Talanta 84 (2011) 1323–1328

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A fast and highly sensitive detection of cholesterol using polymer microfluidic devices and amperometric system

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ARTICLE INFO

Article history:
Available online 4 March 2011

Keywords:
Cholesterol
Poly(dimethylsiloxane)
PDMS microchip
Electrochemical detection
Enzymatic assays
Microchip capillary electrophoresis

ABSTRACT

In this work, the rapid detection of cholesterol using poly(dimethylsiloxane) microchip capillary electrophoresis, based on the coupling of enzymatic assays and electrochemical detection, was developed. Direct amperometric detection for poly(dimethylsiloxane) (PDMS) microchip capillary electrophoresis was successfully applied to quantify cholesterol levels. Factors influencing the performance of the method (such as the concentration and pH value of buffer electrolyte, concentration of cholesterol oxidase enzyme (ChOx), effect of solvent on the cholesterol solubility, and interferences) were carefully investigated and optimized. The migration time of hydrogen peroxide, product of the reaction, was less than 100 s when using 40 mM phosphate buffer at pH 7.0 as the running buffer, a concentration of 0.68 U/mL of the ChOx, a separation voltage of +1.6 kV, an injection time of 20 s, and a detection potential of +0.5 V. PDMS microchip capillary electrophoresis showed linearity between 38.7 μg/dL (1 μM) and 270.6 mg/dL (7 mM) for the cholesterol standard; the detection limit was determined as 38.7 ng/dL (1 nM). To demonstrate the potential of this assay, the proposed method was applied to quantify cholesterol in bovine serum. The percentages of recoveries were assessed over the range of 98.9–101.8%. The sample throughput was found to be 60 samples per hour. Therefore, PDMS microchip capillary electrophoresis, based on the coupling of enzymatic assays and electrochemical detection, is very rapid, accurate and sensitive method for the determination of cholesterol levels.

1. Introduction

One of the most important and frequently quantified substrates in clinical analysis is the blood cholesterol. Cholesterol is a steroid metabolite of waxy nature constituting an essential of mammalian cell membranes and is transported in the blood plasma of all animals. In addition, cholesterol is an important component in the manufacture of bile acids, steroid hormones, and several fat-soluble vitamins. Cholesterol is routinely monitored for the risk assessment of cardiovascular conditions, such as atherosclerosis and hypertension, which can develop to coronary heart disease, myocardial and cerebral infarction (stroke). A high level of cholesterol above the physiological norm can lead to the conditions of hypothyroidism, nephrosis, diabetes mellitus, myxedema, and obstructive jaundice. In contrast to that, decreased amount levels are found in patients suffering from hyperthyroidism, anaemia, malabsorption and wasting syndromes [1,2]. Hence, the development of quantitative methodology of cholesterol is extremely important in clinical laboratories.

Over the past decades, the conventional method for the determination of cholesterol utilized the reaction between cholesterol and cholesterol oxidase enzyme (ChOx), and cholest-4-en-3-one produced, although the consumption of oxygen, or the production of hydrogen peroxide are detected by spectrophotometry [3,4]. However, these techniques are neither suitable for rapid nor cost effective detection because complicated principle and requires an expensive instrument as well as reagents. Therefore, alternative amperometric biosensors have been proposed based on the immobilization of cholesterol oxidase enzyme with respect to the cholesterol reaction sequence [3–11]. Unfortunately, various matrix species such as ascorbic acid and uric acid interfere with these biosensors.

Since a microfluidic platform was described, a number of applications were quickly and continuously increased. Microfluidic

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doi:10.1016/j.talanta.2011.02.040

devices have been shown to have potential of biological applications, including biomolecular separations, enzymatic assays, the polymerase chain reaction, and immunohybridization reactions [12–29].

