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One-step electrochemical detection of cholesterol in the presence of suitable $K_3Fe(CN)_6$/phosphate buffer mediator by an electrochemical approach

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ABSTRACT

One-step approach of cholesterol biosensor was fabricated onto smart micro-chips based on cholesterol oxidase (ChOx) co-immobilized thioglycolic acid self-assembled monolayer (TGA-SAM) for biomedical applications. The selective cholesterol biosensor was investigated with modified tiny micro-chip (Au/SAM/ChOx) by the facile and reliable cyclic voltammetric (CV) method in a $K_3Fe(CN)_6$/phosphate buffer (PB) system. The modified micro-chip displayed a large dynamic range (1.0 nmol L^{-1} to 1.0 mmol L^{-1}), lower detection limit ($\sim$0.49 nmol L^{-1}, based on $S/N\sim3$), higher sensitivity ($\sim$93.75 μA μmol L^{-2} cm^{-2}), good linearity (correlation coefficient r^2, 0.9995), lower sample volume ($<$ 50.0 μL), and good stability as well as reproducibility. The Au/TGA system was implemented for a facile and simple approach to the immobilization of ChOx onto micro-chip, which can offer analytical access to a large group of enzymes for a wide range of bio-molecule applications in health-care and biomedical fields. This integrated microchip provides a promising low-cost platform for the sensitive and rapid detection of biomolecules using miniatured samples.

1. Introduction

To assess health status, diseases onset, and monitoring treatments through a non-invasive technique, it is a key attempt to be accomplished in health care promotions and research works. There are three prerequisites to achieve the goals: specific biomarkers that indicates a healthy or diseased state, a non-invasive approach to detect and monitor the biomarkers, and the technologies to discriminate the biomarkers. The early disease diagnosis is crucial for patient survival and successful prognosis of the diseases, so that sensitive and specific techniques are required. Among the numerous health diseases, these are relevant because of their worldwide incidences, prevalences, morbidity and mortality, diabetes, cardiovascular diseases and cancer [1a]. The ability to scrutinize selected biomolecules in real samples with real time approaches using microchips or micro-total analytical system has achieved substantial attention from point of care diagnostic society. The small assembly of a microchip offers many advantages over macro-electrode-based biological devices owing to their developed mass transport and diffusion, high signal-to-noise ratio, and low detection limit. The characteristics of composites and the intrinsic benefits of microchips, including their laminar-flow, low-consumption of costly reagents and power, portability, minimal need for handling biohazardous materials, fast response time, multiplexing, and parallelization are advantageous for the fabrication of microchips [1c]. The biological compound cholesterol (Ch) is a fat that is produced in the liver and intestines. Ch is used to produce hormones and cell-membranes and transported in the blood plasma in mammals. It is a significant structural component of mammalian cell membranes, which is required to establish proper membrane permeability and fluidity. In addition, Ch is an essential component for the manufacture of bile acids, steroid hormones, and vitamin D. Cholesterol is the principal sterol synthesized by animals; however, small quantities can be synthesized in other eukaryotes such as plants and fungi. It is almost completely absent among prokaryotes (bacteria) [1b]. Although cholesterol is essential and important for mammals, high levels of cholesterol in the blood have been linked to damage to arteries and potentially linked to diseases such as those associated with the cardiovascular system (heart disease). Development of cholesterol biosensors in therapeutic diagnostics has newly gained much attention in health care and biomedical fields. With the different experimental parameters, detection of cholesterol in blood sample has been considered incredibly significant since it

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enhances relating with diabetes, heart diseases, nephrosis, and obstructive jaundice, whereas reduced level of cholesterol is owing to mal-absorption wasting syndrome, hypothyroidism, and anemia etc. [2–4]. Among the different detection techniques of cholesterol, biosensing technique has been introduced recently [5]. In the development of a cholesterol sensor, immobilization of an enzyme on a device or chip is usually the primary step in the fabrication of biosensor. The selection of an immobilization method is essential for the performance of biosensor and the future development for fabrication in biosensor design will predictably focus upon the equipment of innovative devices or nanomaterials [6–8] that recommends assurance to resolve the bio-compatibility and bio-fouling problems. Nevertheless, there is a substantial chance for fabrications of cholesterol biosensor with developing sensitivity, large dynamic range, detection limit, stability etc.

