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Development of disposable lipid biosensor for the determination of total cholesterol

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ABSTRACT

A prototype chronoamperometric biosensor for the determination of total cholesterol was developed that consists of a homemade potentiostat and disposable strips immobilized with Fe_3O_4, cholesterol oxidase (ChOx), and cholesterol esterase (ChE). The principle of sensing cholesterol is based on the detection of reduction signal of hydrogen peroxide generated in two enzymatic reactions. The co-immobilization of ChE and ChOx allows the sensor to detect both concentrations of esterified and free cholesterol. The effects of biosensor on catalyst, enzymes, applied potential, and buffer pH was investigated, and the operation conditions were optimized. The detection of cholesterol can be accomplished in one step, a 10 μL of sample was dropped onto the area of sensing strip and the reduction signal was obtained at an applied potential of −200 mV (vs. Ag/Ag^+). The pre-reaction time was set at 15 s before applying potential on the strip and the sampling time was 5 s. The sensing device displays a linear response over the range of 100–400 mg/dL (R^2 = 0.999) for cholesteryl oleate. The coefficient variation was determined as 5.06% (N = 20) for 100 mg/dL cholesteryl oleate and the detection limit is 19.4 mg/dL (S/N = 3). The probable interferences in bio-matrix were selected to test the selectivity and no significant response was observed in the biosensor.

1. Introduction

Cholesterol is an important lipid, a steroid alcohol, and often found to be esterified with a fatty acid in the body. The accumulation of cholesterol in blood may lead to lipid metabolism disorder. Therefore, the determination of total cholesterol is critically important in clinical diagnosis and cardiac diseases prevention. Many medical evidences show that a high serum cholesterol level is related to arteriosclerosis, cerebral thrombosis, and coronary heart diseases (Libby, 2002; Libby et al., 2002), whereas the patients with deficient cholesterol levels are found to suffer from hyperthyroidism, anaemia, malabsorption and wasting syndromes. The desired total cholesterol plasma for an individual is less than 200 mg/dL, and a high level is considered as greater than 240 mg/dL (National Heart Lung and Blood Institute, 1994). Beside, the quality control and nutritional labeling of foods in the foodstuff industry is another application for the measurement of cholesterol (Bragagnolo and Rodriguez-Amaya, 2002; Hwang et al., 2003).

In this paper, a simple process for fabricating a disposable cholesterol biosensor based on the cathodic measurement principle is illustrated. The recognition components, ChOx and ChE together with Fe_3O_4 compound were integrated in the biosensor to improve its specificity. The novel catalyst Fe_3O_4 is a mixed-valence compound with the conductivity and ferrimagnet properties. In our previous studies, Fe_3O_4 was shown to provide a suitable energy surface for the electrocatalytic reduction of hydrogen peroxide at low potential (Lin and Leu, 2005).

Various analytical strategies for cholesterol assay have been proposed that can be categorized into chemical and enzymatic methods. In general, the enzymatic method provides more selective than chemical method. In clinical diagnosis, the total cholesterol contains a mixture of free and esterified cholesterol in serum that must be assayed simultaneously. The enzymatic reactions of cholesterol esterase and cholesterol oxidase have been employed in routine clinical test. In these reactions, the cholesteryl esters are hydrolyzed by cholesterol esterase to produce free cholesterol and fatty acids. The free cholesterol is then oxidized by the presence of cholesterol oxidase to produce 4-cholestene-3-one and hydrogen peroxide. Finally, the hydrogen peroxide is measured to quantify the total cholesterol.

In the past decade, a number of enzyme-based cholesterol biosensors have been developed, for example, spectrophotometry, fluorometry (Amundson and Zhou, 1999), chemiluminescence (Hung et al., 1999), thermometry (Raghavan et al., 1999), electrochemical method (Bongiovanni et al., 2001), electrochemiluminescence (Marquette et al., 2000), and quartz crystal acoustic

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wave (Martin et al., 2003). Several matrixes were applied to immobilize the enzymes of ChOx and ChE for cholesterol biosensor, such as carbon paste (Gilmartin and Hart, 1994), nylon mesh (Mascini et al., 1983; Moody et al., 1988), conducting polymers (Matharu et al., 2007; Singh et al., 2006; Wang and Mu, 1999; Vidal et al., 1999), silicate sol–gel (Tan et al., 2005; Li et al., 2003; Kumar et al., 2000; Yao and Takashima, 1998), graphite–Teflon (Pena et al., 2001), and Formvar (Kumar et al., 1999). Besides, polyanions such as poly(styrene sulfonate) and polycations such as poly(ethylene imine) can also be applied to immobilize ChOx by layer-by-layer technique (Ram et al., 2001).

