Chinese Journal of Chemistry, **2004**, *22*, 360—364 **Article**

Amperometric Biosensor for Hydrogen Peroxide Based on Electrodeposited Sub-micrometer Gold Modified Glassy Carbon Electrode

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A new type of hydrogen peroxide amperometric biosensor was fabricated based on electrochemically deposited sub-micrometer Au particles (sm-Au) on a glassy carbon electrode (GCE). Electrochemical deposition condition was optimized for obtaining uniformly distributed sub-micrometer sized Au array on the electrode surface. The hydrogen peroxide sensor was fabricated by adsorbing phenothiazine methylene blue (MB) molecules on the surface of sm-Au and covering a cross-linked horseradish peroxidase (HRP) layer, labeled as HRP/MB/sm-Au/GCE. The characteristics of this biosensor were evaluated with respect to applied potential and pH. The amperometric response of the sensor was linear to the H_2O_2 concentration over a wide range of 9.9×10^{-6}—1.11×10^{-2} mol/L. A detection limit ($s/n=3$) of 3.0×10^{-6} mol/L H_2O_2 was estimated for a sampled chronoamperometric detection at 1.5 min after potential step of 200 to −400 mV vs. SCE. The immobilized MB molecules shuttled electrons at $\alpha=0.77$ and an apparent electron transfer rate constant of $k_s^{0'}=0.053\ s^{-1}$. Interference of ascorbic acid, dopamine and uric acid was investigated. This sensor has very good stability and reproducibility for long-term use.

Keywords hydrogen peroxide, amperometric biosensor, sub-micrometer gold, glassy carbon electrode, methylene blue, horseradish peroxidase

Introduction

Amperometric biosensor of hydrogen peroxide is of practical importance because of its wide applications in chemical, biological, clinical, environmental and many other fields. For improvement of sensor's quality, various kinds of chemical modification methods have been developed for reducing redox overpotentials of H_2O_2 at electrode surfaces, increasing the detection sensitivity, linear range, stability and live time. It has been shown that the use of sub-micrometer sized metal particles such as Pt-black can significantly improve the quality of the biosensor.[1] These advantages can be attributed to the enlarge surface area and micro array structure, the bio-compatibility and catalytic activity of the particles.

On the other hand, the use of suitable mediators can facilitate the electron transfer between electrode surface and modified enzyme molecules, which is promising for fabricating high quality amperometric sensors.[2-4] Phenothiazine methylene blue (MB) has been used as mediator for fabricating H_2O_2 biosensors.[5,6] It was demonstrated that MB can be firmly immobilized on the waxed graphite electrode and accelerate the electron transfer at an apparent electron transfer rate constant of $0.12\ s^{-1}$.[7]

Horseradish peroxidase (HRP) has been mostly used as the immobilized enzyme for construction of the biosensor because of its many advantages.[8-22] The cross-linking method is often chosen due to its simplicity and reliability.[23,24] In this work, MB molecules were successfully incorporated in the surface of sub-micrometer Au particles (sm-Au) modified glassy carbon electrode (GCE), and a novel hydrogen peroxide amperometric biosensor, HRP/MB/sm-Au/GCE, was fabricated. The results indicated that the immobilized MB on sm-Au shuttles electron very well for highly sensitive, stable and reproducible H_2O_2 sensing over a wide linear rage.

Experimental

Chemicals and Apparatus

Horseradish peroxidase (EC 1.11.1.7, 250 U/mg) was obtained from the Sino-American Biotechnology Company. Bovine serum albumin (BSA) was from the Shanghai Blood Research Institute of the Chinese Academy of Agriculture Sciences. Methylene blue (MB) (AR) was purchased from the Guangzhou Factory of Chemical Reagents. Glutaraldehyde (25% *V*/*V* solution in water), hydrogen peroxide (30% *V*/*V* solution in water), uric acid and ascorbic acid were all stained from the Chemical Reagents Company of Shanghai. Dopamine (3-hydroxytyramine hydrochloride), serotonin and epinephrine were purchased from Fluka. The 0.1 mol/L

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Received August 11, 2003; revised and accepted December 10, 2003.
Project supported by the National Natural Science Foundation of China (No. 20075025).

phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock solutions of KH_2PO_4 and K_2HPO_4. All other chemicals were analytical regents. All the solutions were prepared with doubly distilled water. High purity nitrogen was used for solution deoxygenation.

