Appl Biochem Biotechnol (2010) 162:2189–2196
DOI 10.1007/s12010-010-8993-1

Enzyme Biosensor Based on the Immobilization of HRP on SiO_2/BSA/Au Composite Nanoparticles

Shirong Yuan · Ruo Yuan · Yaqin Chai · Ying Zhuo · Xia Yang · Yali Yuan

Received: 10 March 2010 / Accepted: 11 May 2010 /
Published online: 9 June 2010

Abstract In this work, an enzyme biosensor based on the immobilization of horseradish peroxidase (HRP) on SiO_2/BSA/Au/thionine/nafion-modified gold electrode was fabricated successfully. Firstly, nafion was dropped on the surface of the gold electrode to form a nafion film followed by chemisorption of thionine (Thi) as an electron mediator via the ion-exchange interaction between the Thi and nafion. Subsequently, the SiO_2/BSA/Au composite nanoparticles were assembled onto Thi film through the covalent bounding with the amino groups of Thi. Finally, HRP was immobilized on the SiO_2/BSA/Au composite nanoparticles due to the covalent conjugation to construct an enzyme biosensor. The surface topographies of the SiO_2/BSA/Au composite nanoparticles were investigated by using scanning electronic microscopy. The stepwise self-assemble procedure of the biosensor was further characterized by means of cyclic voltammetry and chronoamperometry. The enzyme biosensor showed high sensitivity, good stability and selectivity, a wide linear response to hydrogen peroxide (H_2O_2) in the range of $8.0\times10^{-6}\sim3.72\times10^{-3}$ mol/L, with a detection limit of 2.0×10^{-6} mol/L. The Michaelies-Menten constant K_M^{app} value was estimated to be 2.3 mM.

Keywords SiO_2/BSA/Au composite nanoparticles · Nafion (Nf) · Thionine (Thi) · HRP · Hydrogen peroxide (H_2O_2)

List of symbols

v	Scan rate(mV s^{-1})
E	Potential (V)
I	Current (μA)
C	Concentration(mM)
T	Time (s)

S. Yuan · R. Yuan (✉) · Y. Chai · Y. Zhuo · X. Yang · Y. Yuan
Chongqing Key Laboratory of Analytical Chemistry, College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, People's Republic of China
e-mail: yuanruo@swu.edu.cn

Introduction

The development of highly sensitive and selective methods for H_2O_2 determination is a very important analytical task in both clinical and environmental fields. Biosensors based on horseradish peroxidase (HRP) have emerged as the most convenient tools for hydrogen peroxide determination [1–4]. It is well-known that immobilizing a biomolecule on the sensing electrode surface, and maintaining the enzyme activity is a crucial step in enzyme biosensor design [4–7]. Therefore, new materials have been researched for getting more sensitive and stable biosensors [8, 9]. Up until now, a lot of materials have been used as electrode-modified matrices for immobilization of HRP, such as polymers, sol–gel, and nanoparticles [10, 11].

Nanoparticles have often been used in constructing electrochemical biosensors due to their special features including their large surface area, high surface free energy, and good biocompatibility [12, 13]. For example, metal nanoparticles can be used to promote electron transfer between proteins and electrodes. Particularly, Au nanoparticles (nano-Au) have been extensively used as an immobilized matrix to promote the direct electron transfer of the immobilized redox proteins [10–16]. Xiao et al. developed a biosensor based on HRP immobilized on nano-Au. However, the lifetime of the biosensor was not satisfied [17]. Additionally, SiO_2 nanoparticles have also been found to be a good biocompatible solid support for biomolecule immobilization to construct biosensor [11, 12]. Although the lifetime of the biosensor based on HRP immobilized on SiO_2 nanoparticles was in the range of several months, the conductivity was poor, which resulted in the low sensitivity [17]. Actually, the composite nanomaterials have a synergistic effect of each component [18]. In comparison to the individual components, the composites could exhibit better physical, chemical properties, and performance characteristics [19, 20]. Therefore, the SiO_2/Au composite nanoparticles were hopeful to obtain good conductivity and favorable environment to retain the enzyme activity.

