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Short communication

A biosensor for cholesterol based on gold nanoparticles-catalyzed luminol electrogenerated chemiluminescence

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ABSTRACT

A novel cholesterol biosensor was prepared based on gold nanoparticles-catalyzed luminol electrogenerated chemiluminescence (ECL). Firstly, L-cysteine-reduced graphene oxide composites were modified on the surface of a glassy carbon electrode. Then, gold nanoparticles (AuNPs) were self-assembled on it. Subsequently, cholesterol oxidase (ChOx) was adsorbed on the surface of AuNPs to construct a cholesterol biosensor. The stepwise fabrication processes were characterized with cyclic voltammetry and atomic force microscopy. The ECL behaviors of the biosensor were also investigated. It was found that AuNPs not only provided larger surface area for higher ChOx loading but also formed the nano-structured interface on the electrode surface to improve the analytical performance of the ECL biosensor for cholesterol. Besides, based on the efficient catalytic ability of AuNPs to luminol ECL, the response of the biosensor to cholesterol was linear range from 3.3 μM to 1.0 mM with a detection limit of 1.1 μM (S/N = 3). In addition, the prepared ECL biosensor exhibited satisfying reproducibility, stability and selectivity. Taking into account the advantages of ECL, we confidently expect that ECL would have potential applications in biotechnology and clinical diagnosis.

1. Introduction

Among various analytical techniques, chemiluminescence and electrogenerated chemiluminescence (ECL) have wide applications in immunoassays, clinical analysis and food safety fields (Qi et al., 2011; Robin et al., 2011; Sun et al., 2010; Xu et al., 2011). Especially, ECL is a means of converting electrochemical energy into radiative energy at the surface of an electrode through an applied potential. Luminescence signals can be obtained from the excited states of an ECL luminophore generated at the electrode surfaces during the electrochemical reaction (Chen et al., 2011). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) produces strong ECL at a wavelength of 425 nm under appropriate experimental conditions. Luminol ECL system requires a lower oxidizing potential and less expensive reagent compared with $Ru(bpy)_3^{2+}$ ECL system (Ren et al., 2005). Therefore, luminol is considered as an efficient reagent in ECL measurement for the detection of hydrogen peroxide (H_2O_2) and one of the most widely used ECL luminophores or labels especially in chemical and biochemical analysis such as enzyme biosensors (Blum et al., 1989; Borisov and Wolfbeis, 2008; Qiu et al., 2009). Enzyme biosensors based on luminol ECL involve appropriate enzymatic reaction between substrate and enzyme and the subsequent ECL reaction between luminol and enzymatically produced H_2O_2 or related biological compounds (Knight, 1999; Zhu et al., 2002a; Zou and Ju, 2004). Moreover, many biological active substances, which can be metabolized or transformed into H_2O_2 by the catalytic action of enzymes, can be detected by luminol ECL system. Based on this principle, in combination with an appropriate enzymatic reaction, some efficient ECL systems were reported for glucose, lactate and choline measurements (Tsafack et al., 2000a,b; Zhu et al., 2002b). Therefore, luminol-based ECL biosensors greatly extend the applications of enzyme biosensors.

The ECL behaviors of luminol have been reported. Luminol generates strong chemiluminescence or ECL signals in alkaline media, but its signals in neutral media are very weak (Fähnrich et al., 2001). To overcome this problem, several efforts have been devoted to apply and sensitize luminol ECL reaction for the determination of biologically important compounds with high sensitivity in their physiological conditions (Chen et al., 2009; Cui et al., 2004; Dong et al., 2006; Lu et al., 2011). The catalytic effect of metal nanoparticles, such as gold nanoparticle (AuNPs) and silver nanoparticles on ECL reactions, has been demonstrated for luminol–H_2O_2 system (Cui et al., 2003; Wang et al., 2007). Among these nanoparticles, AuNPs have excellent catalytic performance to directly enhance the ECL intensity of luminol–H_2O_2 system. For example, Liu's group (Liu et al., 2008) reported a glucose biosensor based on AuNPs-catalyzed luminol ECL, and the proposed biosensor showed satisfying performance towards glucose detection. What is more, AuNPs, owing to

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high surface-to-volume ratio, good biocompatibility and facilitate electron transfer between biomolecules and electrode surface, have been used as the immobilization matrix for fabrication of biosensor with enhanced analytical performance (Gupta and Chaudhury, 2007). Besides the increasingly concern in ECL analysis, AuNPs are widely used in biosensors and bioelectronic applications.

