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Lipase-nanoporous gold biocomposite modified electrode for reliable detection of triglycerides

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ABSTRACT

For triglycerides biosensor design, protein immobilization is necessary to create the interface between the enzyme and the electrode. In this study, a glassy carbon electrode (GCE) was modified with lipase-nanoporous gold (NPG) biocomposite (denoted as lipase/NPG/GCE). Due to highly conductive, porous, and biocompatible three-dimensional structure, NPG is suitable for enzyme immobilization. In cyclic voltammetry experiments, the lipase/NPG/GCE bioelectrode displayed surface-confined reaction in a phosphate buffer solution. Linear responses were obtained for tributyrin concentrations ranging from 50 to 250 mg dl^{-1} and olive oil concentrations ranging from 10 to 200 mg dl^{-1}. The value of apparent Michaelis–Menten constant for tributyrin was 10.67 mg dl^{-1} and the detection limit was 2.68 mg dl^{-1}. Further, the lipase/NPG/GCE bioelectrode had strong anti-interference ability against urea, glucose, cholesterol, and uric acid as well as a long shelf-life. For the detection of triglycerides in human serum, the values given by the lipase/NPG/GCE bioelectrode were in good agreement with those of an automatic biochemical analyzer. These properties along with a long self-life make the lipase/NPG/GCE bioelectrode an excellent choice for the construction of triglycerides biosensor.

1. Introduction

Total triglycerides (TGs) level in serum is a clinically important indicator of abnormalities in lipid metabolism, atherosclerosis, and hypertension (Pundir, 2008; Vijayalakshmi et al., 2008). Besides, the TGs level in food has become more significant due to increased health awareness and stringent regulatory laws (Reddy et al., 2001; Solanki et al., 2009). Therefore, a sensitive and selective determination method of TGs is needed for diagnosis and food supervision.

Among various methods available for TGs detection, biosensor has received increasing attention due to its intrinsic advantage in high sensitivity and selectivity, fast response, easy operation, and continuous on-line detection (Qiu et al., 2012). Reddy et al. fabricated porous silica based potentiometric pH dependant triglyceride biosensor (Reddy et al., 2001). Fernandez et al. compared potentiometric triglyceride biosensor with micromechanical triglyceride biosensor (Fernandez et al., 2009). TGs biosensors based on muti-enzymes were also reported (Basu et al., 2005; Pundir, 2008). For TGs biosensor, the denaturation and leaching of enzyme as well as the facilitation of electron transfer between enzyme and electrode are still of great challenges.

With the development of nanomaterials, higher sensitivity and the intimate attachment of enzymes are achievable due to their high surface as well as their unique physical, electronic, and chemical properties (Vijayalakshmi et al., 2008; Solanki et al., 2009). Applications of nanoporus materials such as activated carbon (Sotiropoulou et al., 2003), fullerenes (Gavalas and Chaniotakis, 2000), and carbon nanotubes (Sotiropoulou et al., 2005) have been attracting great attention in the design of high performance biosensors. Recently, nanoporous gold (NPG) has attracted particular interest in the enzyme immobilization and enzyme-based biosensor construction due to its three-dimensional spongy morphology (Qiu et al., 2008 and 2009; Ahmadalinezhad and Chen, 2011; Wang et al., 2011). Especially, NPG has unique physicochemical properties such as biocompatibility, active surface, and excellent conductivity, which are critical for electrochemical biosensors (Ding and Chen, 2009).

In our previous work, lipase/NPG biocomposite was successfully constructed by assembling lipase onto the surface of NPG (Wang et al., 2011). In this work, further studies were performed on the fabrication of high performance lipase-based biosensor for TGs detection. The resulting lipase/NPG/GCE bioelectrode had excellent stability, sensitivity, selectivity, and anti-interference ability.

2. Experimental

2.1. Chemicals

Lipase (Solarbio L8070 from *porcine pancreas*) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tributyrin was purchased from Tokyo Kasei Kogyo Co. Ltd. (Japan). Olive oil (density is 0.915 g l^{-1})

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was purchased from Guoyao Chemical Reagent Co., Ltd. (China). All other chemicals used are of analytical grade.