Microchip capillary electrophoresis (MCE) is a technique that integrates with laboratory functions on a single chip. It has been developed for the separation of various biological compounds including DNA and proteins [12–23]. Enzyme sensor chips based on electrochemical detection are widely studied by many researchers. Anyhow, almost all the studies seem to be focused on the fabrication of small electrodes and chambers as well as the possibility of applying the optimized method to the fabrication of micro devices by immobilizing the enzyme on the surface of the electrode and on chips. There is a few works reported on the development of a fast and sensitive analysis assay.

The general material for microchip fabrication is glass, such as quartz and borosilicate, but the method of glass fabrication is complicated and relatively difficult for fabrication [12–16,18]. Compared to other materials, PDMS is an attractive material for microchip fabrication due to its excellent optical transparency, easy replica molding, non-toxicity and biocompatibility [19–22,29]. Thus, PDMS has become popular material for in the manufacture of microchip devices and there is a significant interest in the development and the use of polymers as substrates for MCE. Hence, MCE fabricated from polymer has potential to be an alternative assay for the determination of biological compounds including cholesterol because it is easy to use, require small volumes of reagents and sample, and provide rapid analysis. Moreover, electrophoresis separation in MCE should be an efficient way to minimize the matrix effect by the specific migration time of the analyte.

The current detection methods for MCE are laser-induced fluorescence detection [22–25], and electrochemical detection [12–19,21,26]. Among these detection schemes, electrochemical detection is an attractive for the microchip systems owing to its high sensitivity and its easy operation. Amperometric detection is the most useful mode for microchip systems due to its fast response, high sensitivity, and miniaturized instrumentation.

Such miniaturization and speed advantages of MCE and sensitivity of electrochemistry, the goal of the work is to explore the possibility of carrying out bioanalytical assays based on enzymatic reactions for diagnostic applications, by combining the attractive performance of polymer MCE and the high sensitivity of electrochemical principles for the rapid detection of cholesterol. Cholesterol and ChOx enzyme were flowed through the separation channel and then sample was introduced to mix at the channel intersection and in the separation channel using electrokinetic flow. Then, the hydrogen peroxide produced from the enzymatic reaction was immediately detected at the downstream gold (Au) wire working electrode. The performance characteristics of the polymer MCE/amperometric microsystem are reported in the following sections.

2. Experimental

2.1. Reagents and solutions

Sylgard 184 silicone elastomer and curing agent were obtained from Dow Corning (Midland, MI, USA). Dopamine, 2-(N-morpholino)-enthanesulfonic acid (MES), cholesterol, ChOx both from *Streptomyces* sp. (34 U/mg), and lipid cholesterol rich from adult bovine serum were obtained from Sigma (St. Louis, MO). Catechol was obtained from Fluka (Switzerland). All standard and sample solutions were prepared by using high purity water from Milli Q Water System (Millipore, USA, $R \geq 18.2$ MΩ/cm). Stock solution of cholesterol was daily prepared in 5% (w/v) Triton X-100 and high purity water and stored at 4 °C. This stock solution was further diluted to make the different concentrations of the cholesterol in high purity water. Stock solution of ChOx was prepared freshly by dissolving in 40 mM phosphate buffer (PBS, pH 7.0). All the solutions were filtered with 0.45 μM membrane prior to be used. A 25 μm of 99.99% gold wire (Goodfellow, England) was used as working electrode. The lipid cholesterol rich from adult bovine serum was purchased from Sigma (St. Louis, MO). The stock solution of cholesterol in bovine serum (450–600 mg/dL) prepared by dissolving 75 mg of bovine serum in 1.00 mL in 40 mM PBS (pH 7.0).

2.2. Apparatus of PDMS microchip capillary electrophoresis

The details of microchip capillary electrophoresis are briefly described here. The microchip consisted of two crossed channels and four reservoirs, including a four-way injection cross (connected to the three reservoirs). The microchip had the channel with the width and depth of 50 μm and the length of separation channel was 50 mm. The electrode channels were 50 μm wide and 50 μm depth. Schematic of the microchip capillary electrophoresis is shown in Fig. 1.