Inspired by the useful applications of AuNPs in the fields of biosensor and luminol ECL, we reported a cholesterol biosensor based on AuNPs-catalyzed luminol ECL. In the present work, AuNPs were adsorbed on L-cysteine-reduced graphene oxide composites. Cholesterol oxidase (ChOx), chosen as the model enzyme, was adsorbed on the surface of AuNPs to construct a cholesterol biosensor. Such fabrication of biosensor exhibited good accuracy, high sensitivity and long-term stability. Details of the preparation, characterization, optimal conditions and possible application of biosensor electrodes were described as follows.

2. Experimental

2.1. Chemicals

Graphene oxide was obtained from Nanjing xianfeng nano Co. (Nanjing, China). Cholesterol oxidase (ChOx, EC 1.1.3.6, ≥50 units/mg, from Brevibacterium sp.), cholesterol ($C_{27}H_{46}O$, M_r: 386.67, ≥99% purity, from lanolin), Triton X-100 ($C_{34}H_{62}O_{11}$, MW: 646.85), gold chloride ($HAuCl_4$), sodium citrate, poly(ethylene imine) (PEI) and chitosan (CS, MW: 100,000–300,000, deacetylating grade: 70–85%, from crab shells) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The L-cysteine was purchased from Kangda Amino Acid Company (Shanghai, China). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC) and N-hydroxy succinimide (NHS) were purchased from Shanghai Medpep Co. Ltd (Shanghai, China). According to the literature (Enustun and Turkevich, 1963), AuNPs were produced by reducing $HAuCl_4$ with sodium citrate at 100 °C for 0.5 h. The diameter of AuNPs was about 16 nm (Fig. 1S). Phosphate buffer solutions (PBS, containing 0.9% NaCl) with pH 7.4 were prepared with 0.05 M KH_2PO_4 and 0.05 M Na_2HPO_4. The stock solution was prepared by dissolving cholesterol in the mixture of 2-propanol and Triton X-100, and then diluted it only with Triton X-100 solution for preparing standard solutions. Other chemicals used were of analytical grade and were used as received. Double distilled water was used throughout this study.

2.2. Apparatus

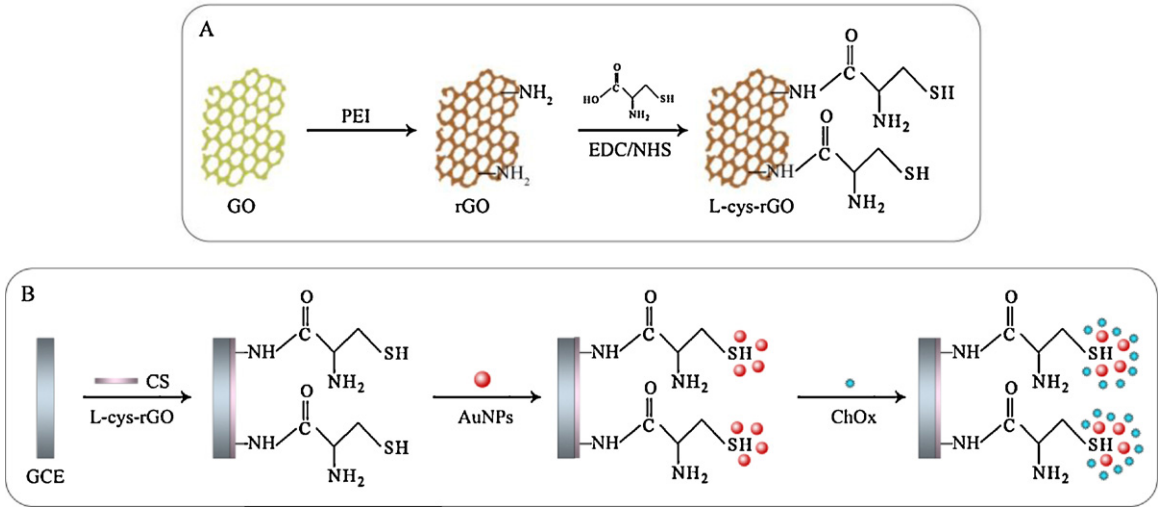
Cyclic voltammetry (CV) was performed with a CHI 600D electrochemical work station (Shanghai CH Instruments Co., China). The ECL emission was monitored with a model MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., China) with the voltage of the photomultiplier tube (PMT) set at 600 V in the process of detection. All experiments were performed with a conventional three-electrode system. The modified glassy carbon electrode (GCE) as working electrode, a platinum wire as counter electrode and a saturated calomel electrode (SCE) or Ag/AgCl (sat. KCl) as reference electrode. The size of the prepared AuNPs was estimated by transmission electron microscopy (TEM, H600, Hitachi Instrument, Japan). The topographs of the GCE modified with different materials were investigated with atomic force microscopy (AFM, Veeco, Woodbury, NY, USA).

