Korean J. Chem. Eng., **29**(12), 1766-1770 (2012)
DOI: 10.1007/s11814-012-0078-0

A new electrochemical biosensor for hydrogen peroxide using HRP/AgNPs/cysteamine/p-ABSA/GCE self-assembly modified electrode

Ali Shokuhi Rad*,†, Mohsen Jahanshahi, Mehdi Ardjmand***, and Ali-Akbar Safekordi******

*Chemical Engineering Group, Faculty of Engineering, Qaemshahr Branch, Islamic Azad University, Tehran, Iran
**Nanotechnology Research Institute, School of Chemical Engineering, Babol University of Technology, Babol, Iran
***Chemical Engineering Group, Faculty of Engineering, South Tehran Branch, Islamic Azad University, Tehran, Iran
****Chemical Engineering Group, Faculty of Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran
(*Received 5 November 2011 • accepted 25 May 2012*)

Abstract−An electrochemical hydrogen peroxide biosensor was designed by immobilizing horseradish peroxidase (HRP) on Ag nanoparticles/cysteamine/p-aminobenzene sulfonic acid/glassy carbon (GC) electrode. Ag nanoparticles can act as tiny conduction centers on electrodes that adsorb redox enzymes, facilitating the transfer of electrons with no requiring any loss of biological activity. The forerunner film was first electropolymerized on the glassy carbon electrode with p-aminobenzene sulfonic acid (p-ABSA) by cyclic voltammetry. The cysteamine (CA) was bound on the surface of the film by electrostatic force, then Ag nanoparticles were immobilized on the cysteamine monolayer, and lastly HRP was adsorbed onto the surfaces of the Ag nanoparticles. A dramatic decrease in the overvoltage of H_2O_2 was observed with improved sensitivity, which makes the modified electrodes of great promise for oxidase-based amperometric biosensors. The biosensor responded to H_2O_2 in the linear range from 1.2×10^{6} mol/L to 9.8×10^{3} mol/L with a detection limit of 1.1×10^{8} mol/L. Moreover, the obtained biosensor exhibited good accuracy and high sensitivity.

Key words: AgNP, Hydrogen Peroxide, Cycteamamin, Biosensor, Cyclic Voltammetry

INTRODUCTION

The most challenging in developing enzyme-based biosensors is the incorporation of sensing molecules in proper matrix as well as monitoring the interactions between the analytes and those molecules. The development of a suitable matrix to immobilize electroactive compounds for biosensors is thus a significant task [1]. The immobilization of enzymes has been an active research topic during the fabrication of biosensors. Conventional immobilizing approaches adopted for the biosensor comprise self-assembly [2], as well as different kinds of sol-gel/hydrogel [3], cross-linking [4], and each of these mentioned methods above has its drawback: there are still numerous challenges related to effortlessness of fabrication, efficient retention of enzyme activity and stability of biosensors. To advance the performance of the biosensor, it is necessary to find an easy-handling immobilization technique. Fast and reliable determination of hydrogen peroxide is very important in fields of food, industry, environmental protection, clinical control and so on. The rapid and accurate determination of hydrogen peroxide (H_2O_2) is of great importance in pharmaceutical, clinical, industrial, and environmental analyses, so numerous techniques such as chemiluminescence [5], titrimetry [6], spectrophotometry [7], and electrochemistry [8] have been employed for detection of H_2O_2. Among these techniques, electrochemical methods are significant for the determination of H_2O_2 because of their simplicity and high sensitivity. To develop and to design electrochemical biosensors, an increasing number of active compounds have been used [9] for detecting H_2O_2 and some other target materials. For these biosensors, horseradish peroxidase (HRP) is the most commonly used enzyme [10].

We selected HRP as a model because it is well-studied and it is commercially available in a highly purified form [11], and colloidal silver nanoparticles (AgNPs), which are easy to synthesize, have been widely used to incorporate HRP because of their good biocompatibility and catalytic activity. Also, colloidal AgNPs can provide an environment similar to the native environment of redox HRP and be used to immobilize HRP for their direct electrochemistry [12].

