Amperometric biosensor for cholesterol based on novel nanocomposite array gold nanoparticles/acetone-extracted propolis/multiwall carbon nanotubes/gold

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In this reported work, the electrochemical behaviour of gold electrodes modified with gold nanoparticles, acetone-extracted propolis, multiwall carbon nanotubes and cholesterol oxidase was established for the detection of hydrogen peroxide by using cyclic voltammetry and amperometric techniques. The obtained results confirmed that the current enzymatic biosensor exhibits a fast, highly sensitive, and cost-effective detection of cholesterol. Cholesterol in the concentration range of 0.15–0.55 mmol l^{-1} was determined with a detection limit of 4.9×10^{-5} mol l^{-1} by the amperometric method, and the sensitivity of the proposed method was found to be 17.38 μA/mmol l^{-1}. Normal electroactive species such as ascorbic acid and glucose in the presence of the constant concentration of cholesterol in the samples do not interfere with the determination.

1. Introduction: Cholesterol is one of the most essential fatty compounds found in biological systems, and it plays a vital role in the bodies of humans and animals. These molecules that are an important lipid in biological membranes, nerve and brain cells, steroid hormones, bile acid and fat soluble vitamins [1] are produced naturally in the liver from meat. Since cholesterol is insoluble in blood, it is transferred through the circulatory system with lipoprotein or its analogues which are soluble in the bloodstream. Higher concentrations of this compound (>2.6 mg ml^{-1}) in human serum, however, can cause several diseases such as hypertension, myocardial infarction and arteriosclerosis, any of which can ultimately lead to death [2, 3]. The determination of the cholesterol concentration is of great importance in clinical diagnosis and medical screening. Several techniques have been developed for the estimation of the cholesterol levels in blood and food such as high performance liquid chromatography [4], gas chromatography [5], spectrometry [6], electrochemistry [7] and colorimetric methods [8]. Some traditional cholesterol detection methods have disadvantages such as low specificity and poor selectivity. Among various electrochemical procedures for this purpose, electrochemical sensors, especially biosensors, have been met with more interest because of the simplicity, speed, portability and miniaturisation capabilities of these techniques [9].

In biosensors, the bioreceptor must be immobilised on an appropriate substrate, which is a main step in the fabrication of biosensors [10]. For this purpose, a number of routine types of enzyme-immobilisation have been reported in the medical literature, including adsorption, entrapment/encapsulation, cross-linking and covalent attachment [11]. Different substrates have been used to develop biosensors such as hydrogels, synthetic and natural polymers, Langmuir-Blodgett film and self-assembled monolayers [12]. Cholesterol oxidase (ChOx), cholesterol sterase (ChEt), horseradish peroxidase or combinations of these were used for the fabrication of the cholesterol biosensors [13]. In a recent study, Turkarslan *et al.* [13] used ChOx physically entrapped in poly(3, 4-ethylenedioxypyrrole) to construct an amperometric cholesterol biosensor. Also, Khan *et al.* [14] have fabricated an amperometric cholesterol biosensor with sol–gel film on indium tin oxide coated glass substrate. In addition, Basu *et al.* [9] have developed a fast, economic and easy-to-use cholesterol biosensor based on immobilised ChEt and ChOx on a polycarbonate membrane attached to the tip of the oxygen electrode for the determination of cholesterol in the food samples. Moreover, Ahmadalinezhad and Chen [15] have reported a new biosensor with the co-immobilising of the ChOx, ChEt and HRP enzymes using the adsorption method on the nanoporous gold networks which were grown directly on a titanium substrate for total cholesterol measurement. Other studies have demonstrated that carbon nanotubes (CNTs) could be a reliable choice for electrode modification owing to their electrical conductivity, unusual increased surface to volume ratio and their ability to promote the electron-transfer reaction on their surface [16, 17]. Moreover, the chemical functionalisation of multiwall CNTs (MWCNTs) can be used to attach the desired chemical components to their surfaces, which leads to the increased solubility and biocompatibility of the CNTs because of the fact that the nanostructures are able to load large amounts of bioreceptors with no change in their bioactivity [14, 18–21].