High-level of Ch in serum is directly related to different health diseases, like arteriosclerosis, heart diseases, hypertension, cerebral thrombosis, coronary artery disease etc. [9,10]. Ch and its fatty acids are the main components of nerve and brain cells in human health, which are caused by the abnormal levels of Ch in blood. They are also the precursors for other biological chemicals, such as bile acid and steroid hormones, [11]. Hence, the progress of reliable and high sensitive technique for the active and fast detection of Ch is an interesting topic nowadays. It is also enviable to build up a reliable and sensitive Ch biosensor, which can allow a suitable and fast detection of cholesterol from blood sample. Various methods have been commenced for the recognition of cholesterol such as biochemical investigation using radioactive labels [12,13], HPLC analysis [14,15], and electrochemical detection [16,17]. The key drawbacks of these methods are their pitiable sequential and spatial resolutions and the difficulty of the supplementary technical arrangement. Utilization of implantable micro-biosensors could overcome these difficulties with carbon-fiber-based electrodes looking particularly promising [18–21]. General methods for biochemical recognition include HPLC on micro-dialysis samples where an immobilized enzyme column and combined electrochemical detectors are used [22,23]. A recent study was performed on pH sensitive poly (vinylchloride)membrane with a plasma-polymerized film as a potentiometric biosensor for biochemical recognition, where the detection parameters were not satisfactory. However, for this plasma-polymerized film fabricated device, the characteristic curve was not linear and calibration was required. There are a lot of applications for Enzyme-Field-Effect-Transistors for glucose [24,25], urea [26,27], acetylcholine [28,29], and alcohol [30], using various kinds of enzymes. The technique of enzyme on microchips is very essential, where experimental immobilization background is concerned on sensitivity and stability. Sensors based on the principle of field effect in semiconductor structures have been comprehensively premeditated in recent years [31–33]. Recently, a charge-transfer-type pH sensor based on a charge-coupled devices [34,35] was successfully developed, where sensitivity of the sensor was not reached in satisfactory level. In a different methodology, the cholesterol biosensors attained a substantial interest owing to their sensitivity, selectivity, fast response time, repeatability, and stability. The mediator free electrochemical biosensors which are based on the appropriate immobilization of selective enzyme on proper matrixes offer a portable, economical, disposable, and fast technique for the detection of different bio-molecules. In recent times, researchers are persuaded to exploit bio-compatible composite materials as appropriate matrixes for the enzyme immobilizations for the efficient recognition of different biological molecules [36,37]. Among different immobilization methods, the ChOx fabricated micro-chips are one of the most promising matrixes which can be used for the immobilization of different enzymes owing to their numerous interesting properties such as non-toxicity, mediator-free detection, high-surface area, low-sample volume needed, fast-response time, chemical stability, highly-sensitive, ease of handling, and ease of enzymatic fabrication.

In this report, a highly sensitive Ch biosensor based on self-assembled monolayer modified micro-chips is developed at room conditions, which is investigated by single-step CV approaches in phosphate buffer system. The micro-chip is designed and fabricated successfully using photolithographic technique. The most significant characteristics of developed Ch-biosensor using micro-chip are highly sensitivity, large-linear dynamic range, very low-detection limit, highly stability and reproducibility, which required a microlevel sample volume.

2. Experimental

2.1. Reagents and equipments

N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), thioglycolic acid (TGA), monosodium phosphate, cholesterol (Ch), disodium phosphate, and cholesterol oxidase (ChOx) were obtained from Sigma-Aldrich company (USA). All other chemicals were of analytical grade and used without further purification. 0.1 mol L^{-1} PB (pH ~ 7.1) is prepared by mixing the uni-molar proportion of 0.2 mol L^{-1} NaH_2PO_4 and 0.2 mol L^{-1} Na_2HPO_4. All solutions were prepared with de-ionzed distilled water, which was obtained from a water purifying apparatus (12.0 MΩ cm) (AQUA MEDIA). The electrochemical experiments were performed using a votammetric analyzer (CV-50 W, BAS, USA). All investigations were carried out onto micro-chip (5.0×5.0 mm^2), of which the sensing area is 0.0805 cm^2. The total examinations were done with enzyme modified micro-chips composed as working, Pt layer as counter, and an Ag/AgCl (saturated KCl) as reference electrodes. Cyclic voltammetry (CV) was recorded at ChOx/TGA/AuE electrode from −0.1 to +0.5 V (versus Ag/AgCl) in a 0.1 mol L^{-1} PB (pH 7.1) at 100.0 mV s^{-1} scan rates. The total experimental setup is presented with camera-view photographs below (Fig. 1). Here, the potential analyzer (A, CV-50 W) is controlled and interfaced with SOTECH-PC (B), which is directly connected with the lab-set electronic system (C) with micro-chips (magnified view, D).