Some research has been done based on electrochemical biosensor for hydrogen peroxide biosensor. Yanxia et al. [13] made a hydrogen peroxide biosensor based on the direct electrochemistry of Hb in Hb-Ag sol on GC electrode. This modified electrode showed a sensitive response to the reduction of H_2O_2 without any electron mediator. Chuan et al. [14] made a hydrogen peroxide biosensor based on the direct electrochemistry of myoglobin immobilized on AgNPs doped carbon nanotubes film. Fengxian et al. [10] fabricated a hydrogen peroxide biosensor with good accuracy and high sensitivity by self-assembling of hemoglobin, gold nanoparticles and L-cysteine on the precursor film formed by electropolymerization of p-ABSA on the platinum disk electrode. AgNps were selected due to their excellent conductivity, and also the synthesis of AgNPs has been well-demonstrated.

AgNps were self-assembled to the electrode binding with cysteamine via strong Ag S covalent bond to fabricate the nano-Ag self-assembled modified electrode. In other side, Ag nanoparticles can act as tiny conduction centers on electrodes that adsorb redox enzymes, facilitating the transfer of electrons with no requiring any loss of biological activity.

In many earlier works, a drawback to physical adsorption and

†To whom correspondence should be addressed.
E-mail: A.Shokuhi@gmail.com

entrapment was that the distribution of nanoparticles and then the enzyme molecules was not uniform on the surface of electrode and also was sometimes unstable, tending to leach with time. To our knowledge, covalent binding of HRP on the bonded AgNPs to cysteamine has longer reaction time. The ideal immobilization method should employ mild chemical conditions and a short immobilization time to allow for large quantities of enzyme to be immobilized, provide a large surface area for enzyme-substrate contact within a small total volume, minimize barriers to mass transport of substrate and product, and provide a chemically and mechanically robust system. To our knowledge, the fabrication of an HRP biosensor based on AgNPs immobilized on a cysteamine monolayer has been reported only by Ren et al. [15]. They did immobilization of HRP on AgNPs adsorbed on a cysteamine monolayer-modified gold electrode toward H_2O_2 via cyclic voltammetry and amperometry. At our current research, we used GC modified electrode, which is more convenient and also more sensitive than gold modified electrode used by Ren et al. For doing this we had to use an intermediate between the surface of GC electrode and cysteamine because there isn't any tendency among them. So at the first stage we electropolymerized p-ABSA to obtain negatively charged poly-(p-ABSA) film to immobilize cysteamine. Here, immobilization of cysteamine was attributed to the electrostatic force between positively charged cysteamine and the negatively charged sulfonic acid groups in p-ABSA polymer. AgNps attached to the CA monolayer through electrostatic adsorption and finally HRP adsorbed on AgNP in three dimensional. HRP was selected as a model enzyme, which immobilized on AgNP adsorbed on a CA monolayer-modified GC to prepare the biosensor. AgNP has large specific surface area and good biocompatibility. Additionally, it can act as tiny conduction centers which make easy the transfer of electrons [16]. The biosensor obtained shows electrocatalytic activity, good accuracy, and a low cost.

MATERIALS AND METHODS

1. Apparatus and Chemicals

Ivium state (The Netherlands) was used to carry out the electrochemical analysis as well as cyclic voltammetric, amperometric measurements and differential pulse voltammetry. A conventional three-electrode system was employed with a modified GCE (2.0 mm in diameter) as a working electrode, a saturated calomel electrode (SCE) as a reference electrode, and a platinum wire as an auxiliary electrode. All the potentials given in this paper were referred to the SCE. The experimental solutions were deaerated by high pure nitrogen for 15 min, and nitrogen atmosphere was kept over the solution during measurements.

Cysteamine (CA) and $AgNO_3$ were obtained from Merck (Germany). p-ABSA was obtained from Sigma Co. (USA). HRP (250 U/mg), and Sodium borohydride and sodium citrate were from Fluka. All other reagents were of analytical grade and used as received. A magnetic stirrer was used for the convective transport when necessary. Phosphate buffer solutions (PBS) with different pH values were prepared by mixing aqueous solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH. A fresh solution of Hydrogen peroxide (30% w/v solution) was obtained from Chemical Reagent Company, Chongqing (China).