Gold nanoparticles (AuNPs) play important roles in several reactions because of their unique catalytic properties [22]. The combination of the interesting properties of AuNPs and two types of CNTs (multiwalled and single walled) for use in a biosensor structure has been reported by many research teams [17, 23–27]. The decoration of the CNT with AuNPs is performed by physical, chemical and electrochemical methods [28]. Propolis is a viscous and natural resin-like substance that is produced by worker bees from tree buds. It is used in folk medicine and cosmetic products because of its biological activities including antioxidant, antibacterial, antiviral, antifungal and other beneficial activities. Scientists have determined that this bio-material is composed of about 45% resin, 35% wax and 20% inert material [29], respectively. Some reports have shown that the identification of the propolis components is achieved by using organic solvents, because it is generally insoluble in polar solvents [30]. Propolis has attractive properties, including its excellent membrane-forming ability in water, good adhesion, non-toxicity, high mechanical strength, excellent biocompatibility and susceptibility to chemical modification because of the presence of a large number of reactive functional groups [31, 32]. In our previous paper [33], we reported a modified gold electrode for the detection of the HIV-1 p24 antigen with a novel nanocomposite film consisting of AuNPs, MWCNTs and acetone-extracted propolis (AEP). In this Letter, we used the AuNPs deposited on a functionalised MWCNT's surface for obtaining a composite established by AEP as a thin film on the electrode's surface. This arrangement improved the electrical conductivity of the fabricated hybrid film.

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This prepared nanocomposite was attached to the ChOx to catalyse the oxidation of cholesterol to cholest-4-en-3-one. The byproduct of this reaction is hydrogen peroxide [34]

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholest} - 4\text{-en} - 3\text{one} + H_2O_2 \quad (1)$$

The obtained results confirmed that the current enzymatic biosensor exhibits a fast, highly sensitive and cost-effective cholesterol detection. The other parameters, such as pH, temperature and storage stability, that could affect the efficiency of the fabricated biosensor were also investigated.

2. Experimental

2.1. Reagents and chemicals: ChOx enzyme was purchased from Sigma. Cholesterol, dimethylformamide and acetone were obtained from Merck (Darmstadt, Germany). Sodium tetrachloroauric acid, potassium hydroxide, sodium borohydride ($NaBH_4$) and Triton X-100 (99%) were purchased from Sigma-Aldrich. The MWCNTs were prepared by the Chemical Reagent Co. (Institute of Nanotechnology, Shiraz University, Iran). The propolis samples were obtained from settled hives in the mountains around Urmia, located in West Azerbaijan, Iran, during the fall of 2012. All the other reagents were of analytical grade, and double distillated water was used throughout the experiments.

2.2. Apparatus: A scanning electron micrograph (SEM) was recorded for the further characterisation and morphology of the proposed surface modifier using a Philips XL-30 electron microscope operated at 30 kV. Purification and functionalisation were investigated using a Fourier transform infrared spectroscopy (FTIR) instrument (Thermo Nicolet Nexus® 670, USA) in the transmittance mode between 400 and 4000 cm^{-1}. The samples were freeze dried for the SEM and the FTIR tests using an ALPHA 1-4 freeze dryer (CHRIST, Germany) under vacuum conditions at −50°C for 48 h. Electrochemical experiments were performed using an Autolab PGSTAT30 Potentiostat/Galvanostat and controlled General Purpose Electrochemical System (GPES4.9) software (Eco Chemie, Utrecht, The Netherlands). Gold, a working electrode (2 mm diameter), a platinum wire counter electrode and an Ag/AgCl (3 mol l^{-1} KCl) reference electrode were obtained from Azar Electrode Instruments (Urmia, Iran).