Cyclic voltammetry (CV) and amperometric measurement were carried out at a voltametric analyzer (CV-A, USTC) and an X-Y recorder (LM20A, Dahua Science & Technology). A conventional three-electrode system with a saturated calomel reference electrode (SCE) was used. Scanning electron micrograph (SEM) was obtained at HITACHI X-650 (Japan). Surface analysis of the electrodes was performed by X-ray photoelectron spectroscopy (XPS) on ESCALab MK2 (VG) with Mg K-Al α source. The analyzer energy was 50 eV with 0.1 eV steps.

Electrode fabrication

After carefully polishing and cleaning, a homemade glassy carbon disk electrode (GCE) (ϕ=6 mm) was pretreated electrochemically in 1 mol/L NaOH solution at 1.8 V (vs. SCE) for 2 min, which was similar to the method reported in the literature.[25,26] Then, it was transferred to 7.35×10^{-4} mol/L chloraurate for electrolysis at −0.20 V for 5 min. After rinsing with water, this electrode was soaked in 1.0×10^{-4} mol/L MB solution for 24 h at room temperature. After rinsing with water, the prepared MB/sm-Au/GCE was ready for use. The MB/sm-Au/GCE can be further modified by spreading a droplet of 20 μL 10 g/L HRP (pH=7.0 PBS)+5 μL 5.0% (*W*/*W*) BSA+5 μL 10% (*V*/*V*) glutaraldehyde on its surface for obtaining HRP/MB/sm-Au/GCE. The enzyme matrix on the electrode surface was dried and solidified slowly in the air at room temperature. This electrode was immersed in PBS (pH=7.0) at 4 ℃ for storage.

The same procedure except the electrochemical placing sm-Au particles was used for preparing MB/GCE and HRP/MB/GCE for comparison.

Results and discussion

Fabrication of sm-Au/GCE

The pretreated GCE showed a reduction current at potentials more negative than about −0.10 V in 7.35×10^{-4} mol/L chloraurate. Experimental results showed that −0.20 V was the optimized potential for the deposition. A miniaturation gold layer can be formed with slow rates at potentials more positive than −0.20 V. However, the deposition speed was too fast to control and the deposited particles were too big in size and easy to leach out into the bulk at potentials more negative than −0.20 V.

At −0.20 V, 5 min deposition time was found as the most suitable for obtaining the sm-Au/GCE. As shown in Figure 1a, the scanning electron micrograph showed that about 0.3 μm in size and well distributed Au particles were deposited on the GCE at 5 min, however, oversized particles and close-grained deposition layer was obviously formed if the time was longer than 5 min (Figure 1b).

Characterization of HRP/MB/sm-Au/GCE

Cyclic voltammetry was used for characterization of this prepared biosensor. As shown in Figure 2, a couple of CV peaks appeared at $E_{1/2}$ of −250 mV in the blank PBS solution, which correspond to the redox reactions of the immobilized MB. The peak-to-peak potential difference (E_p) was about 140 mV at 20 mV/s, however, the cathodic peak current (i_{pc}) and anodic peak current (i_{pa}) were linearly proportional to the scan rate (v) in the rage of 20—200 mV/s, indicating a surface electron transfer phenomenon. The E_p value was increased obviously with increase of the scan rate, indicating a kinetic process of negative and positive ions penetrating the modified film.

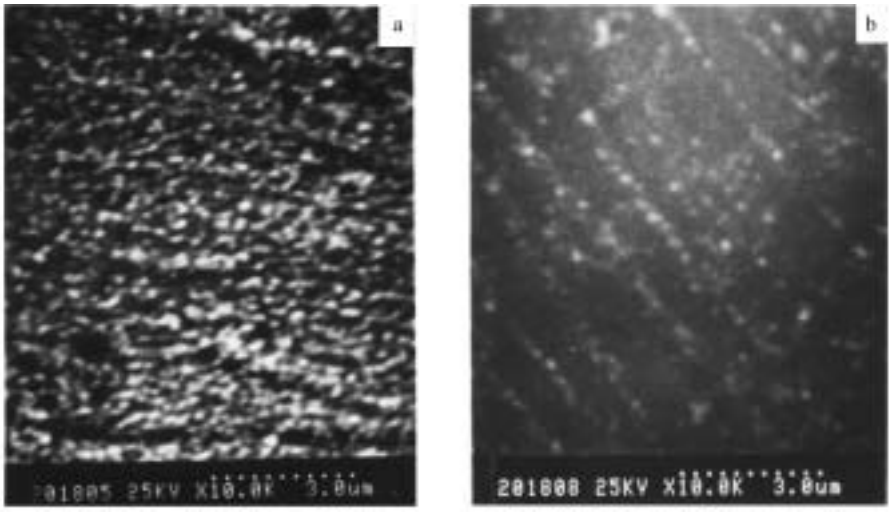

Figure 1 Scanning electron micrograph of the Au/GCE (×10,000). Electrodeposition time: (a) 5 min, (b) 10 min.