2. Preparation of AgNps

Following the descriptions in the literature [20], AgNps were prepared by reducing metallic ions in aqueous solution with freshly prepared $NaBH_4$ solution in ice-cold conditions and stored at 4 °C. An aqueous solution of 2 mM $NaBH_4$ (2 mL) was cooled with ice and then 1 mL of 2 mM $AgNO_3$ aqueous solution was added to it under vigorous stirring, resulting in the light-brown Ag colloidal solution.

3. Preparation of Modified Electrode as Biosensor

The bare GCE was polished with 0.05 μm Al_2O_3 before modification, rinsed with double-distilled water, sonicated in acetone, and double-distilled water for 5 min, respectively.

After electrochemical cleaning of the freshly polished GC electrode by sending it through several potential cycles between −0.5 and 1.2 V versus SCE in 0.1 M H_2SO_4, the electrode was immersed in 2.0×10^{-3} mol/L p-ABSA solution containing 0.1 mol/L NaCl and was conditioned by cyclic sweeping between −1.5 and +2.5 V at 100 mV/s for 10 scans. After that, the electrode was washed by water, dried in air, and immersed in a solution of 2 mM CA for 12 h. Fig. 1(a) shows the AFM image coated cysteamine monolayer which seems very smooth. After being cleaned with distilled water, the electrode was immersed in Ag colloids for 8 h to form a nano-Ag layer. Fig. 1(b) shows the immobilized AgNPs. Subsequently, the resulting electrode was immersed in HRP solution (1 mg/ml, 0.1 mol/L pH 6.0 PBS) for 12 h to obtain the hydrogen peroxide biosensor, as shown in Fig. 1(c). The biosensor was stored in a refrigerator (4 °C) until further use.

4. Experimental Measurements by Biosensor

The electrochemical characteristics of the modified electrode were

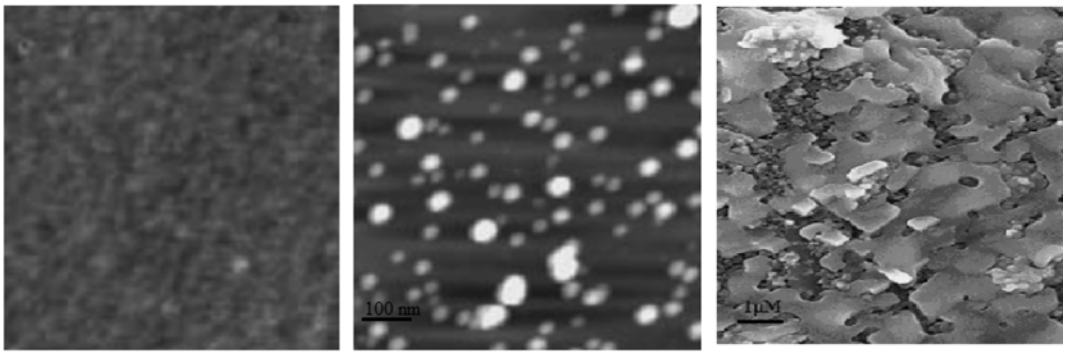

Fig. 1. AFM images of coated cysteamine monolayer (a), silver nanoparticles/cysteamine (b). The SEM of HRP Covered on GCE (c).

characterized by amperometry at applied potential of −0.4 V as well as cyclic voltammetry (CV) with potential swept from −0.8 to 0.15 V (vs. SCE) at a sweeping rate of 100 mV/s, both in 5 ml pH 7.0 PBS. Electrochemical experiments were performed in a conventional electrochemical cell containing a three-electrode arrangement. All experiments were conducted at room temperature, except an experiment to survey the effect of temperature which was done in artificial baths.