Recently, the composite nanoparticles of SiO_2/Au were used for immobilization of proteins [21–24]. In this work, an inert protein with many reactive amino groups, bovine serum albumin (BSA) [25], was chosen to functionalize silica nanoparticles. BSA was easily absorbed on SiO_2 surface through the hydrogen bonding between -NH group of BSA and -OH group of SiO_2 [26]. In comparison with the PEI-functionalized silica and APTMS-functionalized silica [27], BSA-functionalized silica could possess better biocompatibility to retain the enzyme activity. Furthermore, the BSA-covered SiO_2 could load gold nanoparticles by the reactive amino groups of BSA, the resulting SiO_2/BSA/Au composite nanoparticles could be used to immobilize the enzyme and efficiently avoid the inactive of the enzyme.

Thus, the enzyme biosensor based on the composite nanoparticles of SiO_2/BSA/Au was constructed by employing the self-assembly technique to form HRP/SiO_2/BSA/Au/Thi/Nf-modified gold electrode. The surface morphology on the SiO_2/BSA/Au composite nanoparticles was observed by using scanning electron microscopy (SEM), the modified biosensors were characterized by means of cyclic voltammetry and chronoamperometry. The performance and factors influencing the resulting biosensor were studied in detail. The resulted biosensor exhibited high sensitivity, good repeatability, and selectivity.

Experimental

Reagent and Materials

Thionine (Thi), Nafion (Nf), HRP, bovine serum albumin (BSA, 96–99%) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). H_2O_2 (30%, *w*/*v* solution) was purchased

from Beijing Chemical Reagent (Beijing, China). The prepared Au colloids (16 nm) were kept at 4 °C before use. All chemicals and solvents used were of analytical grade and were used as received. Double-distilled water was used throughout this study. The working buffer (pH 5.5) was composed of 0.1 M HAc–NaAc buffer solution containing 0.1 M KCl.

Apparatus

Cyclic voltammetric measurements (CVs) and chronoamperometry were performed with CHI 610B electrochemistry workstation (Shanghai CH Instruments Co, China). All experiments were carried out with a conventional three-electrode system with the modified gold electrode as the working electrode, a platinum wire as the counter electrode, and a saturated calomel reference electrode (SCE).

Preparation of SiO_2/BSA/Au Composite Nanoparticles

SiO_2 nanoparticles were prepared according to the following steps: first, 100.0 mL ethanol was added into 12.0 mL $NH_3 \cdot H_2O$ (25.0 wt%), after the solution became homogeneous, 6.0 mL tetraethylorthosilicate was dropped slowly into solution under vigorous stirring. After a few minutes, the white precipitate was washed, centrifuged, and dispersed in distilled water, until the supernatant solution turned neutral, the opaque and white silica sol–gel is formed [28].

Subsequently, the obtained silica sol–gel was surface chemically functionalized by BSA. Finally, the functionalized silica sol–gel and Au colloid were mixed and vigorously stirred for 4 h. Then, the SiO_2/BSA/Au composite nanoparticles were obtained. In Fig. 1, the micrographs (morphologies) of SiO_2/BSA/Au composite nanoparticles are shown. As shown, the silicon substrate was covered with a great deal of small spherical gold nanoparticles, which demonstrated that the SiO_2/BSA/Au composite nanoparticles were prepared successfully.

Fabrication of the Enzyme Biosensor

A gold electrode (Φ=4 mm) was polished repeatedly with 1.0, 0.3 μm alumina powder, followed by successive sonication in bi-distilled water and ethanol, and dried in air. The

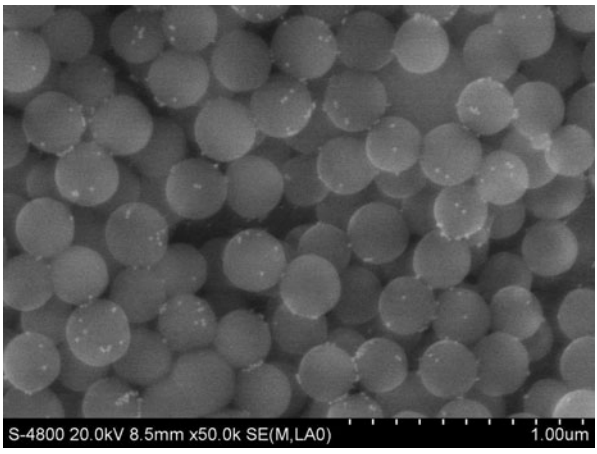

Fig. 1 SEM images of SiO_2/BSA/Au nanoparticles

cleaned electrode was coated with Nf ethanol solution (*v*/*v*, 2%) firstly and then soaked in Thionine (Thi) solution (3 mM) for about 10 min. Following that, it was immersed in 0.5 mL solution of the SiO_2/BSA/Au composite nanoparticles for 4 h to form a SiO_2/BSA/Au composite nanoparticles monolayer. Finally, the modified biosensor was coated with HRP solution about 4 h at 4 °C. The schematic illustration of the stepwise self-assembly procedure of the biosensor was shown in Fig. 2.

