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Full Paper

Fabrication of Molecularly Imprinted Polymer Coated Carbon Nanotubes Modified Gold Electrode for Determination of Cholesterol

Mohammad Reza Milani Hosseini,[1,2,*] Shiva Hemmati,[1] Ali Ghaffarinejad[1,2] and Ali Motaharian[1]

[1]*Department of Analytical Chemistry, Faculty of Chemistry, Iran University of Science and Technology, Tehran 1684613114, Iran*

[2]*Electroanalytical Chemistry Research Center, Iran University of Science and Technology, Tehran 1684613114, Iran*

* Corresponding Author, Tel.: +98 21 73912750; Fax: +98 21 77491204

E-Mail: drmilani@iust.ac.ir

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Abstract- A novel molecularly imprinted polymer (MIP) electrochemical sensor for selective detection of cholesterol has been developed by grafting a very thin polymer film on the surface of the functionalized-carbon nanotube (f-CNT) modified gold electrode. This sensor was made by electropolymerization of 2-mercaptobenzimidazole in the presence of cholesterol as a template on the surface of carbon nanotube modified gold electrode. Since cholesterol did not have any electroactivity on MIP/MWCNT/Au electrode in the phosphate buffer; indirect method was used for the determination of cholesterol. In this method, $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox couples was used as an electrochemical probe to characterize the sensor using cyclic voltammetry, differential pulse voltammetry and chronoamperometry methods. The linear response range for cholesterol detection was between 2 and 350 $mg.dL^{-1}$ with a detection limit of 1 $mg.dL^{-1}$. The sensor exhibited good selectivity for cholesterol, with a satisfactory reproducibility and repeatability. The proposed electrode was successfully applied for determination of cholesterol in human blood serum.

Keywords- Cholesterol sensor, Molecularly imprinted polymer, Carbon nanotube, Electropolymerization, Gold electrode

1. INTRODUCTION

Cholesterol is an important component in the manufacture of bile acids, steroid hormones, and several fat-soluble vitamins [1]. Its level in the blood is an important parameter in the diagnosis and prevention of diseases. However, high cholesterol accumulation in blood serum can cause blood vessel damage and resulting diseases such as coronary heart disease, arteriosclerosis, myocardial infarction, brain thrombosis, lipid metabolism dysfunction, hypertension, etc. [2-4]. On the other hand, a low cholesterol level may result in hyperthyroidism, anemia, malabsorption and wasting syndromes [1,5]. The desired total plasma cholesterol for an individual is less than 5.2 mM (200 $mg.dL^{-1}$), and it poses a potential health threat when the level is greater than 6.2 mM (240 $mg.dL^{-1}$) [5,6].

To date, many analytical systems and methods have been reported for measuring cholesterol, such as colorimetric [7], spectrometric [8,9], electrochemical methods [10], high-performance liquid chromatography (HPLC) [11,12], gas–liquid chromatography [13], cholesterol biosensor (that enzymes are used as molecular recognition materials) [14,15], and molecularly imprinted polymer (MIP) sensors [16]. Among these, some methods often suffer from poor selectivity [17,18], use of unstable and corrosive reagents, very intensive pretreatment of the samples, time consuming, instability of reagents and high cost [19-21].Thus, there has been continuous effort to develop reliable and sensitive biosensors, which can permit a cost-effective, convenient, rapid and sensitive determination of cholesterol level in clinical laboratories.

Biological recognition materials such as antibodies, enzymes and aptamers have been used as specific receptors for detection of a target molecule in a wide variety of sensors or biosensors. However many difficulties exist in their application due to their sensibility to various conditions (such as instability against high temperature, organic solvents, and pH), lack of reusability, cost and not easy to obtain [22,23]. Recently, to overcome the above limitations, scientists have replaced biological receptors with synthetic counterparts as a recognition element in chemo/biosensors. Among various approaches, molecular imprinting technique has become a powerful method for the preparation of polymeric materials that have the capability of specific adsorption and recognition of the template molecule [22,24].

Molecular imprinting, first constructed by Wulff et al. and Mosbach, is a method for making artificial binding sites in a polymer [25-27]. Molecular imprinting typically involves the copolymerization of functional monomers in the presence of template molecules. The removal of template molecules from the polymer matrix generates the recognition sites complementary to the shape, size and functionality of the template [24,28]. Because of high mechanical and chemical stability, ease of preparation and low cost to produce of molecularly imprinted polymers [23,29], this technology has considerable applications in many scientific and technical fields, such as chromatography [30,31], catalyst [32,33], drug delivery [34,35], artificial antibody [36,37], and sensing devices [38–42].

The efficiency of the MIP-based sensors largely depends on the selectivity and sensitivity of the used MIP materials to target species [43,44]. However, the low binding capacity, slow mass transfer, poor site accessibility, heterogeneous distribution of the binding sites and slow binding kinetics because of most imprinted sites to be embedded in high rigid polymer matrix interior [45] are the most important problems with the use of MIPs prepared by conventional method [22,24,27,46].

In recent years, scientists suggested that the use of surface imprinting methods and nanoparticles could overcome the above difficulties. To achieve surface imprinting, the best method is electropolymerization. This method allows the generation of a rigid, uniform, and compact MIPs film with good adherence onto the solid supports of any shape and size. Moreover, the thickness and density of the film are adjustable by controlling polymerization conditions (e.g. applied voltage and cyclic scan) [47-51].

In addition, various nanoparticles, such as carbon nanotube (CNT) [52,53], silica nanoparticles [54-56], magnetite nanoparticles [57-59] or other substrates [60,61] have been introduced to combine with MIPs [62]. Also the use of various nanoparticles on the surface of the electrode provides a higher surface area and thus increases the amount of effective binding sites. In addition, combining of nanoparticles with MIPs enhances the intensity of the electrochemical signal and improves the sensitivity of the sensor [63,64]. Among the various nanomaterial supports, carbon nanotube is an excellent candidate [65,22] as substrates to resolve the mentioned problems, owing to their extremely high surface area, high electrical and thermal conductivity [66] and good stability [24,27,46]. Thus, with these approaches, the thin layer of MIPs was polymerized onto the surface of nano dimension solid supports. The binding cavities in the outer thin MIPs layer can effectively improve the accessibility capacity for template molecules and reduce the binding time [22].

