Anal. Bioanal. Electrochem., Vol. 4, No. 1, 2012, 70 - 82

Analytical &
Bioanalytical
Electrochemistry

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Full Paper

Application of Coulometric FFT Cyclic Voltammetry for Determination of Cholesterol Based on Cholesterol Oxidase Nano-Biosensor

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Received: 20 July 2011 / Accepted: 1 February 2012 / Published online: 28 February 2012

Abstract- In this work, a new electrochemical system was developed for determination of cholesterol based on coulometric fast Fourier transformation cyclic voltammetry (CFFTCV) in a flow injection analysis system. In this detection method, also a new cholesterol oxidase biosensor was designed by immobilizing the enzyme into a cross-linked matrix of chitosan–room-temperature ionic liquid (1-butyl-3-methylimidazolium tetra fluoroborate). Initially, the surface of a glassy carbon electrode was modified with CeO_2 NPs and then by the electrodeposition of Au particles onto the thiol functionalized multiwalled carbon nanotubes. Scanning electron microscopy and impedance spectroscopy was use to characterize the biosensor. The presence of Au and CeO_2 nanoparticles in the matrix of provides an environment for the enhanced electrocatalytic activities. Under optimal conditions, the biosensor exhibited a linear response to cholesterol in the concentration range of 0.01–10 μM with a correlation coefficient of 0.99, good sensitivity, a low response time, repeatability (R.S.D value of 1.9%) and long term stability, 45 days with a decrease of 4% response .

Keywords- Cholesterol, Biosensor, Au Nanoparticles, Coulometric FFT Cyclic Voltammetry

1. INTRODUCTION

It is well known that cholesterol is a structural component of biological membranes and it can be found in nerve tissues, brain, skin, adrenal glands and liver and is the main ingredient in the fatty sheet that insulates the nerve. Therefore, the development of a biosensor for

determination of cholesterol concentration in human blood is important due to the prevalence of cardiovascular diseases as a major health threat and clinical diagnosis of heart disease, brain thrombosis around the world. However, the classical spectrometry method of determination of cholesterol by suffered from poor specificity, instability of color forming agent and standardization difficulties. Electrochemical biosensors have shown to be very effective tools for analysis of biological important molecules. Immobilization of an enzyme onto the substrate (matrix) is a crucial step for the construction of enzyme based biosensors. They are very simple, fast, inexpensive, portable and capable of reliable response in wide concentration ranges [1,2].

Different matrices have been used to immobilize enzymes on the surface of electrodes, towards fabrication of cholesterol biosensors. Support matrices consisting of either a single component or multi components have been tried. A recent review presents the details on varieties of matrices used for the immobilization of cholesterol oxidase (ChOx) [3] and characteristics of the fabricated cholesterol term biosensors. Nanomaterials prepared from metals, shaped into nanoparticles have been widely used for their ability as electrode modification materials to enhance the efficiencies of electrochemical biosensors. CeO_2 NPs can catalyst the decomposition of hydrogen peroxide. Therefore, the produced O_2 from the decomposition of hydrogen peroxide can be reloaded in the cholestrol oxidation reaction and the dynamic range of biosensor is extended [4,5].

Carbon nanotubes have many properties that make them ideal as components in electrical circuits, including their unique dimensions and their unusual current conduction mechanism. Using MWCNTs can improves significantly the conductivity and conversion of the chemical signal to noise. By increasing the dynamic working range and response time of the sensor improve [6-8].

This work introduces a new electrochemical method for determination of cholesterol combine with coulometric FFT cyclic voltammetry (CFFTCV) technique in a flow injection analysis system [9-13] and using a new biosensor. To the best of our knowledge, this is the first application of CFFTCV method for cholesterol biosensor based on cholesterol oxidase (ChOx). Fig. 1 shows the enzymatic reactions in the use of ChOx as a receptor can be described as follows:

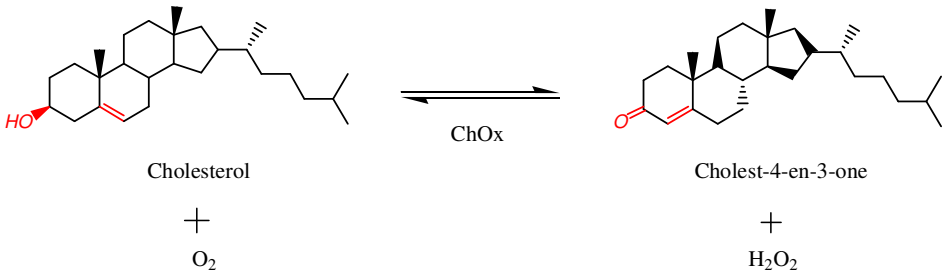

Fig. 1. Schematic diagram of ChOx enzymatic reaction

This is figure shows that the cholesterol is catalyzed by ChOx in the presence of oxygen, and accordingly hydrogen peroxide is produced at the enzyme belong to the family of reductases. The oxidation current of hydrogen peroxide, which is produced at the same time, is detected after application of a suitable potential to the system.

In this biosensor, initially, the surface of bare glassy carbon (GC) electrode was modified with the CeO_2 nanoparticle and electrodeposition of Au nanoparticles onto thiol functionalized multiwalled carbon nanotubes and then ChOx was immobilized onto a cross-linked matrix of chitosan–room-temperature ionic liquid (1-butyl-3-methylimidazolium tetra fluoroborate). The important parameters were optimized for improving the detection method.

2. EXPRIMENTAL

2.1. Materials and methods

Cholesterol oxidase (ChOx) (100U/mg) was purchased from Sigma-Aldrich. Cholesterol (95%) was purchased from Aldrich. 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM-BF_4, ionic liquid, IL) were of analytical grades and from Merck Co. CeO_2 particles purchased from SHBI & Exp. Co., Ltd. The multi-wall carbon nanotubes (MWCNTs) with 10-40 nm diameters, 5-20 μm length, and 700 m^2/g and with 95% purity were purchased from Research Institute of the Petroleum Industry (Iran). Chitosan (85%) (Chi) and Triton-X 100 (polyoxyethylene isooctylphenyl ether) were obtained from Sigma-Aldrich. All other chemicals are of analytical grade form Merck Co. and are used without further purification. The solutions are prepared in deionized distilled water. 0.5 g of Chi was dissolved in 100 mL of 1.0% (v/v) acetic acid and ultrasonicated for 30 min. Cholesterol (3 mM) was prepared in phosphate buffer (PB) 0.02 M; pH 7.0 in a 50 mL volumetric flask containing 1 mL isopropanol and 5 mL Triton X-100 and the solution was kept at 50 °C for 1 h .

2.2. Biosensor preparation

A glassy carbon electrode, GCE, (3 mm in diameter) were polished well with 1.0, 0.3 and 0.05 μm alumina slurry and then it was washed thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water,

and then allowed to dry at room temperature. The fabrication of CeO_2NPs/MWCNT (SH) Au NPs/ChiIL/ChOx biosensor electrode involves three sequential stages. First, formation of CeO_2NPs/MWCNT (SH) AuNPs, in which, MWCNTs were thiol (SH) functionalized to MWCNT(SH) using 4-aminothiophenol as the linker. In the second step, for formation of CeO_2NPs/MWCNT(SH) AuNPS/ChiIL, 8 mg of MWCNT(SH) was suspended in 10 mL of dimethylformamide and sonicated for 5 min. Then, 100 μL of the suspension was dropped onto the surface of GC which is previously modified by CeO_2NPs and casted as a film by drying at 50 °C for 14 h. Au NPs were then deposited electrochemically onto the CeO_2NPs /MWCNT(SH) from a solution of 5.0×10^{-4} M $HAuCl_4$ by cycling the potentials with a scan rate of 100 mV/s between 1.15 and 0 V (*vs*. Ag/AgCl). Finally, ChiIL mixture was drop coated onto GC/CeO_2NPs /MWCNT(SH) Au electrode. After being washed with water and dried, 8 μl of ChOx was dropped onto the surface of GC/CeO_2NPs/MWCNT(SH) Au NPs/ChiIL to fabricate the biosensor. The modified electrodes were washed thoroughly with PB solution at pH 7 containing 1.0% NaCl and 1% Triton-X 100 and stored at 4 °C.