Electrochemical Measurements

Electrochemical measurements were performed at 25 °C (room temperature) and the potential swept from −0.6 to 0.2 V (versus SCE) with the scan rate of 100 mV s^{-1}. The electrochemical measurements were performed in 0.1 M HAc–NaAc buffer solution (pH 5.5).

Results and Discussion

Electrochemical Characterization of Differently Modified Electrodes

Cyclic voltammograms of differently modified electrodes were presented in Fig. 3. No obvious electrochemical peaks were found at the bare gold electrode (Fig. 3a), or the Nf-modified gold electrode (Fig. 3b) due to the lack of electron exchange. It was not until positively charged Thi^+ and negatively charged SiO_2/BSA/Au composite nanoparticles were assembled on the Nf-modified gold electrode that the current increased substantially (Fig. 3c, d). The reason may be the fact that nanometer-sized gold colloids play an important role similarly to a conducting wire or electron-conducting tunnel. However, when HRP was adsorbed onto the SiO_2/BSA/Au composite nanoparticle monolayer, a further decrease of the peak currents may be contributed HRP hindering the transmission of electrons toward the electrode surface (Fig. 3e).

The CVs of the modified electrode in HAc–NaAc buffer solution (pH 5.5) at different scan rates were investigated. It was found that the biosensor represented reversible waves in cyclic voltammetry, indicating that the film modified was quite stable. Also, both the anodic and cathodic peak currents were directly proportional to the potential scan rates in the range of 20–300 mV s^{-1}, suggesting a surface confined redox process.

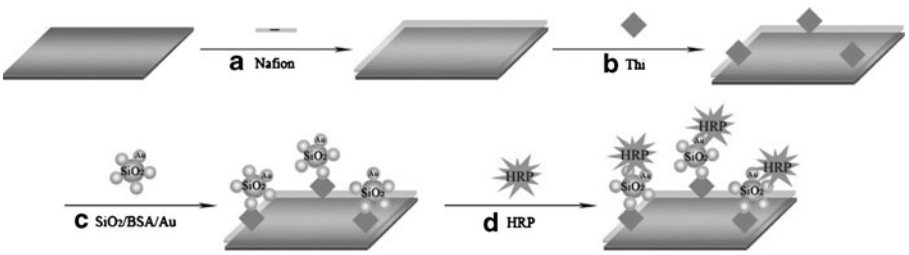

Fig. 2 Schematic illustration of the stepwise enzyme biosensor fabrication process: **a** Dropping 2% nafion on the electrode surface, **b** Adsorption of Thi, **c** assembly of SiO_2/BSA/Au nanocomposites, **(d)** loading of HRP

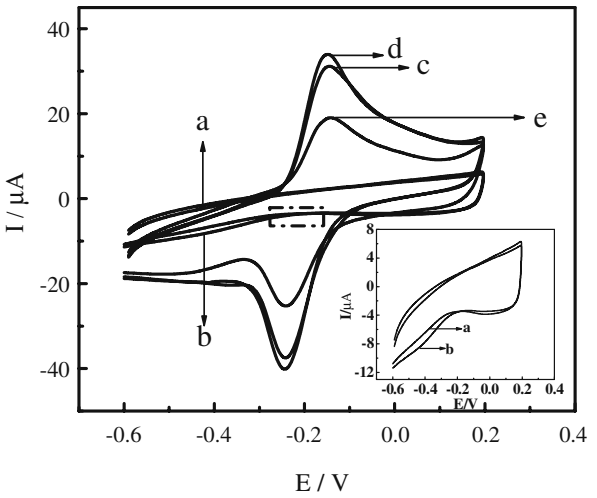

Fig. 3 Cyclic voltammograms of the electrode in the HAc–NaAc buffer solution: **a** bare electrode, **b** adsorption Nafion, **c** adsorption Thi, **d** SiO_2/BSA/Au/Thi/nafion electrode, **e** HRP will be fixed to the modified SiO_2/BSA/Au/Thi/nafion electrode surface, modification of the electrode in HAc–NaAc buffer solution, scanning rate is 100 mV s^{-1}. Insert: amplified response curve

The Amplification Properties of HRP

The finding that HRP had good catalytic activity towards H_2O_2 can be seen in the cyclic voltammograms obtained in working buffer (pH 5.5) with and without H_2O_2. In the absence of H_2O_2, oxidation-reduction peaks were observed, due to Thi being a good redox probe. In the presence of H_2O_2, the cathodic peak current significantly increased 52.10% and the anodic current decreased 6.40%. The phenomena indicated that HRP attached to the electrode surface retains high enzymatic catalytic activity and Thi is a suitable electron-transfer mediator.