Electrochemical method is the most frequently applied in the fabrication of enzyme-based cholesterol biosensors. These detection schemes are usually based on the monitoring of the consumption of oxygen or the production of hydrogen peroxide during the enzymatic reactions. Amperometric measurement of hydrogen peroxide is frequently monitored, but the interference is usually a problem due to the anodic measurement of hydrogen peroxide at high overvoltage. For example, some easily oxidizable species, such as ascorbic acid and uric acid in blood sample may be oxidized at the same time. In order to minimize these interferences, some possible schemes have been used to modify a mediator on the electrode surface for changing the electron transfer pathway, such as ferrocene derivatives (Charpentier and El Murr, 1995) and phenothiazine derivatives (Nakaminami et al., 1997), flavin nucleotides (Vidal et al., 2000); or to immobilize a catalyst for cathodic determination of hydrogen peroxide, such as rhodium (Wang et al., 1994), iridium (Tian and Zhu, 2002), prussian blue (Ricci and Palleschi, 2005; Itaya et al., 1984), metal hexacyanoferrate (Wang et al., 1999; Lin et al., 1998, 1999; Milardovic et al., 1997), titanium dioxide (Cosnier et al., 1999), and copper nitrate (Ravi Shankaran et al., 2003).

Recently, cytochrome P450scc was investigated and applied to develop cholesterol biosensor (Shumyantseva et al., 2004). Some nanomaterials, such as carbon nanotubes (Li et al., 2005), gold nanoparticles (Shumyantseva et al., 2005), platinum nanoparticles (Yang et al., 2006a,b), magnetic nanoparticles (Kouassi et al., 2005), and cobalt hexacyanoferrate nanoparticles (Yang et al., 2006a,b), have been applied in cholesterol biosensor to improve the sensitivity, selectivity, and stability.

There are only few reports that focused on the development of disposable cholesterol biosensor. In this study, we demonstrated the feasibility of utilizing Fe_3O_4 as a catalyst to develop an interference-free electrochemical cholesterol biosensor. The disposable strips were constructed on polypropylene (PP) sheet by screen-printing technique and the electrochemical measurements were done by a homemade portable potentiostat connecting to a personal computer.

2. Materials and methods

2.1. Materials

All reagents were prepared in deionized water, which was purified by a deionization system (EASYpure, Barnstesd, USA) before use. Cholesterol oxidase (EC 1.1.3.6, from *Streptomyces* sp., 22.4 units (U)/mg) and cholesterol esterase (EC 3.1.1.13, from *Pseudomonas* sp., 118 U/mg) were obtained from Toyobo Co. (Osaka, Japan). The activities of ChOx and ChE were measured by a UV–vis spectrometer using procedures modified from those provided by Toyobo Co. Cholesterol, cholesteryl oleate, ascorbic acid, uric acid, dopamine, aspirin, creatine, creatinine, lactic acid and acetaminophen were purchased from Sigma (St. Louis, MO, USA). Triton X-100, Span 20, Tween 85, potassium chloride, hydrogen peroxide, disodium hydrogenphosphate-2-hydrate, sodium dihydrogenphosphate-2-hydrate, urea, and potassium ferricyanide were obtained from RiedeldeHaen (Seelze, Germany). Glucose was purchased from Merck (Darmstadt, Germany). Carbon ink (C10903D14) and silver/silver chloride paste (C71105R5) were obtained from Gwent Electronic Materials Ltd. (Pontypool, UK).

