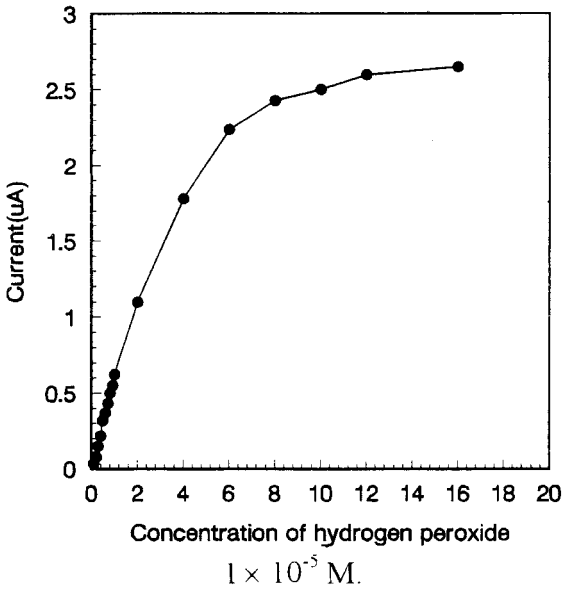


Figure 5. Calibration plot for TH/HRP/SAME with eight layers of HRP and three layers of TH at an applied potential of −0.18 V.

linear current increase was observed up to a hydrogen peroxide concentration of 4.0×10^{-5} mol/L. The sensor achieved 95% of the steady-state current within 4 s.

The stability of TH/HRP/SAME is controlled not only by the stabitily of the Schiff base bond (−C═N−) but also by the stability of the self-assembled monolayer itself.

The background currents at the L-cysteine monolayer electrode were determined every day in order to examine the stability of the SAMs. The results showed that the background currents increased rapidly over time. After 7 days, the current was the same as that of the bare electrode, which showed that the L-cysteine molecules modified onto gold electrode surface leached out completely. It has been reported that the reaction of glutaraldehyde with amino groups is a complicated one,[27] and the mode of coupling depends on the purity grade of glutaraldehyde solution. Use of glutaraldehyde from Sigma improved the stability of the Schiff base bond.

CONCLUSION

TH/HRP/SAME, a reagentless hydrogen peroxide biosensor, based on the multilayer enzyme and the multilayer mediator, was constructed. The sensitivity of TH/HRP/SAME was controlled by the number of the linked HRP layers and the number of the tethered TH layers. Such a resulting enzyme biosensor with the multilayer enzyme and the multilayer mediator responded rapidly to low hydrogen peroxide concentration. This novel and efficient strategy, that is, successful tethering of multilayer horseradish peroxidase as a base enzyme with multilayer thionine as a mediator in the same self-assembled monolayer configuration, opens up a new approach to construct reagentless enzyme biosensors, owing to the multiformity of available mediator derivatives and redox enzymes. Further studies in this direction are underway in our laboratory.

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(27) Kirkeby, S.; Jakobsen, P.; Moe, D. *Anal. Lett.* **1987**, *20*, 303−315.