2.3. Instrumentation

The electrochemical measurement system used, for cyclic voltammetric measurements, was a homemade potentiostat, which was connected to a PC PIV outfitted with an analog to digital data acquisition board (PCL-818H, Advantech Co.), which was used to generate an analog waveform and acquire current readings. In the measurements, the memory and CPU requirements of the computer were dictated by the condition of the data acquisition requirements electrochemical software was developed using Delphi 6.0. The potential waveform was repeatedly applied to the working electrode and then the data was acquired, and stored by the software. Also, in this electrochemical setup, the data could be processed and plotted in real time, or the stored data could be loaded and reanalyzed to generate voltammogram. EIS measurements were performed in 0.5 mM $K_3Fe(CN)_6$ in PB at pH 7 .

2.4. Flow Injection Setup

The flow injection analysis equipment integrated with an eight roller peristaltic pump (UltrateckLabs Co., Iran). Four way injection valve (Supelco Rheodyne Model 5020) with 200 μL sample injection loop. The analyte solutions were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flow injection analysis is shown in Fig. 2.

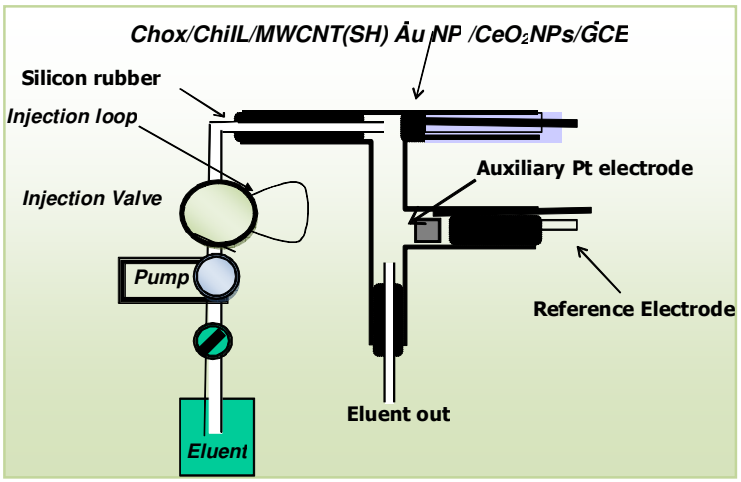

Fig. 2. The diagram of cholesterol biosensor and the electrochemical cell used in flow injection analysis

3. RESULTS AND DISCUSSION

Fig. 3 shows results of the SEM studies carried out on. This SEM image of GC/CeO_2NPs/MWCNT(SH) AuNps/ChiIL/ChOx the presence of uniformly distributed spherical clusters of Au Nps with cluster diameters in the range 200-800 nm, which reveals the presence of interconnected of ChiIL composite on the surface of electrodes.

Fig. 4 shows results of electrochemical impedance spectroscopic (EIS) measurements carried out on CeO_2Nps/MWCNT(SH) AuNPs/ChiIL and CeO_2Nps/MWCNT(SH) AuNPs /ChiIL/ChOx electrode in PB solution containing 3 mM $[Fe(CN)_6]^{3-/4-}$. In Randles circuit, it is assumed that resistance to electron transfer and the diffusion impedance is parallel to the interfacial double layer capacitance.

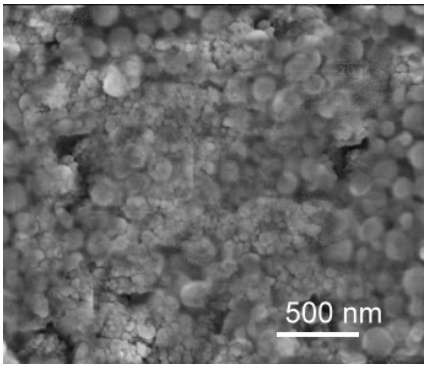

Fig. 3. SEM images of GC/CeO_2NPs/MWCNT(SH) Au NPs/ChiIL/ChOx

In this scheme, the semicircle portion, observed at higher frequencies, corresponds to electron-transfer-limited process, whereas the linear part is characteristic of the lower frequencies range and represents the diffusion-limited electron-transfer process. It can be seen that semicircle diameter of curve b is larger than that of curve a, indicating higher electron transfer resistance at the electrode interface. This increased value of resistance to electron transfer indicates hindrance to the electron transfer indicating immobilization of insulating ChOx.