RESULTS AND DISCUSSION

After electropolymerization of p-ABSA on the GC surface, the CA was immobilized on polymer because of the electrostatic force between positively charged CA and the negatively charged sulfonic acid groups in p-ABSA polymer. On the other hand, the end side of CA was protonated in order to load positive charges. Therefore, the AgNPs, which were stabilized by negative ions could attach to the CA monolayer through electrostatic adsorption. Electrostatic repulsion between similar charges caused the AgNPs to be distributed on the CA monolayer homogeneously. HRP was either adsorbed on the surface of a silver nanoparticle or buried in the interstices between AgNPs, due to the interaction between the NH_4 + -lysine residues of HRP and the silver surface. One silver nanoparticle was capable of linking several HRP molecules; in other words, the two-dimensional electrode surface was modified to be three dimensional, so that more binding sites were provided by the AgNPs for the immobilization of HRP. Therefore, the presence of AgNPs allows efficient electron tunneling and electron transfer between the enzyme and the bulk electrode surface. Furthermore, the AgNPs also act as binding centers that increase the number of binding sites for HRP [17], resulting in a higher catalytic response to the catalysis of H_2O_2.

1. Electrochemical Response to Hydrogen Peroxide

The electrocatalytical behavior of the modified electrode towards the electrochemical reduction of H_2O_2 was studied using cyclic voltammetry. As shown in Fig. 2, in blank PBS, the biosensor only exhibited the electrochemical behavior of CA, a pair of anodic and cathodic waves (curve a). With the addition of H_2O_2, the cathodic peak current increased and the anodic peak current decreased significantly (curves b and c). The calibration plot of the hydrogen peroxide biosensor under optimal experimental conditions is illustrated in the inset of Fig. 2. As expected, the response of the biosensor to H_2O_2 was linear in the range from 1.2×10^{-6} mol/L to 9.8×10^{-3} mol/L with a detection limit of 1.1×10^{-8} mol/L.

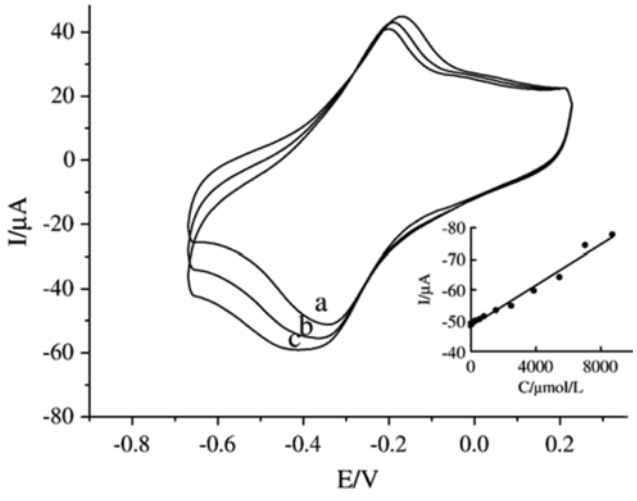

Fig. 2. Cyclic voltammograms of the biosensor in 0.1 mol/L pH 7.0 PBS (curve a) and in the presence of 1.5×10^{-3} mol/L H_2O_2 (curve b) and 3.9×10^{-3} mol/L H_2O_2 (curve c). Scan rate: 100 mV/s. Inset: the calibration curve of the biosensor.