2.2. Fabrication of the screen-printed strips

Three-electrode strips were printed by using a semi-automatic screen printer (SMTech 100MV, MPM, UK) onto a 0.4 mm thickness PP sheet (36 cm × 36 cm). The counter electrode and a conductive wire of working electrode were printed by carbon ink, and reference electrode was also screen-printed by silver/silver chloride paste. The printing paste for the working electrode was prepared by mixing Fe_3O_4, carbon ink, and cyclohexanone, which is required to obtain the optimum viscosity for printing purpose. The paste, including Fe_3O_4, was printed on working electrode with a surface area of 1.77 mm^2. The printed strip was dried at 40 °C for 30 min after each printing process described above and then the area of working electrode was controlled by insulating tape. Both enzymes of ChOx and ChE were prepared in distiller water and then 10 μL of the enzyme solution was applied to a small piece of filter paper whose diameter was about 7 mm cut from a 90-mm filter paper (ADVANTEC, Japan) using a puncher. The small filter paper with enzyme solution was dried for 2 h at 4 °C. Finally, the working electrode was covered with the filter paper coated with enzymes. The design of sensing strip is illustrated in Fig. 1. All strips were cut from the PP sheet at the size of 1 cm × 4 cm and stored at 4 °C until use.

2.3. Electrochemical measurements

All chronoamperometry experiments were carried out by using a homemade potentiostat and the screen-printed cholesterol biosensor strips. The potentiostat can give an output voltage of 1.0 V when the electrochemical reaction generates current of 2.5 μA, where the measuring system was built in an aluminum box (9 cm × 6 cm × 5 cm) with a connection port for inserting the three-electrode strip. At the beginning of the measurement, the potentiostat was kept on the open circuit condition when the test strip was inserted and applied 10 μL sample solution. After an enzymatic event for the occurring of the enzymatic reaction, a constant potential was then applied by the potentiostat and the output signal was recorded. All measurements were controlled by a computer system including the software of Labview program (Version 6.0, National Instrument, USA) and a data acquisition card (PCI-MIO-16E-4, National Instrument, USA). A commercial potentiostat (750A, CHI, USA) was used for the cyclic voltammetry and the temperature of all measurements was controlled at 25 °C.

3. Results and discussions

3.1. Principle

In this paper, we developed a portable electrochemical cholesterol biosensor for measuring total cholesterol and demonstrated the feasibility of utilizing the catalytic property of Fe_3O_4 with no interference observed. The design of biosensor strip is illustrated in Fig. 1(A). Three layers, including carbon ink, catalyst, and two enzymes were integrated on the sensing strip. The sensing principle for the determination of cholesterol and cholesteryl ester is based on the formation of hydrogen peroxide during the enzyme-

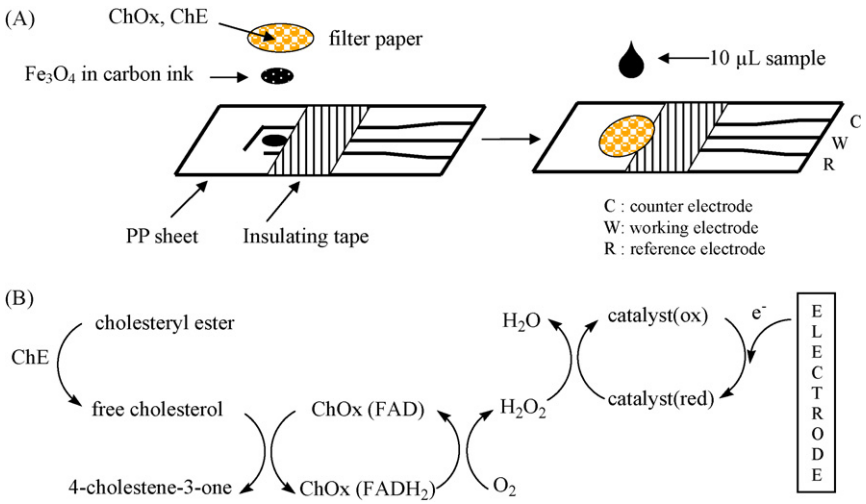

Fig. 1. An illustration of (A) the disposable cholesterol strip and (B) the mechanism of catalyst-based total cholesterol biosensor.

catalyzed reactions as follows:

$$\text{Cholesteryl esters} + H_2O \xrightarrow[\text{EC 3.1.1.13}]{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{(EC 1.1.3.6)}]{\text{cholesterol oxidase}} \text{cholesterol-4-en-3-one} + H_2O_2$$

In enzymatic reaction, the hydrogen peroxide is generated in the subsequent reduction of Fe_3O_4 at low cathodic potential. In our previous studies, the properties of Fe_3O_4 were examined, and the feasibility of utilizing the catalytic properties of Fe_3O_4 to fabricate a hydrogen peroxide chemical sensor was investigated. Combining both enzymatic and catalytic reactions, a possible measurement scheme for total cholesterol is shown in Fig. 1(B).