For determination of cholesterol, the current passing through the biosensor was sampled during the potential ramp, and then, coluometric signal was calculated by integrating of net current changes is applied over the selected scanned potential range. The biosensor response (charge change under the peak) was calculated as;

$$\Delta Q = Q - Q_0 \quad (1)$$

Where, Q is the electrical charge obtained by integration of cyclic voltammetric curve between 0 and -800 mV in the chatodic scan, and Q_0 represents Q in the absence of the adsorbent. The peaks in this method, ΔQ are calculated based on the -current changes at the CV in the integration potential range. Moreover, the results indicate that with increasing the concentration of cholesterol in the injected sample, ΔQ increases proportionally .

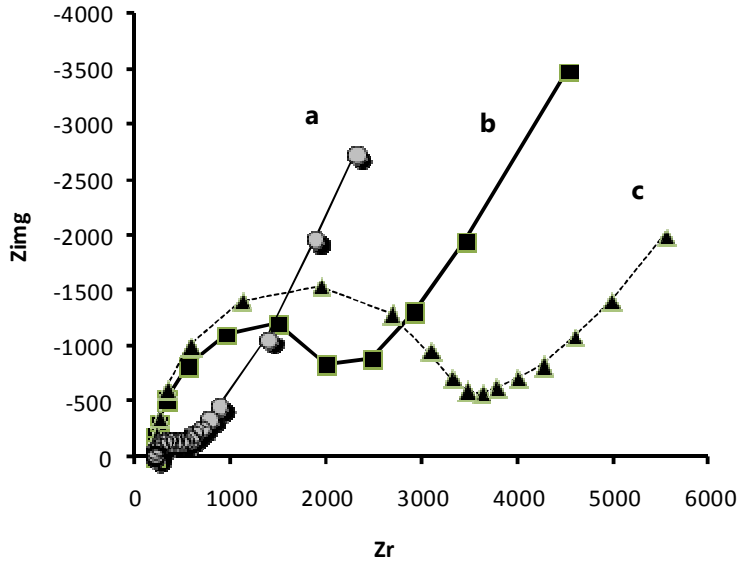

Fig. 4. EIS plots of modified electrode in 3 mM $K_3Fe(CN)_6$ with 0.02 M KCl: (a) bare GCE (b) CeO_2NPs/MWCNT(SH) AuNPs/ChiIL and (c) CeO_2NPs/MWCNT(SH) AuNPs/ChiIL /ChOx

The cyclic voltammetric studies for GC/CeO_2NPs/MWCNT(SH) AuNPs/ChiIL/ChOx electrodes in PB solution containing 3 mM $[Fe(CN)_6]^{3-/4-}$ shows a well-defined reversible redox peak with increased value of the peak current, which was not seen for the GC electrode. It was seen that the peak current increases due to electrostatic repulsion between ChOx immobilized on the CeO_2NPs/MWCNT(SH) AuNPs/ChiIL electrode and the anionic redox couple ions. It is likely seems these nano structures facilitate immobilization of ChOx. Consequentiality, under such condition the active sites expose for its easier access to the substrate leading to improved coulometric response of the biosensor. The results obtained using cyclic voltammetry measurements are in agreement with the results obtained from EIS. Fig.5 shows CFFTCV and the changes in voltammetric of the GC/CeO_2NPs/MWCNT(SH) AuNPs /ChiIL/ChOx biosensor in the potential range of -600 to 1200 mV at potential sweep rate is 4 V/s. The potential axis on this graph represents potential applied to the working electrode during each sweep. The time axis represents the time passing between the beginning of the flow injection experiment and the beginning of a particular sweep (i.e. it represents a quantity proportional to the sweep number) [14-16]. The figure shows that after injection of 250 μL of 5.0×10^{-5} M cholesterol in the PB buffer solution, at pH 7, a signal appears at potential 680 mV. The integration range for the current is in range of -100 to 600 mV. The reference cyclic voltammogram was calculated by averaging of a 5 cyclic voltammograms, recorded before injection of the analyte. The increase in the current at potential 500 mV can be due to the generation of H_2O_2 during the oxidation of cholesterol at electrode surface by ChOx. However, as mentioned above the attachment of the ChOx to high surface area of MWCNT/CeO_2NPs facilitates a higher rate of direct electron transfer between the active sites of immobilized ChOx. This can increase the peak current at the recorded cyclic voltammograms when the sample was injected.