Optimization of the Assay Conditions

pH of the Working Buffer

The CVs of the biosensor in HAc–NaAc buffer at various pHs ranging from 3.5 to 6.0 are shown in Fig. 4. It was observed that both the cathodic and anodic potential shifted in a

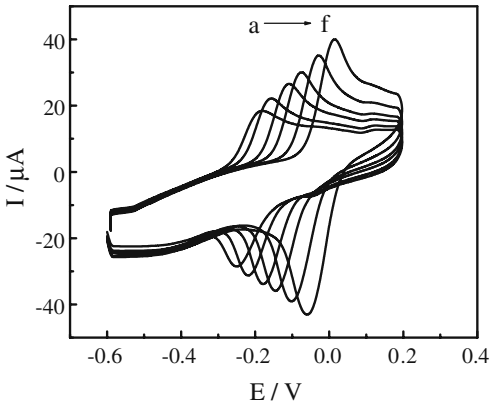

Fig. 4 Cyclic voltammograms of the enzyme-based biosensor in HAc–NaAc buffer at various pHs: **a** 6.0, **b** 5.5, **c** 5.0, **d** 4.5, **e** 4.0, **f** 3.5 at 100 mV s^{-1} scan rate under 25 °C

negative direction with an increase of pH. And the current response decreased with increasing pH value 3.5 to 6.0. The reason may be due to the fact that the Thi is easily protonized in the solution containing enough H^+. The pH influences the biosensor response in two ways: one is that pH affects the activity of HRP, with increasing solution acidity its activity decreases greatly. The other one is that pH affects the current response and the potential of Thi. Therefore, while considering the response and the lifetime of the biosensor, an optimal pH of the working buffer was 5.5.

Performance of the Biosensor

Figure 5 showed the chronoamperometric response of the biosensor to different concentrations of H_2O_2 at an applied potential of −200 mV. Additions of H_2O_2 to the solution resulted in an apparent increase of the reduction current. The enzyme biosensor had a satisfactory response within the concentration range tested from 8.0×10^{-6} to 3.72×10^{-3} mol L^{-1} with a correlation coefficient of 0.997; the detection limit is 2.0×10^{-6} mol L^{-1}. Table 1 shows the linear range and detection limit of hydrogen peroxide biosensors with previous reports [29–33]. Compared with other methods, the biosensor has a relative large linear range and low detection limit.

The apparent Michaelis–Menten constant (K_M^{app}), which shows an indication of the enzyme-substrate kinetics, can be obtained from the electrochemical version of the Lineweaver-Burk equation [34]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max} C}$$

Where, I_{ss} is the steady current after the addition of substrate, $I_{\max}$ is the maximum current under saturated substrate condition, and C is the H_2O_2 concentration, K_M^{app} stands for the apparent Michaelis–Menten constant of the system. The value of the apparent Michaelis–Menten constant can be calculated from the slope and the intercept for the plot of the reciprocals of the steady-state current versus H_2O_2 concentration. The K_M^{app} value for the HRP/SiO_2/BSA/Au/Thi/Nafion/Au electrode was determined to be 2.3 mM. This K_M^{app} value is smaller than that obtained for incorporated in Au nanoparticles and chitosan

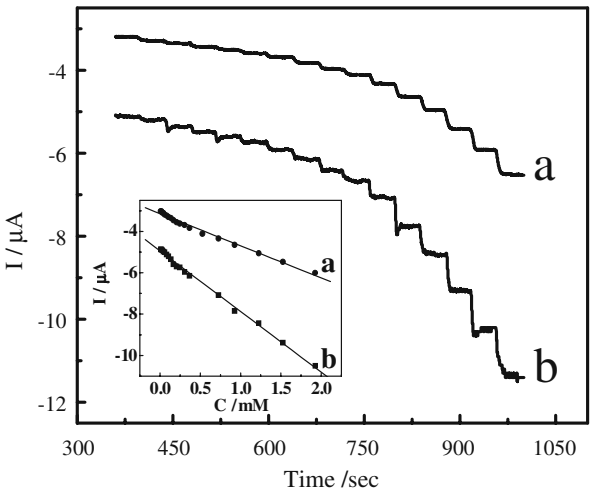

Fig. 5 The chronoamperometry response of the enzyme-based biosensor at an applied potential of −200 mV: **a** HRP/Au/Thi/nafion; **b** HRP/SiO_2/BSA/Au/Thi/nafion. *Insert* is the calibration curve of the enzyme-based biosensor

Table 1 Comparison of linear range and detection limit of the biosensor with previous reports.