The catalytic characteristics of Fe_3O_4 for the reduction of hydrogen peroxide were evaluated by cyclic voltammetry. Fig. 2 shows the changes of cathodic current after three successive additions of H_2O_2 in 0.05 M phosphate buffer solution (pH 5.5) under anaerobic condition. The response of 1–5 mM H_2O_2 indicates that the cathodic current becomes significant when the applied potentials are less than 0 V (vs. Ag/Ag^+). The result clearly indicates that the reduction of hydrogen peroxide occurs at low applied potential on the Fe_3O_4 modified strip.

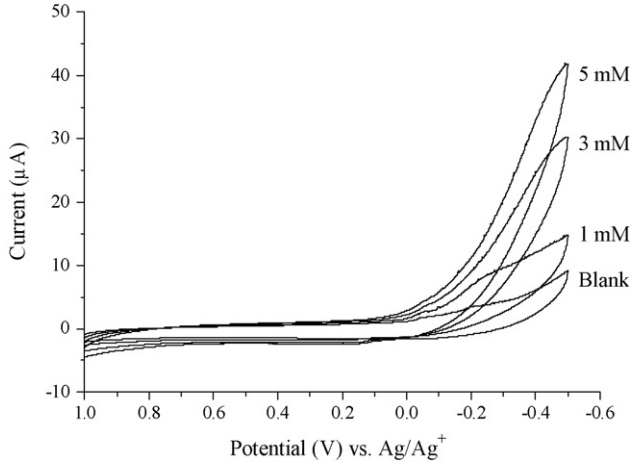

Fig. 2. Cyclic voltammograms of three successive additions of H_2O_2 on a 40% Fe_3O_4 modified strip. The voltammograms were obtained in 0.05 M phosphate buffer (pH 5.5) containing 0.1 M NaCl under anaerobic condition; scan rate 50 mV/s.

3.2. Sample preparation

In order to prepare a higher concentration of cholesterol and cholesteryl oleate in aqueous solution, some surfactants were usually employed due to their amphiphilic property. A series of surfactants were carefully investigated for the purpose of sample dissolution and stability issues. Triton X-100, a non-ionic surfactant, is often used for cholesterol and cholesteryl oleate preparation in aqueous solution. However, surfactant contents above 3% (m/v) will inhibit the enzyme activity, and greater than 5% (m/v) can lead to the formation of dense micelles around cholesterol molecules and interfere with the enzymatic oxidation of the substrate. Another surfactant, Tween 85 which is a more hydrophobic surfactant than Triton X-100 and can improve the solubility of cholesterol and cholesteryl oleate in aqueous buffer solution. In this study, the standard solutions were prepared by first dissolving cholesteryl oleate in the mixture of Triton X-100 and Tween 85 at 60 °C, and then adding 0.05 M phosphate buffer (pH 5.5) containing 0.1 M NaCl. The final standard solution contained 1% (w/v) Triton X-100 and 2% (w/v) Tween 85.

3.3. Optimization of the total cholesterol biosensor

3.3.1. Effect of catalyst and enzyme composition

The contents of catalyst and enzyme are the important factors for enhancing the sensitivity of the biosensor. The results show that cathodic response increases proportionally with the amount of Fe_3O_4 within the working range from 10 to 40%. Moreover, a markedly increase of variation was observed due to the rise of carbon ink viscosity in strip printing procedure when the percentage of Fe_3O_4 exceeded 40%. Hence, the 40% of catalyst was chosen for working electrode modification. The amount of the immobilized ChOx and ChE was also optimized within the range from 0.1 to 1 U/strip and 0.25 to 1.5 U/strip, respectively. Fig. 3(A) shows the response increases for the amount of ChOx at a range from 0.1 to 0.5 U/strip and then reaches a plateau after applying 100 mg/dL cholesterol solution. In the study, each data was obtained from six replicates and the coefficients of variation (CVs) were less than 8.8%. Subsequently, the ChE was optimized and ChOx was immobilized at 0.5 U/strip. The curves in Fig. 3(B) represent the response increases with the ChE at a concentration range from 0.25 to 0.75 U/strip and