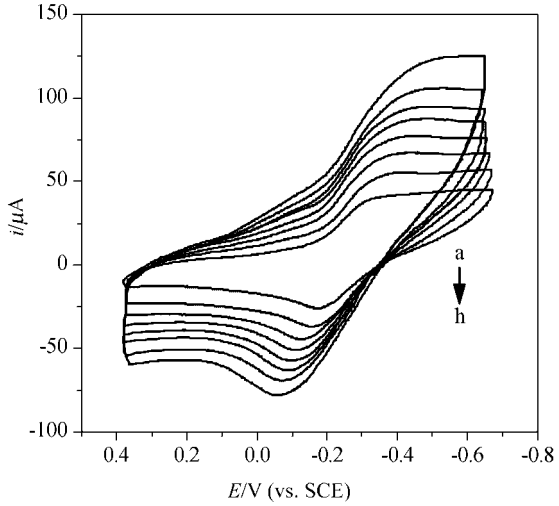

Figure 2 Cyclic voltammograms of the HRP/MB/sm-Au/GCE at scan rates of (a) 20, (b) 40, (c) 60, (d) 80, (e) 100, (f) 130, (g) 160 and (h) 200 mV/s respectively in 0.1 mol/L pH 7.0 PBS.

Figure 3 shows the scan rate dependence of the anodic peak potential. A linear relationship with regression equation of $E_{pa}=0.03484+0.05623\ln v$ ($r=0.997$) was observed at higher scan rates. From the slope, a value of 0.77 was determined for the charge transfer coefficient, α, based on the equation of $E_{pa}=K+[RT/(1-\alpha)nF]\ln v$,[27] where K is a constant relative to the electrode process. In comparison with the equation:[28]

$$\log k_s^{0'}=\alpha\log(1-\alpha)+(1-\alpha)\log\alpha-\log(RT/nFv)-\alpha(1-\alpha)nF\Delta E_p/(2.3RT),$$

an apparent surface electron transfer rate constant, $k_s^{0'}=0.053\ s^{-1}$ was obtained.

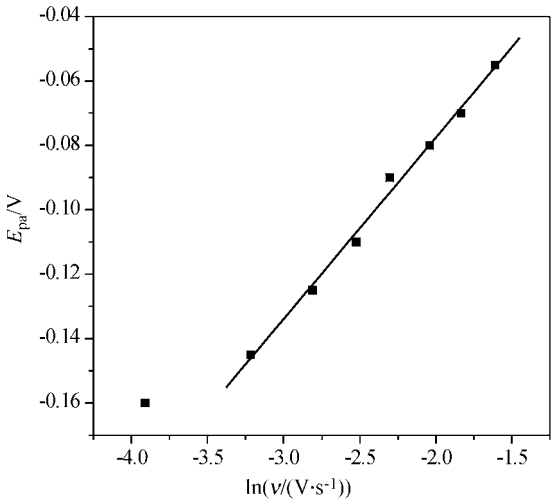

Figure 3 Plot of E_{pa} vs. ln v for the HRP/MB/sm-Au/GCE in 0.1 mol/L pH=7.0 PBS.

The MB/GCE and MB/sm-Au/GCE were also characterized by XPS (ESCA), as shown in Figure 4. The adsorption band of sulfur appeared at 164.5 eV for MB/GCE and 164.2 eV for MB/sm-Au/GCE. Because the adsorption band of Au 4f was appeared at 84.1 eV for MB/sm-Au/GCE, in comparison with the value of 83.8 eV for pure gold the variable value was in good agreement with that of sulfur. The result indicated that the sulfur in MB molecules built up molecular bond power with the gold particles. In addition, the relative mass of sulfur was 0.78% for MB/GCE and 1.52% for MB/sm-Au/GCE by increasing 98%. The value could also agree with the result mentioned above.