2.3. Synthesis of L-cysteine-reduced graphene oxide composites

The synthesis of L-cysteine-reduced graphene oxide (L-cys-rGO) composites was performed according to the literature (Li et al., 2008): firstly, a stable dispersion of exfoliated graphene oxide (GO) sheets (0.5 mg/mL, 50 mL, pH 1.1) was mixed with PEI (3%, 1 mL) and heated under reflux at 135 °C for 3 h. The reduced graphene oxide (rGO) was obtained by centrifugating and washing several times by double distilled water. Secondly, EDC and NHS were used as coupling agents which catalyzed the formation of amide bond between the carboxyl of the L-cysteine and the amino of rGO. After the L-cysteine solution (0.02 M, 0.1 M NaOH) was activated by the aid of EDC and NHS, rGO was added, and the mixture was allowed to stay overnight at room temperature under continuous stirring to produce the L-cys-rGO composites. Centrifugation for purification was conducted by acetone for at least three times. Then the L-cys-rGO composites were dispersed in CS solution (1 mg/mL in 1% HAc) for future use. The schematic diagram of the stepwise procedure of the synthesis was shown in Scheme 1A.

2.4. Fabrication process and detection principle of the biosensor

Scheme 1B showed the schematic diagram of the fabrication of the cholesterol biosensor. The GCE (Φ = 4 mm) was polished with 0.3 and 0.05 μm alumina slurry, and then rinsed in ethanol and water by sonication. Then 10 μL L-cys-rGO composites were



Scheme 1. (A) Procedure for the fabrication of L-cys-rGO composites; (B) illustration of the preparation process of the modified electrode.

dropped on the surface of the bare GCE. After dried in air, it was immersed in AuNPs colloid solution for 10 h. Subsequently, 6 μL ChOx (1 mg/mL in 0.1 M PBS, pH 7.0) solutions were dropped on the surface of the electrode to construct a cholesterol biosensor (noted as ChOx/AuNPs/L-cys-rGO/GCE). For comparison, ChOx/L-cys-rGO/GCE was prepared similarly. The modified electrodes were stored at 4 °C for future use.

The principle of the biosensor involved in the detection of cholesterol occurs as the following:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholest-4-en-3-one} + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{luminol} \xrightarrow{\text{AuNPs}} \text{aminophthalate} + \text{hI} \quad (2)$$

The cholesterol is oxidized by ChOx with formation of cholest-4-en-3-one and H_2O_2 (Eq. (1)). Under positive potential and with the help of AuNPs, the luminol reacts with enzymatically produced H_2O_2 to produce the signal of ECL (Eq. (2)). The intensity of the ECL signal is directly proportional to the concentration of cholesterol. So cholesterol can be detected quantitatively on the modified electrode.

3. Results and discussion

3.1. Characterization of the biosensor fabrication

AFM technique is employed to confirm the fabrication process of the biosensor. Typical AFM images of Au substrate modified with different materials were shown in Fig. 1. As can be seen from Fig. 1A, the image of L-cys-rGO film presented a homogeneous surface and its root mean square (RMS) roughness was 43.703 nm. After AuNPs were assembled on the L-cys-rGO film (Fig. 1B), the surface became rough and numerous globular particles were observed in the AFM images with its RMS of 73.543 nm, which was ascribed to the assembly of AuNPs. The AFM image of ChOx/AuNPs/L-cys-rGO film (Fig. 1C) exhibited a smoothing effect as compared to that of AuNPs/L-cys-rGO film. The globular particles disappeared and the random cloud-like structure could be seen (RMS was 52.383 nm), which might be due to ChOx molecules filling the interstitial places between AuNPs, indicating that the ChOx was successfully absorbed on the surface of the AuNPs/L-cys-rGO.