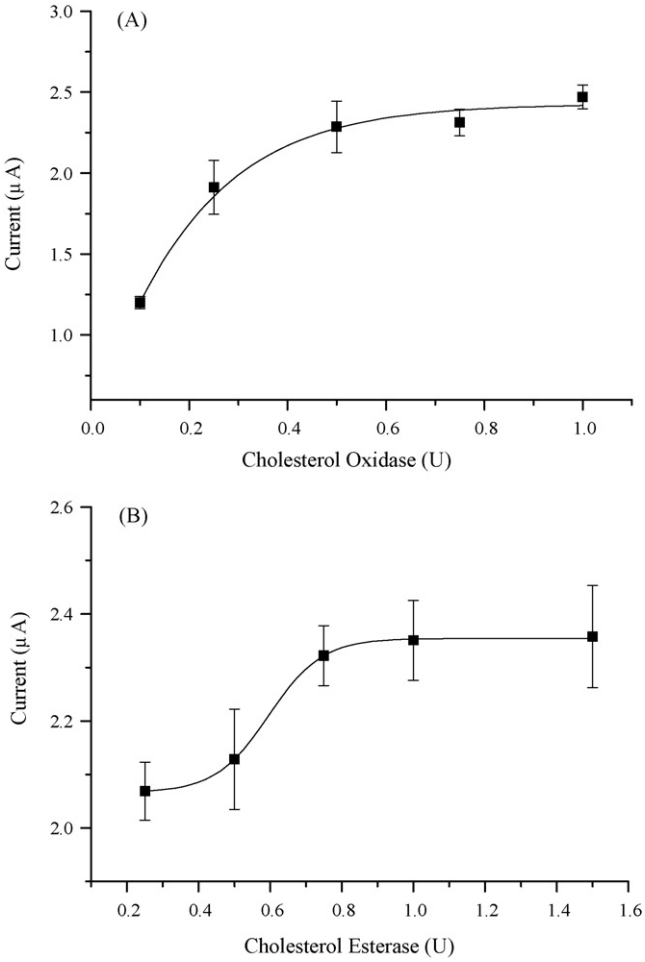

Fig. 3. Effect of the enzyme composition. The Fe_3O_4-based strips were immobilized with (A) ChOx (B) 0.5 U/strip ChOx and ChE for determining 100 mg/dL cholesterol and cholesteryl oleate in 0.05 M phosphate buffer (pH 5.5) containing 0.1 M NaCl, respectively; applied potential −200 mV.

then reach a plateau after the addition of 100 mg/dL cholesterol oleate ($N = 6$, CVs < 4.5%). The optimum concentration for ChOx and ChE was determined as 0.5 and 0.75 U/strip, separately.

3.3.2. Selection of applied potential

The specificity of electrochemical biosensor depends on the immobilization of the recognition element and the selection of the applied potential. In order to selectively detect the reduction of hydrogen peroxide in biological samples, the possible interference from easily oxidizable species such as ascorbic acid, uric acid, and acetaminophen must be minimized. An effective strategy for improving the selectivity is to modify the working electrode with a catalyst and measure the electrochemical response under an appropriate and usually a relatively low applied potential.

In the printed electrodes, the applied potential is expected to shift with the change of chloride concentration in sample solution because a silver/silver chloride paste was used as a reference electrode. We found that the potential of reference electrode was shifted about −108.7 mV as compared to a commercial reference electrode (Ag/AgCl, 3 M [Cl^-]) when the sample solution contained 0.1 M NaCl. The concentration of chloride was chosen at 0.1 M for the standard solution preparation because the normal concentration range of chloride in serum is about 0.1 M.

In our study, the increase of catalytic current for the reduction of hydrogen peroxide is proportional to the applied cathodic potential, but the background current was also shown to increase when a more negative potential was applied. Fig. 4 shows the differences of response from 0 to −250 mV (vs. Ag/Ag^+) for 100 mg/dL cholesteryl oleate with a blank solution. Although the results show that the sensitivity can be improved at a more negative potential, the interference of ambient oxygen at the cathodic potential is another concern. In the Fe_3O_4-based biosensor, the oxygen can be reduced at an applied potential of less than −250 mV and the easily oxidizable interferences present in the blood samples can be oxidized when the applied potential is greater than 0 V. Therefore, a potential window with interference-free behavior was found between 0 and −250 mV. On the basis of sensitivity and selectivity, the optimum operational potential for the biosensor was chosen at −200 mV.