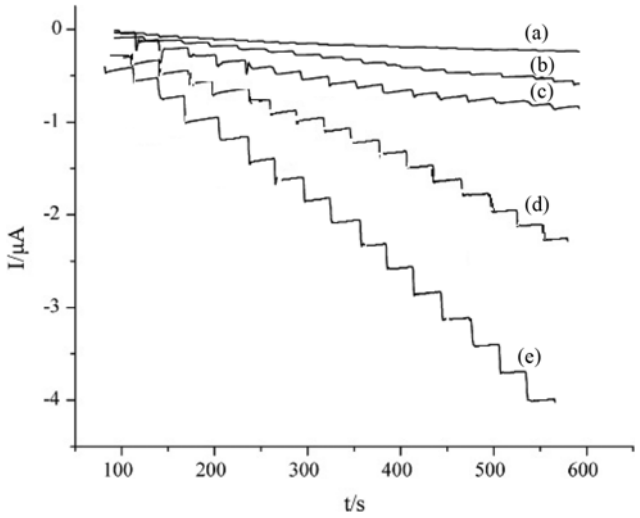

Fig. 3. The chronoamperometry response of differently prepared electrodes in the assembly process with successive injections of 1.0×10^{-2} mol/LH_2O_2 to the PBS (pH 7.0) under stirring. (a) Poly(p-ABSA), (b) CA/poly(p-ABSA), (c) nano-Ag/CA/poly(p-ABSA), (d) HRP/GCE and (e) HRP/nano-Ag/CA/poly(p-ABSA) modified GC electrode. Applied potential: −0.4 V.

To confirm whether the current was due to the nonenzymatic reduction of H_2O_2, the electrode behavior was studied using chronoamperometry after each modified step. We compared the chronoamperometry response of differently modified electrodes in the assembly process with successive injections of 1.0×10^{-2} mol/L H_2O_2 to the PBS (pH 7.0) at applied potential of –0.4 VV. The GC electrode electropolymerized with only p-ABSA hardly responded to hydrogen peroxide as shown in Fig. 3(a). According to Fig. 3(b), when CA was adsorbed into p-ABSA electropolymerized film via the opposite-charged adsorption technique, a small amperometric response to H_2O_2 was observed. Furthermore, AgNP can act as tiny conduction centers, which facilitated the transfer of electrons, so the current response increased after the nano-Ag was immobilized on the electrode as shown in Fig. 3(c). However, it had maximal response to H_2O_2 when HRP was chemisorbed on the electrode surface, as shown in Fig. 3(e). The effect of only HRP coated GC electrode is shown in Fig. 3(d) to compare with the performance of AgNP-HRP coated GC electrodes toward H_2O_2 sensing to clearly demonstrate the effect of the use of AgNPs. It can be seen that the presence of AgNPS increased the activity of HRP enzyme because of increased active surface.

2. Optimum of Analytical Condition

2-1. The Effect of the pH

Differential pulse voltammetry (DPV) was used for finding the effect of the pH value of the solution on the modified electrode response. The PH value extended from 5.0 to 8.0, with the corre-

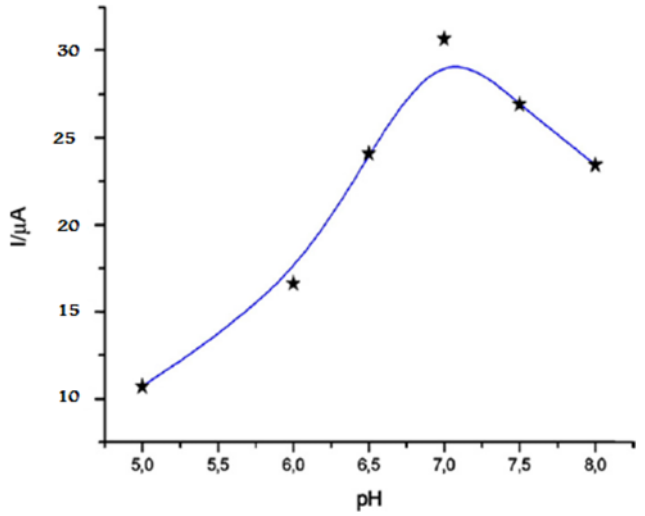

Fig. 4. Influences of pH values of PBS solution on the peak current of the response current to 0.1 mM H_2O_2 at the enzyme electrode. Data obtained from DPV which recorded from −0.7 to 0.0 V in 0.1 M pH 7.0 PBS.

sponding results shown in Fig. 4. From Fig. 4, the response current of the modified electrode is observed to increase with an increase in the pH value, and the maximum response current is seen at pH 7.0, in agreement with the results reported in the literature [18]. However, the neutral pH is close to the physiological environment and can retain HRP the activity. Thus, pH 7.0 was chosen for the following investigations, and therefore, pH 7.0 is used in further experiments and determination of H_2O_2.

