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Short communication

Glucose biosensor based on covalent immobilization of enzyme in sol–gel composite film combined with Prussian blue/carbon nanotubes hybrid

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ABSTRACT

A novel electrochemical glucose biosensor was developed based on in situ covalent immobilization of glucose oxidase (GOx) by one-pot chitosan (CS)-incorporated sol–gel process in the presence of Prussian blue deposited multi-walled carbon nanotubes hybrids (PB/MWNTs) using 3-isocyanatopropyltriethoxysilane (ICPTES) as both a sol–gel precursor and a covalent coupling agent for GOx and CS. The electrode modified with the PB/MWNTs-GOx-CS-ICPTES sol–gel composite film showed good electrical conductivity and effective low-potential electron transfer mediation toward H_2O_2 reduction attributed to the incorporation of PB/MWNTs. The biosensor exhibited a linear response to glucose in the concentration range from 2.5×10^{-5} to 1.3×10^{-3} M with a correlation coefficient of 0.9998, a detection limit of 7.5×10^{-6} M, a low response time (10 s), good sensitivity and high anti-interference ability. Compared with the control biosensor based on the traditional tetraethoxysilane derived sol–gel composite film, the biosensor showed a similarly small apparent Michaelis–Menten constant of 3.67 mM but much higher electrochemical and biosensing stability.

1. Introduction

Despite the impressive progress in the development of electrochemical glucose biosensors due to their rapid response, high sensitivity and intrinsic selectivity, there are still many challenges and obstacles related to the achievement of a highly stable and reliable continuous glycemic monitoring (Wang, 2008). Most glucose biosensors are based on the oxidation or reduction of enzymatically produced H_2O_2, so the preparation of biosensing materials with both efficient enzyme immobilization and good electrochemical properties is of prime importance (Kang et al., 2008; Yu et al., 2008; Zou et al., 2008; Kang et al., 2009). The chitosan–silica (CS–SiO_2) based organic–inorganic sol–gel composite films as biocompatible enzyme immobilization matrix have showed great promise due to their tunable porosity, high thermal stability and good film forming ability under mild conditions (Wang et al., 2000; Wang et al., 2003; Zou et al., 2008). However, enzymes immobilized by physical adsorption in the CS–SiO_2 composite films have to be confronted with the problem of enzyme leakage, leading to poor stability of the fabricated biosensors (Kato et al., 2004; Molvinger et al., 2004; Coradin et al., 2006).

As a particular silane coupling agent, 3-isocyanatopropyltriethoxysilane (ICPTES) has attracted increasing interest in the synthesis of SiO_2-based organic–inorganic hybrid materials with polymers, such as CS (Biazzotto et al., 2000; Silva et al., 2005; Garcia-Sanchez et al., 2009; Kumbar et al., 2010). The good covalent coupling reactivity of the isocyanate group toward the amino group without severe conditions and the spontaneous sol–gel process of the triethoxysilane group allow the convenient preparation of covalently cross-linked organic–inorganic hybrid materials (Li et al., 2009). Furthermore, the isocyanate group has been widely applied in enzyme immobilization (Ozmen et al., 2009a; Ozmen and Yilmaz, 2009; Ozmen et al., 2009b; Romaskevic et al., 2010). Thus, covalently cross-linked enzyme–CS–SiO_2 sol–gel composite films with synergistic benefits of good biocompatibility and effective immobilization of enzyme might be obtained using ICPTES as both the covalent coupling agent for enzyme, CS and the sol–gel precursor. Nevertheless, the poor electrical conductivity and the required relatively high working potentials of most SiO_2-based sol–gel materials remain as problems to be solved (Li et al., 2004).