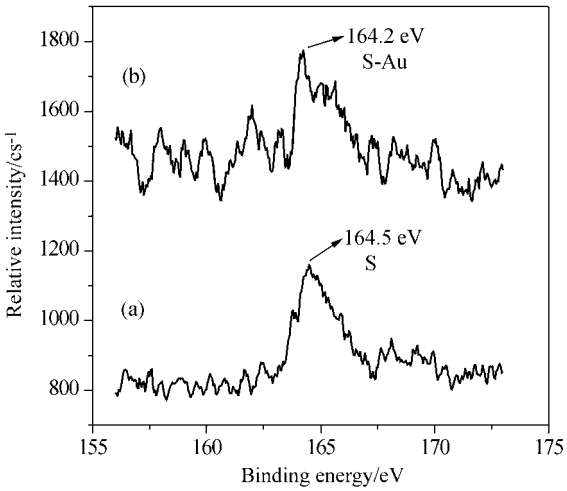

Figure 4 XPS (ESCA) spectra of S_{2p} for MB/GCE (a) and MB/sm-Au/GCE (b).

The cathodic peak current of the MB increased in the presence of H_2O_2 in the solution, which is the principle of the H_2O_2 sensing. Experimental data showed that the current response to 4.76 mmol/L H_2O_2 was 30 μA for the HRP/MB/GCE and 56 μA for the HRP/MB/sm-Au/GCE. The 87% increase was in agreement with that the amount of immobilized MB molecules significantly increased due to the binding of MB and Au, and was essential for increased sensitivity of the sensor.

During the dryness, the surface of the HRP/MB/GCE began to burst. In comparison with the HRP/MB/GCE, the live-time of the HRP/MB/sm-Au/GCE was much longer. This is understandable because the deposition of sm-Au not only increased the roughness of the electrode but also provided a large area of additional immobilization interface for both MB and HRP molecules.

Figure 5 shows the CVs for HRP/MB/GCE and HRP/MB/sm-Au/GCE in the presence and absence of H_2O_2. As seen in this figure, the cathodic peak current increased significantly in the presence of H_2O_2. The H_2O_2 was reduced at enzyme HRP, which was regenerated by electron mediator MB.[29]

$$H_2O_2+HRP\rightarrow H_2O+HRP\text{-}I$$

$$HRP\text{-}I + (MB)_{red} \rightarrow HRP\text{-}II + (MB)_{ox}$$

$$HRP\text{-}II + (MB)_{red} \rightarrow HRP + (MB)_{ox}$$

$$(MB)_{ox} + 2e \rightarrow (MB)_{red}$$

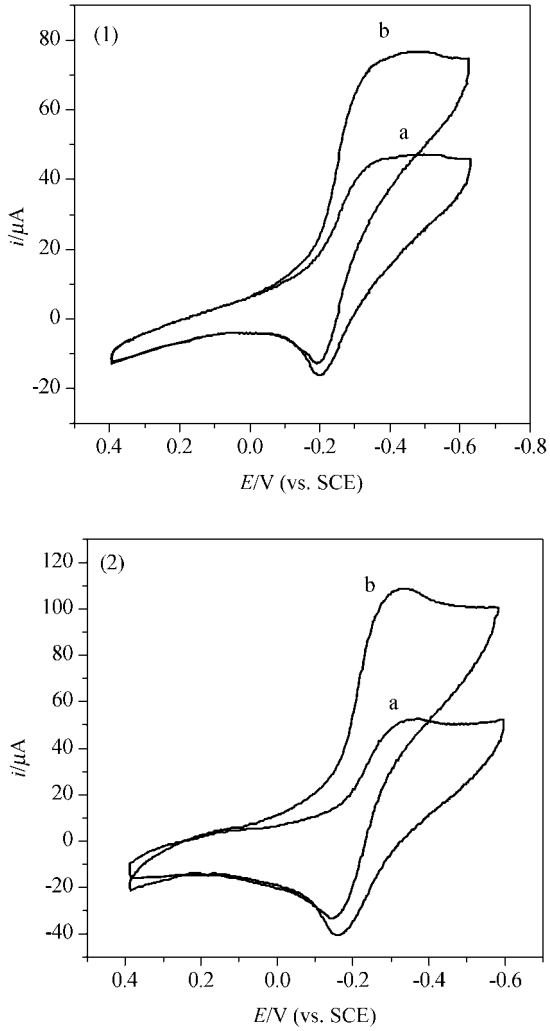

Figure 5 Cyclic voltammograms of the HRP/MB/GCE (1) and HRP/MB/sm-Au/GCE (2) at 20 mV/s in 0.1 mol/L pH 7.0 PBS (a) and 4.76 mmol/L H_2O_2 (b).