2-2. The Effect of the Temperature and Determination of Activation Energy

The outcome of temperature on the steady state response of amperometric was also investigated in the range of 25-50 °C, shown in Fig. 5. With temperature increase it can be seen increasing in the response, reaching a maximum at 45 °C, and then decreasing. This probably has been caused by denaturation of HRP or film instability at the higher temperatures. The dependence of amperometric current on temperature in an initial region can be expressed as an Arrhenius relationship:

$$i(T) = i_0 \exp\left\{\frac{-E_a}{RT}\right\}$$

Where i_0 represents a collection of currents, R is the gas constant, T is the temperature in degrees Kelvin, and Ea is the activation energy. The activation energy for enzymatic reaction is calculated to be 1.42 kJ mol^{-1} from the slope of I– 1/T in the adoptive region of temperature (inset of Fig. 5). This E value attained is smaller than that reported by Xu et al. [19] for HRP immobilized on the polyaniline films. The smaller E_a value means that the HRP immobilized on the modified electrode possesses higher enzymatic activity.

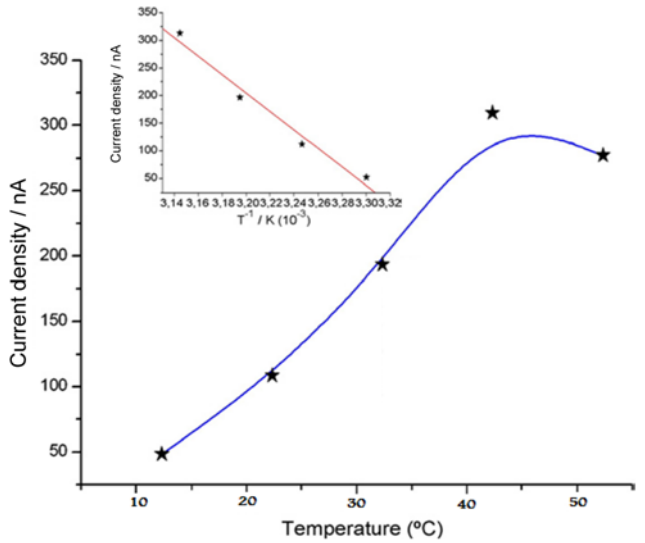

Fig. 5. The effect of the temperature on the response of the enzyme electrode to 0.1 mM H_2O_2 in 10 mM PBS, pH 7.0 solution. Inset was i vs. 1/T plot for the enzyme electrode. Data obtained from DPV recorded from –0.35 to –0.0 V in 0.1 M pH 7.0 PBS.

3. Repeatability and Stability of the Hydrogen Peroxide Biosensor

A relative standard deviation (RSD) of 3.2% was obtained when the present biosensor was repeated for eight succeeding measurements at a hydrogen peroxide concentration of 1×10^{-4} mmol/L (the result not shown). RSD was 4.1% for four electrodes prepared in the same conditions. The stability of the biosensor was investigated by amperometric measurements in the presence of 1.0×10^{-4} mol/L H_2O_2 periodically. When not in use, it was stored under dry conditions at 4 °C in a refrigerator. The biosensor lost only 5.8% of the initial response after ten days and maintained more than about 75% of the initial values after storage for more than 1 month.

4. Selectivity of the Hydrogen Peroxide Biosensor

Discrimination of interfering species having electro-activities similar to the target analyte is one of the most important analytical features for an amperometric biosensor. Ascorbic acid (AA) and uric, acid (UA) are the most common interfering electroactive species during the amperometric detection of H_2O_2. In the experiment, seven interfering substances (tryptophan, dopamine, acetic acid, ascorbic acid, uric acid glucose and l-tyrosine) were used to assess the selectivity of the biosensor. The current ratios were considered by reading the current of the biosensor in the assay solution containing 1.0×10^{-4} mol/L H_2O_2 and 1.5×10^{-4} mol/L interfering substance and comparing it with the current from the biosensor in the same assay solution containing only 1.0×10^{-4} mol/L H_2O_2. Therefore, the degree of interference from the substances can be judged from the current ratios. In our experiments, the seven tested substances did not interfere significantly with the resulting biosensor. This was largely due to the low working potential; no electrochemical reactions occurred at this potential.