In this study Au electrode was modified with carboxyl functionalized multi-walled carbon nanotubes (MWCNT/Au), and by electropolymerization of 2-mercaptobenzimidazole (2-MBI) in the presence of cholesterol a thin layer of MIP was deposited on the MWCNT/Au (MIP/MWCNT/Au). Cyclic voltammetry (CV), differential pulse voltammetry (DPV) and chronoamperometry were used to characterize the electrochemical sensor and determination of cholesterol. MIP/MWCNT/Au electrode also showed specific recognition selectivity for cholesterol molecules in presence of some important biological molecules and similar compounds. Eventually, the capability of this method was applied to determine cholesterol in human serum sample.

2. EXPERIMENTAL

2.1. Materials

Multi-walled carbon nanotube (MWCNT) (with the purity of >95%) was purchased from Plasma Chem GmbH company(Berlin, Germany). Cholesterol ($C_{27}H_{46}O$, extra pure,

M=386.67)was purchased from Scharlau (Barcelona, Spain). 2-Mercapto benzimidazole (2-MBI), sodium perchlorate, phenol, tryptophan, ethanol, 2-propanol, dimethyl formamide (DMF), potassium hexacyanoferrate (KHCF), sulfuric acid and nitric acid were purchased from Merck (Germany). All chemicals were of analytical grade, and were used without further purification. All solutions were prepared with deionized water.

2.2. Apparatus and software

Electrochemical measurements and electropolymerization were performed with a Autolab PGSTAT 100 controlled by a Nova 1.7 software (Echo Chemie, B.V., Netherlands, NOVA software), and a conventional three-electrode configuration. The three-electrode system consisted of bare or modified gold disk electrode (2 mm i.d.), Ag/AgCl (3 M KCl) and platinum wire as working, reference and counter electrodes, respectively. The pH measurements were performed with a Metrohm 691 pH meter. The formation of the functional groups on the MWCNT was confirmed by FT-IR spectrometer (Shimadzu 8400S, Japan).

2.3. Preparation of carbon nanotubes modified gold electrode

MWCNTs functionalized with the carboxylic group were prepared as follows: 1 g of the MWCNTs was added into a round bottom flask containing HNO_3: H_2SO_4 (1:3). This mixture was refluxed for 6 h at 140 °C, then the resulting MWCNTs were cooled, filtrated through a 0.45 μm cellulose nitrate membrane, and washed repeatedly with the deionized water until the filtrate became neutral, and finally dried at 110 °C in oven.

For Au electrode modification, the electrode was initially immersed in diluted nitric acid solution for 5 min. Then the surface of Au electrode was polished with 0.05 μm alumina powder for 1 min at polishing cloth, and rinsed with deionized water. Then it successively washed in an ultrasonic bath with the deionized water and ethanol (1:1) for 10 min to remove any particles that might be attached at the surface, and allowed to dry at room temperature. After this, in the 5.0 mM KHCF solution containing 0.1 M KCl supporting electrolyte, cyclic potential sweeps in the range of −0.2 to 0.6 V (*vs.* Ag/AgCl)with the scan rate of 50 $mV.s^{-1}$was applied on the bare Au electrode until a pair of well-defined redox peaks were obtained.

For preparation of MWCNT/Au, 1.5 mg of functionalized MWCNT powder was dispersed in 1 mL of DMF and sonicated for 15 min to obtain a black suspension. For better adhesion of nanotubes to the electrode surface and more reproducibility, 0.5 mg polyvinyl chloride (PVC) was added to the above solution, and then 3 μL of the prepared mixture was dropped on the Au electrode surface, allowed to evaporate solvent and formed resistant MWCNT modified thin film electrode.

2.4. Preparation of molecularly imprinted polymer on MWCNT/Au

The resulting MWCNT/Au was immersed into alkaline ethanol containing 40 mM of2-MBI, 15 mM of cholesterol and 50 mM of sodium perchlorate. Then, it was electropolymerized by applying 7 successive cyclic voltammograms in the potential range of −0.60 to 1.30 V(*vs.* Ag/AgCl)and with the scan rate of 40 $mV.s^{-1}$. To remove the cholesterol template from the imprinted film, the resulting electrode was dipped into a mixture of ethanol/water (4:1 v/v) for 50 min under mild stirring. Then, it was washed with distilled water and dried at room temperature to obtain MIP/MWCNT/Au. As a control, to prepare non-molecularly imprinted polymer (NIP/MWCNT/Au) the above procedure was used without adding cholesterol in the electropolymerization process.

2.5. Electrochemical measurements

In view of the fact that cholesterol did not have any electroactivity on MIP/MWCNT/Au in the phosphate buffer; cholesterol could not be determined by direct method using the MIP/MWCNT/Au electrode. Therefore, indirect method was used for the determination of cholesterol. In this method, KHCF solution (5.0 mM) containing 0.1 M of KCl, was used as an electrochemical probe. When the MIP/MWCNT/Au was incubated in cholesterol solution, the cavities in the imprinted film were occupied by cholesterol, which could lead to the decrease of current signal produced by the probe. By increasing the cholesterol concentration, the probe current is decreased. For electrochemical measurements CV, DPV and chronoamperometry techniques were carried out.