The recycling of MB at the sm-Au/GCE led to an increase of the cathodic current corresponding to the concentration of H_2O_2.

Optimization of H_2O_2 sensing

Selection of applied potential In order to determine the optimized applied potential (E_2) for the H_2O_2 sensing, a curve of sampled chronoamperometric current vs. applied potential was obtained as illustrated in Figure 6.

The initial potential (E_1) was selected as 200 mV at which no electrode reaction was occurred. The current response was sampled at 1.5 min (t_s) after the electrode potential was stepped from E_1 to E_2, where a steady state diffusion current was almost reached. As seen in this figure, the sampled current increased significantly from −50 mV and tended to be steady at −400 mV, showing an S-shaped wave similar to a sampling voltammetric current for a simple redox system. By virtue of considering reproducibility and stability of the sensor, −400 mV was selected as the optimized monitoring potential in the following work.

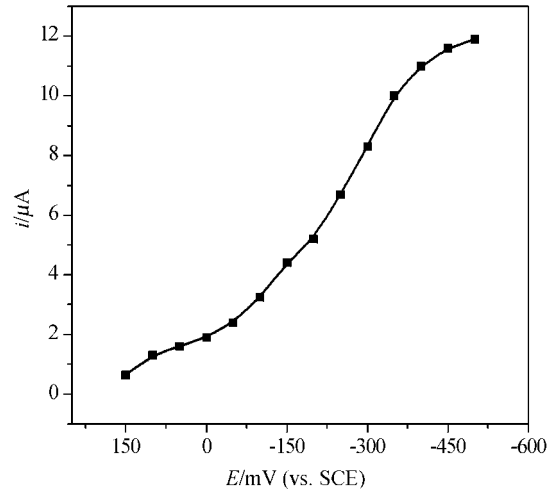

Figure 6 Sampled current of the HRP/MB/sm-Au/GCE related to the applied potential (E_2) in 0.1 mol/L pH 7.0 PBS containing 2.44×10^{-4} mol/L H_2O_2. E_1=200 mV, t_s=1.5 min.

Selection of pH The response current of the sensor towards H_2O_2 is also dependent on pH value. The results showed that the current response was steady in the range of pH=5.0—7.0, however, it dropped down quickly after pH = 7.0. Therefore, considering the physiological pH (7.4) and sensitivity, the value of bulk solution pH was selected as 7.0.

Calibration curve for H_2O_2

Under optimum deposition and determination conditions, the feasibility of using HRP/MB/sm-Au/GCE to determine the concentration of H_2O_2 was investigated. The catalytic peak current exhibits a linear dependence on H_2O_2 concentration over a wide range of 9.9×10^{-6} to 1.11×10^{-2} mol/L. A regression equation was determined as $i(\mu A) = 1.99714 + 6085.5c$ (mol/L) (r = 0.9987). It can also be obtained that the slope of the linear equation is much larger than that of the HRP/MB/WSGE.[7] A detection limit (s/n=3) of 3.0×10^{-6} mol/L H_2O_2 was estimated. Although further optimization on instrumentation and experimental procedures should be done for its application, a simple comparison has shown the higher sensitivity and wider linear rage of the sensor than those of the HRP/MB/WSGE.[7]

Interference and stability of the sensor

Interference from some substances commonly present such as ascorbic acid, dopamine, epinephrine, serotonin and uric acid was investigated. Although the background of the current increased when 5.0×10^{-3} mol/L ascorbic acid was added in the solution of hy-

drogen peroxide, the pure current of hydrogen peroxide and ascorbic acid was as same as that of 4.76×10^{-4} mol/L of hydrogen peroxide. It is known that ascorbic acid can interact with the mediators of HRP.[10] This may be the reason of the increase of the background. However, the presence of the same concentration of dopamine, epinephrine, serotonin and uric acid did not interfere with the determination of 4.76×10^{-4} mol/L H_2O_2. These compounds are not electrochemically active at the potentials, although they may be oxidized much slowly with some of the intermediates of sensing reactions. On the other hand, the stability of this sensor was determined. It was found that the current response decreased by 6% after its storage for 10 d in pH=7.0 PBS at 4 ℃, however, only about 19% for one month.