In addition, CV is also a convenient and valuable technology to monitor the characteristics of the process of electrode modification. Fig. 1D showed the CVs of differently modified electrodes in 5 mM $[Fe(CN)_6]^{4-/3-}$ solution (0.1 M KCl). As can be seen, a well-defined oxidation and reduction peaks of $[Fe(CN)_6]^{4-/3-}$ (curve a) were observed at the bare GCE. After the L-cys-rGO composites were modified on the surface of GCE, the peak current of the modified electrode decreased (curve b), indicating that the L-cys-rGO composites had been immobilized successfully. However, when AuNPs were adsorbed on the electrode, the peak current increased markedly (curve c) for the reason that AuNPs could facilitate electron transfer between biomolecules and electrode surface. When the ChOx was adsorbed on the electrode (curve d), the peak current clearly decreased for the hindrance of non-conductive ChOx.

3.2. Optimization of analytical conditions

In order to optimize the performance of the prepared ECL biosensor towards cholesterol detection, the concentrations of luminol and AuNPs on the intensity of the ECL signal were investigated. Studies on the effect of the concentration of luminol on the intensity of the ECL signal showed that the ECL intensity increased with the increasing of luminol concentration. A further increase in

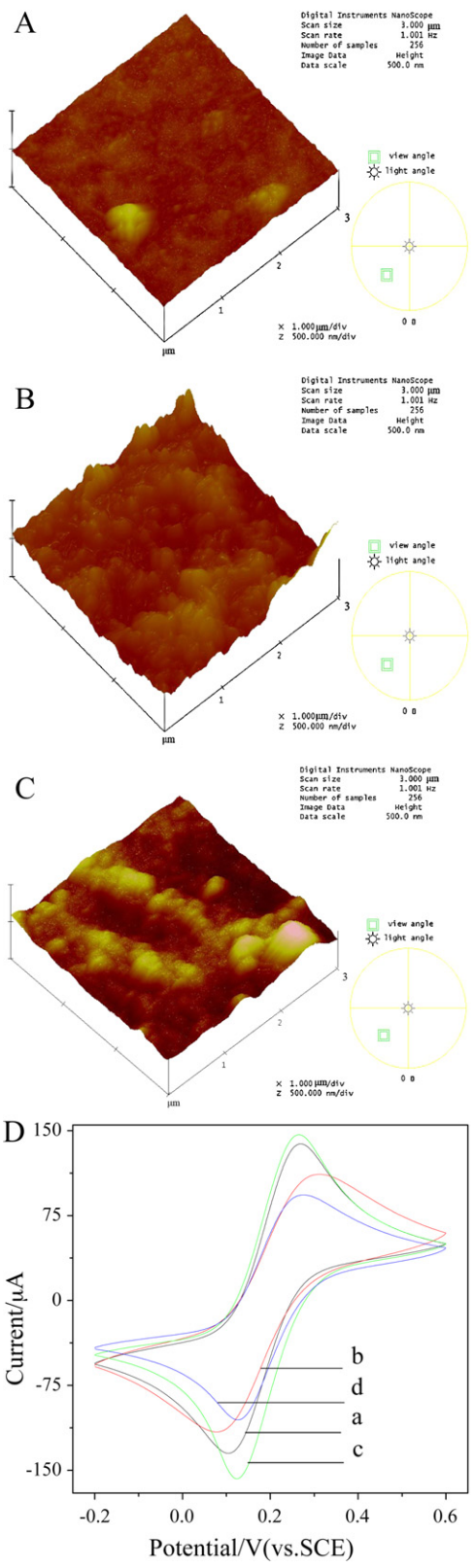


Fig. 1. AFM images of different modified Au substrates: (A) L-cys-rGO/Au, (B) AuNPs/L-cys-rGO/Au, (C) ChOx/AuNPs/L-cys-rGO/Au, and (D) CVs of different electrodes (a) GCE, (b) L-cys-rGO/GCE, (c) AuNPs/L-cys-rGO/GCE, and (d) ChOx/AuNPs/L-cys-rGO/GCE in 5.0 mM $[Fe(CN)_6]^{4-/3-}$ solution (0.1 M KCl). Scan rate: 100 mV/s.