Prussian blue deposited carbon nanotubes hybrids, combining the efficient facilitation effect of CNTs on electron transfer (Tang et al., 2004; Guan et al., 2005; Huang et al., 2006; Tsai and Tsai, 2009) with the effective low-potential electron transfer mediation effect of Prussian blue toward the reduction of H_2O_2 (Ahmadalinezhad et al., 2009; Chiu et al., 2009; Wang et al., 2009; Li et al., 2010), are attracting particular interest in the area of electrochemistry (Zhai et al., 2009; Zhang et al., 2009). To the best of our knowledge, no attempt has been made to fabricate biosensors with

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synergistic merits of the covalently cross-linked enzyme–CS–SiO_2 sol–gel composite film and Prussian blue deposited carbon nanotubes hybrids. This paper developed a novel strategy to fabricate glucose biosensors based on in situ covalent immobilization of glucose oxidase (GOx) by one-pot CS-incorporated sol–gel process in the presence of Prussian blue deposited multi-walled carbon nanotubes hybrids (PB/MWNTs). ICPTES was used as both the sol–gel precursor and the covalent coupling agent for GOx and CS. Due to the good electrochemical properties of the PB/MWNTs-modified electrode, as well as the improved effectiveness of enzyme immobilization provided by the sol–gel covalent immobilization process, the fabricated biosensor is highly sensitive, selective and stable.

2. Experimental

2.1. Materials and reagents

GOx (EC 1.1.3.4, Type II, lyophilized powder, 109 units/mg, from *Aspergillus niger*) was obtained from Amresco. D-(+)-glucose (99.5%) and CS (90%) were obtained from Sigma. MWNTs (carboxyl functionalized, 95%, 20–40 nm) were purchased from Shenzhen Nanotech. Port. Co., Ltd. (Shenzhen, China). ICPTES (99%) was obtained from Diamond Advanced Material of Chemical Inc. (Hubei, China). Tetraethoxysilane (TEOS) was purchased from Shanghai Reagent Co. (Shanghai, China). Phosphate buffer solution (PBS, 0.05 M) containing K_2HPO_4, KH_2PO_4 and 0.1 M KCl was employed as the supporting electrolyte. Aqueous solutions were prepared with deionized water (18.2 MΩ cm) from a Milli-Q purification system.

2.2. Instruments

Electrochemical measurements were performed on a PARSTAT 2273 electrochemical workstation (PE Co., USA) with a standard three-electrode system comprising a platinum wire as the counter electrode, a saturated calomel electrode (SCE) as the reference electrode and a modified glassy carbon electrode (GCE, 4 mm in diameter) as the working electrode. The electrochemical impedance spectroscopy (EIS) was carried out in pH 6.9 PBS containing 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 0.5 M KCl in the frequency range from 10 KHz to 10 MHz. The measurements of glucose concentration in serum samples were carried out by adding the serum with a certain volume into the electrochemical cell with 30 mL PBS under stirring. Scanning electron microscopy (SEM) measurements were made on a Quanta 200 FEG scanning electron microscope (FEI Co., USA) at an accelerating voltage of 30 kV. Absorption spectra were recorded on a Cary 4000 UV–vis spectrophotometer (USA).

2.3. Fabrication of the composite film modified GCE

Prussian blue deposited multi-walled carbon nanotubes hybrids (PB/MWNTs) were synthesized according to the literature (Zhai et al., 2009). 0.25% CS solution was prepared by dissolving 0.25 g CS in 100 mL 1.0% acetic acid with sonication for 1 h. Before electrode modification, GCE was successively polished with 0.3 and 0.05 μm alumina slurry, followed by rinsing thoroughly with deionized water. After successive sonication in ethanol and deionized water, the electrode was dried at room temperature.

2.3 mg PB/MWNTs were dispersed in 500 μL 0.25% CS solution with sonication for 15 min, then 80 μL ICPTES was added. The mixture initially composed of two phases was vigorously stirred for 4 h at room temperature, followed by the addition of 2.9 mg GOx. After the mixture was gently stirred for 1.5 h at 4 °C, a homogenous sol–gel solution was obtained. Then, the sol–gel solution (7 μL) was casted on the surface of GCE, dried at 4 °C in a refrigerator for 12 h, resulting in the sol–gel composite film modified GCE (PB/MWNTs-GOx-CS-ICPTES-GCE). Finally, the modified electrode was immersed in pH 6.9 PBS at 4 °C for 24 h before the electrochemical measurements. For the comparative study, a control biosensor based on the PB/MWNTs-GOx-CS-TEOS sol–gel composite film was prepared in parallel using TEOS with the same content of silica to that of ICPTES. All modified electrodes were stored in pH 6.9 PBS at 4 °C in a refrigerator when not in use.