CV measurements were performed in the potential range between -0.20 and 0.60 V (*vs.* Ag/AgCl) with the scan rate of 50 $mV.s^{-1}$. DPV measurements were performed in the potential range between -0.20 and 0.50 V (*vs.* Ag/AgCl). To record differential pulse voltammograms, instrumental parameters were as follows: step potential 5 mV, modulation amplitude 25 mV, and scan rate 10 $mV.s^{-1}$.Since dissolved oxygen present in the cell did not affect the current response, de-aeration of the cell content was not necessary.

The results were compared with similar experiments for NIP/MWCNT/Au, to ensure that the observed effects are only due to the imprinting features, and not to the subsequent treatments undergone by the electrode.

2.6. Real sample preparation

Human serum sample was taken from Daneshpathobiology laboratory and primary amount of cholesterol in the sample was determined 40 $mg.dL^{-1}$in the laboratory. This sample was placed into a glass vial containing perchloric acid and centrifuged at 13500 rpm for 15 min. A series of sample solutions were prepared by adding specified volumes of cholesterol to human serum to give working concentration of 50, 100 and 125 $mg.dL^{-1}$. Then the solution

was diluted to 10 mL with phosphate buffer solution (PBS) (pH 7.4). The MIP/MWCNT/Au electrode was incubated in the above solution for 20 min to adsorb the cholesterol and then it was washed with water. DPV was applied using the MIP/MWCNT/Au electrode in KHCF(as a probe) to determine the peak current.

3. RESULTS AND DISCUSSION

3.1. FTIR study of functionalized CNT

FT-IR was used to detect the functional group that formed by oxidation treatment of MWCNTs (Fig. 1). The vibrations bands at about 3400 cm^{-1} were attributed to O–H stretching at about 1716 $cm^{-1}$was also observed in functionalized MWCNT spectrum, which is related to C=O in the carboxylic acid group. Thus, the FT-IR spectrum indicates that carboxylic acid groups were grafted onto the surface of the MWCNTs.

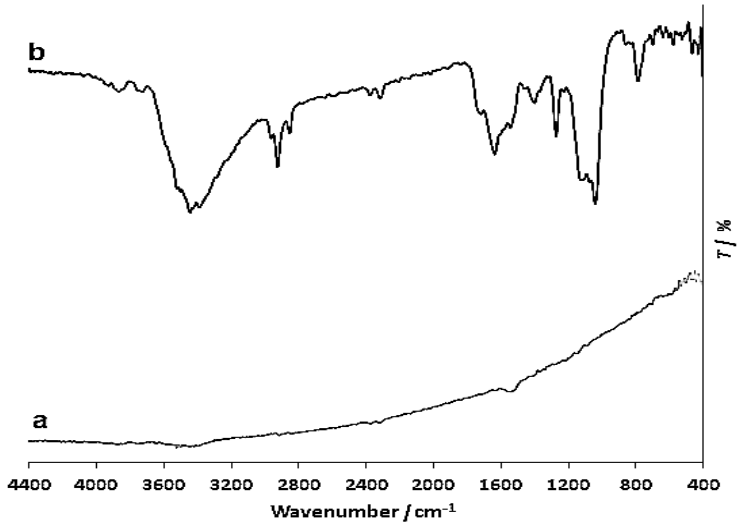

Fig. 1. FT-IR spectra of MWCNT (a), and functionalized MWCNT (b)

3.2. Stability improvement of MWCNT/Au electrode

Perhaps, the simplest way for preparation of CNT based electrodes is to coat the electrode surface with a CNT suspension in a proper solvent such as THF. But in this method the CNT adherence on the electrode may be poor. In this work to reach better CNT adhesionon the Au surface, PVC was added to the suspension of MWCNT in DMF. To obtain the proper amount of PVC and MWCNT in the suspension, various electrodes were prepared (Table 1), and the CV response of each electrode was investigated in 0.1 M KCl containing KHCF (5 mM) as probe. After each measurement in KHCF, the electrode was washed for 60 min, and another CV was obtained in probe solution at the same conditions. The lowest change in the CV response between before and after washing was chosen as the best composition of the

suspension mixture. Table 1 shows that among examined mixtures the lowest decrease in the CV response is for 1.5 mg MWCNT, 0.5 mg PVC and 1 mL THF, so this amounts were used for the remained experiments.

Table 1. Effect of suspension mixture composition on the MWCNT/Au electrode stability

Composition No.	MWCNT (mg)	PVC (mg)	DMF (mL)	ΔI_p[a] (μA)	ΔI_p[b](μA)
1	1	0.5	1	28.2	14
2	1	1	1	15.3	10
3	1.5	1	1	21.4	12
4	1.5	0.5	1	35.2	3
5	2	0.5	1	39.5	20

[a] difference of KHCF peak current on bare Au and MWCNT/Au electrodes

[b] KHCF peak current change on the MWCNT/Au after 60 min washing

Fig. 2 shows a sample CV of the bare Au and MWCNT/Au (with the selected composition) in the probe solution. According to this figure, the surface area in the MWCNT/Au electrode increased significantly, which is related to the deposited CNTs on the electrode surface. The active surface area of the bare and MWCNT modified gold electrode was calculated with chronoamperometry and Cottrell equation in KHCF standard solution, which was 0.041 and 0.112 cm^2 respectively.