2.3. Preparation of the AuNPs/CNT/AEP nanocomposite: For the preparation of the AuNPs/CNT/AEP nanocomposite, the procedures previously published by our team were followed [34]. The propolis solution (2% w/v) was prepared in acetone and stirred at room temperature for 4 h until all the wax ingredients were completely dissolved. Then, the AEP solution was filtered through a 0.45 µm Millex-HA syringe filter unit (Millipore). When not in use, the solution was stored in a refrigerator at 4°C. Chemical functionalisation of the MWCNTs with the carbonyl (C = O) groups was achieved by an ultrasonic agitation in a mixture of sulphuric acid and nitric acid (3:1) for 4 h in order to complete the oxidation reaction (Fig. 2*b*). The amino-functionalised MWCNTs ($MWCNTNH_2$) and the AuNPs/CNT/AEP were prepared by procedures similar to those reported in our previous papers [35]. The functionalised MWCNTs were isolated and washed several times with distillated water by centrifugation until the media was neutralised to a pH of about 7 and dried at 80°C overnight. Subsequently, the produced activated MWCNTs were treated in 10 ml thionyl chloride at 65°C for 12 h to obtain acyl chloride-modified MWCNTs (MWCNT-COCl), which were then purified by centrifugation and allowed to dry overnight at room temperature. For the effective attachment of the Au nanoparticles on the MWCNTs, 100 mg of MWCNT-COCl were dispersed into 10 ml ethylene diamine as a modifier, stirred at 80°C for 24 h and then separated by centrifugation, washed repeatedly with ethanol and deionised water, and eventually dried at 80°C for 12 h in order to obtain the $MWCNTNH_2$. 1 ml of 1% $NaBH_4$ was added to 50 ml of a 0.05% $HAuCl_4$ solution containing 0.01 g of $MWCNTNH_2$, stirred for 60 min and finally freeze-dried for 24 h. The Au nanoparticles were immobilised on the surface of the functionalised MWCNTs via electrostatic adsorption. The AuNPs/CNT/AEP nanocomposite was constructed by mixing 2 ml of the prepared AEP solution with the AuNPs/CNT powder and then gently stirred for 3 h until a

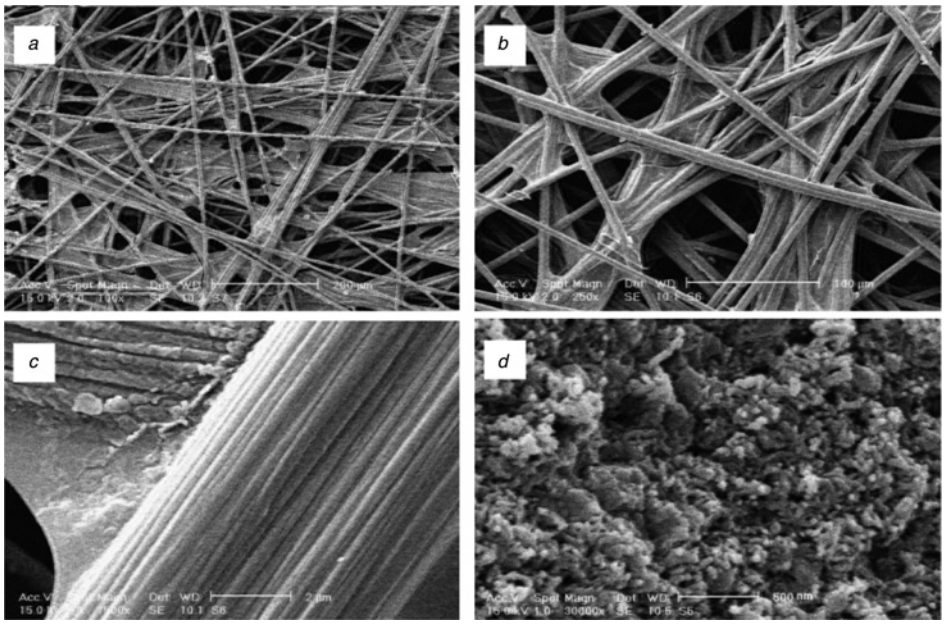

Figure 1 *Scanning electron micrograph of prepared nanocomposite film structure at different magnifications*
a 100×
b 250×
c 7500×
d 30 000×