2.3. Fabrication of the PDMS microchips

PDMS capillary electrophoresis microchips were fabricated using standard soft lithographic techniques [21,26,29]. Briefly, PDMS was mixed with curing agent in the ratio of 10:1 (w/w) to form a PDMS mixture and degassed under vacuum until bubbles were all out. The PDMS mixture was poured over a molding master and a blank wafer, and cured for at least 2 h at 65 °C in the oven. After that, the cross-linked PDMS was peeled off the mold, the reservoirs were created by a circular punch and a 25 μm gold wire working electrode was placed in the electrode channel at the end of separation channel. Next, the PDMS layers were placed in an air plasma cleaner, oxidized for 60 s, and immediately brought to form an irreversible seal with a second piece of cross-linked. The end-point of the electrode channel was sealed with silicone glue. Finally, an electrical connection was made using silver paint and silver wire. The

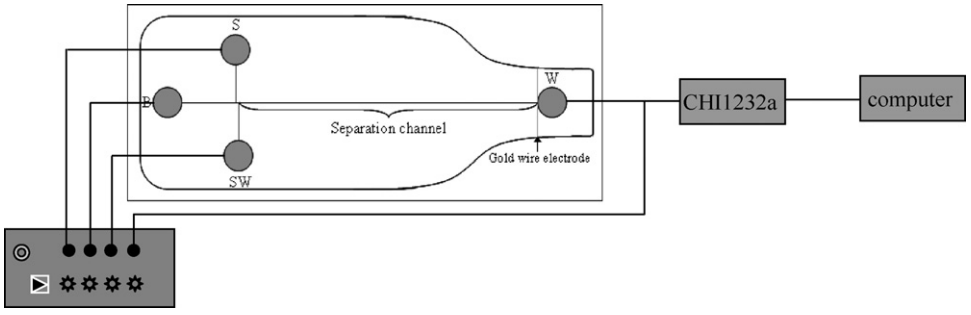

Fig. 1. Schematic of PDMS microfluidic device. Channels: 50 μm width, 50 μm depth, 50 mm length of separation channel and 25 μm Au wire working electrode (B = PBS + ChOx, S = sample, SW = sample waste, and W = PBS).

Table 1
Potentials applied and solution in each reservoir on the PDMS microchip during either the injection or the separation step.

Reservoir	Containing	Separation (V)	Injection (V)
B	PBS + ChOx enzyme	+1600	+450
S	Sample	+450	+450
SW	PBS	+450	−160
G	PBS	Ground	Ground

electrical connections made from platinum electrodes were placed into reservoirs.

2.4. Electrophoresis procedure

Prior to use, the channels were treated by rinsing with 0.1 M sodium hydroxide and high purity water for 20 min and 5 min, respectively. This treatment changed a silanol groups (Si-OH) to negative form ($Si\text{-}O^-$) on the surface of the PDMS microchip. To perform the electrophoretic separation, waste reservoir was filled with the phosphate buffer solution, while the buffer reservoir was filled with phosphate buffer containing the cholesterol ChOx. The sample reservoir was also filled with cholesterol. For injection step, the sample and buffer reservoirs were held at +450 V while the sample waste reservoir was held at −160 V for loading the sample plug into the double-T injector for 20 s. Separation was performed by switching the high voltage contacts and applying the corresponding separation voltages to the running buffer reservoir with the detection reservoir grounded, all other reservoirs floating. Subsequently, a separation voltage of +1.6 kV was then applied across the buffer reservoir and the grounded detection reservoir. Cholesterol in separation channel was reacted with the ChOx in the running buffer to generate the hydrogen peroxide. Finally, hydrogen peroxide was detected at the downstream gold wire electrode by amperometric detection. The potential at separation step and injection step applied to the reservoir are summarized in Table 1.