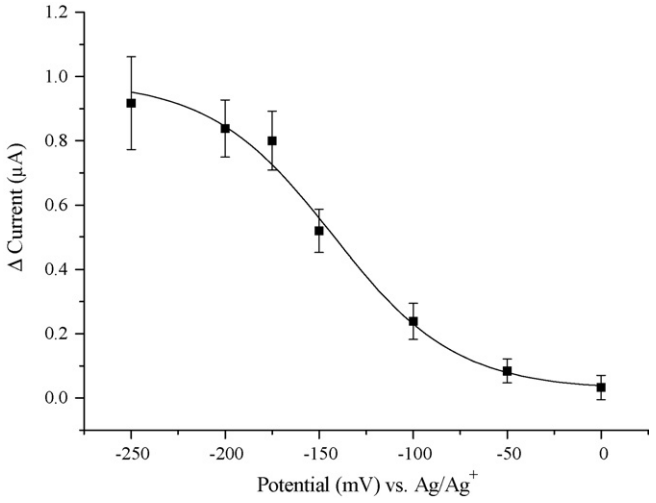

Fig. 4. Hydrodynamic voltammogram for 100 mg/dL cholesteryl oleate (pH 5.5) in the Fe_3O_4-based cholesterol biosensor. The strips were immobilized with 40% Fe_3O_4, 0.5 U of ChOx and 0.75 U of ChE.

3.3.3. Effect of buffer pH

The pH of buffer has a great influence on enzyme activity and the catalytic capacity of Fe_3O_4 for hydrogen peroxide reduction. The change in ionization state of amino acid side chain can perform critical functions in the enzyme active site, thus the enzyme activity should change with the pH of buffer. The optimum pH for ChOx and ChE was determined at pH 6.5 and pH 7, respectively, but the catalytic reaction for reducing hydrogen peroxide is more sensitive at low pH. In our previous study, the sensitivity was reduced proportionally with pH increasing from 3 to 8 (Lin and Leu, 2005). Therefore, nine standard solutions of 100 mg/dL of cholesteryl oleate were prepared in phosphate buffer with pH adjusted from 4 to 8 to investigate the pH effect ($N = 6$, CVs < 6.3%). The differences between signal and background response are shown in Fig. 5. The results show that the pH profile is based on both the characteristics of enzyme and catalyst and the maximum of response can be obtained at pH 5.5, which was employed in the subsequent studies.

3.4. Analytical performances

3.4.1. Calibration curve and validation

The calibration curves for cholesteryl oleate are shown in Fig. 6. The results show two linear regions: (1) from 25 to 100 mg/dL with a regression equation of $y = 1.544 + 0.00878x$ ($R^2 = 0.9998$) and (2) from 100 to 400 mg/dL with the regression equation of $y = 2.021 + 0.00397x$ ($R^2 = 0.999$) ($N = 6$, CVs < 6.9%). The correlation

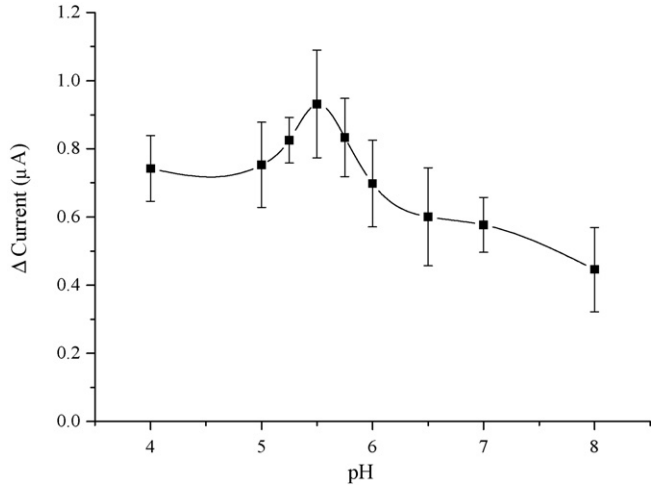

Fig. 5. Effect of pH in the Fe_3O_4-based cholesterol biosensor for determining 100 mg/dL cholesteryl oleate. The applied potential is at −200 mV. Other conditions are as in Fig. 4.

between concentrations (x) range (from 25 to 500 mg/dL) and currents (y) can also be expressed by a nonlinear regression equation: $y = 4.304 - 2.71 \times (0.9968)^x$ ($R^2 = 0.993$). The nonlinear correlation curve was shown in Fig. 6 and the actual responses were also shown in inset.