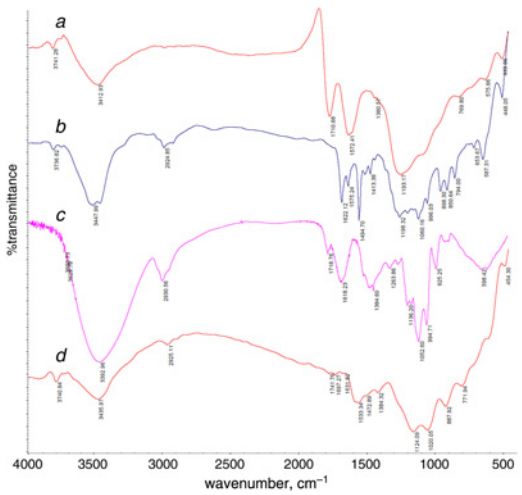

Figure 2 *FTIR spectra of functionalisation steps of MWCNTs spectra a after purification and carboxylation; spectra b after acylation of carboxylic groups on surface of MWCNTs; spectra c after amino-functionalisation of MWCNTs-COCl; spectra d after modification of MWCNTs with AuNPs*

homogeneous solution formed. In the next step (modification of the Au electrode with the prepared nanocomposite), 10 µl of this mixture was added onto the (smooth) oxidised gold electrode in 0.05 mol l^{-1} H_2SO_4, and allowed to dry for 3 h in a vacuum desiccator at room temperature. Finally a known amount of the ChOx solution was injected onto the surface of the modified electrode, dried at 4°C in a refrigerator and stored in a 0.1 mol l^{-1} phosphate buffer solution (PBS) at 4°C for all the experiments.

3. Results and discussion

3.1. Surface morphological studies: Figs. 1*a–d* display a typical SEM of the structure of the prepared nanocomposite (AuNPs/AEP/MWCNTs) in different magnifications. As can be seen in Figs. 1*a* and *b*, the AEP was found to have a fibrous composition and structure. The Au nanoparticles attached to the MWCNTs were captured in the pores of the AEP structure, and the obtained nanocomposite film was actually composed of dispersed AuNPs. Moreover, the results exhibited in Fig. 1*d* confirmed that the desired coating of the MWCNTs on the AEP surface was obtained.

3.2. FTIR analysis: The recorded FTIR spectra of the functionalisation steps of the MWCNTs are shown in Figs. 2*a–d*. The newly-appeared FTIR peaks at 1710.6 cm^{-1} (Fig. 2*a*) are related to the stretch vibrations of the C = O bond, and the peak at 3412 cm^{-1} could be assigned to the stretch vibration of the carboxylic acid OH that can be confirmed successfully with the preparation of carboxilated nanotubes. It is well known that the peaks at 1622.1 and 1060 cm^{-1} (Fig. 2*b*), respectively, are probable indicators of the –(C = O)–N– and the –C–O–C– stretching vibration that is related to the acylation step of the functionalised MWCNTs [36]. It should be noted that the new peaks presenting about 2850–2924 cm^{-1} and the strong band between 3300 and 3500 cm^{-1} shown in (Fig. 2*c*) after the MWCNTs-COCl were amino-functionalised are normally attributed to the stretching vibration mode of the OH and the NH group [36]. With these results, it can be concluded that the CNT surface has been modified and functionalised successfully. Also, Fig. 2*d* shows the FTIR spectra of the AuNPs-modified MWCNTs. In comparing the FTIR spectra recorded in Figs. 2*c* and *d*, it has been demonstrated that the strong band at 3392 cm^{-1} related to the O–H and the N–H group was weakened, and the minor shift on 3392–3435 cm^{-1} may be related to the attachment of the AuNPs to the surface of the modified MWCNTs from the N–H and the O–H bonds, respectively.