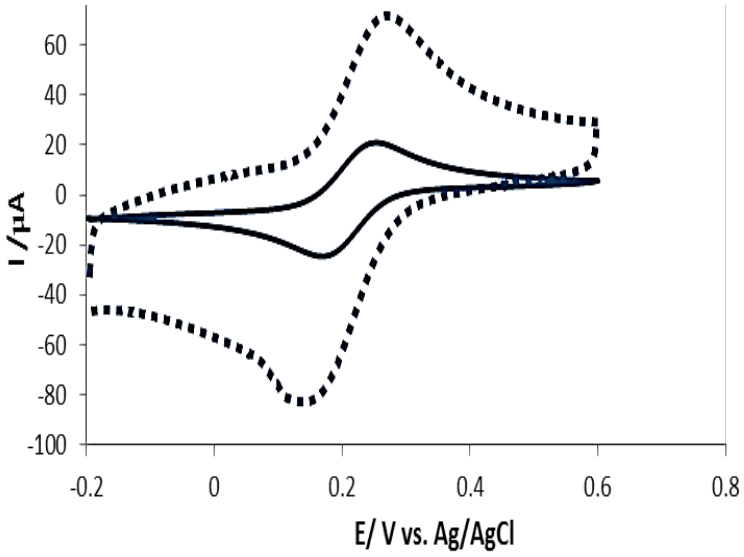

Fig. 2. Cyclic voltammograms of Au (solid line) and MWCNT/Au (dash line) in 5 mM KHCF solution containing 0.1 M KCl (scan rate 50 $mV.s^{-1}$)

3.3. Parameter optimization for preparation of MIP/MWCNT/Au electrode

The preparation procedure of the MIP/MWCNT/Au electrode is a three step procedure: a) Au electrode modification with MWCNTs, b) electropolymerization of 2-MBI on the surface of MWCNT/Au electrode, and c) removal of the imprinting cholesterol molecules from the imprinted polymeric film. During the electropolymerization of 2-MBI, cholesterol template molecules were trapped in the polymer matrix. This complex defined the size and orientation of the chemical functions of the imprinted cavity, which could cause the specific cavity to cholesterol after removal of the template.

To prepare thin layer of imprinted polymer on the MWCNT/Au electrode which has maximum active sites on the surface of polymer, some parameters such as template molecules concentration, monomer concentration, scan rate, number of cyclic voltammograms and extraction time of the template from the synthesized polymer were optimized by DPV technique. For this reason, difference of oxidation peak current of the probe before and after extraction of cholesterol from polymeric film on the surface of electrode was obtained, and the maximum difference value was selected as the optimum response.

3.3.1. Effect of cholesterol concentration

To initiate the electropolymerization 50 mM of sodium perchlorate , 25 mM of 2-MBI, cholesterol concentration 2.5–25 mM,8 CV cycles , scan rate of 25 $mV.s^{-1}$ and extraction time of the template 60 min were used to determine the best concentration of cholesterol during electropolymerization, for each electrode the differential pulse peak current in KHCP solution (5.0 mM) containing 0.1 M of KCl was recorded before and after cholesterol extraction, and the difference of the peak heights(ΔI_p) was calculated. Fig. 3 shows the plot of ΔI_p*vs*. cholesterol concentration. As it is seen in figure 3, the highest value of ΔI_p is obtained at 15 mM of cholesterol. At the lower concentrations of cholesterol the appropriate imprinting is not occurred, and at higher concentrations the polymer layer may be thicker so the removal of cholesterol is not performed completely. So for the rest of experiments, concentration of 15 mM was used for the cholesterol solution.

3.3.2. Effect of 2-MBI concentration

For optimization of 2-MBI monomer concentration at electropolymerization of MIP all parameters were kept constant (sodium perchlorate: 50 mM, cholesterol: 15 mM, number of CV cycles: 8, scan rate: 25 $mV.s^{-1}$, extraction time of the template: 60 min) and the concentration of 2-MBI was changed from 10 to 50 mM. Then as the previous section for each electrode the value of ΔI_pin 5 mM of KHCF was obtained. The results of this experiment are shown in Fig. 4. The results show that, 40 mM was the best concentration for

2-MBI. At higher concentration of 2-MBI thick polymeric film is produced, and the cholesterol removal from MIP is not performed completely. Also at lower concentrations of 2-MBI the electropolymerization was not completely performed. Therefore, concentration of 40 mM was chosen as the suitable concentration for the rest experiments.

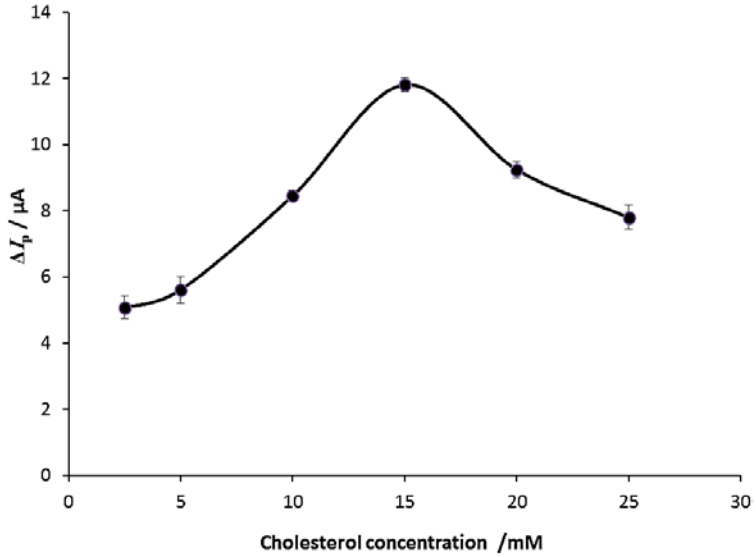

Fig. 3. Effect of cholesterol concentration (2.5, 5, 10, 15, 20 and 25 mM) on the MIP/MWCNT/Au electrode response in KHCF solution (5.0 mM) containing 0.1 M of KCl. (Each point was repeated 3 times)

3.3.3. The potential sweep effect on the MIP formation

In this experiment the effect of potential sweep in MIP electropolymerization step from two aspects (the number and rate of sweeps) was studied. To determine the best scan rate during electropolymerization, a series of electrodes in the various scan rates(5–100 $mV.s^{-1}$)were prepared, while the other parameters were kept constant (sodium perchlorate: 50 mM, cholesterol: 15 mM, 2-MBI: 40 mM, number of CV cycles: 8 and extraction time of the template: 60 min).Then the response of each electrode (ΔI_p) was determined in KHCF solution (5.0 mM) containing 0.1 M of KCl before and after removing cholesterol from MIP. Fig. 5A shows the plot of ΔI_p *vs*. scan rate forthese MIP/MWCNT/Au electrodes. The figure shows that the maximum difference in I_p is obtained at the scan rate of 40 $mV.s^{-1}$.At the faster scan rates it was possible that the polymerized film would be relatively porous and thick, which was not desirable for the subsequent use when the scan rate was low, the polymeric film would be too dense, resulted in difficult removal of template from the polymer. Therefore, the optimized scan rate was chosenat40 $mV.s^{-1}$.