2.2. Construction of micro-chips

Electrochemical chips were constructed by conventional photolithographic technique, where electrodes and passivation layers of chips are fabricated on silicon wafer followed by dicing and packaging. Nitrogen-doped silicon wafers are prepared and overflowed by extra-pure de-ionized water. Here, the contaminants on the surface as well as native SiO_2 layer are removed. At first, the

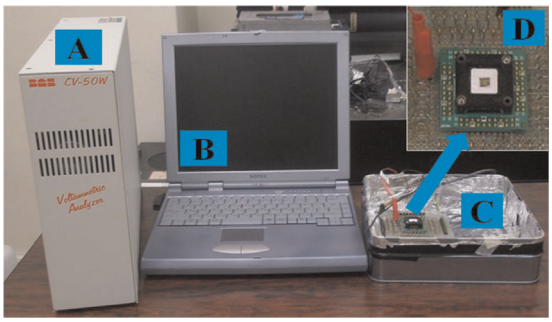

Fig. 1. Schematic diagram of micro-chip setup with all assembly. Camera view experimental setup of (A) potential analyzer (CV-50 W), (B) controlled and interfaced by PC, (C) micro-chip installed with electronic board for data collection, (D) real and magnified view of micro-chips.

wet oxidation technique is processed and then dry oxidation is executed. Wafers are annealed in the condition of nitrogen system, and then Aluminum is sputtered with Al-1% Si target. After that the photolithograph processes are applied. By Kanto chemicals, resist coating, baking, exposure, and development are executed, and then it is rinsed thoroughly by ionic water. Aluminum is etched by etching solution. Resist is removed by plasma etching instrument. Then wafers are cleaned by acetone, methanol, and finally by plasma simultaneously. Silicon nitride layer is deposited by chemical vapor deposition. Surface of pad electrodes is etched by reactive ion etching. Finally residual resist layer is removed by plasma acing. After photolithographic process, Pt is sputtered by SP150-HTS. Then it is patterned by lift-off technique, in which wafers are immersed into the remover, and then washed with isopropyl alcohol. Photolithographic process is again investigated, where Ti is sputtered as a binding layer, and then Au is evaporated by deposition. Finally, gold layer is patterned by lift-off procedure. Palylene passivation layer is formed for the protection of the chip from water. Photolithographic technique is carried out again for pad protection. Then palylene-dimer is evaporated by deposition apparatus. Photolithography process is made again for patterning. Palylene layer is patterned by etching. Finally, unnecessary resists are removed by acetone and then wafer is cleaned by isopropyl alcohol. Resist is coated on a whole surface of the wafer for protection during dicing process. Si wafer is diced into pieces by dicing apparatus and stored into the desiccators. Resist on chip surface is removed by acetone and cleaned with IPA. The backside of the chip is roughed by a sheet of sandpaper for better adhesion and electrical stability. The chip is bonded with die and packaged by silver paste. It is dried in a drying oven. Pads on chip are connected to the package through gold wire with bonding machine. Finally, Si-based adhesive is put on the periphery of the chip to protect pads and gold wire from sample solution. Adhesive is dried at room temperature for 24 h. The composition with thickness of each fabricated layer into micro-chips is Si-wafer material (500.00 μm), SiO_2 insulation (0.40 μm), Al electric wiring (1.00 μm), SiN protection/separation (1.00 μm), Ti/TiN binding (0.15 μm), Ti binding (0.10 μm), Pt counter electrode (0.25 μm), Au working electrode (0.30 μm), and Palylene passivation/protection (1.00 μm).

The semiconductor micro-chips were made on silicon wafer. Aluminum was sputtered to fabricate as wiring and bonding pads. Pt/Ti/TiN was sputtered on thermal silicon oxide and patterned by photolithography to fabricate counter electrode (CE). Ti/TiN layers were used for strong adhesion. Au/Ti was sputtered and lithographed, which made circular working electrode (WE) with a diameter of 1.6 mm in the center of the chip. After electrodes fabrication, palylene layer was fabricated by evaporation method as a passivation layer. The wafer was diced to 5.0 × 5.0 mm^2 chips. This chip was bonded to a package by silver paste. Aluminum pads were connected to the package by gold wire. Finally, adhesive (Araldite, Hantsman, Japan) was put on the periphery of the chip to prevent target solution from contacting pads, which is presented in Fig. 2A. The magnified construction view of internal chip-center (sensing area) is shown in Fig. 2B. A cross section of the total sensor chip and sensing area is presented in Fig. 2C and D respectively.