Tributyrin was dissolved in absolute ethanol to form 100 g dl^{-1} tributyrin stock solution. The tributyrin stock solution was stored at 4 °C refrigerator. One volume of the 100 g dl^{-1} tributyrin stock solution in absolute ethanol was mixed just before use with nine volumes of a 50 mM phosphate buffer solution (PBS, pH 8.0) containing Triton X-100 with the final concentration of 0.2% (w/v).

Olive oil (2 ml) was mixed with 33 ml of 100 g l^{-1} arabic gum and 5 ml ultrapure water, then followed by homogenization at 10,000 rpm for 30 min using a homogenizer (HJ-1, Jintan Ronghua Instrument Manufacturing Co., Ltd., China) at 4 °C.

2.2. Preparation of NPG/GCE electrode

NPG was made by dealloying 12-carat white gold leaves (Au50Ag50 wt%, Sepp Leaf Products, USA) in concentrated HNO_3 at 30 °C for 30 min. After that, NPG was washed by ultrapure water until pH to neutral, and then kept in ultrapure water.

Glassy carbon electrodes (GCEs) were polished with 0.05 μm alumina slurry on a piece of chamois leather. Before use, the GCEs were cleaned ultrasonically in HNO_3 (1:1, v/v), and washed with ultrapure water and absolute ethanol, respectively. Then the GCE was coated by a piece of NPG in ultrapure water to form NPG/GCE. The NPG/GCE was kept in a vacuum drier for an hour to dry.

2.3. Lipase immobilization

Lipase solution (1 mg ml^{-1}) is freshly prepared in PBS (50 mM, pH 7.0) prior to being used. The freshly prepared NPG/GCE was immersed immediately in lipase solution. After 72 h immobilization, the lipase/NPG/GCE bioelectrode was immersed in PBS (50 mM, pH 7.0) and stored in a refrigerator at 4 °C until use.

2.4. Apparatus and measurement

The morphology of NPG was characterized using a JEOL JSM-6700F field emission scanning electron microscope (SEM).

All electrochemical measurements were performed in a conventional three-electrode cell at room temperature. Cyclic voltammograms (CVs) were measured using a three-electrode conventional cell with a CHI 760D electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China). The modified GCE was used as a working electrode, and a Pt sheet (1 cm × 1 cm) and a saturated calomel electrode (SCE) were used as counter and reference electrodes, respectively. All potentials were referred to the SCE. The electrolyte solutions were deaerated by N_2 bubbling for 10 min prior to electrochemical measurements, and a blanket of N_2 was maintained throughout each experiment.

2.5. Detection of TG concentration in serum

Serum samples were offered by school hospital of Shandong University (Jinan, China). The sample was mixed with equal volume absolute ethanol, and then centrifuged at 12,000 rpm for 2 min to remove protein. The treated sample (700 μl) was added to 15 ml deaerated PBS (50 mM, pH 8.0) in a three-electrode system with the lipase/NPG/GCE as working electrode. Simultaneously, the serum samples were detested by an automatic biochemical analyzer (HITACHI 7080, Japan).

3. Results and discussion

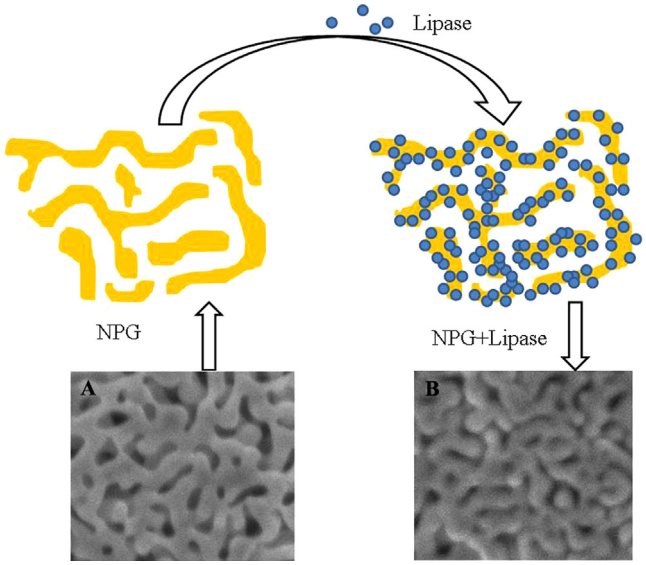
3.1. Construction and characterization of lipase/NPG/GCE bioelectrode