3.3. Electrochemical characterisation: It is well known that the AEP is a non-conductive material and blocks electron transfer in the constructed enzymatic film. Furthermore, the MWCNTs have a semi-conductive behaviour and have been found to be a suitable choice for application in modified electrodes, because their use in the electrode modification process could help to compensate for the occurred resistance because of a non-deployed electron cloud. The AuNPs attached to the MWCNTs in the introduced film correspond with the conductivity roles and facilitate the electron transfer. The cyclic voltammetry (CV) technique was used to investigate the possible occurring mechanisms on the surface of the proposed biosensor. According to (1), cholesterol oxidase can catalyse the oxidation of cholesterol, with hydrogen peroxide being generated as a side product. Fig. 3 presents the CV curves of a bare Au electrode at any modification step in a buffer solution (pH = 6.5) in the potential range of −0.6 to 0 V in the presence of H_2O_2 (0.2 mmol l^{-1}). Cyclic voltammograms of the Au electrodes in the presence of the known concentrations of the cholesterol solution were performed, too. As seen in Fig. 3*a*, the cathodic peak of H_2O_2 was observed on the bare Au electrode, whereas a very weak peak was recorded in the presence of cholesterol. When the AEP were placed on the surface of the Au electrodes, the cathodic waves disappeared. This result may be ascribed to the non-conductive nature of the AEP (Fig. 3*b*). By attaching the MWCNTs/AEP as a modifier on the electrode surface, the reduction peak was again discernible in the buffer content of 0.2 mmol l^{-1} of H_2O_2 (Fig. 3*c*). It should be noted that, when the MWCNTs decorated with the AuNPs were replaced in the composite (Fig. 3*d*), the cathodic currents in both the media significantly increased. Such a result shows the electrical conductivity of the Au/MWCNTs. This phenomenon may be explained by the use of the CNTs, because of the large specific surface area and the ability to load high quantities of the

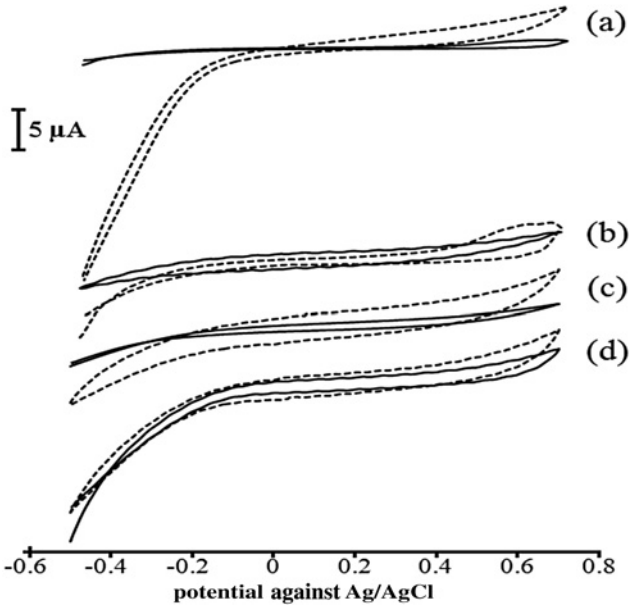

Figure 3 *Cyclic voltammograms of (a: solid line) in presence of 0.3 mmol l^{-1} cholesterol; (a: dashed line) in presence of 0.1 mmol l^{-1} H_2O_2 in 0.1 mol l^{-1} PBS (pH = 6.5) at unmodified gold electrode; (b: solid line) in presence of 0.3 mmol l^{-1} cholesterol; (b: dashed line) in presence of 0.1 mmol l^{-1} H_2O_2 in 0.1 mol l^{-1} PBS (pH = 6.5) at AEP modified gold electrode; (c: solid line) in presence of 0.3 mmol l^{-1} cholesterol; (c: dashed line) in presence of 0.1 mmol l^{-1} H_2O_2 in 0.1 mol l^{-1} PBS (pH = 6.5) at AuNPs/MWCNT/AEP modified gold electrode; (d: solid line) in presence of 0.3 mmol l^{-1} cholesterol; (d: dashed line) in presence of 0.1 mmol l^{-1} H_2O_2 in 0.1 mol l^{-1} PBS (pH = 6.5) at ChOx/AuNPs/MWCNT/AEP modified gold electrode*
Scan rate: 50 mV s^{-1}