H_2O_2 biosensor	Linear range (M)	Detection limit (M)
HRP/SiO_2/BSA/Au/Thi/nafion/Au	8.0×10^{-6}~3.72×10^{-3}	2.0×10^{-6}
HRP/silica sol–gel/chitosan/CPE [29]	2.5×10^{-6}~3.4×10^{-3}	3.0×10^{-6}
HRP/SiO_2-MB/gelatine/GCE [30]	1.0×10^{-5}~1.2×10^{-3}	4.0×10^{-6}
HRP/nano-Au/thio-CCE [31]	1.22×10^{-5}~1.10×10^{-3}	6.1×10^{-6}
HRP/Hb/chitosan/GCE [32]	3.9×10^{-4}~8.9×10^{-3}	1.2×10^{-4}
HRP/FMC-BSA/MWNTs/ormosil composite/GCE [33]	2.0×10^{-5}~4.0×10^{-3}	5.0×10^{-6}

CPE carbon paste electrode, *CCE* carbon composite electrode

composite film ($K_M^{app} = 4.51$ mM) [35] in poly(thionine)film ($K_M^{app} = 28$ mM) [36], indicating that HRP immobilized in SiO_2/BSA/Au/Thi/Nafion film retains its bioactivity and has a high biological affinity to H_2O_2.

A comparative study of the chronoamperometric response of the biosensors was carried out using SiO_2/BSA/Au composites or Au nanoparticles to immobilize HRP. Figure 5a was the response curve of the HRP/Au/Thi/Nafion modified electrode. It was found that the sensitivity of the studied electrode was enhanced by the SiO_2/BSA/Au composite nanoparticles. Further studies showed that the electrode in this study exhibited high stability, when it was kept at 4 °C for a period of 4 weeks, the electrode retained more than 87.5% of its initial response.

Selectivity of the Biosensor

Selectivity is also important in practical use of the biosensors. In order to investigate the effect of some possible interfering substances on the biosensor, five interfering substances were used for measurement in our experiments. The results are listed in Table 2. The current ratios were calculated by reading the current of the biosensor in the solution containing 1.7 mM interfering substance and 1.7 mM H_2O_2 and comparing it with the current from the biosensor in the same solution containing only 1.7 mM H_2O_2. According to the results of the current ratio, we can find that the five tested substances did not interfere significantly with the resulting biosensor, indicating that the biosensor has a sufficient selectivity.

Conclusions

In this study, the SiO_2/BSA/Au composites were prepared and used for the immobilization of HRP. The results showed that the immobilized HRP kept its bioactivity with the

Table 2 Possible interferences tested with the biosensor.

Possible interferences	Current ratio
L-cysteine	0.98
L-tyrosine	0.97
Ethanol	1.03
Glucose	1.01
Ascorbic acid	0.99

modified electrode exhibiting high sensitivity, good stability and selectivity, and a wide linear response to H_2O_2. Thus, this assembly method was hopeful for making a stable, high sensitivity biosensor and the strategy can be used in other fields.

Acknowledgements This work was supported by the NNSF of China (20675064), the Ministry of Education of China (Project 708073), the Natural Science Foundation of Chongqing City (CSTC-2009BA1003) and High Technology Project Foundation of Southwest University (XSGX02), China.