3. Results and discussion

Cyclic voltammetry (CV) is a type of conventional potentiodynamic electrochemical measurement. In CV experiment, the working electrode potential is ramped linearly versus time. A standard CV experiment generally uses a reference electrode (here, Ag/AgCl in saturated KCl), working electrode (here, AuE surface of microchips), and counter electrode (here, Pt-electrical line into microchip). These

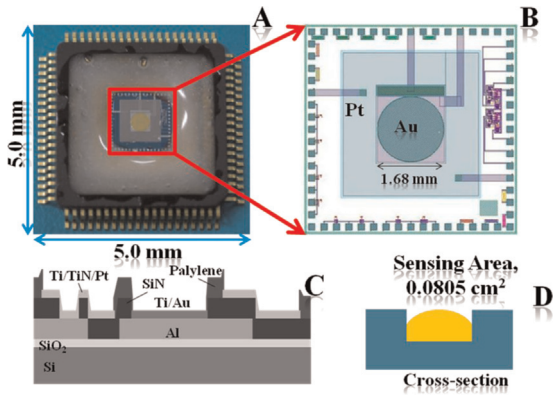

Fig. 2. Schematic diagram of micro-chip situation Schematic diagram of (A) top camera-view, (B) magnified view of sensing area, (C) total cross-sectional view, and (D) cross-section of sensing area of micro-chip.

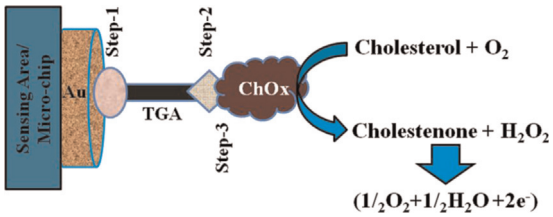

Fig. 3. Fabrication pattern. Schematic view is presented about the fabrication procedure of cholesterol biosensor on micro-chips.

combinations are referred to as a three-electrode setup. In our experimental approach, reference electrode (Ag/AgCl) is vertically contacted onto the SAM/ChOx fabricated center of microchip. An electrolyte/mediator is usually used ($K_3Fe(CN)_6$/PB) to the target sample solution to ensure sufficient conductivity. The PB-solvent, electrolyte, and material composition (TGA-SAM/ChOx) of the working electrode are determined in the potential range that can be accessed during the experiment. Here, Fig. 3 sketches the sensing procedure using the ChOx/TGA/Au-modified micro-chip by single step approach. It is used for covalent bond formation to immobilize the ChOx enzyme on the TGA-SAM via peptide conjugation in the presence of activating agent (EDC). Firstly, the self-assembled monolayer of TGA is formed by dipping the micro-chips into TGA solution for two hours. Then ChOx enzyme is immobilized onto TGA-SAM by the amide bond formation between the terminal-unbound carboxylic acids groups on the TGA film and the amine groups of the ChOx enzyme.

For the constant covalent attachment of ChOx onto TGA-SAM, the micro-chip was kept for 24 h into the refrigerator at 4.0 °C. The enzymatic reactions involved in the bio-sensing system for the detection of cholesterol are as follows (see Fig. 3):

$$\text{Cholesterol} + O_2 \rightarrow \text{Cholestenone} + H_2O_2 \text{ (in presence of ChOx)} \quad (1)$$

$$H_2O_2 \rightarrow 1/2O_2 + 1/2H_2O + 2e^- \quad (2)$$

In reaction (1), it was directly dependent on cholesterol concentration in the reaction medium. On micro-chip, Ch is oxidized to form cholestenone and H_2O_2 in the presence of ChOx. Then H_2O_2 is auto-dissociated to produce the current (presented in reaction (2)). This current is directly proportional to the cholesterol concentration in the mediator/PB solution system, which measured by single step CV approach.