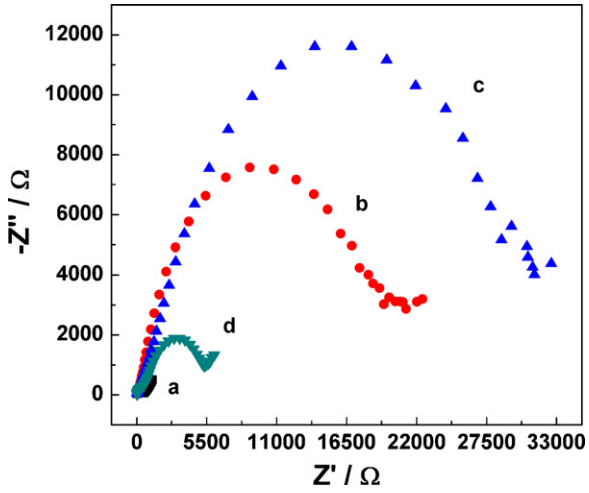

Fig. 1. Electrochemical impedance spectra of (a) GCE, (b) CS-ICPTES-GCE, (c) GOx-CS-ICPTES-GCE and (d) PB/MWNTs-GOx-CS-ICPTES-GCE in 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.5 M KCl.

3. Results and discussion

3.1. Characterization of the composite film modified electrode

The morphology of the composite film was characterized by SEM. After the deposition of PB on MWNTs, a large number of closely arranged PB nanoparticles with diameter around 50 nm were randomly deposited on MWNTs surfaces. The PB/MWNTs-GOx-CS-ICPTES sol–gel composite film showed a typical compact and dense morphology of sol–gel composite films with uniformly incorporated PB/MWNTs (see SFig. 1 in Supplemental materials).

The PB/MWNTs-GOx-CS-ICPTES-GCE showed a pair of well-defined redox peaks with a formal potential of +0.193 V, which corresponded to the redox conversion between PB and Prussian white (PW) (see SFig. 2 in Supplemental materials). Both the cathodic and anodic peak currents were linearly dependent on the square root of the scan rate in the range from 10 to 260 mV/s, indicating a diffusion-controlled electrochemical process. In addition, PB in the composite film showed high stability under neutral and basic pH attributed to the synergistic protective effect of the Si–O–Si sol–gel networks, MWNTs and CS (see SFig. 3 in Supplemental materials).

Fig. 1 shows the typical Nyquist plots of the modified electrodes in $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The electron transfer resistance (R_{et}) for the redox process of the probe increased drastically after the CS-ICPTES sol–gel composite film was modified on the GCE (b). When GOx was immobilized in the above film, a further increased R_{et} was observed (c). Significantly, after the further incorporation of PB/MWNTs, the R_{et} decreased dramatically (d), indicating that the incorporated PB/MWNTs facilitated the electron transfer of the probe to the electrode attributed to the well known facilitation effect of MWNTs.

The PB/MWNTs-GOx-CS-ICPTES-GCE showed a typical chronoamperometric response upon the successive addition of H_2O_2 at −0.1 V (see SFig. 4 in Supplemental materials). The reduction current increased steeply and then reached 95% of the

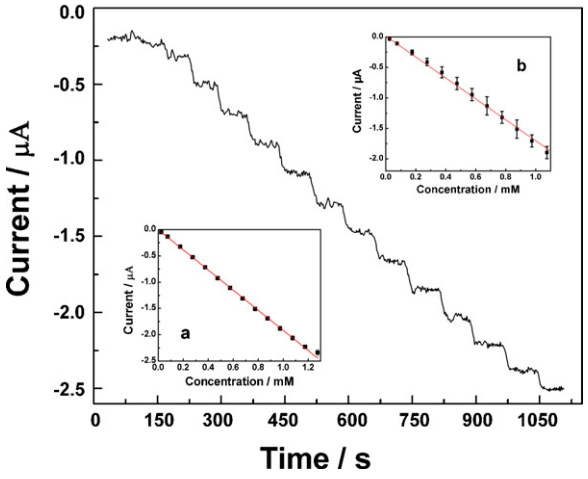

Fig. 2. Chronoamperometric response of PB/MWNTs-GOx-CS-ICPTES-GCE upon the successive addition of glucose at −0.1 V. (Inset) Calibration plots of chronoamperometric responses of (a) PB/MWNTs-GOx-CS-ICPTES-GCE and (b) PB/MWNTs-GOx-CS-TEOS-GCE *vs.* glucose concentration.

steady-state current within 10 s after the addition of H_2O_2 with a wide linear response range from 1.3×10^{-4} to 6.9×10^{-3} M and a sensitivity of 38.1 μA mM^{-1} cm^{-2}. The result indicated that the incorporated PB/MWNTs can act as the effective low-potential electron transfer mediator for H_2O_2 reduction, which was of particular importance for the sensitive and selective biosensing of glucose.