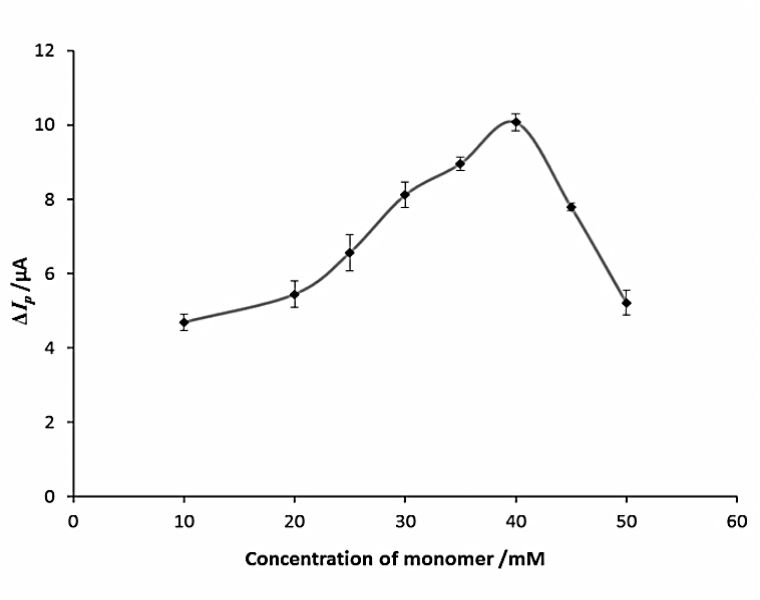

Fig. 4. The average ΔI_p (n = 3) of MIP/MWCNT/Au electrodes in KHCF solution (5.0 mM) containing 0.1 M of KCl. The electrodes are prepared at various concentration of 2-MBI monomer (10, 20, 25, 30, 35, 40, 45 and 50 mM).

Fig. 5B illustrates the effect of scan number on the formation of MIP while other parameters including sodium perchlorate (50 mM), cholesterol (15 mM), 2-MBI (40 mM), scan rate (40mV.s^{-1}), and extraction time of the template (60 min) were kept constant. The higher value of the ΔI_p with DPV in KHCF solution (5.0 mM) containing 0.1 M of KCl was obtained for the electrode which prepared with the 7 applied successive cycles in electropolymerization step. At higher number of cycles, the thickness of polymeric film increased inordinately, so the cholesterol molecules was hardly extracted from imprinted polymer. When the lower number of cycles was used, the formation of polymeric film was not complete. Therefore, 7 cycles was chosen as the optimum cycle number.

3.3.4. Effect of extraction time of the template from the MIP

The MIP/MWCNT/Au electrode was prepared at optimal conditions (sodium perchlorate: 50 mM, cholesterol: 15 mM, 2-MBI: 40 mM, scan rate: 40 mV.s^{-1}, and number of CV cycles 7). Then to find the best washing time, the imprinted polymer on the electrode was immersed in the mixture of alkaline ethanol/water (4:1 v/v, pH 13) under mild stirring, and after various elution times (10, 20, 30, 40, 50, 60, 70 and 80 min) the electrode signal was recorded by DPV in probe solution. Then the difference of this signal with the one before removal of cholesterol was plotted *vs*. elution time. The results of this study showed that, 50 min elution time has the highest ΔI_p, thus this time was selected as the optimum elution time for removal of template (cholesterol). At times lower than 50 min, the extraction of cholesterol from the

MIP was not completed, and at the times greater than 50 min the structure of MIP may be corrupted.

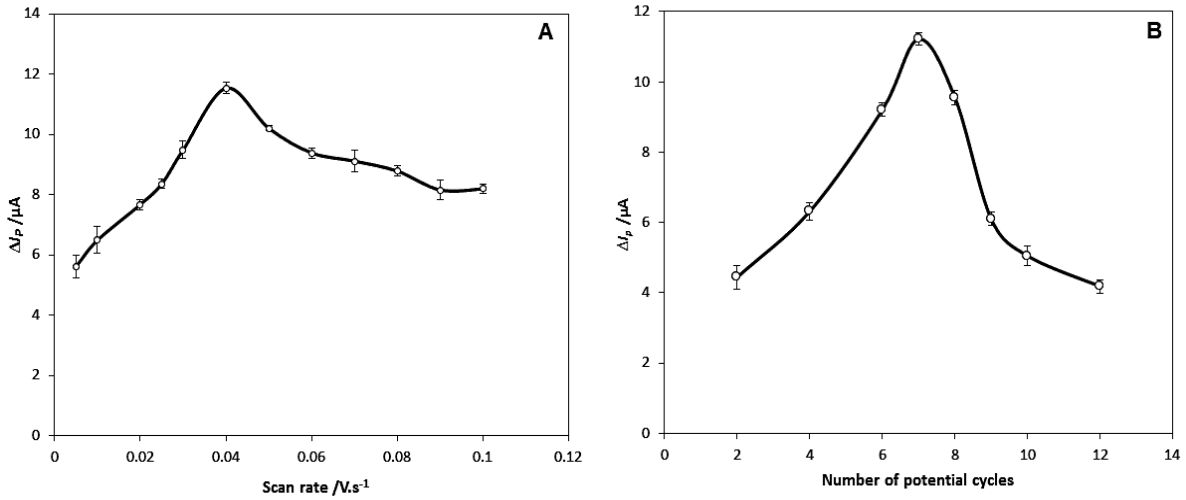

Fig. 5. The average ΔI_p (n=3) of MIP/MWCNT/Au electrodes in KHCF solution (5.0 mM) containing 0.1 M of KCl before and after removal of cholesterol. The effect of scan rate (5, 10, 20, 25, 30, 40, 50, 60, 70, 80, 90 and 100 mV.s^{-1}) (A), and the number of potential cycles (2,4, 6, 7, 8, 9, 10 and 12) (B) on the electropolymerization of MIP.

