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Short communication

Study of carbon nanotube modified biosensor for monitoring total cholesterol in blood

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Abstract

A carbon nanotube modified biosensor for monitoring total cholesterol in blood was studied. This sensor consists of a carbon working electrode and a reference electrode screen-printed on a polycarbonate substrate. Cholesterol esterase, cholesterol oxidase, peroxidase and potassium ferrocyanide were immobilized on the screen-printed carbon electrodes. Multi-walled carbon nanotubes (MWCN) were added to prompt electron transfer. Experimental results show that the carbon nanotube modified biosensor offers a reliable calibration profile and stable electrochemical properties.

Keywords: Total cholesterol; Biosensor; Carbon nanotube

1. Introduction

Normal human blood serum contains less than 200 mg/dl cholesterol, of which two third is esterified with fatty acids and one third is present as sterol (White et al., 1978). There is a strong positive correlation between high serum cholesterol level and arteriosclerosis, hypertension and myocardial infarction. So, the determination of cholesterol is important in clinical diagnosis.

As awareness of the importance of total cholesterol levels has increased, numerous methods for human blood cholesterol assays have been developed (Crumbliss et al., 1993; Charpentier and Murr, 1995; Martin et al., 2003; Tatsuma and Watanabe, 1991; Situmorang et al., 1999; Shumyantseva et al., 2004), including colorimetric spectrometric and electrochemical methods. Mainly enzymatic procedures are employed in clinical diagnosis due to their rapid, selective, sensitive nature and the great accuracy.

Former amperometric methods work at relatively high potentials so that many other species may be oxidized. To minimize the effect of interferences, techniques have been developed using the redox mediator ferricyanide to reduce hydrogen peroxide, making the product ferrocyanide detectable at relatively low potentials. However, this measuring system was affected by air oxidation of ferrocyanide that takes place as a competitive reaction during the enzymatic oxidation.

Carbon nanotubes are a novel type of carbon material and can be considered as the result of folding graphite layers into carbon cylinders. There are two groups of carbon nanotubes, multi-walled carbon nanotubes and single-walled carbon nanotubes (Zhao et al., 2002). Carbon nanotubes have been recognized as one of the most promising electrode materials since the first electrode application in the oxidation of dopamine in 1996 (Britto et al., 1996). Since then, the research has been focused on their electrocatalytic behaviors toward the oxidation of biomolecules and their performance has been found to be much superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit. Rubianes (Rubianes and Rivas, 2003) have reported carbon nanotubes paste electrode could decrease the

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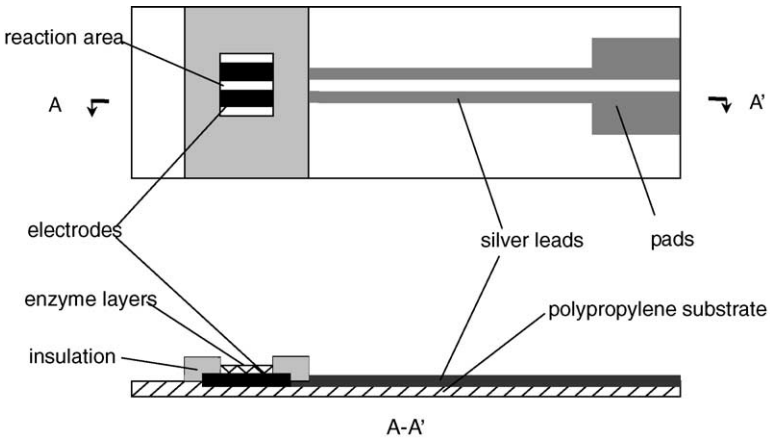

Fig. 1. The structure of the cholesterol biosensor.

overvoltage for the redox of ascorbic, uric acid and hydrogen peroxide (H_2O_2), while H_2O_2 is an important chemical material in biochemical assay, many substrate will convert to H_2O_2 to be determined on electrode surface. Davis et al. (Davis et al., 1997, 1998) have described the high surface area possessing abundant acidic sites that may offer special opportunities for the immobilization of enzymes in biosensors. Guiseppi-Elie (Guiseppi-Elie et al., 2002) has researched the direct electron transfer of glucose oxidase on carbon nanotubes. The similarity in length scales between nanotubes and redox enzymes suggests interactions that may be favorable for biosensor electrode applications. However, the detection mechanism of carbon nanotubes is not fully understood. A systematic study has not been reported so far. In this paper, we used carbon nanotubes to modify carbon paste electrode, which can promote electron transfer and enhance the response current.