In our previous work, lipase/NPG biocomposite was successfully constructed by assembling lipase onto the surface of NPG (Wang et al., 2011). To investigate the potential of the lipase/NPG biocomposite for biosensor, further studies were performed on the fabrication of lipase-based bioelectrode for TGs detection. NPG with a pore size of ca. 35 nm was used for bioelectrode construction. The highly conductive, porous, and biocompatible three-dimensional structure of NPG is suitable for enzyme immobilization, which acts as a bridge to link the active sites of enzyme and the underlying electrode (Wang et al., 2011; Qiu et al., 2012). A procedure was developed by encapsulating lipase into NPG to construct lipase/NPG/GCE bioelectrode as shown in Scheme 1 and Scheme S1 (Supporting Information). The samples of NPG before (Scheme 1A) and after (Scheme 1B) lipase loading were characterized using SEM. Compared with the bare NPG, smaller pore size and relatively smoother surface morphology were observed for the lipase/NPG biocomposite, which suggested a preferential immobilization of lipase over the ligament site with high radial curvatures (Wang et al., 2011).

Triglycerides can be hydrolyzed into glycerol, fatty acids, and protons by lipase. Scheme S1 demonstrated the electrochemical reaction (Supporting Information). As the fatty acid produces, the pH of solution in three-electrode cell changes. The redox peak currents of NPG can be sensitively changed with the changing of solution pH (Qiu et al., 2008 and 2009). A linear relationship is shown between the redox currents of NPG and the concentrations of triglycerides. Therefore, TGs detection is achieved by the detection of the changes of NPG redox currents using the lipase/NPG/GCE bioelectrode.

3.2. Electrochemical behavior of lipase/NPG/GCE bioelectrode

Electrochemical behaviors of NPG/GCE electrode and lipase/NPG/GCE bioelectrode were compared in deaerated PBS (50 mM, pH 7.0) at the scan rate of 50 mV s^{-1}. It was obvious that the current response of the NPG/GCE electrode was higher than that of the lipase/NPG/GCE bioelectrode as shown in Fig. 1A. The lower



Scheme 1. Schematic illustration of lipase immobilized onto NPG and SEM images of NPG before (A) and after (B) lipase loading .

current response after lipase loading may be due to the insulating characters of the coating lipase (Solanki et al., 2009). These results were in accordance with the previous report that the immobilized glucose oxidase behaved as a barrier layer to block the electron transfer involved in the redox process of the benzoquinone/hydroquinone in a row couple (Qiu et al., 2012). Furthermore, the NPG/GCE electrode and lipase/NPG/GCE bioelectrode were compared in deaerated PBS (50 mM, pH 8.0) with 50 mg dl^{-1} TB at the scan rate of 50 mV s^{-1}. The obvious negative shift of the onset potential from +0.44 V to +0.37 V and enhancement of peak current density of the lipase/NPG/GCE bioelectrode indicated a superior catalytic activity toward TB as shown in Fig.1B.

Fig. 2A showed a group of CVs for the lipase/NPG/GCE bioelectrode at different scan rates in deaerated PBS (50 mM, pH 7.0). It was clear that the redox peak current density was dependent on the scan rate. This was further proven by plotting the peak current density against scan rate as shown in the inset of Figs. 2A. A perfect linear relationship between the peak current density and the scan rate from 20 mV s^{-1} to 300 mV s^{-1} were obtained. The regression coefficients for cathodic and anodic peak were 0.970 and 0.988, respectively. Besides, the ratio of the cathodic peak current density and the anodic one (*I*pc/*I*pa) approximates to 1 and the formal potential keeps almost unchanged. These typical characteristics confirmed that the electron transfer process was a surface-controlled process (Liu et al., 2007; Li et al., 2011).