The results show that with increasing the concentration of cholesterol in the injected sample, the current increases. This confirms a good electrocatalytic and fast electron exchange behavior of modified electrode at high potential sweep rate .Once CFFTCV is used to monitor a flowing system, cholesterol electrochemical processes will cause a measurable change in the peak current at the voltammogram. According to Fig. 1, the electrochemical reaction for the detection of cholesterol in presence of ChOx is proposed and the enzymatic reaction, which produce H_2O_2.

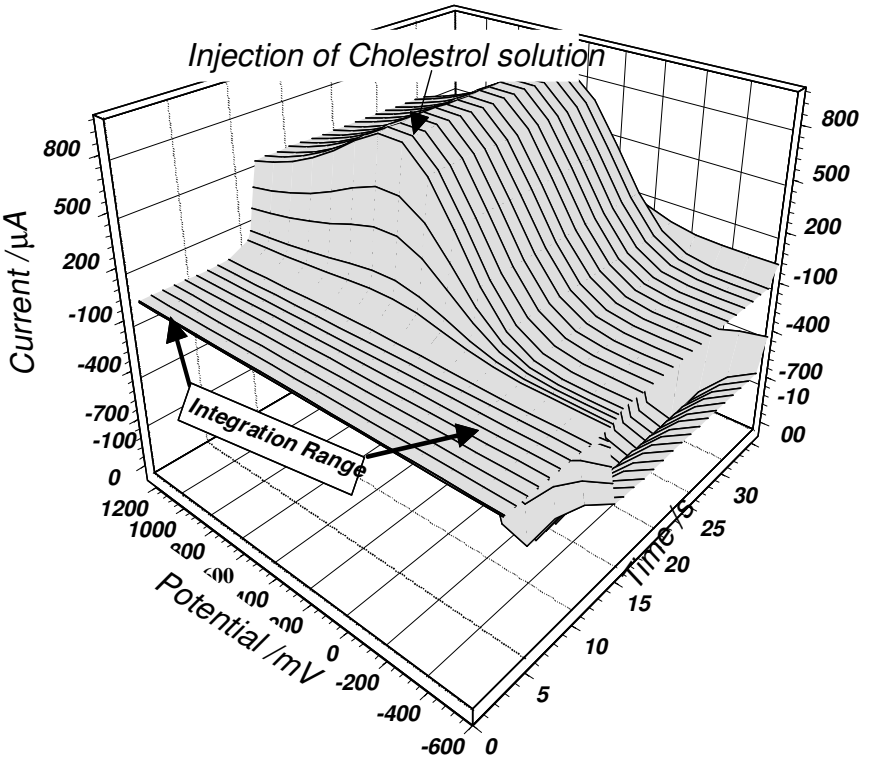

Fig. 5. FFT cyclic voltammogram of the GC/CeO_2NPs/MWCNT(SH) AuNPs/ChiIL/ChOx biosensor without (in absent) and with injection of 250 µL of 5.0×10^{-5} M cholesterol in PB solution at pH 7 in the potential range of -600 to 1200 mV at 4 V/s, and the potential Integration range for the current

3.1.Optimizing the experimental parameters

In order to investigate the influence of scan rates and the elunet flow rate on the sensitivity of the detector response, at different scan rates (from 0.5 to 20 V/s) and the eluent flow, solutions having a concentration of 2.0×10^{-6} M of Cholesterol were injected, and the responses of the detector were recorded. From this point of view, checking how the sensitivity of the method is affected by the sweep rate is necessary, while the sensitivity of the biosensor mainly depends on the potential scan rate, principally, due to kinetic factors of the electrochemical processes at the biosensor surace, and instrumental limitations in data acquisition rate [17,18].