3.2. Amperometric response of the composite film based biosensor to glucose

The PB/MWNTs-GOx-CS-ICPTES-GCE showed a typical chronoamperometric response upon the successive addition of glucose at −0.1 V as shown in Fig. 2. The time to reach 95% of the steady-state current was less than 10 s. The linear response range to glucose was from 2.5×10^{-5} to 1.3×10^{-3} M with a correlation coefficient of 0.9998 and a sensitivity of 15.2 μA mM^{-1} cm^{-2} (Inset a). The detection limit was calculated to be 7.5×10^{-6} M defined from a signal/noise ratio of 3. It was noteworthy that the sensitivity was much higher than 5.94 μA mM^{-1} cm^{-2} of the previously reported GOx-CS-methyltrimethoxysilane/PB-GCE (Tan et al., 2005). Meanwhile, the PB/MWNTs-GOx-CS-TEOS-GCE as the control biosensor also showed a typical chronoamperometric response to glucose with a similar linear response range from 2.5×10^{-5} to 1.1×10^{-3} M, a correlation coefficient of 0.9986, a detection limit of 5.3×10^{-6} M, a response time less than 10 s, and a similar sensitivity of 13.5 μA mM^{-1} cm^{-2} (Inset b).

3.3. Michaelis–Menten constant of the composite film based biosensor

According to the Lineweaver–Burk equation (Zou et al., 2008; Wei et al., 2010), the apparent Michaelis–Menten constant (K_m) of the biosensor (PB/MWNTs-GOx-CS-ICPTES-GCE) and the control biosensor (PB/MWNTs-GOx-CS-TEOS-GCE) were calculated to be 3.67 and 2.75 mM, respectively. The slightly higher K_m of the biosensor than that of the control biosensor indicated that the covalent coupling reaction between GOx and ICPTES could impair the biological activity of immobilized GOx to a small extent. Nevertheless, the K_m of the biosensor based on the PB/MWNTs-GOx-CS-ICPTES sol–gel composite film was smaller than reported ones of immobilized GOx (Kang et al., 2008; Zou et al., 2008; Liang et al., 2008; Liu et al., 2009; Wang et al., 2009), indicating that the PB/MWNTs-GOx-CS-ICPTES sol–gel composite film can still provide a biocompatible microenvironment for immobilized GOx.

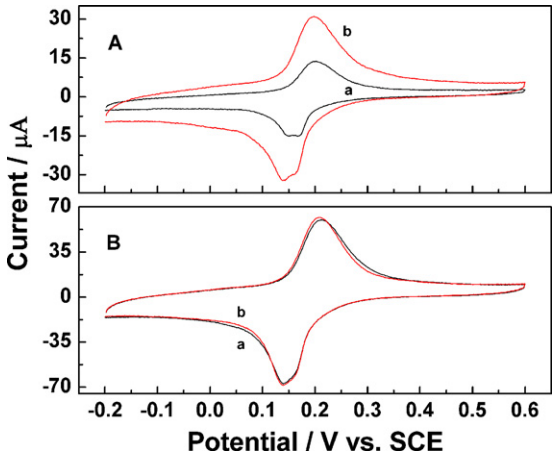

Fig. 3. Cyclic voltammograms of (A) PB/MWNTs-GOx-CS-TEOS-GCE and (B) PB/MWNTs-GOx-CS-ICPTES-GCE in pH 6.9 PBS at 50 mV/s before (a) and after (b) the storage in vigorously stirred PBS for 24 h at 4 °C.