The surface concentration of lipase on the NPG/GCE is an important parameter to characterize the quality of the bioelectrode. According to Laviron (1979) study, the loading of lipase on NPG can also be investigated by the voltammetric method. The peak current density can be expressed as:

$$I_p = \frac{nFQv}{4RT} = \frac{n^2F^2vA\Gamma^*}{4RT} \quad (1)$$

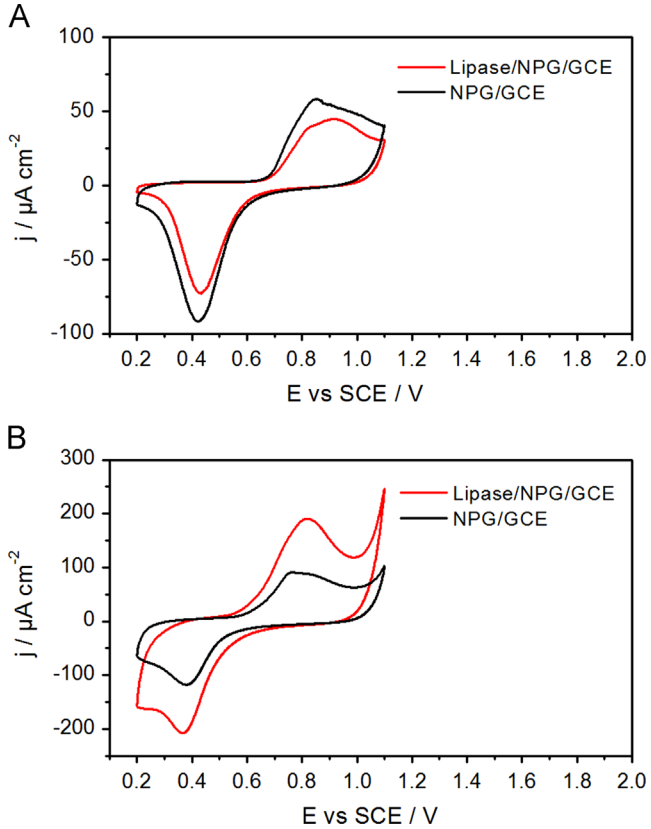


Fig. 1. CVs of NPG/GCE electrode and lipase/NPG/GCE bioelectrode in PBS (50 mM, pH 7.0) at the scan rate of 50 mV s^{-1} (A) and in PBS (50 mM, pH 8.0) with 50 mg dl^{-1} TB at the scan rate of 50 mV s^{-1} (B).