Fig. 6 shows the obtained results for the CFFTCV measurements of standard solution of cholesterol, which indicate that the detector exhibits the maximum sensitivity at 4 V/s and 2.5 mL/min of the flow rate. As mentioned above, the dependent of the detection limit of biosensor on the potential scan rate on can be taken into consideration speed of current sampling, second, kinetic factors of the enzymatic processes. In addition, as expected for any

flow injection analysis finally flow rate of the eluent which controls the time retention of the solution zone in the detector, as well as the peak height of the cholesterol signal. However, the main reason for application of very high potential scan rates can have a negative effect on detection processes during the potential scanning.

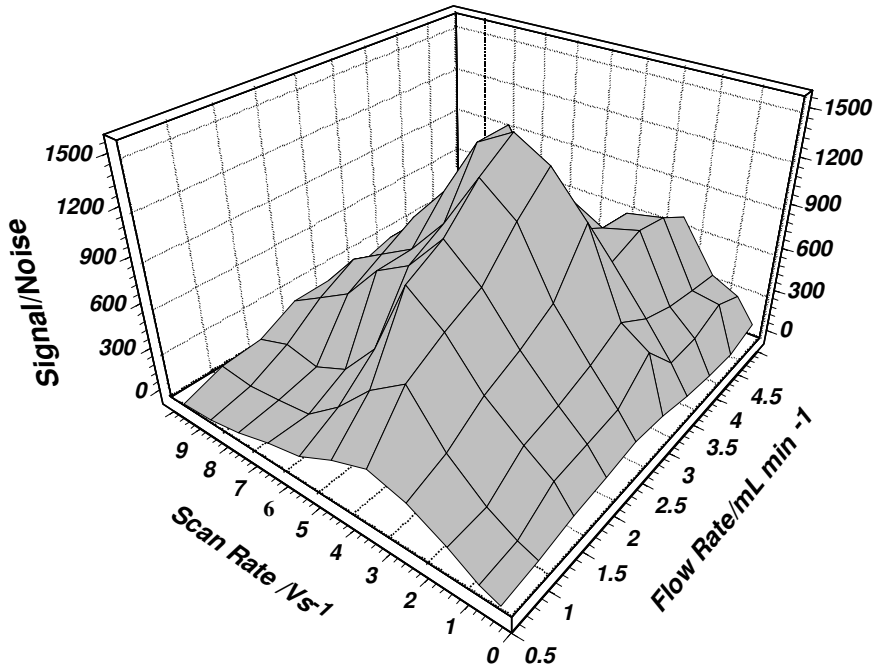

Fig. 6. Effect of the potential scan rate and eluent flow rate on the sensitivity of the response of the GC/CeO_2NPs/MWCNT(SH) Au NPs/ChiIL/ChOx biosensor to injections of 2.0×10^{-6} M cholesterol

3.2 .Calibration curve and biosensor characterization

As mentioned above the electrode response could be expressed in various ways as peak heights (in μC). For this reason, the magnitude of the flow-injection peaks depends on the choice of the integration range. Fig. 5 illustrates a typical ΔQ response of the biosensor on a standard solution of cholesterol (from 1.0 to 25.0 nM in PB solution, pH 7), where the experimental parameters were set at optimum values in order to obtain the best detection limits for the biosensor.

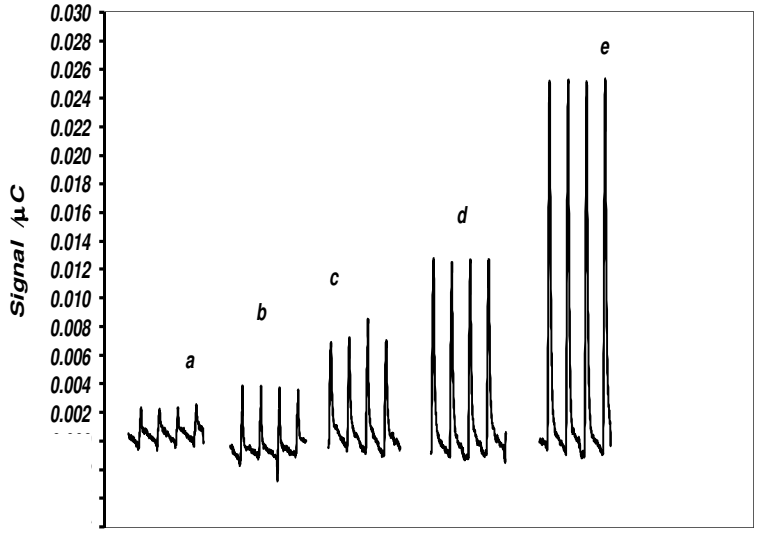

Fig. 5. Response of the GC/CeO_2 NPs/MWCNT(SH)Au NPs/ChiIL/ChOx biosensor to cholesterol standard solutions, with concentrations: a, 1; b, 2; c, 4; d, 8; e, 15 nM