2.5. Electrochemical detection

Amperometry is a one technique of electrochemical detection and it is popular to be detection for microchip capillary electrophoresis. Amperometric detection was performed with an electrochemical analyzer (CHI1232a, CH-instrument, USA) using the "amperometric *i*–*t* curve" mode. The electropherograms were recorded with a time resolution of 0.2 s while applying a desired detection potential versus platinum wire. Sample injections step were performed after stabilization of the baseline. Amperometry was used to detect a mixed standard of dopamine and catechol for the characterization of PDMS microchip and detect the cholesterol as well. For characterization of PDMS microchip, the constant detection potential of +0.8 V was applied to the working electrode.

3. Results and discussion

As mentioned previously, the ultimate goal of this work was to develop miniaturized clinical analyzers by coupling enzymatic assays in the PDMS microchips channel, the ChOx enzyme prepared in running buffer and sample solutions (cholesterol) were mixed at the channel intersection and in the separation channel using electrokinetic flow. The enzymatic reaction occurred along the separation/reaction channel. The neutral peroxide species (H_2O_2) was generated from the reaction between cholesterol and ChOx enzyme and was detected electrochemically at gold wire electrode by amperometry. In the following parts, the influence of some experimental parameters such as the buffer pH, buffer concentration, solubility of cholesterol, enzyme concentration and interferences on the separation efficiency and detection sensitivity are reported in detail.

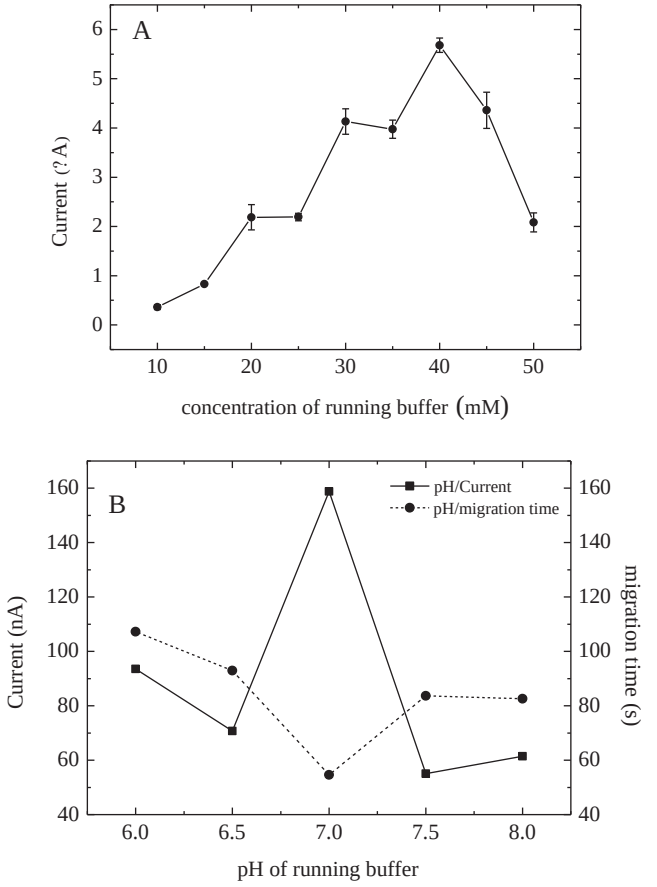

Fig. 2. (A) The effect of running buffer concentration on 1 mM cholesterol. (B) The effect of running buffer pH on current and migration time of 0.1 mM cholesterol. Conditions: running buffer, a PBS (40 mM, pH 7.0) solution containing 0.034 U/mL; detection potential, +0.5 V, separation potential, +1.3 kV; injection potential, +450 V; injection time, 20 s; at 25 μm Au wire working electrode.