CONCLUSIONS

We have introduced a novel method for fabrication of an electrochemical third-generation hydrogen peroxide biosensor based on self-assembly technology. The biosensor shows an electrochemical activity to the reduction of H_2O_2 with regard to low potential requirement. During fabrication of the biosensor, HRP, as a model protein, was adsorbed successfully on nano-Ag/CA/p-ABSA/GC-

modified electrode. The resulting system has a promising potential in constructing the third-generation electrochemical biosensor based on the direct electrochemistry of HRP. In addition, the low determination limit, and wider response range point to a promising future in various biosensing processes. The proposed biosensor shows favorable reproducibility, stability, selectivity and accuracy, and can be used to determine H_2O_2 in real samples with favorable recoveries.

REFERENCES

1. J. J. Gooding, R. Eibowo, J. Q. Liu, W. R. Yang, D. Losie, S. Orbons, F. J. Mearns, J. G. Shapter and D. B. Hibbert, *J. Am. Chem. Soc*., **125**, 9006 (2003).
2. J. J Sun, H. Q Fang and H. Y Cheng, *Analyst*, **123**, 1365 (1998).
3. Y. Liu, R. Yuan, Q. F. Li, Y. Q Chai, X. Zhong and D. P Tang, *South African Journal of Chemistry*, **580**, 4 (2005).
4. T. Tatsum, Y. Okawa and T. Watanabe, *Anal. Chem*., **61**, 2352 (1989).
5. C. Fan, H. Wang, S. Sun, D. Zhu, G. Wagner and G. Li, *Anal. Chem*., **73**, 2850 (2001).
6. J. Zhang and M. Oyama, *Electrochim. Acta*, **50**, 85 (2004).
7. V. V. Shumyantseva, Y. D. Ivanov, N. F. Bistolas, W. Scheller, A. I. Archakov and U. Wollenberger, *Anal. Chem*., **76**, 6046 (2004).
8. Z. Dai, S. Liu and H. Ju, *Electrochim. Acta*, **49**, 2139 (2004).
9. A. Morrin, A. Guzman, A. J. Killard, J. M. Pingarron and M. R. Smyth, *Biosens. Bioelectron.*, **18**, 715 (2003).
10. F. Gao, R. Yuan, Y. Chai, M. Tang, S. Cao and S. Chen, *Colloids Surf. A: Physicochem. Eng. Aspects*, **295**, 223 (2007).
11. K. Chattopadhyay and S. Mazurdar, *Bioelectrochemistry*, **53**, 17 (2001).
12. S. Zhao, K. Zhang, Y. Y. Sun and C. Q. Sun, *Bioelectrochemistry*, **69**, 10 (2006).
13. Y. Xu, C. Hu and S. Hu, *Sensors and Actuators B*, **130**, 816 (2008).
14. C. Y. Liu and J. M. Hu, *Biosens. Bioelectron.*, **24**, 2149 (2009).
15. C. Ren, Y. Song, Z. Li and G. Zhu, *Anal. Bioanal. Chem.*, **381**, 1179 (2005).
16. D. P. Tang, R. Yuan, Y. Q. Chai, X. Zhong, Y. Liu and J. Dai, *Biochem. Eng. J*., **22**, 43 (2004).
17. Y. Cao, R. Jin and C. Mirkin, *J. Am. Chem. Soc*., **123**, 7961 (2001).
18. G. S. Lai, H. L. Zhang and D. Y. Han, *Sens. Actuators B: Chem.*, **129**, 497 (2008).
19. Q. Xu, C. Mao, N. N. Liu, J. J. Zhu and J. Shen, *React. Funct. Polym*., **66**, 863 (2006).
20. G. Wang, W. Wang, J. Wu, H. Liu, S. Jiao and B. Fang, *Microchim Acta*, **164**, 149 (2009).