The fabrication of cholesterol biosensor by using ChOx onto sensing area of tiny micro-chip followed by the TGA-SAMs self-assembly has been confirmed. The electrochemical technique, CV is the most versatile electro-analytical technique for the study of

bioactive materials and species, which is extensively used in industrial applications and academic research works as well as R&D approaches. CV is also a significant method to assess the blocking property of the monolayer-coated electrodes using diffusion controlled redox couples. Chip surface is cleaned by Piranha solution [H_2SO_4:H_2O_2 (3:1)] and washed with pure water, and then dried adequately by nitrogen. TGA was dissolved in ethanol to make 10.0 mmol L^{-1} solution. TGA solution was dropped onto micro-chip sensing area and then kept wet for two hours at room conditions. Fig. 4 shows the CVs of un-modified and TGA-SAM modified micro-chip electrodes in 5.0 mmol L^{-1} $K_3Fe(CN)_6$ with 0.1 mol L^{-1} PB as the supporting electrolyte at 0.1 V/s scan rates. It can be seen from Fig. 4A that the micro-chip electrode (black-curve) shows a reversible voltammogram for the redox couple indicating that the electron transfer reaction is completely diffusion controlled. In contrast, the absence of any peak formation in the CVs of the TGA monolayer modified electrodes (blue-curve) shows that the redox reaction is either inhibited or totally blocked. The CVs for TGA signified a good blocking behavior for the electron transfer reaction, which means that a TGA-monolayer which is highly ordered, and compact formed on the sensing surface of micro-chips uniformly [38,39].

Selective enzyme (ChOx) was immobilized onto the TGA-SAM surface by the phenomena of peptide conjugation. First 10.0 mmol L^{-1} EDC in 0.1 mol L^{-1} PB was put onto the TGA-SAM micro-chip and then it was kept in refrigerator at 4.0 °C to activate carboxylic group of TGA for 24 h. Then EDC-treated electrode was washed slowly with 0.1 mol L^{-1} PB to eliminate excess EDC. Then ChOx solution was dropped onto the sensing area (center) of micro-chip and incubated in refrigerator at 4.0 °C for 24 h. ChOx was effectively immobilized onto TGA SAM via covalent attachment, which was confirmed by the current change in Fig. 4B. It showed that the CVs were recorded for the bare-surface (black curve), ChOx/TGA/Au (blue curve), and 0.1 mmol L^{-1} cholesterol solution (green curve) of fabricated micro-chip in 0.1 mol L^{-1} PB at 0.1 V/s scan rates. According to the control experiment, no significant change was executed when the CV was recorded with the bare-surface for 0.1 mmol L^{-1} cholesterol in PB owing to the absence of ChOx enzyme. A small current change was observed at approximately +0.32 V versus Ag/AgCl (saturated KCl) for 0.1 mmol L^{-1} cholesterol solution and this was due to the current of enzymatic reaction with cholesterol in the presence of ChOx on the sensing surface of the micro-chips. The enzymatic current of approximately +0.32 V was executed to be increased with the increasing cholesterol concentration in the PB buffer solution. The experimental conditions affecting the performances (detection limit, sensitivity, and response time) of the sensor were optimized in term of pH and presented in Fig. 4C. The pH of the buffer shows a strong effect on the activity of the sensing layer on ChOx enzyme fabricated micro-chips. The effect of the pH of the buffer on the current change was studied over the pH range of 3.0–9.1. Fig. 4C shows the CV peak currents measured at various pH values for

