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Fabrication of sensitive potentiometric cholesterol biosensor based on Co$_3$O$_4$ interconnected nanowires

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Abstract: Highly sensitive, selective, reliable and inexpensive cholesterol biosensors are highly demanded for the routine monitoring of cholesterol molecules in order to prevent the heart failure incidents. In this study, Co$_3$O$_4$ nanostructures are synthesized using polyvinyl pyrrolidone surfactant as a growth template by a low temperature aqueous chemical growth method. The morphology of nanostructures was investigated by scanning electron microscopy and X-ray diffraction techniques. The nanostructures exhibit interconnected nanowires like morphology with interconnected network of nanowires. The nanostructures of Co$_3$O$_4$ are polycrystalline. The cholesterol oxidase was physically adsorbed on the interconnected nanowires of Co$_3$O$_4$ for the chemical sensing of cholesterol molecules. The sensor device detected a wide range of cholesterol from 1×10^{-7} M to 1×10^{-3} M concentrations with sensitivity of -94.031 mV/decade. A detection limit of 0.035×10^{-7} M cholesterol concentration was observed and a fast response time of 10 s was also noticed. The fabricated device is highly stable, selective, sensitive, reproducible and repeatable. All the collected information about presented cholesterol biosensor indicates its potential application for the monitoring of cholesterol concentrations from human blood serum and real-life samples.

Keywords: Biosensor; Co$_3$O$_4$ interconnected nanowires; cholesterol oxidase; potentiometric response; sensitive

1. Introduction

Cholesterol is a lipid compounds, and present in all the cells of human body [1]. The physiological level of cholesterol in the human serum is around 5.17 mM [2]. The elevated concentration of cholesterol could lead to life threatening diseases such as diabetes, cancer, cardiac, brain vascular, etc... Therefore, the determination of cholesterol concentration in human body is vital task [3–5]. Different analytical tools have been used for the quantification of cholesterol levels including calorimetric, spectroscopic, and electrochemical techniques [6-8]. Meanwhile, potentiometric sensors are well-known electro-analytical devices over the past five decades. Their versatile chemical selectivity could easily be tuned with lipophilic ionophore [9]. The first potentiometric enzyme sensor was reported in 1969 [10] since then fast advances in the field of potentiometric sensing have been demonstrated e.g. [11-13]. Generally, potentiometric sensors do not require external power source and no current passes during detection; hence they are very attractive for developing sensors for biological systems [12].

Due to emerging of nanotechnology science in the development of novel devices with more selectivity, fast response time and sensitivity has provided a solid platform for the generation of new sensors [14] which could measure accurate concentration of cholesterol. The properties of nanostructures are largely depending on their morphology and size of the nanoparticles [15, 16], which in turn play an important role for the sensor fabrication and their performance [17-22]. Among these several nanomaterials, cobalt oxide (Co_3O_4) nanostructures, the cobalt (II, III) oxide is a p-type magnetic semiconductor. Co_3O_4 exhibits spinal structure in which Co^{2+} ions occupy the tetrahedral sites and Co^{3+} ions occupy the octahedral sites in the cubic close packed lattices of oxide ions [23]. In addition to this, Co_3O_4 has a high isoelectric point (IEP) value $\sim$ 8 and it can be little higher when the temperature rises [24] and biocompatibility, since cobalt itself is an essential element for plants and animals (as vitamin B12). The high IEP of Co_3O_4 makes it a good matrix to immobilize an enzyme such as cholesterol oxidase having low isoelectric point ($\sim$ 4.6) through electrostatic interaction [19]. Co_3O_4 has been used for various applications including heterogeneous catalysts, solid state sensors and electrochemical based device applications [25-31]. Several nanostructures of Co_3O_4 such as nanowires/nanorods [31], nanotubes [32], nanocubes [33], nano spheres [34], and nano plates [35] have been fabricated through various growth techniques. Co_3O_4 nanocrystals have been synthesized by a solubility method using surfactant

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as growth template [33] and by a combustion method have been used for the synthesis of Co_3O_4 nanocrystals [36].

Irrespective of these growth methods, other more facile growth methods including sol-gel, polyol process, solvothermal, and polymer assisted method, reflux/microwave assisted techniques and low temperature aqueous method have been used to prepare Co_3O_4 nanostructures [37-39]. These growth methods can produce high quality and diverse morphologies of cobalt oxide nanostructures; however the high temperature (>100 °C) of these growth methods limits the growth of cobalt oxide nanostructures on organic flexible substrates. The hydrothermal growth method is widely used for the synthesis of cobalt oxide nanostructures on several substrates particularly of its low temperature (<100 °C), simple methodology, non-toxicity, inexpensive and yields high amount of desired nanomaterial. At large scale, Co_3O_4 nanowires have been synthesized by low temperature aqueous chemical growth method [40]. The poly (vinyl pyrrolidone) (PVP) was used as a template for the growth of Co_3O_4 nanostructures in the present study. Several parameters such as reproducibility, stability, repeatability and selectivity were investigated and discussed in detail.

This study describes the synthesis of cobalt oxide interconnected nanowires using PVP surfactant as a growth template by hydrothermal method on a gold coated glass substrate. The morphology was studied by the scanning electron microscopy and crystal structure was investigated by X-ray diffraction technique. The cholesterol oxidase was immobilized on the Co_3O_4 interconnected nanowires for the development of highly sensitive cholesterol biosensor using potentiometry technique.