References

1. Wan, J., Bi, J. L., Du, P., & Zhang, S. S. (2009). *Analytical Biochemistry, 386*, 256–261.
2. Lupu, A., Lisboa, P., Valsesia, A., Colpo, P., & Rossi, F. (2009). *Sensors and Actuators B, 137*, 56–61.
3. Li, F., Chen, W., Tang, C. F., & Zhang, S. S. (2009). *Talanta, 77*, 1304–1308.
4. Camacho, C., Chico, B., & Cao, R. (2009). *Biosensors & Bioelectronics, 24*, 2028–2033.
5. Gopalan, A. I., Lee, K.-P., & Ragupathy, D. (2009). *Biosensors & Bioelectronics, 24*, 2211–2217.
6. Shimoumura, T., Itoh, T., Sumiya, T., Mizukami, F., & Ono, M. (2009). *Talanta, 78*, 217–220.
7. Pingarron, J. M., Yanez-Sedeno, P., & Gonzalez-Cortes, A. (2008). *Electrochimica Acta, 53*, 5848–5866.
8. Pang, X. Y., He, D. M., Luo, S. L., & Cai, Q. Y. (2009). *Sensors and Actuators B, 137*, 134–138.
9. Kang, X. H., Wang, J., & Tang, Z. W. (2009). *Talanta, 78*, 120–125.
10. Guo, S. J., & Dong, S. J. (2009). *Trend in Analytical Chemistry, 28*, 96–109.
11. Zhu, Y. H., Cao, H. M., Tang, L. H., Yang, X. L., & Li, C. Z. (2009). *Electrochimica Acta, 54*, 2823–2827.
12. Zhuo, Y., Yu, R. J., Yuan, R., & Chai, Y. Q. (2009). *Journal of Electroanalytical Chemistry, 628*, 90–96.
13. Libor, Z., & Zhang, Q. (2009). *Materials Chemistry and Physics, 114*, 902–907.
14. Kerman, K., Saito, M., Yamamura, S., & Takamura, Y. (2008). *Trends in Analytical Chemistry, 27*, 585–592.
15. Zhu, A. W., Tian, Y., Liu, H. Q., & Luo, Y. P. (2009). *Biomaterials, 30*, 3183–3188.
16. Shimomura, T., Itoh, T., Sumiya, T., Mizukami, F., & Ono, M. (2008). *Sensors and Actuators B, 135*, 268–275.
17. Di, J. W., Shen, C. P., & Peng, S. H. (2005). *Analytica Chimica Acta, 553*, 196–200.
18. Zhang, Y. Z., Zhang, K. Y., & Ma, H. Y. (2009). *Analytical Biochemistry, 387*, 13–19.
19. Wang, Y. Y., Chen, X. J., & Zhu, J. J. (2009). *Electrochemistry Communications, 11*, 323–326.
20. Lamarre, J.-M., Billard, F., Kerboua, C. H., & Lequime, M. (2008). *Optics Communications, 281*, 331–340.
21. Wang, Y., Qian, W. P., Tan, Y., Ding, S. H., & Zhang, H. Q. (2007). *Talanta, 72*, 1134–1140.
22. Liang, Y., Gong, J. L., & Huang, Y. (2007). *Talanta, 72*, 443–449.
23. Jung, K.-H., Yoon, J.-W., & Koshizaki, N. (2008). *Current Applied Physics, 8*, 761–765.
24. Qi, Y. L., Chen, M., Liang, S., & Yang, W. (2008). *Applied Surface Science, 254*, 1684–1690.
25. Fang, L. Y., Lu, Z. Z., Wei, H., & Wang, E. K. (2008). *Biosensors & Bioelectronics, 23*, 1645–1651.
26. Kandimalla, V. B., Tripathi, V. S., & Ju, H. X. (2006). *Biomaterials, 27*, 1167–1174.
27. Xue, J. G., Wang, C. G., & Ma, Z. F. (2007). *Materials Chemistry and Physics, 105*, 419–425.
28. Zhang, L., Feng, Y. G., & Wang, L. Y. (2007). *Material Research Bulletin, 42*, 1457–1467.
29. Miao, Y., & Tan, S. N. (2001). *Analytica Chimica Acta, 437*, 87–93.
30. Yao, H., & Li, N. (2005). *Biosensors and Bioelectronics, 21*, 372–377.
31. Lei, C. X., & Hu, S. Q. (2004). *Bioelectrochemistry, 65*, 33–39.
32. Xiong, H. Y., & Chen, T. (2007). *Electrochemistry Communications, 9*, 2671–2675.
33. Tripathi, V. S., & Kandimalla, V. B. (2006). *Biosensors and Bioelectronics, 21*, 1529–1535.
34. Chen, S. H., Yuan, R., & Chai, Y. Q. (2007). *Biosensors and Bioelectronics, 22*, 1268–1274.
35. Tangkuaram, T., & Ponchio, C. (2007). *Biosensors and Bioelectronics, 22*, 2071–2078.
36. Xiao, Y., & Ju, H. X. (1999). *Analytica Chimica Acta, 391*, 299–306.