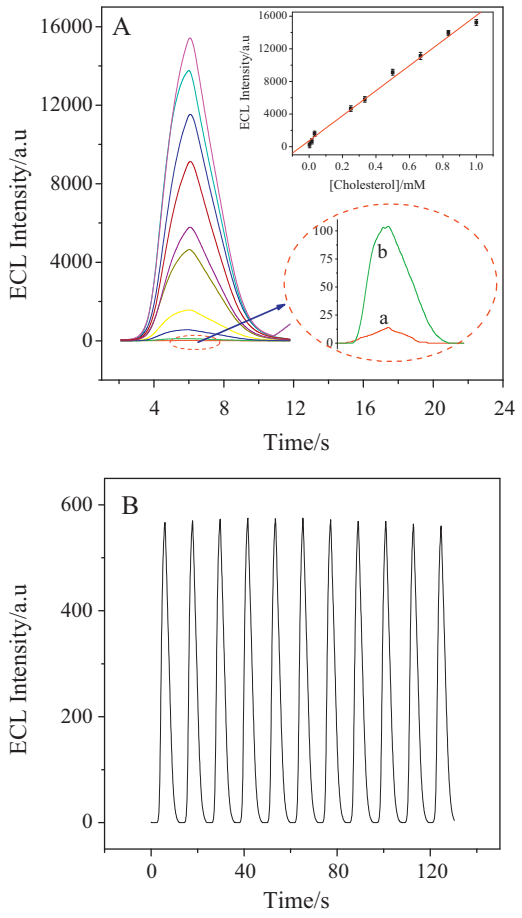


Fig. 2. (A) ECL responses of luminol (0.25 mM) at ChOx/AuNPs/L-cys-rGO/GCE in the presence of 0 mM (a), 0.0033 (b), 0.017 (c), 0.033 (d), 0.25 (e), 0.33 (f), 0.50 (g), 0.67 (h), 0.83 (i) and 1.00 (j) mM cholesterol. Inset showed linear relationship between the ECL signal intensity and the concentration of cholesterol; (B) reproducibility of ECL responses of luminol at ChOx/AuNPs/L-cys-rGO/GCE in 0.05 M PBS (pH 7.4). Scan rate: 100 mV/s.

the concentration of luminol (from 0.25 mM to 1.5 mM) did not cause obvious enhancement on the intensity of the ECL of the biosensor (Fig. 2S). Therefore, 0.25 mM was selected as the optimal concentration of luminol for further experiments.

The effect of the amount of AuNPs on the intensity of the ECL signal of the biosensor was also evaluated. The amount of AuNPs depended on the adsorbing time. So we studied the influence of different adsorbing time of AuNPs on the performance of the biosensor, and the adsorbing time of 10 h was selected for the biosensor fabrication.

3.3. Performance of the biosensor

In order to check up the effect of AuNPs on the intensity of the ECL signal of the biosensor, a comparative study was carried out by using two kinds of biosensor ChOx/L-cys-rGO/GCE (a) and ChOx/AuNPs/L-cys-rGO/GCE (b). As shown in Fig. 3S, the biosensor using AuNPs showed much greater response to cholesterol. The reasons were as follows: AuNPs had excellent catalytic performance to directly enhance the ECL intensity of luminol–H_2O_2 system; the favorable microenvironment provided with AuNPs kept the activity of ChOx; and AuNPs facilitated electrons transfer to electrode surface, thus the biosensor showed a much better response to cholesterol.

The main aim of the present investigation was to determine cholesterol, so the performance of the prepared biosensor was evaluated by detecting standard cholesterol solutions with ECL technique under optimal experimental conditions. Fig. 2A revealed the ECL behaviors of the biosensor at determining cholesterol, and the calibration graph of intensity of the ECL versus cholesterol concentration was linearly from 3.3 μM to 1.0 mM, and the limit of detection (S/N = 3) was 1.1 μM. The regression equation was I (a.u.) = 1.53×10^4 C (mM) + 731 with a correlation coefficient of 0.992. The relative standard deviation (RSD) for repetitive measurements (n = 11) of the biosensor response to 16.7 μM cholesterol (Fig. 2B) was 4.6%. Compared with other cholesterol sensors (Table 1S) with different determined methods, the ECL biosensor established in this work had low detection limit and high sensitivity. The reasons may be ascribed as follows: first, metal nanoparticles, especially AuNPs, usually cause high electron transfer rate and enhance their currents towards the compounds. Second, AuNPs perform a catalytic property to enhance the ECL intensity of luminol–H_2O_2 system. Third, it is due to the high sensitivity of ECL technique.

The stability of the biosensor was also tested by monitoring of its ECL response towards cholesterol. It was found that the response of the ECL biosensor decreased 19.8% after one week. Some compounds that commonly existed and possibly interfered with determination of cholesterol in biological samples were tested. After 50-fold concentration of dopamine and glycine were added into 0.05 M PBS (pH 7.4) containing 16.7 μM cholesterol, the intensity of the ECL signal generated from dopamine and glycine were negligible, which indicated the biosensor had a strong anti-interference ability.