The results demonstrated in this figure correspond to the averaged signal for 4 consecutive injections of the cholesterol standard solutions. Under optimized conditions, the steady-state voltammetry showed a linear dynamic range of 1 to 100 nM (Fig. 6). A correlation coefficient of R=0.998 with %R.S.D. values. Typically, the regression equation for the calibration curve was found to be y=0.63y+2.53. Measurements carried out for small analyte concentrations allow the estimation of the detection limit and the linearity was evaluated by linear regression analysis. The detection limit, estimated based on signal to noise ratio (S/N=3), was found to be 0.2±0.01 nM .

The long-term storage stability of the sensor was tested. The sensitivity retained 91.3% of initial sensitivity up to 50 days which gradually decreases afterwards might be due to the loss of the catalytic activity. In evaluation, the performances of the fabricated biosensor is compared with some of the best previously reported cholesterol biosensors based on the utilization of different materials as the working electrode and different detection techniques (Table 1) and it was confirmed that the presented MWCNTs and CeO_2 NPs based cholesterol biosensor with CFFTCV exhibited an excellent and reproducible sensitivity [18-22].

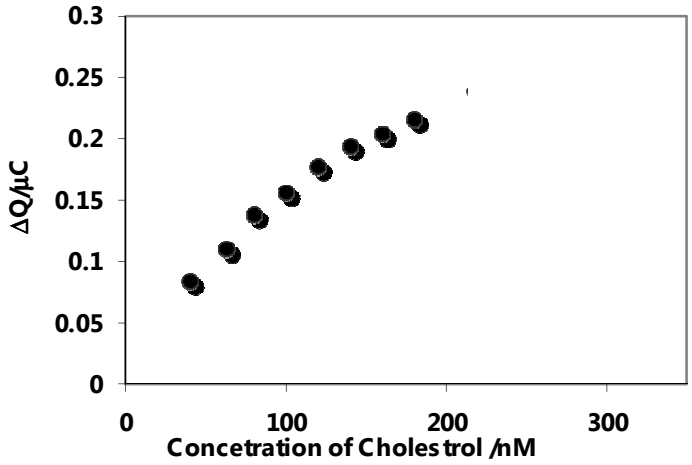

Fig. 6. The calibration curve for cholesterol determination

Table 1. The comparison of the proposed biosensor with the best previously reported ones based on the utilization of different materials

Ref.	Detection Method	DL	Materials
18	HPLC, UV-Vis	0.08 mg/mL	5-α-cholestan-3-β-ol
19	Capacitive detection	0.42 μM	Molecularly imprinted polymer
20	Amperometry	0.01 μM	Well-crystallized flower-shaped ZnO nanostructures
21	Amperometry	0.3 nM	ZnO NPs
22	Amperometry	0.3 μM	Conductive poly-3',4'-diamine-2,2',5',2"-terthiophene (PDATT)- Au NPs
This work	CFFTCV	0.2 nM	GC/CeO_2NPs/MWCNT(SH) AuNPs/ChiIL/ChOx

4. CONCLUSIONS

An ultra-sensitive cholesterol biosensor has been fabricated by modifying the GC electrode surface with GC/CeO_2 NPs/MWCNT(SH) AuNPs/ChiIL/ChOx. A higher producible sensitivity of 2.1 μCnM/cm^{-2}, response time less than 9 s and detection limit of 0.2 nM was observed biosensor. The long-term storage stability of the sensor was tested for 50 days. The sensitivity retained 91% of initial sensitivity up to 45 days which gradually decreases afterwards might be due to the loss of the catalytic activity .

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