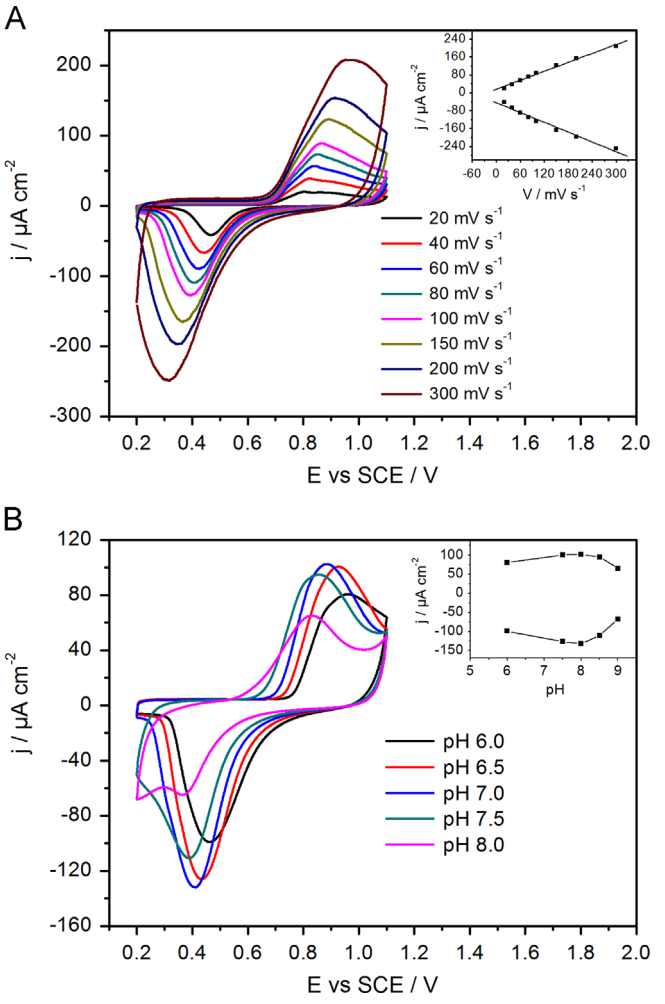


Fig. 2. CVs of lipase/NPG/GCE bioelectrode at different conditions. A is the CVs of lipase/NPG/GCE bioelectrode at different scan rates (20–300 mV s^{-1}); Inset: peak current density as function of scan rate (20–300 mV s^{-1}). B is the CVs of lipase/NPG/GCE bioelectrode as a function of pH in PBS (50 mM) at the scan rate of 50 mV s^{-1}; Inset: the effects of pH on the peak current density of lipase/NPG/GCE bioelectrode.

Where I_p the anodic peak current, n is the number of electrons transferred (1), A is the surface area (0.071 cm^2), Γ^* is the average surface concentration of lipase, T, R, and F are absolute temperature, gas constant, and Faraday constant, respectively. On the basis of Eq. (1), the average surface concentration of lipase adsorbed onto the NPG/GCE is calculated to be 1.41×10^{-8} mol cm^{-2} from the slope of the $I_p - v$ curve. This value is higher than the value previously reported (1.79×10^{-9} g cm^{-2}) for lipase immobilized on silicon nitride (Fernandez et al., 2009). This result can be ascribed to the three-dimensional nanoporous structure of NPG, which facilitated the efficient immobilization of lipase on NPG.

3.3. Effect of pH on lipase/NPG/GCE bioelectrode

The effect of pH (ranging from 6.0 to 8.0) on the lipase/NPG/GCE bioelectrode was investigated using CV technique in deaerated PBS (50 mM) at scan rate of 50 mV s^{-1}. It was clear that the current response of the lipase/NPG/GCE bioelectrode increased with increasing pH, and the maximum current response was found at pH 7.0 as shown in Fig. 2B. After Then, the current response of the lipase/NPG/GCE bioelectrode decreased with increasing pH. These results confirmed that the lipase/NPG/GCE bioelectrode was sensitive to the change in pH, which were consistent with the report that the lipase/porous silicon biosensor was sensitive to

small changes in pH (Reddy et al., 2001). During TGs detection, lipase hydrolyzes TGs to produce glycerol, fatty acid, and protons (Solanki et al., 2009; Fernandez et al., 2009). The generated protons decrease pH of buffer medium, resulting in the electrochemical signal change of the lipase/NPG/GCE bioelectrode. These results were in accordance with the principle of the lipase/NPG/GCE bioelectrode as shown in Scheme S1 (Supporting Information). To get a positive correlation between TGs concentration and the current response of the lipase/NPG/GCE bioelectrode, all subsequent performance detections of the lipase/NPG/GCE bioelectrode were conducted at pH 8.0.

3.4. Amperometric detection of tributyrin and olive oil

The electrochemical response of the lipase/NPG/GCE was investigated as a function of tributyrin concentrations (50–250 mg dl^{-1}) using CV technique at 50 mV s^{-1} scan rate in deaerated PBS (50 mM, pH 8.0). According to previous results, the generated protons decreased the pH of PBS (50 mM, pH 8.0), which in turn resulted in a positive current response of the lipase/NPG/GCE bioelectrode.

As shown in Fig. 3, the anodic and cathodic peak current density of the lipase/NPG/GCE bioelectrode increased with increase in tributyrin concentration, which can be attributed to the pH-sensitive behavior of the lipase/NPG/GCE bioelectrode (Solanki et al., 2009). A well-defined and fast reduction peak response was observed at the potential of +0.37 V, which is more sensitive than the oxidation one. Moreover, the cathodic peak current densities were all proportional to the concentration of tributyrin in the range of 50–250 mg dl^{-1}, and the correlation coefficient was 0.993. The lipase/NPG/GCE bioelectrode showed high sensitivity of 0.30 μA mg^{-1} dl^{-1} cm^{-2} and low detection limit of 2.68 mg dl^{-1}. These results indicated that the lipase/NPG/GCE bioelectrode had much higher sensitivity and lower detection limit than most currently available lipase-based biosensors as shown in Table S1 (Supporting Information).