2. Experiments

2.1. Reagents

All reagents, except multi-walled carbon nanotubes, were commercially available and of analytical-reagent grade chemicals. Cholesterol oxidase (COD), 20 U/mg, TOYOBO), cholesterol esterase (CEH, 100 U/mg, TOYOBO), peroxidase (POD), potassium ferrocyanide, trehalose, TritonX-100 were purchased from the respective companies. Carboxyl modified multi-walled carbon nanotubes were provided by the Department of Material Science, Zhejiang University.

2.2. Electrode preparation

The electrodes were fabricated using screen-printing technique, which is a common method for electrode fabrication (Nagata et al., 1995). Three masks were used to form sliver conducting lead wires, carbon film for electrochemical reaction and insulating film. The structure of the cholesterol sensor is shown in Fig. 1. The sensor consists of two carbon paste electrodes. One electrode was used as working electrode while the other was used as reference electrode. The function of the silver leads was to improve electric conductivity of the electrodes. The reaction area, where the carbon paste film was modified by carbon nanotubes and immobilized enzymes, was defined by the insulating film coated on the carbon paste film. There were 20 sensor bases on each plate. Once the sensor array was ready, sensor strips were cut out of the array to be measured. The size of the reaction area was 2 mm × 2 mm while the sensor strip was 35 mm × 10 mm

2.3. Electrode modification and enzyme immobilization

After dropping 2 μl the carboxyl modified multi-walled carbon nanotubes solution (5 mg/ml) onto the reaction area of the electrode, 1 μl CMC solution (20 mg/ml) was added. It was dried in hot air at 50 °C for 10 min to form a hydrophilic polymer layer. Subsequently, 70 unit cholesterol oxidase, 1.8 mg peroxidase, 8 mg potassium ferrocyanide and 6 mg trehalose were dissolved in 0.125 ml phosphate buffer of pH 7.0. Then 2 μl solution was added on the hydrophilic polymer layer to form a cholesterol oxidase layer. Finally, 200 unit cholesterol esterase, 3 mg trehalose, 1 μl TritonX-100 were dissolved in 0.125 ml phosphate buffer (pH7.0). And 2 μl of the solution was added to form a cholesterol esterase layer.

2.4. Measurement

All measurements were performed at 25 °C room temperature. Amperometric measurements were performed by a CHI660 Electrochemical Work Station from CH Instruments Inc., USA. The working potential is 300 mV. All reaction processes were recorded using an IBM PC compatible computer via a RS232 series port communicating to the electrochemical analytic station. The current values were read 180 s after the sample solution was dropped onto the reaction area.

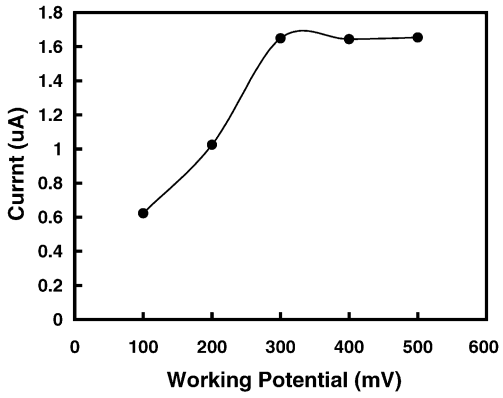

Fig. 2. Effect of working potential on response current of the sensor.