3.1. Effect of concentration and pH of running buffer

The concentration of running buffer and the pH of running buffer are an important parameter, which affects on the sensitivity of the peak current of cholesterol, the EOF velocity, enzyme activity, and migration time. The concentration of running buffer was studied over the concentration range of 5 mM to 50 mM. The effect of concentration on the peak current of cholesterol is illustrated in Fig. 2A. The peak current of 1 mM cholesterol was found to be increased when the concentration of running buffer increased from 5 mM to 40 mM and the peak current then decreased with any further increase in the concentration of running buffer. With regard to sensitivity of the peak current, the concentration of running buffer at 40 mM was selected for the determination of cholesterol. For the influence of buffer pH, the optimal pH values were determined by varying the pH of the running buffer from 6.0 to 8.0 as shown in Fig. 2B. First, the effect of the running buffer pH on the migration time was monitored. It was found that the migration time of 0.1 mM cholesterol decreased when the running buffer pH increased from 6.0 to 7.0 and then increased with a further increase of the running buffer until 7.2 after that the migration time seem to be constant. Second, the effect of the running buffer pH on peak current was investigated. It can be seen that the peak current increased until pH 7.0, after that the peak current was dropped and became constant. This phenomenon may be con-

cerned on the activity of ChOx enzyme in pH medium that also caused on the sensitivity. Generally, the enzyme activity will be decreased in more acid or basic conditions. Hence, decreasing of peak current at pH higher than 7.0 was observed. Therefore, the running buffer pH 7.0 was selected as the optimal pH and used in all experiments.

3.2. Effect of solvent on the cholesterol solubility and ChOx enzyme concentration

To obtain the suitable solvent for dissolving the cholesterol, in comparison, water, 10 mM PBS and running buffer (40 mM PBS) were used as cholesterol solvents. The effect of cholesterol solvent on the peak current of 1 mM of cholesterol was investigated. The peak current of 1 mM cholesterol was increased when using water as the solvent because the sample becomes more concentrated inside the channel, and efficiency of peak current is increasing by principle of sample stacking. Sample stacking occurred when the sample was dissolved in a lower ionic strength solution than in separation electrolyte [30]. Compared to all used solvents, the highest current was observed when water was used as the solvent. Therefore, water was chosen as a suitable solvent to dissolve the cholesterol standard before injection into the channel.

The effect of ChOx concentration in the running buffer upon the response to the 1 mM cholesterol substrate was also studied. The current increased rapidly when the ChOx concentration was raised from 0.034 U/mL to 0.68 U/mL. Then, signals increased more slowly and finally leveled off above 0.85 U/mL (data not shown). This due to high levels of enzyme resulted in increased background noise and adsorption onto the channel walls. So, the optimal concentration was found at 0.68 U/mL.

3.3. Effect of interferences

Fig. 3 demonstrates the separation of 1 mM of standard cholesterol and 0.5 mM of standard ascorbic acid at the following detection potentials: +0.5 V, +0.6 V, and +0.8 V, respectively. Ascorbic acid was chosen as an interference agent in this work because it is generally found and is a major interference in serum. The results showed that the interference peak will be increased when the detection potential was increased. The peak current of ascorbic acid significantly increased when the detection potential was higher than +0.5 V. This indicated that using a detection potential of +0.5 V can reduce this interference effect, which allows the utilization of the proposed method for the determination of cholesterol in real sample.

3.4. Linear range and detection limit

The optimized conditions used for PDMS microchip capillary electrophoresis consisted of a 40 mM phosphate buffer solution (pH 7.0) containing 0.68 U/mL of the ChOx, a detection potential of +0.5 V, a separation voltage of +1.6 kV, and an injection time of 20 s. Under the selected conditions, calibration curves were obtained in the range of 38.7 μg/dL (1 μM) to 270.6 mg/dL (7 mM), with a coefficient of determination of 0.9984, as shown in Fig. 4. The limit of detection (LOD) and limit of quantitation (LOQ) were found to be 38.7 ng/dL (1 nM) and 38.7 μg/dL (1 μM), respectively. Compare to previous works, surprisingly, the proposed assay provided a very low detection limit of 1 nM (Table 2). This means that the developed method is very highly sensitive.

The expert panel has suggested that the total blood cholesterol level found in an adult should be lower than 200 mg/dL for normal blood, cholesterol between 200 mg/dL and 239 mg/dL for borderline-high blood cholesterol, and higher than 240 mg/dL for high blood cholesterol [1,2]. This information strongly supported that our proposed method can be applied for detection of cholesterol in real sample since all blood cholesterol levels are within the linear range.