The apparent Michaelis–Menten constant (K_m), which gives an indication of the enzyme-substrate kinetics, can be estimated from the Lineweaver–Burk equation as followings (Kamin and Wilson, 1980):

$$\frac{1}{i_s} = \frac{K_m}{i_{max}} \frac{1}{C} + \frac{1}{i_{max}} \quad (2)$$

where i_s is the steady state current, i_{max} is the current measured for the enzymatic product when electrode surface is saturated and C is the concentration of tributyrin. From the Eq. (2), the apparent Michaelis–Menten constant (K_m) of the lipase/NPG/GCE bioelectrode was calculated to be 10.67 mg dl^{-1}, which was also lower than that of the triglyceride biosensors reported as shown in Table S1, (Supporting Information). The lower K_m value indicated that the immobilized lipase possessed high catalytic activity and that the fabricated biosensor exhibited a high affinity with tributyrin. Additionally, olive oil as a long chain triglyceride was also investigated using the lipase/NPG/GCE bioelectrode at 50 mV s^{-1} scan rate in deaerated PBS (50 mM, pH 8.0) as shown in Fig. S1 (Supporting Information). The cathodic peak current densities were all inversely proportional to the concentration of olive oil in the range of 10–200 mg dl^{-1} as shown in the inset of Fig. S1, and the correlation coefficient was 0.996. These results were in accordance with the previous report that a negative linear relationship was observed between current density and olive oil concentration due to the low diffusion efficiency of long chain products (Liu et al., 2012). These reliable detections indicated that the lipase-NPG/GCE bioelectrode was an excellent choice for the construction of triglycerides biosensor.

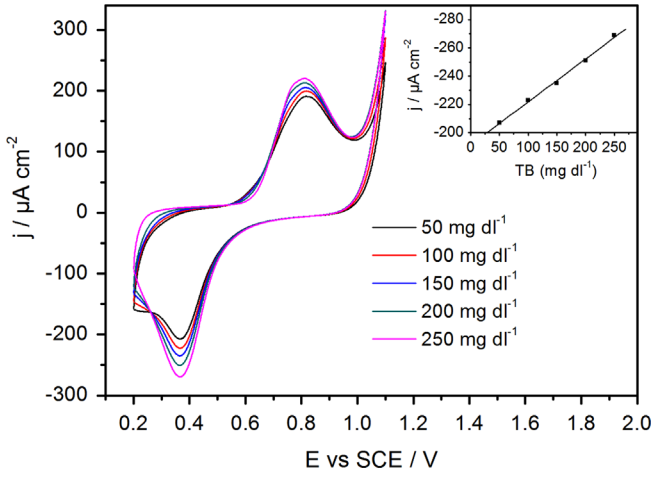


Fig. 3. Electrochemical response of lipase/NPG/GCE bioelectrode as function of tributyrin concentration. Inset: calibration curve between peak current density and tributyrin concentration (50–250 mg dl^{-1}).

3.5. Stability and repeatability of lipase/NPG/GCE bioelectrode

Stability is a basic requirement for the fabrication of a biosensor. The stability of the lipase/NPG/GCE bioelectrode was investigated. The lipase/NPG/GCE bioelectrode was preserved in ultrapure water at 4 °C. After 28 day storage, it remained 91% of its initial current response. Even after 55 days, there was still 95% of its initial current response remained for the lipase/NPG/GCE bioelectrode. These results suggested that the lipase/NPG/GCE bioelectrode was quite efficient for retaining the catalytic activity of lipase. The good stability may be attributed to the biocompatibility of NPG networks, which involved the adsorption of the enzyme with its active site oriented away from the porous surface with little leaching yet sufficient mobility to retain catalytic activity (Ravindra et al., 2004; Sotiropoulou et al., 2005; Hudson et al., 2008; Wang et al., 2011).