3.4. Comparing MIP and NIP polymerization at optimized conditions

At the optimized conditions for MIP electropolymerization, NIP was electropolymerized on the MWCNT/Au electrode, but in the absence of cholesterol template. No considerable difference was observed between the cyclic voltammograms obtained during the electropolymerization (7 CVs between -0.6 to 1.3 V with the scan rate of 40 mV.s^{-1}) in the presence and in the absence of cholesterol, which can be due to the fact that cholesterol did not have any electroactivity on MWCNT/Au. As a result, the structure of cholesterol was not electrochemically changed in the polymerization process.

For comparing the response of the MIP and NIP/MWCNT/Au electrodes in the absence and presence of cholesterol, both electrodes were examined at the same conditions. Fig. 6 illustrates the result of this experiment. It shows that the response of NIP in the both cases did not change very much, which can be explained by the lack of specific binding sites and cavities for cholesterol on the NIP film.

3.5. Comparing of MIP deposited on MWCNT/Au and bare Au

In another experiment the MIP was electrodeposited on the bare Au electrode at the same conditions as MWCNT/Au electrode. The results showed that, by applying successive CVs during polymerization the conductivity of the MIP on the bare gold electrode (MIP/Au) was

considerably reduced and was smaller than MIP on the MWCNT/Au. One possible reason may be due to direct interaction of thiol groups on the monomer with Au surface, which reduce the conductivity of the polymer. While on the MWCNT/Au, CNT coating prevents binding thiol groups with the gold surface.

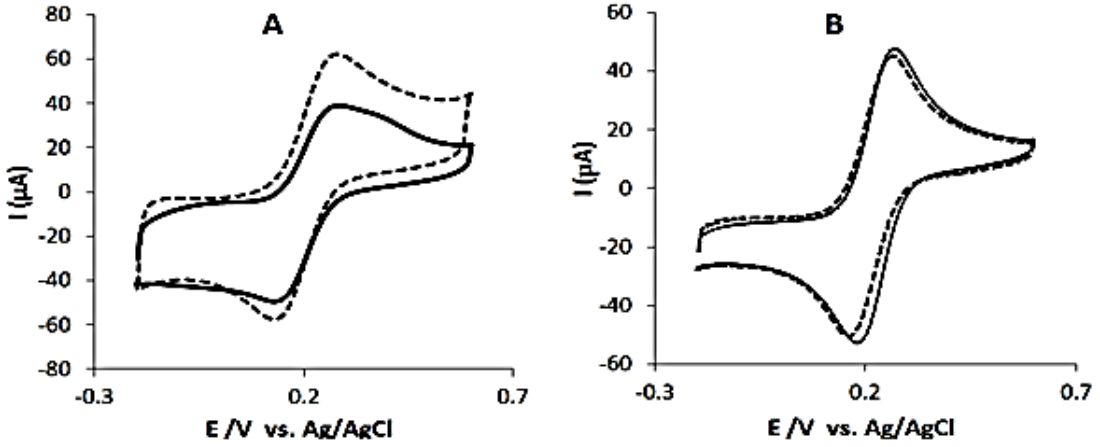

Fig. 6. CV response obtained in 5.0 mM KHCF containing 0.1 M of KCl for MIP (A) and NIP (B) modified electrodes after incubation in 150 mg.dL^{-1} cholesterol solution for 20 min (dash line) and after 50 min washing electrode for removal of adsorbed cholesterol (solid line)

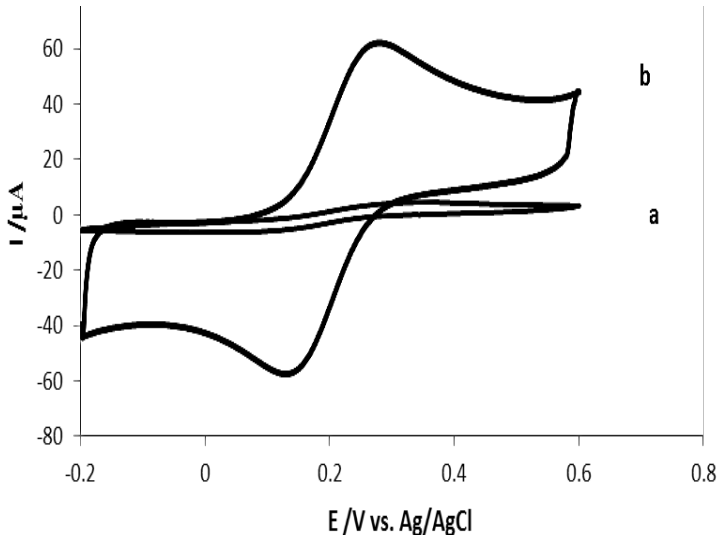

Fig. 7. CVs of KHCF (scan rate=50 mV.s^{-1}) on the MIP/Au (a) and MIP/MWCNT/Au (b) electrodes

More detailed studies are illustrated in Fig. 7, which compares the CVs of KHCF on the MIP/Au and MIP/MWCNT/Au electrodes. The results show an obvious difference between conductivity of MIP/MWCNT/Au related to MIP/Au electrode.