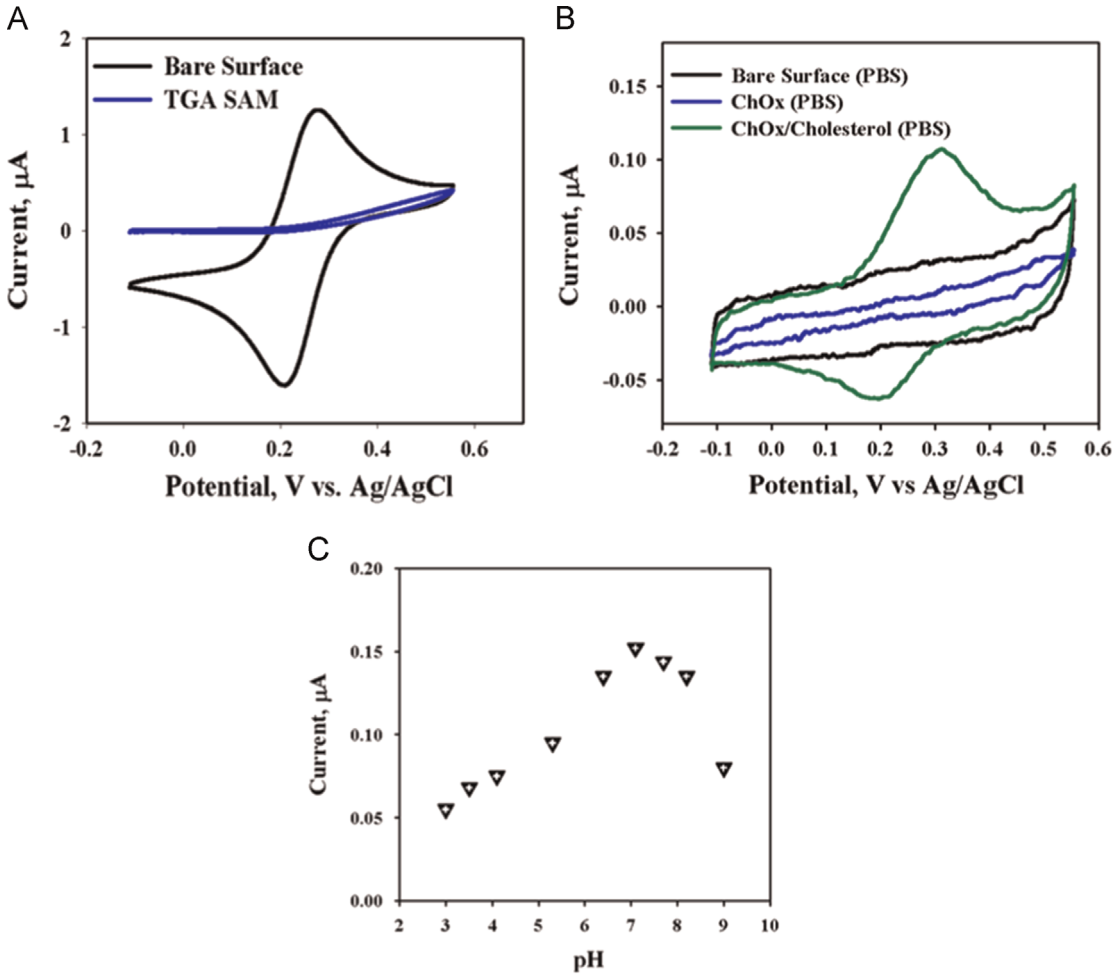

Fig. 4. Optimization of biosensors. (A) CVs of 5.0 mmol L^{-1} $K_3Fe(CN)_6$/PBS (0.1 mol L^{-1}) for bare (black curve) and TGA-SAM (blue curve) modified electrodes; (B) CVs recorded in 0.1 mol L^{-1} PBS of bare chip (black curve), ChOx modified chip (blue curve), and in presence of 0.1 mmol L^{-1} cholesterol (green curve) solution; (C) Effect of pH of ChOx/TGA/Au electrode in 0.1 mmol L^{-1} cholesterol solution with the micro-chip. Scan rate: 0.1 V s^{-1}, RE: Ag/AgCl (saturated KCl), supporting electrolytes: 0.1 mol L^{-1} PBS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

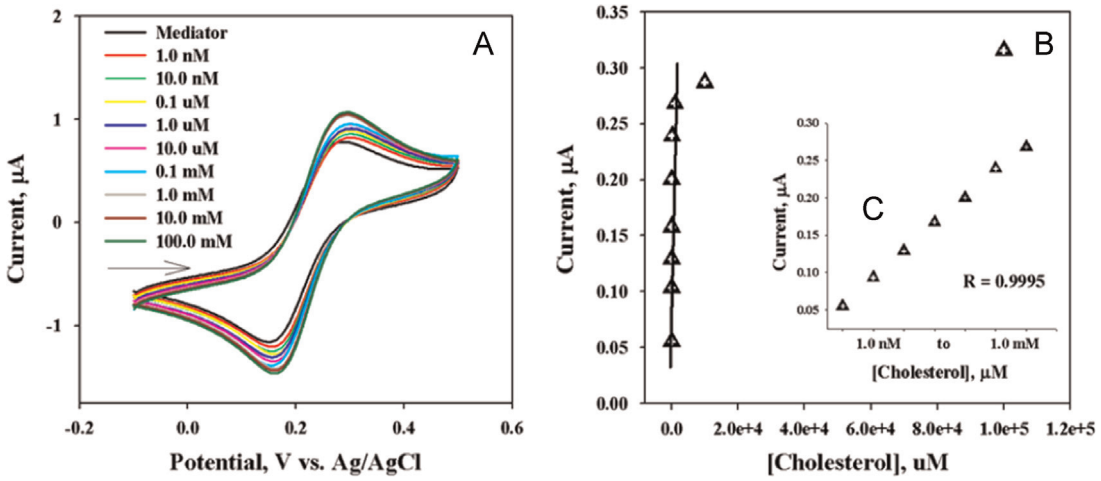

Fig. 5. Performances of fabricated biosensors by electrochemical approach. Electrochemical sensor responses in 0.5 mmol L^{-1} $K_3Fe(CN)_6$/0.1 mol L^{-1} PBS; (A) variation of cholesterol concentrations (1.0 nmol L^{-1} to 100.0 mmol L^{-1}), (B) calibration curve, and (C) linearity of developed on micro-chip at room conditions.