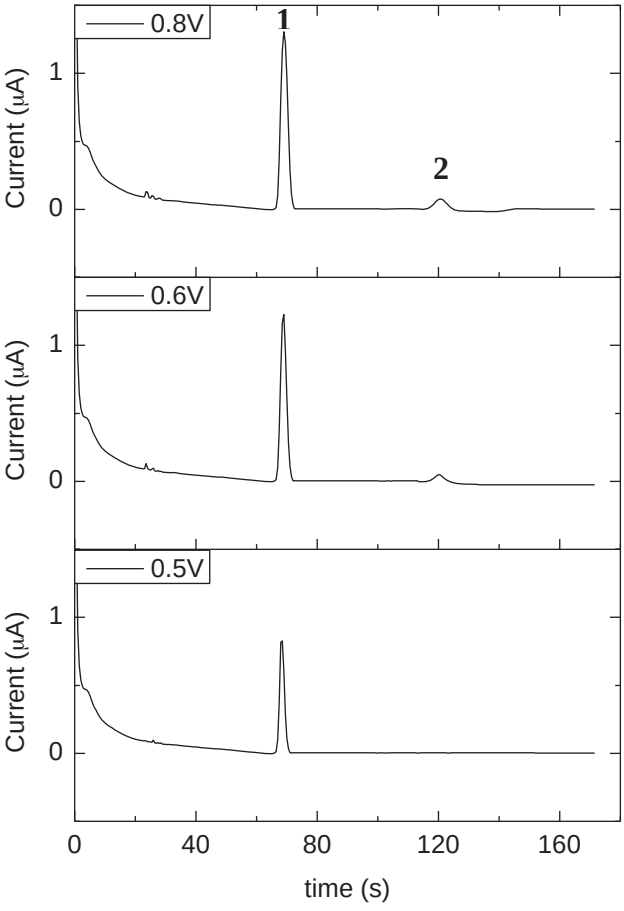

Fig. 3. The effect of interference for PDMS Microchip capillary electrophoresis. The electropherogram of (1) 1 mM of cholesterol and (2) 0.5 mM ascorbic acid at different detection potentials (a) +0.5 V, (b) +0.6 V, and (d) +0.8 V. Conditions: running buffer, a PBS (pH 7.0) solution containing 0.68 U/mL, separation potential, +1.6 kV; injection potential, +450 V; time, 20 s; at 25 μm Au wire working electrode.

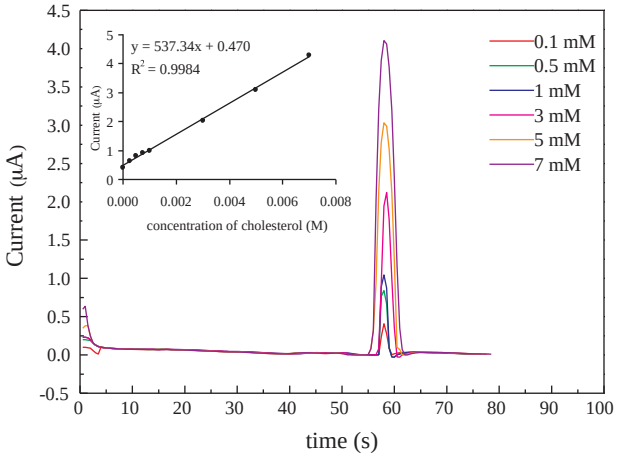

Fig. 4. The calibration curve of cholesterol. Conditions: running buffer, a 40 mM PBS (pH 7.0) solution containing 0.68 U/mL, separation potential, +1.6 kV; injection potential, +450 V; injection time, 20 s; at 25 μm Au wire working electrode. The linear range is shown in the inset.

Table 2
Comparison of data for cholesterol determination.