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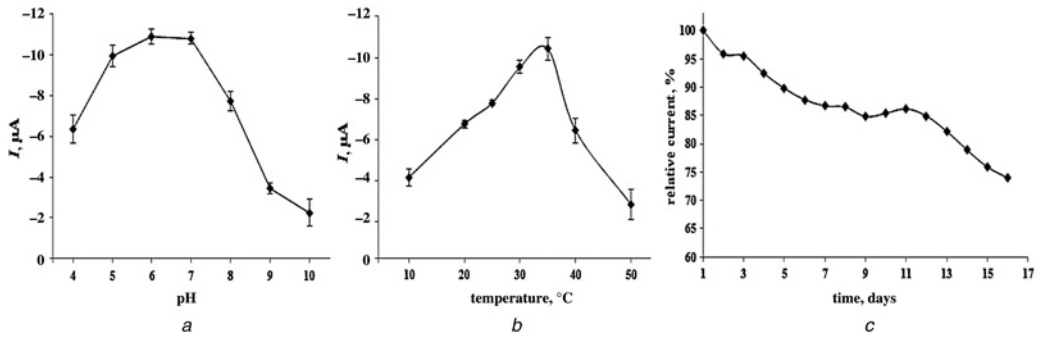

Figure 4 *Influence of pH, temperature, and shelf-life effect on response of ChOx/AuNPs/MWCNT/AEP modified gold electrode in 0.1 mol l^{-1} PBS containing 0.3 mmol l^{-1} cholesterol*
a Influence of pH
b Influence of temperature
c Shelf-life effect

nanoparticles, followed by an extensive enzyme binding. Finally, after the attachment of a cholesterol oxidase enzyme on the film, a reduction in H_2O_2 occurs with a maximum current in the solution content of cholesterol.

3.4. Optimising the experimental conditions on the biosensor response: The dependence of various parameters on the modified enzyme electrode signal was also studied. Fig. 4*a* illustrates the response of the pH with a constant concentration of cholesterol (0.3 mmol l^{-1}) at a phosphate buffer (0.1 mol l^{-1}). As can be seen, the optimum response was achieved at pH = 6.5, which is also compatible with the optimum pH range (5.3–7.5) for a free enzyme activity. Therefore the PBS at pH = 6.5 was selected for further experiments. The temperature dependence of the modified electrode was studied, because the enzymes and the other biological molecules are strongly influenced by temperature; the electro-catalytic activities of the enzymes are also greatly dependent on temperature. Fig. 4*b* shows that the biosensor's maximum response was achieved at 35°C. It was observed that by increasing the temperature of the 0.3 mmol l^{-1} cholesterol solution from 10 to 50°C under an optimised pH value, a significant increase in the current response was seen around 35°C. Meanwhile, a strong decrease in the current value was observed at high temperatures, which may be most probably because of enzyme deactivation as well as the fact that the AEP is deformed at around 35°C. Thus, room temperature was chosen as the optimum temperature. The lifetime of a fabricated enzyme biosensor was investigated by measuring the amperometric response under the same operating conditions of amperometric detection during two weeks. The modified electrode stayed at 4°C in the buffer solution when not in use. As shown in Fig. 4*c*, the relative current of the chronoamperometric measurement performed in the 0.3 mmol l^{-1} cholesterol solution retained 82% of its initial response after 13 days, and after that was observed to decrease gradually. The relative standard deviation (RSD) was found to be 4.2%.