Table 1
Evaluation of the ChOx/TGA/Au-chip cholesterol sensor performances compared with various enzyme–material conjugated sensors.

Electrode materials	Sensitivity	Detection limit	Sample volume (µL)	Response time (s)	Linear dynamic range (LDR)	Linearity (R)	References
ZnO nanoparticles + chitosan composite	14.1 µA µmol L^{-2} cm^{-2}	0.125 × 10^3 µmol L^{-1}	–	15	(0.125–7.76) × 10^6 µmol L^{-1}	–	[40]
ZnO nanoporous thin film	–	–	–	15	(0.65–10.35) × 10^6 µmol L^{-1}	–	[41]
Tertraethylorthosilicate	–	0.50 µmol L^{-1}	–	50	(2.0–10.0) × 10^3 µmol L^{-1}	–	[42]
ZnO nanorods	61.7 µA µmol L^{-2} cm^{-2}	0.012 µmol L^{-1}	–	5	1.0–15.0 µmol L^{-1}	0.9979	[43]
Polypyrrole films	15.0 µA µmol L^{-2} cm^{-2}	–	–	–	1.0–8.0 × 10^3 µmol L^{-1}	–	[44]
ChOx/NanoPt/CNT	–	–	–	< 20	4.0 × 10^{-6}–1.0 × 10^{-4} mol L^{-1}	–	[45]
ZnO nanoparticles	23.7 µA µmol L^{-1} cm^{-2}	0.37 nmol L^{-1}	–	5	0.001–0.5 µmol L^{-1}	–	[46]
Nanoporous CeO_2 film	5.98 µA µmol L^{-2} cm^{-2}	–	–	15	1.3–10.35 × 10^6 µmol L^{-1}	–	[47]
ChOx/CS/MWCNT	1.55 µA mmol L^{-2}	–	–	< 20	4.0 × 10^{-6}–7.0 × 10^{-4} µmol L^{-1}	–	[48]
Ni/$K_3Fe(CN)_6$/CNT	–	–	–	< 20	0.005–3.0 mmol L^{-1}	–	[49]
ChOx/NanoZnO-CHIT	1.41 × 10^{-4} A mg dL^{-1}	–	–	15	05–300 mg dL^{-1}	–	[50]
ChOx/TGA/Au-micro-chip (mediator)	**93.75 µA µmol L^{-2} cm^{-2}**	**0.49 nmol L^{-1}**	**50.0**	**10**	**1.0 nmol L^{-1} to 1.0 mmol L^{-1}**	**0.9995**	**Current work**

DL: ~3N/S nmol L^{-1}; S: slope of the calibration curve; N: noise of the signal; sensitivity: µA µmol L^{-2} cm^{-2}

0.1 mmol L^{-1} cholesterol in 0.1 mol L^{-1} PB system. The peak height increased from pH 5.6 to 7.1 and then decreased above pH 7.1 until 9.1. The decrease of peak current above pH 7.1 might have been owing to the poor ChOx enzyme activity at higher basic medium. Therefore, the pH of the PB system was kept constant at 7.1 throughout the experiment.

Cyclic voltammetry was investigated to confirm the detection of Ch concentration with mediators in simple 0.1 mol L^{-1} PB electrolyte system. 50.0 µL of each cholesterol solution with mediator/electrolyte was dropped on the sensing area of the micro-chip and the sensing oxidation current was measured. Fig. 5A shows a typical CV (current–voltage) plot for the addition of varying amounts of cholesterol in a 0.5 mmol L^{-1} $K_3Fe(CN)_6$/0.1 mol L^{-1} PB (pH 7.1). The currents increased steadily with increase of the cholesterol concentration (1.0 nmol L^{-1} to 100.0 mmol L^{-1}) in stable and saturated value. The ChOx/TGA/Au modified micro-chip electrode achieved 96% of steady state currents within 10.0 s. The increase of oxidation current was presented due to the cholesterol oxidized in the presence of ChOx and the current change leads to the higher current value. Fig. 5B shows the calibration plots for the cholesterol found from the current–voltage responses with fabricated micro-chips. Under the optimized conditions, the steady-state currents showed a linear relationship with the cholesterol concentration in the range from 0.1 nmol L^{-1} to 1.0 mmol L^{-1}, which is shown in Fig. 5C.