Conclusions

By means of a simple and reproducible procedure, a new type of hydrogen peroxide biosensor, HRP/MB/sm-Au/GCE, has been successfully developed based on electrochemically deposited sub-micrometer Au particles modified GCE. The current response of this biosensor has a wide linear range of 9.9×10^{-6} to 1.11×10^{-2} mol/L and detection limit ($s/n=3$) of 3.0×10^{-6} mol/L H_2O_2. The electrode has very good stability and reproducibility for long-term use. Based on its high efficiency of electrocatalytic activity of H_2O_2 and coupling with other enzyme system, it is believed that this type of sensor can be extended for many other biological analytes and has potentially applications in the field of biosensors.

References

1 Chen, J.; Lin, X.-Q.; Chen, Z.-H.; Wu, S.-G.; Wang, S.-Q. *Anal. Lett.* **2001**, *34*, 491.
2 Frew, J. E.; Harmer, M. A.; Hill, H. A. O.; Libor, S. I. *J. Electroanal. Chem.* **1986**, *201*, 1.
3 Armstrong, F. A.; Lanon, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7211.
4 Tatsuma, T.; Okawa, Y.; Watanabe, T. *Anal. Chem.* **1989**, *61*, 2352.
5 Ye, J.; Baldwin, R. P. *Anal. Chem.* **1988**, *60*, 2263.
6 Ruan, C.; Yang, F.; Xu, J.; Lei, C.; Deng, J. *Electroanalysis* **1997**, *9*, 1180.
7 Lin, X.-Q.; Chen, J.; Chen, Z.-H. *Electroanalysis* **2000**, *12*(4), 306.
8 Jonsson, G.; Gorton, L. *Electroanalysis* **1989**, *1*, 465.
9 Razumas, V. J.; Gudavicius, A. V.; Kulys, J. J. *J. Electroanal. Chem.* **1983**, *151*, 311.
10 Ruzgas, T.; Csoregi, E.; Emneus, J.; Gonton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1996**, *330*, 123.
11 Green, M. J.; Hill, H. A. O. *J. Chem. Soc., Faraday Trans.* **1986**, *82*, 1237.
12 Wang, J.; Lin, M.-S. *Electroanalysis* **1989**, *1*, 43.
13 Pantano, P.; Morton, T. H.; Kuhr, W. G. *J. Am. Chem. Soc.* **1991**, *113*, 1832.
14 Tatsuma, T.; Gondaria, M.; Watanable, T. *Anal. Chem.* **1992**, *64*, 1183.
15 Zhao, J.; Henkens, R. W.; Stonehuemer, J.; O'Daly, J. P.; Crumbliss, A. L. *J. Electroanal. Chem.* **1992**, *327*, 109.
16 Navaratne, A.; Rerchnitz, G. A. *Anal. Chim. Acta* **1992**, *257*, 59.
17 Deng, Q.; Dong, S. *Electroanalysis* **1994**, *6*, 878.
18 Deng, Q.; Dong, S. *J. Electroanal. Chem.* **1994**, *377*, 191.
19 Ferri, T.; Poscio, A.; Santucci, R. *Bioelectrochem. Bioenerg.* **1998**, *45*, 221.
20 Kane, S. A.; Iwuoha, E. I.; Smyth, M. R. *Analyst* **1998**, *123*, 2001.
21 Rajendran, V.; Csoregi, E.; Okamota, Y.; Gorton, L. *Anal. Chim. Acta* **1998**, *373*, 241.
22 Gong, J.-M.; Lin, X.-Q. *Chin. J. Chem.* **2003**, *21*, 761.
23 Tatsuma, T.; Okawa, Y.; Wayanable, T. *Anal. Chem.* **1989**, *61*, 2352.
24 Kulys, J.; Bilitewski, U.; Schmid, R. D. *Bioelectrochem. Bioenerg.* **1991**, *26*, 277.
25 Zhang, H.-C.; Zuo, X.-B.; Ji, M.-R.; Wu, S.-G. *Acta Phys.-Chim. Sin.* **1996**, *12(7)*, 649 (in Chinese).
26 Yang, Y.-F. *Chin. J. Anal. Chem.* **1999**, *27(3)*, 346 (in Chinese).
27 Dong, S.-J.; Che, G.-L.; Xie, Y.-W. *Chemically Modified Electrodes*, Science Press, Beijing, **1995**, p. 129 (in Chinese).
28 Laviron, E. *J. Electroanal. Chem.* **1974**, *52*, 355.
29 Aoki, K.; Kaneko, H. *J. Electroanal. Chem.* **1988**, *247*, 17.

(E0308112 ZHAO, X. J.)