The coefficient of variation for 20 measurements of 100 mg/dL cholesteryl oleate is 5.06%. The detection limit is 19.4 mg/dL (S/N = 3) determined by performing 20 blank measurements. The potential profile for measurement was also optimized. The total time for one sample assay is 20 s that includes 15 s of enzyme reaction time before applying potential and 5 s of electrochemical detection.

The stability of strips was also tested up to 1 month and the responses were stable throughout the study period, which were 100.4 ± 1.8% ($N = 7$, 95% confidence level) compared to the fresh strips. In the study, each data was obtained from three replicates and the CVs were less than 7.2%.

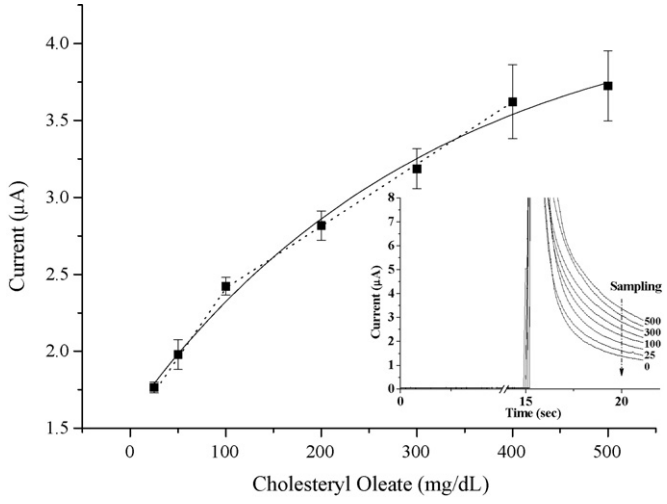

Fig. 6. Calibration curve of the cholesterol biosensor for cholesteryl oleate. The two linear regressions are showed as dash lines and the nonlinear regression as solid line. The typical chronoamperometric responses of the biosensor are also shown in inset.

Table 1
Interference study

Test compound[a]	Concentration (mg/dL)	Interference[b] (%)
Ascorbic acid	20	+3.08
Uric acid	20	+1.25
Glucose	100	+1.53
Urea	20	−1.35
Creatine	20	−0.55
Creatinine	20	−2.20
Dopamine	20	+1.88
Lactic acid	20	−1.20
Aspirin	20	+2.82
Salicylic acid	20	+3.18
Acetaminophen	20	+2.20

[a] Test compounds were prepared in 100 mg/dL cholesterol solution.
[b] Interference = $(I_1 - I_0)/I_0 \times 100\%$; where I_1 is the response of the test compound and I_0 is the response of 100 mg/dL cholesterol solution.

3.4.2. Interference study

Interference is always an important issue for the development of an electrochemical biosensor. In order to evaluate the specificity of the cholesterol biosensor, the effects of possible interferences in blood samples such as ascorbic acid, uric acid, urea, creatine, creatinine, dopamine, acetaminophen, aspirin, salicylic acid and glucose were investigated. These interferences were added into 100 mg/dL cholesterol standard solution, separately, and then measured by the biosensor. The results are summarized in Table 1 and show no significant interferences were observed in aforementioned interferences. This interference-free behavior may attribute to the design of biosensor by using Fe_3O_4. The interference is calculated as the difference in percentage of response between the test compound and cholesterol over that of cholesterol.

4. Conclusions

In summary, we have demonstrated the feasibility of utilizing Fe_3O_4 as a catalyst in an interference-free electrochemical cholesterol biosensor. The screen-printing technology was suitable for the mass production of sensing strip with the advantages of simple, rapid, and low cost. The catalyst of Fe_3O_4 possesses the catalytic property of reducing hydrogen peroxide at low overvoltage, which resulted in no observed interference from the easily oxidizable compounds. An integrated recognition layer is especially innovative in the development of disposable total cholesterol biosensors that includes cholesterol oxidase, cholesterol esterase and catalyst layer. Besides, the one step chronoamperometric measurement can offer the convenience for the development of point of care diagnostic system in the future. We are currently developing a disposable biosensor scheme, which is suitable for the measurement of total cholesterol in a whole blood sample in our laboratory.

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