Method	Detection mode	Liner range	LOD	Reference
Flow injection analysis	Amperometry	0.025–0.3 mM	5.7 μM	[10]
Flow injection analysis	Amperometry	0–0.35 mM	12 μM	[9]
Batch analysis	Amperometry	–	0.4 mM	[6]
Batch analysis	Amperometry	10–100 μM	0.6 μM	[7]
Batch analysis	Chronoamperometry	0.5–5 mM	0.5 mM	[8]
Batch analysis	UV–VIS	0.65–10 mM	0.65 mM	[4]
Batch analysis	UV–VIS	1.3–13 mM	0.65 mM	[3]
Batch analysis	Cyclic voltammetry	0.13–7.8 mM	0.13 mM	[5]
Microchip capillary electrophoresis	Amperometry	1 μM to 7 mM	1 nM	This method

Table 3
Results on the determination of cholesterol by the standard addition method in a bovine serum sample ($n = 3$).

Sample	Labeled concentration (mg/dL)	Determined value (mg/dL)	Relative error	% Recovery
A	20.0	19.81	−0.95%	99.1
B	40.0	39.57	−1.07%	98.9
C	60.0	61.06	+1.77%	101.8

3.5. Sample analysis

As mentioned, cholesterol is an important part of a healthy body because it is used to form cell membranes and some hormones; however, high levels of cholesterol in the blood can adhere to the walls of the arteries, thereby increasing the risk of coronary heart disease. Therefore, it is very important to quantify cholesterol in the blood. PDMS microchip capillary electrophoresis was used for the determination of cholesterol in bovine serum. The standard addition method was used to determine the amount of cholesterol in bovine serum. From these results, the relationship between the current response and the concentration of analyte under the selected condition was presented. The standard addition was investigated by spiking three difference standard cholesterol concentrations ($n = 3$) into the diluted bovine serum. The labeled concentrations of cholesterol in bovine serum were 20.0 mg/dL, 40.0 mg/dL, and 60.0 mg/dL, respectively. The cholesterol concentration in the bovine serum sample was calculated from the standard addition curve. It can be observed that the determined values are highly correlated with the labeled values, as shown in Table 3. Using the proposed methodology, percentages of recoveries of were between 99.1% and 101.8%. The results demonstrated that this method can be used to efficiently determine cholesterol in bovine serum without any sample preparation. Therefore, the PDMS microchip capillary electrophoresis offers a fast, high specificity, accuracy and very high sensitivity for real sample analysis. Apart from the advantages of this method, there are some limitations. Especially, microchip CE made from polymers is the poorly understood and poorly controlled surface chemistry of these materials. In addition, the stability of chips is depended upon the maintenance.

4. Conclusions

For the first time, we have demonstrated an alternative assay to the rapid detection of cholesterol in serum using MCE fabricated from PDMS based on the coupling of enzymatic bioassays and amperometric detection. In this method, EOF was used to flow cholesterol and ChOx enzyme through the separation channel. Cholesterol concentrations were measured by comparing the current response in the presence and absence of ChOx enzyme. The hydrogen peroxide product was detected at the gold wire working electrode. The method provided a very high sensitivity (1 nM) and rapidness for cholesterol detection (less than 100 s) compared to those of previous reports. In addition, the developed method was also successfully applied to measure cholesterol in a sample of bovine serum by standard addition. While compared to large-scale conventional instruments, the developed polymer microfluidic biochips with coupling of enzymatic assays and electrochemical detection have the advantages of compact size, high sensitivity, high selectivity, low cost, and fast response. Therefore, this performance may have been beneficial for diagnostic studies and for routine analysis in clinical laboratories.

Acknowledgments

The authors would like to acknowledge the support from National Research Council of Thailand, the Thailand Research Fund, Chulalongkorn University Centenary Academic Development Project, the Thai Government Stimulus Package 2 (TKK2555), under the Project for Establishment of Comprehensive Center for Innovative Food, Health Products and Agriculture (PERFECTA), and the National Research University Project of CHE and the Ratchadaphisaksomphot Endowment Fund (project code AM003A and AM1009I).

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