The repeatability of the lipase/NPG/GCE bioelectrode was also investigated by detecting the current responses to 50 mg dl^{-1} tributyrin and 10 mg dl^{-1} olive oil for five consecutive measurements. The relative standard deviations (RSD) were 0.43% and 2.41% for tributyrin and olive oil, respectively. These results indicated that the biosensor had good repeatability.

3.6. Specificity and interference

It is known that enzyme is highly specific for substrate. The selectivity of enzyme electrode is closely related to the property of the enzyme. In this work, 1 mM urea, 5 mM glucose, 0.03 mM cholesterol, and 0.2 mM uric acid were added into the deaerated PBS (50 mM, pH 8.0) containing 10 mg dl^{-1} tributyrin to evaluate the specificity of the lipase/NPG/GCE bioelectrode. Fig. 4 showed that the addition of urea, glucose, and cholesterol caused negligible changes of current signal being of 2.5%, 0.8%, and 2.1%, respectively. However, 6.9% current density decrease was observed when uric acid was added. Considering that the concentrations of uric acid in human serum is much smaller than that of triglyceride (normal range: 35–160 mg dl^{-1}), the presence of uric acid hardly affected the detection of triglyceride in serum samples. These results confirmed that the lipase/NPG/GCE bioelectrode had strong anti-interference capability.

3.7. Determination of TGs in Human serum sample

To detect the practical performance of the lipase/NPG/GCE bioelectrode, attempts have been made to detect TGs concentration in human serum. A rapid and stable current response was acquired at +0.37 V. The concentration of TGs in the human serum sample was calculated from the calibration curve and the obtained results were shown in Table 1. Herein the concentration

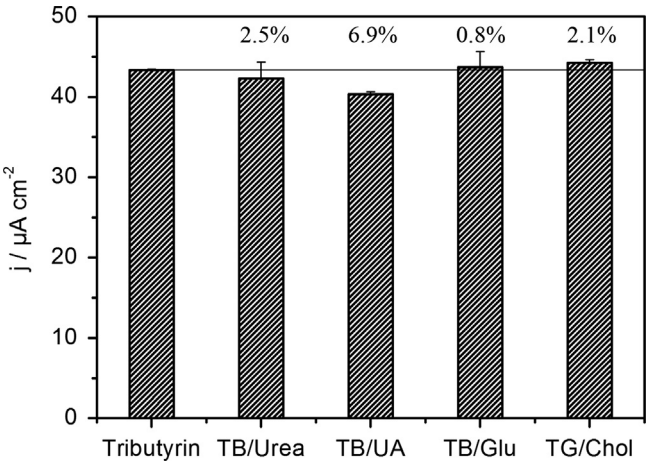


Fig. 4. Effects of interferents on lipase/NPG/GCE bioelectrode.

Table 1
Comparison between the concentration values of TGs measured with lipase/NPG/GCE bioelectrode and automatic biochemical analyzer.

Sample no.	Concentrations of TGs (mg dl^{-1})		Deviation rate (%)
	Determined by lipase/NPG/GCE bioelectrode (mg dl^{-1})	Determined by automatic biochemical analyzer (mg dl^{-1})	
1	283.9	270.8	4.8
2	176.9	169.0	4.7
3	146.0	149.6	2.4

of TGs in human serum sample was also measured using an automatic biochemical analyzer. The values given by the analyzer were in good agreement with our results, thereby demonstrating that the as-prepared biosensor was effective and sensitive for the determination of TGs in actual human serum.

4. Conclusion

In summary, the lipase/NPG/GCE bioelectrode was successfully fabricated by encapsulating lipase into NPG with three-dimensional nanoporous structure, which provided a good interface between the active sites of enzyme and the underlying electrode. The resulting lipase/NPG/GCE bioelectrode presented good catalytic activity and selectivity for TGs in human serum. Due to the excellent sensing performance with high sensitivity, anti-interference ability, and good stability, the lipase/NPG/GCE bioelectrode is expected to act as premising biosensors for the selective detection of TGs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.09.040.

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