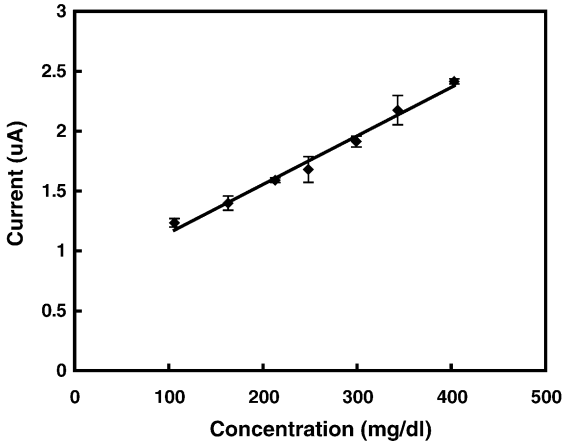

Fig. 3. Calibration curve of the cholesterol sensor.

2.5. Optimization of assay

2.5.1. Optimization of electron mediator concentration

The function of redox mediator is to make the reaction detectable at low potential to minimize the effect of interferences. So, the concentration of potassium ferrocyanide, which plays the role of redox mediator has a certain effect on response current. Proper potassium ferrocyanide increases the response current while too much potassium ferrocyanide inhibits the reaction. The amount of potassium ferrocyanide used is optimized.

2.5.2. Optimization of buffer pH

Enzyme has an optimal pH for its activity, but it will alter after immobilization. The optimal pH was determined by measuring the maximal response current on different pH conditions. Experimental result shows that pH 7.0–9.0 is optimum to obtain the maximal responses. At the same time, considering normal blood is around pH 7.0 and the optimal pH value for both CEH and COD is pH 6.0–8.0, pH 7.0 was chosen in all subsequent studies.

2.5.3. Optimization of working potential

Fig. 2 shows the relationship between the response current of the sensor and the potential applied on the working electrode, when the cholesterol concentration of the sample was 200 mg/dl. The sensitivity evidently increased from 100 to 300 mV, which is due to the increase of electron transfer force. Then the response current changed little with further potential increase. In order to avoid high working potential causing ascorbic acid and uric acid directly oxidized on the electrode surface while obtaining high sensitivity, the working potential of 300 mV was appropriately used.

2.5.4. Operating temperature

Temperature affected the activity of enzymes. We studied the temperature effect on the electrode outputs. The electrode responses to 200 mg/dl cholesterol solution were examined at temperature from 15 to 40 °C. The experimental results show that the response current was fairly stable, especially in the temperature range of 20–30 °C. Theoretically, this is due to the superfluous enzymes. All experiments were carried out at room temperature of 25 °C.

3. Results and discussions

3.1. Calibration

Calibration cure for free cholesterol standard serum solution is presented in Fig. 3. A linear relationship between the cholesterol concentration and the response current of the carbon nanotube modified electrodes was observed.

3.2. Comparison between carbon nanotube modified and unmodified electrodes

The determination of cholesterol by the sensors described in this paper is based on the following sequence of enzyme and electrode reactions:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acids} \quad (1)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \Delta^4\text{-Cholesterol-3+}H_2O_2 \quad (2)$$

$$H_2O_2 + 2[Fe(CN)_6]^{4-} + 2H^+ \xrightarrow{\text{peroxidase}} 2[Fe(CN)_6]^{3-} + 2H_2O \quad (3)$$

$$[Fe(CN)_6]^{3-} + e \rightarrow [Fe(CN)_6]^{4-} \quad \text{(working electrode)} \quad (4)$$

$$[Fe(CN)_6]^{4-} \rightarrow [Fe(CN)_6]^{3-} + e \quad \text{(counter electrode)} \quad (5)$$