3.4. Stability of the composite film based biosensor

Cyclic voltammograms of the modified electrodes in pH 6.9 PBS before (a) and after (b) the storage in vigorously stirred PBS for 24 h at 4 °C were studied to evaluate the immobilization effectiveness of components in the sol–gel composite films (Fig. 3). The cyclic voltammogram of the PB/MWNTs-GOx-CS-TEOS-GCE (A) changed obviously after the storage. The relatively poor electrochemical stability indicated poor structural stability of the sol–gel composite film. In the case of TEOS as the sol–gel precursor, GOx and CS could be only weakly bonded to the Si–O–Si sol–gel networks. Quantities of components, especially GOx, on the film surface might be leached out during the storage, leading to an increase in porosity of the film, which could not only promote the electron transfer but also facilitate the mass transport of glucose into the film (Liang et al., 2008). The resulting promotion of electron transfer could be confirmed by an obvious increase in peak currents of the PB/MWNTs-GOx-CS-TEOS-GCE after the storage (b). On the contrary, the PB/MWNTs-GOx-CS-ICPTES-GCE showed a negligible change of cyclic voltammogram after the storage (B), demonstrating the effective immobilization of components in the PB/MWNTs-GOx-CS-ICPTES sol–gel composite film through strong urea bonds.

In addition, the biosensing stability of the biosensors during the storage in vigorously stirred PBS was investigated (see SFig. 5 in Supplemental materials). The response of the PB/MWNTs-GOx-CS-TEOS-GCE to 0.1 mM glucose increased obviously as the storage time increased within 24 h attributed to the resulting increase in porosity of the composite film during the storage. The response began to decrease after 36 h, and obvious fragmentations around the edge of the composite film were found. Nevertheless, the PB/MWNTs-GOx-CS-ICPTES-GCE showed much more stable response during the storage. This result further confirmed the effective immobilization of components in the PB/MWNTs-GOx-CS-ICPTES sol–gel composite film.

The reproducibility of the biosensor was studied by successively detecting 0.1 mM glucose for 10 times, and the RSD of responses was 3.83%. In addition, the RSD of responses to 0.1 mM glucose at five independently prepared biosensors was 5.08%, indicating good reproducibility of the biosensor.

The addition of 0.1 mM uric acid and 0.1 mM ascorbic acid had no apparent influence on the response to 0.1 mM glucose, indicating high anti-interference ability of the biosensor.

The glucose concentration in a fetal bovine serum sample was determined to be 4.80 ± 0.28 mM, in good agreement with the value of 4.92 ± 0.040 mM obtained using the traditional enzymatic method in a local hospital. The recovery test was carried out by adding 0.1 mM glucose to the serum samples, which produced a recovery of $102.2 \pm 2.72\%$.

4. Conclusions

A new strategy for fabricating glucose biosensors has been developed which features an effective combination of the synergistic merits of the CS-incorporated sol–gel process, in situ covalent cross-linking technique and Prussian blue deposited carbon nanotubes hybrids. The biosensor, based on in situ covalent immobilization of GOx by one-pot CS-incorporated sol–gel process in the presence of PB/MWNTs using ICPTES as both the sol–gel precursor and the covalent coupling agent for GOx and CS, exhibited a good sensitivity, a low detection limit, fast response, high anti-interference ability and excellent stability, attributed to both the efficient enzyme immobilization and good electrochemical properties of the PB/MWNTs-GOx-CS-ICPTES sol–gel composite film. The proposed strategy provided a promising platform for constructing a large number of other biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.03.007.