3.6. Behavior of the modified electrode as a cholesterol sensor

3.6.1. Sensor response time

Response time is an important factor for any sensor. To evaluate the minimum time for achieving maximum cholesterol adsorption on the MIP/MWCNT/Au, after template molecules were removed from the imprinted polymer, the MIP/MWCNT/Au electrode was incubated in a stirring solution of propanol –0.1 M PBS (pH 7.4) containing 100 mg.dL^{-1} cholesterol. For different incubation times in the range of 5–50 min, the DPV responses were recorded in 5.0 mM KHCF containing 0.1 M of KCl. The results demonstrated that the immersion time for maximum adsorption was attained in 20 min, and then was remained almost constant. Therefore, 20 min incubation time was used for the electrochemical determination of cholesterol.

3.6.2. Measurement of cholesterol on MIP/MWCNT/Au electrode (calibration curve)

To investigate the affinity and response of the prepared sensor (MIP/MWCNT/Au and NIP/MWCNT/Au electrodes) to cholesterol, the dependency of the probe ΔI_p on the concentration of cholesterol was examined under the optimized conditions. DPV was used to determine the dependency of the oxidation peak current of the probe versus different cholesterol concentrations at the MIP/MWCNT/Au surface. The corresponding calibration curve is shown in Fig. 8. ΔI_p linearly increases with the cholesterol concentration in the range of 2–350 mg.dL^{-1}with a correlation coefficient of 0.998 (Fig. 8a). When the concentration of cholesterol is greater than 400 mg.dL^{-1}, the peak current gradually leveled off, this indicated that the imprinting sites were saturated.

Additionally, the limit of detection (LOD) was found to be 1.01mg.dL^{-1} of cholesterol, defined as three times the standard deviation of the blank measurement in the absence of target analyte divided by the slope of the calibration plot [67]. Compared with the MIP/MWCNT/Au electrode, the NIP/MWCNT/Au electrode gave a lower current and lower sensitivity (shown in Fig. 8b), which could be concluded that the NIP electrode did not possess considerable active sites for cholesterol.

3.6.3. Reproducibility and repeatability

Five MIP/MWCNT/Au electrodes prepared in the same conditions, and each electrode was applied for determination of 100 mg.dL^{-1} cholesterol, which an acceptable reproducibility with RSD of 4.58% was obtained. The result indicates that the imprinted sensor shows a good electrode-to-electrode reproducibility of the fabrication method.

One important factor related to the MIP film is the stability of the geometry of molecular cavities, created in the polymer matrix, which may affect both selectivity and the sensitivity of the imprinted material in the long term. The stability of the MIP/MWCNT/Au electrode was investigated over a 7 weeks period by measuring the voltammetric responses to 100 mg.dL^{-1} cholesterol with intermittent usage (every 1 week) and by storing at the room temperature when not in use. After storing for 6 weeks at room temperature the response of the MIP/MWCNT/Au electrode only decreased to ~94%, which is a good stability for an electrode.

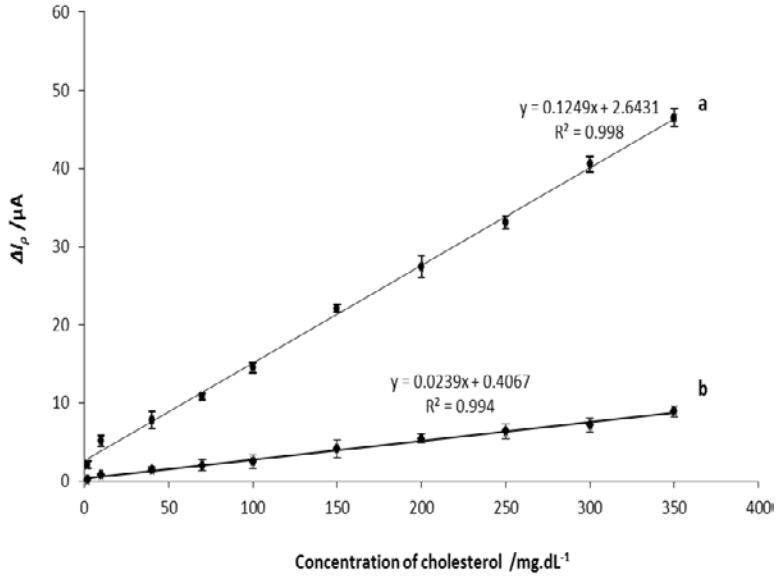

Fig. 8. Calibration curves corresponding to the response of the MIP (a) and NIP (b) sensors to the concentration of cholesterol (each point was repeated 3 times)

The most attractive feature with the use of modified electrodes for determination of cholesterol is the easy renewal of surface for the next use of the method of template extraction by immersing modified electrodes in ethanol:water (4:1) mixture solutions for 50 min. To determine the repeatability of MIP/MWCNTs/Au responses to cholesterol, a 150 mg.dL^{-1} cholesterol solution was analyzed five times by the same electrode. The relative standard deviation (RSD) of the measurements was 2.45%, indicating that the sensor had a good repeatability. Thus the MIP sensor was expected to be regenerated and used repeatedly.

3.6.4. Selectivity

The MIP/MWCNT/Au and NIP/MWCNT/Au response selectivity was studied in the presence of two structurally related compounds (tryptophan and phenol) in PBS (pH 7.4). According to Fig. 9 the MIP/MWCNT/Au electrode showed a very sensitive response to cholesterol in comparison to the other compounds. This can be attributed to the selectivity of

prepared MIP/CNT/Au electrode and the affinity of imprinting sensor to cholesterol. For NIP sensor, the electrode response for cholesterol or similar compounds was very small. This might attribute that there were no suitable sites for cholesterol in NIP. These results confirmed that the MIP/MWCNT/Au electrode had a good selective recognition capacity toward the template molecule as a result of the imprinting effect produced in the presence of cholesterol.