3.5. Amperometric response: The amperometric response of the modified electrode was observed with the ChOx/AuNPs/AEP/MWCNTs/Au electrode in the PBS (0.1 mol l^{-1}, pH = 6.5) in the presence of 1 mmol l^{-1} of the cholesterol solution while being continuously stirred. As shown in Fig. 5*a*, the fabricated electrode showed that the current response of the constructed biosensor at the applied optimum condition immediately rose after each successive injection (0.05 ml) of the cholesterol (50 mmol l^{-1}). Furthermore, the calibration curve (Fig. 5*b*) was plotted, and the limit of detection (LOD) and the sensitivity of the proposed method were found to be 4.9×10^{-5} mol l^{-1} and 17.38 μA mmol l^{-1}, respectively. The response time of the fabricated biosensor was less than 10 s (~8 s) which mainly relates to the fibrous structure of the AEP that can be decreased at the time of cholesterol

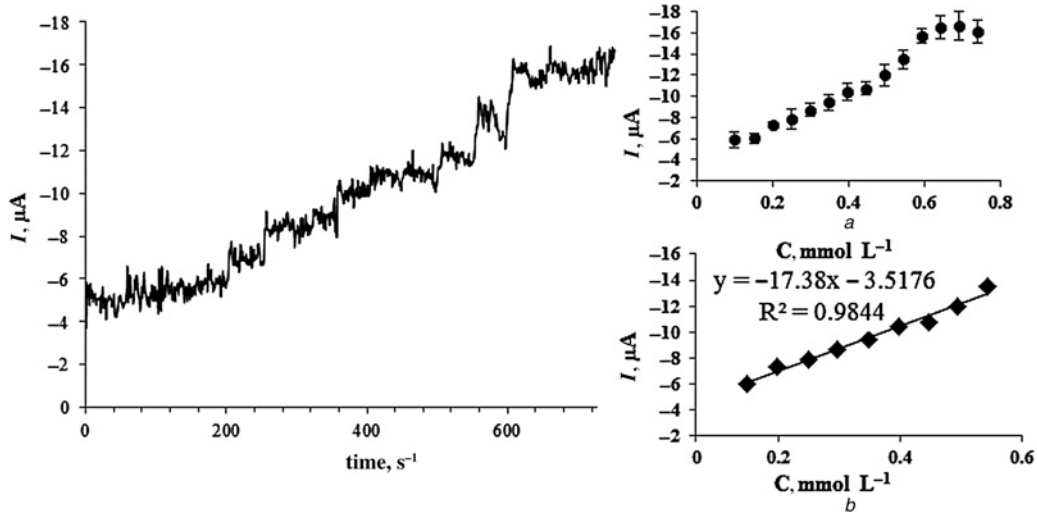

Figure 5 *Chronoamperometric curve of biosensor*
a Plot of current against concentration of cholesterol solution
b Calibration curve with cholesterol solution (50 mmol l^{-1}) in 0.1 mol l^{-1} PBS (pH = 6.5)

diffusion into the composite film, enzymatic reaction time and finally at the electro-reduction of hydrogen peroxide.

To study the selectivity and the applicability of the proposed biosensor for determining the cholesterol level under the optimised conditions, the interference of some electro-active species in the presence of the constant concentration of cholesterol (0.3 mmol l^{-1}) was examined. The effect of glucose (5.6 mmol l^{-1}), ascorbic acid (0.1 mmol l^{-1}), uric acid (0.4 mmol l^{-1}) and L-cysteine (0.02 mmol l^{-1}) as common, potent, interfering metabolites on the amperometric detection of cholesterol with a fabricated electrode was tested. No significant interference was observed.

4. Conclusion: In summary, this study revealed that the nanocomposite array of the AuNPs/AEP/MWCNTs/Au can be used to estimate the cholesterol levels in the solution. A desired sensitivity of 17.38 μA mmol l^{-1}, a response time of less than 10 s and a detection limit of 4.9×10^{-5} mol l^{-1} were observed from the fabricated biosensor. It was demonstrated that some electroactive compounds such as ascorbic acid and glucose in the presence of a constant concentration of cholesterol in the samples do not interfere with the determination. The obtained results confirmed that the current enzymatic biosensor exhibits a fast, highly sensitive and cost-effective detection of cholesterol.

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