References

Ahmadalinezhad, A., Kafi, A.K.M., Chen, A.C., 2009. Electrochem. Commun. 11, 2048–2051.
Biazzotto, J.C., Sacco, H.C., Ciuffi, K.J., Ferreira, A.G., Serra, O.A., Iamamoto, Y., 2000. J. Non-Cryst. Solids 273, 186–192.
Chiu, J.Y., Yu, C.M., Yen, M.J., Chen, L.C., 2009. Biosens. Bioelectron. 24, 2015–2020.
Coradin, T., Allouche, J., Boissière, M., Livage, J., 2006. Curr. Nanosci. 2, 219–230.
Garcia-Sanchez, M.A., De la Luz, V., Estrada-Rico, M.L., Murillo-Martinez, M.M., Coahuila-Hernandez, M.I., Sosa-Fonseca, R., Tello-Solis, S.R., Rojas, F., Campero, A., 2009. J. Non-Cryst. Solids 355, 120–125.
Guan, W.J., Li, Y., Chen, Y.Q., Zhang, X.B., Hu, G.Q., 2005. Biosens. Bioelectron. 21, 508–512.
Huang, J.D., Yang, Y., Shi, H.B., Song, Z., Zhao, Z.X., Anzai, J., Osa, T., Chen, Q., 2006. Mater. Sci. Eng. C 26, 113–117.
Kang, X.H., Mai, Z.B., Zou, X.Y., Cai, P.X., Mo, J.Y., 2008. Talanta 74, 879–886.
Kang, X.H., Wang, J., Tang, Z.W., Wu, H., Lin, Y.H., 2009. Talanta 78, 120–125.
Kato, M., Saruwatari, H., Sakai-Kato, K., Toyo'oka, T., 2004. J. Chromatogr. A 1044, 267–270.
Kumbar, S.M., Selvam, T., Gellermann, C., Storch, W., Ballweg, T., Breu, J., Sextl, G., 2010. J. Membr. Sci. 347, 132–140.
Li, S.Z., Ma, Y., Yue, X.L., Cao, Z., Dai, Z.F., 2009. New J. Chem. 33, 2414–2418.
Li, T., Yao, Z.H., Ding, L., 2004. Sens. Actuators B 101, 155–160.
Li, W.J., Yuan, R., Chai, Y.Q., 2010. Talanta 82, 367–371.
Liang, R.P., Jiang, J.L., Qiu, J.D., 2008. Electroanalysis 20, 2642–2648.
Liu, Y., Chu, Z.Y., Zhang, Y.A., Jin, W., 2009. Electrochim. Acta 54, 7490–7494.
Molvinger, K., Quignard, F., Brunel, D., Boissiere, M., Devoisselle, J.M., 2004. Chem. Mater. 16, 3367–3372.
Ozmen, E.Y., Sezgin, M., Yilmaz, M., 2009a. J. Mol. Catal. B: Enzym. 57, 109–114.
Ozmen, E.Y., Yilmaz, M., 2009. Colloids Surf. B: Biointerfaces 69, 58–62.
Ozmen, M., Can, K., Ersoz, M., 2009b. J. Electroanal. Chem. 633, 228–234.
Romaskevic, T., Viskantiene, E., Budriene, S., Ramanaviciene, A., Dienys, G., 2010. J. Mol. Catal. B: Enzym. 64, 172–176.
Silva, S.S., Ferreira, R.A.S., Fu, L.S., Carlos, L.D., Mano, J.F., Reis, R.L., Rocha, J., 2005. J. Mater. Chem. 15, 3952–3961.
Tan, X.C., Tian, Y.X., Cai, P.X., Zou, X.Y., 2005. Anal. Bioanal. Chem. 381, 500–507.
Tang, H., Chen, J.H., Yao, S.Z., Nie, L.H., Deng, G.H., Kuang, Y.F., 2004. Anal. Biochem. 331, 89–97.
Tsai, M.C., Tsai, Y.C., 2009. Sens. Actuators B 141, 592–598.
Wang, B., Zhang, J., Dong, S., 2000. Biosens. Bioelectron. 15, 397–402.
Wang, G., Xu, J.J., Chen, H.Y., Lu, Z.H., 2003. Biosens. Bioelectron. 18, 335–343.
Wang, J., 2008. Chem. Rev. 108, 814–825.
Wang, X.Y., Gu, H.F., Yin, F., Tu, Y.F., 2009. Biosens. Bioelectron. 24, 1527–1530.
Wei, Z.L., Li, Z.J., Sun, X.L., Fang, Y.J., Liu, J.K., 2010. Biosens. Bioelectron. 25, 1434–1438.
Yu, J.J., Zhao, T., Zeng, B.Z., 2008. Electrochem. Commun. 10, 1318–1321.
Zhai, J.F., Zhai, Y.M., Wen, D., Dong, S.J., 2009. Electroanalysis 21, 2207–2212.
Zhang, W., Wang, L.L., Zhang, N., Wang, G.F., Fang, B., 2009. Electroanalysis 21, 2325–2330.
Zou, Y.J., Xiang, C.L., Sun, L.X., Xu, F., 2008. Biosens. Bioelectron. 23, 1010–1016.