The linear dependence of the fabricated cholesterol sensor is presented with a correlation coefficient (r^2) of 0.9995. The detection limit for cholesterol was calculated from the calibration curve to be 0.49 ± 0.02 nmol L^{-1}, based on signal to noise ratio (3N/S) in the concentration range (1.0 nmol L^{-1}–100.0 mmol L^{-1}). The cholesterol sensor was also shown to have a higher sensitivity as of 93.75 ± 0.5 µA µmol L^{-2} cm^{-2}. The sensitivity is much higher than the previously published cholesterol sensors, where the total comparative performances are concluded in Table 1 [40–50]. The sensor performances of the ChOx fabricated micro-chip are compared with the previously reported cholesterol biosensors based on the enzymes conjugated materials or nanomaterials and it was confirmed that the ChOx/TGA/Au-micro-chip cholesterol biosensor exhibited excellent results.

A series of successive measurements of cholesterol in 0.5 mmol L^{-1} $K_3Fe(CN)_6$/0.1 mol L^{-1} PBS yielded a good reproducibility signal at ChOx/TGA/AuE sensor with a relative standard deviation of (RSD) 3.4%. The sensor-to-sensor and run-to-run reproducibility for 0.1 mmol L^{-1} cholesterol detection was found to be 1.5% and 1.2% respectively. To study the long-term storage stabilities, the response of the ChOx/TGA/AuE sensor was measured with the respect to the storage time. After every experiment, the sensor was washed with the buffer solution and stored in 0.1 mol L^{-1} PB at 4.0 °C until use. The long-term storage stability of the sensor was tested every three days. The sensitivity retained

84% of initial sensitivity up to one month. After one month, the response slowly decreased, probably due to the loss of the enzyme activity on the sensor micro-chip. The above results clearly indicated that the micro-chip of cholesterol sensor can be used for a month without any significant loss in sensitivity. The selectivity (interference effect) of the ChOx/TGA/Au sensor was evaluated in the presence of other electroactive compounds such as lactate, glucose, glutamate, uric acid etc. No significant current response was observed when 0.1 mmol L^{-1} of lactate, glucose, uric acids, and glutamate was introduced into the 0.1 mol L^{-1} PB buffer. But when 0.1 mmol L^{-1} cholesterol solution was added to the electrolyte solution, a clear oxidation response was observed, indicating the selective detection of cholesterol with the ChOx/TGA/Au sensor layer. Significantly, at this concentration level, lactate, glucose, uric acid, and glutamate have no interfering for 0.1 mmol L^{-1} cholesterol detection. Thus, the selectivity of the ChOx/TGA/Au micro-chip sensor is acceptable for cholesterol detection in the presence of the common interfering compounds in normal physiological levels.

4. Conclusions

Successful fabrication of highly sensitive and selective cholesterol sensor by ChOx immobilization onto tiny micro-chip has been examined the in presence of active redox mediators. Sensor micro-chip was constructed by conventional photolithographic technique, which was used to detect the Ch-solution of micro-low-level. The miniaturized microchip based on a Au/TGA-SAM/ChOx allows for the rapid detection of biomolecules at economical approach. The sensor demonstrated higher sensitivity, low-detection limit with satisfactory stability, and a large linear dynamic range as well as reproducibility. The simple self-assembly fabrication process has several advantages over conventional method such as ease of fabrication, enhanced electro-catalysis, and efficiently preserving the activity of bio-molecules. This mediated Ch-biosensor by micro-chip can be used to detect the selective Ch in serum samples. This ChOx-enzyme integrated microchip was highly sensitive to Ch detection, both highly reproducible as well as stable, and allowing the SAM/Chip assembly to be utilized for total cholesterol monitoring. The new approach exhibits lower detection limits and a faster response time. The highly efficient SAM-assembly onto tiny microchip could be used to create an array-based microchip that simultaneously monitors the multi-analytes, including low-density lipoproteins and triglycerides. It would be a promising platform for biomedical applications as well as in biochemical determination in health-care biological and biomedical fields.

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