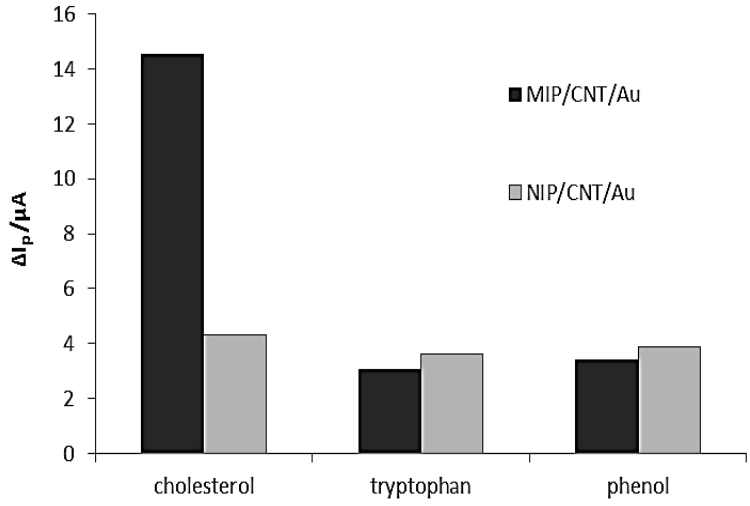

Fig. 9. MIP and NIP/MWCNT/Au response to cholesterol (100 mg.dL^{-1}), tryptophan (100 mg.dL^{-1}) and phenol (100 mg.dL^{-1}) in PBS (0.1 M, pH=7.4).

Also the interference effect of some important compounds which exist in biological samples such as glucose, uric acid and ascorbic acid that had different structures to cholesterol were examined in PBS (pH 7.4). The results of this study is illustrated in Fig. 10. As this figure shows there are no considerable interference for cholesterol detection in the presence of these molecules.

3.6.5. Real sample analysis

In order to evaluate the ability of the fabricated sensor for practical applications, it was applied to determine cholesterol (by standard addition methods) in human serum. Acceptable results and recoveries were obtained, as can be seen in Table 2. The satisfactory results obtained indicate that proposed sensors can be applied to real sample assays.

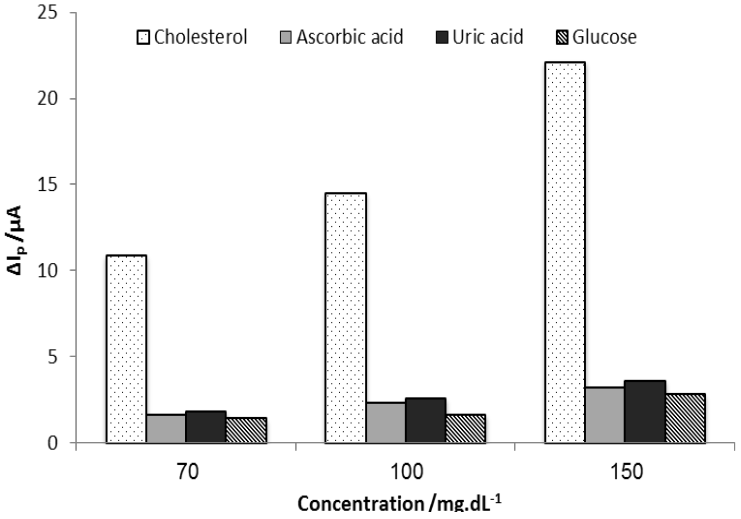

Fig. 10. Interference effect of ascorbic acid, glucose and uric acid on the MIP/MWCNT/Au cholesterol sensor

Table 2. Application of fabricated sensor for the detection of cholesterol in human serum

Sample	Primary amount of cholesterol in the sample[a] ($mg.dL^{-1}$)	Cholesterol added ($mg.dL^{-1}$)	Average of cholesterol found ($mg.dL^{-1}$)	Recovery (%)
1	40	0	37.32	93.3
2	40	50	90.81	100.9
3	40	100	137.06	97.9
4	40	125	161.44	97.84

[a] Determined with a standard clinical spectroscopic method

Table 3. Comparison of different methods for determination of cholesterol

Sensing element	**Linear range ($mg.dL^{-1}$)**	**LOD ($mg.dL^{-1}$)**	**Shelf life**	**Ref.**
MIP capacitive biosensor	0.2 -1.15	0.02	3 weeks	[4]
Nanostructured nickel oxide-chitosan	10-400	43.4	10 weeks	[68]
ChOx– ChE–HRP- MWCN-potassium ferrocyanide –CPE	100-400	-	8 weeks	[69]
Ti/ NPAu/ ChOx– HRP– ChE	Up to 300	0.5	-	[70]
MWCNT(SH)–Au/Chi–IL/ ChOx	20–200	-	20 days	[71]
polyaniline/cholesterol oxidase/cholesterol esterase	Up to 500	25	6 weeks (at 4 °C)	[72]
MIP/MWCNT/ Au electrode	2- 350	1.01	5 weeks (at 25 °C)	This work

ChOx: Cholesterol oxidase, ChE: cholesterol esterase, HRP: peroxidase, CPE: carbon paste electrodes, Chi–IL: chitosan–room-temperature ionic liquid

Table 3 shows the characteristics of some cholesterol sensors and biosensors reported in the literature in comparison with the present cholesterol sensor. The linear range or detection limit of the proposed sensor is superior to other reports presented in the table.

4. CONCLUSION

In this work a novel molecularly imprinted poly 2-MBI modified MWCNT/Au electrode was prepared and applied in the electrochemical determination of cholesterol, resulting in excellent synergistic effect, good reproducibility, strong stability, low cost, and high selectivity as well as quick response to cholesterol.

The prepared sensor displayed a good recognition capacity for template molecule in the presence of other structurally similar molecules and some important biological molecules. Furthermore, this sensor has a wide linear rang with a good detection limit.

The MIP/MWCNT/Au electrode was successfully applied for the determination of cholesterol in blood serum samples, exhibiting its potential as a fast and accurate sensor.

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