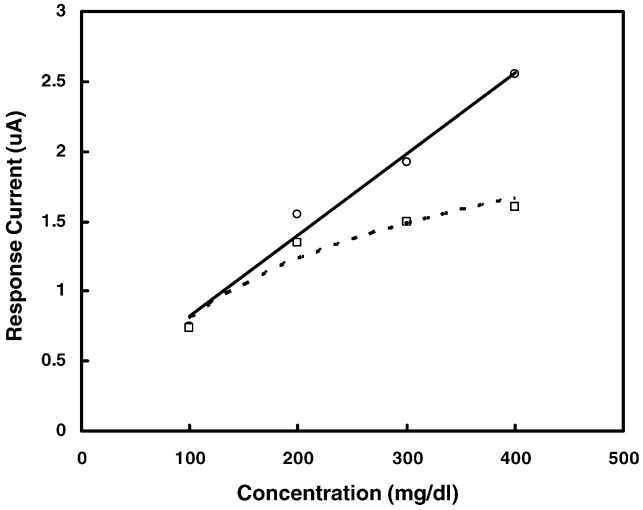

Fig. 4. The comparison of the cholesterol determinations of the carbon nanotube modified (solid line) and unmodified (dash line).

When the enzymes were abundant, the electrode reaction was the control step. Because hydrogen peroxide is difficult to be oxidized directly on the carbon paste electrode, ferrocyanide is used as redox mediator to reduce hydrogen peroxide. We tried to use carbon nanotubes to promote the electrons transfer further.

Using both the carbon nanotube modified and unmodified sensors, the responses to cholesterol were determined over the range of 100–400 mg/dl. An almost linear relationship between the cholesterol concentration and the response current of the carbon nanotube modified electrodes was observed, while a curve for unmodified electrodes was observed (Fig. 4). The average sensitivities are 0.0059 and 0.0032 μA/mg/dl, respectively. It is seen that the carbon nanotubes promote the electron transfer and almost double the sensitivity. Additionally, the carbon nanotube modification also improved the linearity of the electrode.

3.3. Clinical trials

We carried out clinical trials using 31 patients' blood samples. After the total cholesterol in blood samples were determined by the cholesterol sensors presented in this paper and routine hospital blood analyzer individually, the results were analyzed and compared. The blood analyzer used in the clinic trials was Olympus AU2700, and the method used was cholesterol oxidase–trinder reaction. The correlation coefficient, $r = 0.9430$ indicated that testing results by the total cholesterol sensors and the routine hospital blood analyzer were agreed closely.

3.4. Repeatability

In order to test the repeatability of cholesterol electrodes, we used three different concentration blood samples. Three sensors were used for each concentration. The testing results and the calculated means and the relative standard deviations are shown in Table 1, which indicates that the repeatability of the biosensors is practically acceptable.

Table 1
Repeatability of the biosensor

Concentration (mg/dl)	Response current (μA)			Mean (μA)	R.S.D. (%)
	Sensor no. 1	Sensor no. 2	Sensor no. 3		
213	1.522	1.531	1.556	1.536	1.259
343	2.038	2.205	2.280	2.174	5.658
403	2.443	2.401	2.412	2.419	8.700

3.5. Interference

Ascorbic acid and uric acid at normal levels expected in blood have no effect on the response current. This indicates that these substances do not interfere during the blood total cholesterol measurements.

3.6. Conservation

Many studies have shown that high trehalose concentration could protect enzymes from broiling, droughty and heavy metal ion conditions. So, we used rehalose to maintain the activity of the enzymes to prolong the life span of the biosensors. Our experimental results indicated that the electrodes keep stable for 2 months at room temperature without significantly losing sensitivity.

4. Conclusion

A biosensor for monitoring total cholesterol in blood is demonstrated with a combination of cholesterol esterase, cholesterol oxidase, peroxidase, potassium ferrocyanide and multi-walled carbon nanotubes immobilized on a carbon paste electrode. The modification of the carbon nanotubes promoted the electron transfer so as to improve the sensitivity of the cholesterol sensor. Within the total cholesterol range of 100–400 mg/dl, the testing results have shown a fairly good correlation with clinical laboratory method while only 2 μl blood sample was required for a test. The screen-printing method provides a way for rapid, economic and reproducible manufacture of sensor electrode. The sensor developed did therefore provide a promising economic and easy method for monitoring the total cholesterol in blood.

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