3.4. Recovery experiment

The analytical applicability of the biosensor was evaluated by standard addition method. Table 1S listed the recovery of three samples of different cholesterol concentrations. The recovery rate was between 97.8% and 106.5%. The satisfying results demonstrated that the biosensor had a great potential for practical application.

4. Conclusions

In conclusion, a cholesterol biosensor was constructed based on AuNPs-catalyzed luminol ECL, which is a promising alternative approach to improving the sensitivity because of the catalytic activity of AuNPs towards luminol–H_2O_2 system. The ECL method based on AuNPs is simple, sensitive and uses relatively inexpensive reagents. Additionally, the proposed method integrated the advantages of ECL detection with specific biosensor, which could provide a promising approach to develop efficient biosensor to detect biologically important compounds in clinical application.

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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.2011.12.008.

References

Borisov, S.M., Wolfbeis, O.S., 2008. Chem. Rev. 108, 423–461.
Blum, L.J., Gautier, S.M., Coulet, P.R., 1989. J. Biolumin. Chemilumin. 4, 543–550.
Chen, X.M., Cai, Z.M., Lin, Z.J., Jia, T.T., Liu, H.Z., Jiang, Y.Q., Chen, X., 2009. Biosens. Bioelectron. 24, 3475–3480.
Chen, X.M., Su, B.Y., Song, X.H., Chen, Q.A., Chen, X., Wang, X.R., 2011. Trends Anal. Chem. 30, 665–676.
Cui, H., Xu, Y., Zhang, Z.F., 2004. Anal. Chem. 76, 4002–4010.
Cui, H., Zou, G.Z., Lin, X.Q., 2003. Anal. Chem. 75, 324–331.
Dong, Y.P., Cui, H., Wang, C.M., 2006. J. Phys. Chem. B 110, 18408–18414.
Enustun, B.V., Turkevich, J.J., 1963. J. Am. Chem. Soc. 85, 3317–3328.
Fähnrich, K.A., Pravda, M., Guilbault, G.G., 2001. Talanta 54, 531–559.
Gupta, R., Chaudhury, N.K., 2007. Biosens. Bioelectron. 22, 2387–2399.
Knight, A.W., 1999. Trends Anal. Chem. 18, 47–62.
Li, D., Muller, M.B., Gilje, S., Kaner, R.B., Wallace, G.G., 2008. Nat. Nanotechnol. 3, 101–105.
Liu, X.Q., Niu, W.X., Li, H.J., Han, S., Hu, L.Z., Xu, G.B., 2008. Electrochem. Commun. 10, 1250–1253.
Lu, C., Li, Q.Q., Chen, S., Zhao, L.X., Zheng, Z.X., 2011. Talanta 85, 476–481.
Qi, H.L., Li, S.G., Liang, L., Gao, Q., Zhang, C.X., 2011. Spectrochim. Acta - A 82, 498–503.
Qiu, B., Lin, Z., Wang, J., Chen, Z., Chen, J., Chen, G., 2009. Talanta 78, 76–80.
Ren, T., Xu, J.Z., Tu, Y.F., Xu, S., Zhu, J.J., 2005. Electrochem. Commun. 7, 5–9.
Robin, C.N., Jyotsna, K., Yogesh, N., Nishima, W., Aman, B., Suri, C.R., 2011. Analyst 136, 2125–2130.
Sun, B., Qi, H.L., Ma, F., Gao, Q., Zhang, C.X., Miao, W.J., 2010. Anal. Chem. 82, 5046–5052.
Tsafack, V.C., Marquette, C.A., Pizzolato, F., Blum, L.J., 2000a. Biosens. Bioelectron. 15, 125–133.
Tsafack, V.C., Marquette, C.A., Leca, B., Blum, L.J., 2000b. Analyst 125, 151–155.
Wang, W., Dong, Y.P., Cui, H., Xu, Y., 2007. Langmuir 23, 523–529.
Xu, S.J., Liu, Y., Wang, T.H., Li, J.H., 2011. Anal. Chem. 83, 3817–3823.
Zhu, L., Li, Y., Tian, F., Xu, B., Zhu, G., 2002a. Sens. Actuators B 84, 265–270.
Zhu, L., Li, Y., Zhu, G., 2002b. Sens. Actuators B: Chem. 86, 209–214.
Zou, G.Z., Ju, H.X., 2004. Anal. Chem. 76, 6871–6876.