2. Experimental Section

2.1. Chemicals

Cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), cobalt acetate tetrahydrate (($CH_3COO)_2 \cdot Co \cdot 4H_2O$), urea ($CH_4N_2O$), cholesterol oxidase, cholesterol, d-glucose, ascorbic acid, uric acid, and ferrous chloride tetrahydrate were purchased from Sigma Aldrich Sweden. All chemicals were used of analytical grade.

2.2. The fabricated of Co_3O_4 nanostructures on a gold coated glass substrate

A gold layer was coated on a glass substrate was done according to the reported work [41]. The hydrothermal method was used for the synthesis of cobalt oxide nanostructures on the gold coated glass substrate using PVP surfactant as a growth template. Firstly, gold (100 nm) coated glass substrates were sonicated in ultrasonic bath for 20 min in isopropanol, then washed with deionized water and dried with flow of nitrogen gas. A seed layer of cobalt acetate was spin coated on the cleaned gold coated glass substrate using a spin coating technique. The seed deposition process was repeated twice at 2500 r.p.m for 20 s. The seed particles containing substrates were annealed at 120 ℃ for 15 min. A growth solution containing equimolar concentration (0.1 M) of cobalt chloride and urea in 100 mL of deionize water and 300 mg of PVP surfactant was added. PVP was used as the substance that would yield a desired morphology of cobalt oxide nanostructures. After annealing the seed particles containing substrate, it was fixed in Teflon sample holder and dipped with downward face in the growth solution, and then the growth solution was kept in a preheated electric oven for 4– 6 h at 95 °C. After the completion of growth duration, samples containing cobalt hydroxide nanostructures were washed with deionized water and dried at room temperature. Finally, the cobalt oxide nanostructures were obtained after annealing the as-grown samples at 450 °C for three hours.

The morphology of cobalt oxide nanostructures was investigated by LEO 1550 Gemini field emission scanning electron microscope (SEM) operating at 5 kV. The nanocrystalline phase was examined by X-ray powder diffraction (XRD) using a Phillips PW 1729 powder diffractometer combined with CuKα radiation ($\lambda = 1.5418$ Å) at a generator voltage of 40 kV and a current of 40 mA.

2.3. Immobilization of Co_3O_4 interconnected nanowires with cholesterol oxidase and electrochemical measurement

A cholesterol oxidase (ChOx) solution was prepared in 10 mM phosphate buffer of pH 7.3 and immobilized on the Co_3O_4 interconnected nanowires through electrostatic physical adsorption method. A stock solution of 50 mM of cholesterol was prepared in few drops of isopropanol and finally mixed with10 mM phosphate buffer of pH 7.3. Low concentrations of cholesterol were obtained by dilution. All electrochemical measurements were performed using a Keithley 2400 model at room temperature.

3. Results and discussion

3.1. The Crystal Structure and Morphological Studies of Co_3O_4 Nanostructures

The crystal structure of annealed Co_3O_4 samples was investigated by XRD and the results obtained are shown in Figure 1. It can be seen that the prepared product exhibits sharp diffraction peaks and all the diffraction peaks could be assigned to the nanocrystalline cubic phase of Co_3O_4 according to the JCPDS 42-1467 and the high intense peak at 38^0 is assigned to gold substrate. No diffraction peaks were observed other phase of Co_3O_4. The morphology of PVP assisted cobalt oxide nanostructures was studied by scanning electron microscopy and distinctive SEM images has shown that nanostructures possess interconnected nanowires like morphology with interconnected network of nanowires. Various SEM images at different magnification were taken as shown in Figure 2(a–c). In the growth of Co_3O_4 interconnected nanowires, PVP plays a key role in the controlling the Co_3O_4 size and morphology. In this study, PVP was used as a growth template, which resulted in achieved chain structures. In the presence of polymer template, it was possible for Co_3O_4 to grow up along these polymeric chains to give nanostructures of Co_3O_4. Moreover, PVP has the ability to form a shell surrounding the nanoparticles to prevent them from aggregation together, which would otherwise produce larger nanoparticles due to its steric effect. Previously, the presence of a capping molecule, for example, PVP, was reported [42, 43] to change the surface energy of crystallographic surfaces for enhancing the anisotropic growth of nanostructures. The PVP accumulated on the crystal nuclei cobalt oxide behaved as a growth directing agent.

3.2. The Chemical Sensing Application of Co_3O_4 Interconnected Nanowires for the Detection of Cholesterol Molecules

The potentiometric response [44] of the proposed cholesterol biosensor (Figure 3 (a)) was measured in phosphate buffer solution of pH 7.3. The effect of pH on the ChOx activity has been examined in the range of pH values between 4 and 8. The range of 7.0–7.5 has been found to yield the best enzymatic activity of the ChOx/ Co_3O_4 nanowires /Au electrode. The chemical sensing of cholesterol molecules was performed at room temperature by immobilizing cholesterol oxidase using physical adsorption method on the Co_3O_4 interconnected nanowires. The developed cholesterol biosensor showed a robust response for the dynamic range of cholesterol concentration from 1×10^{-7} M to 1×10^{-3} M with sensitivity

of -94.031 mV/decade as shown in Figure 4. The wide range of detection and high sensitivity of presented cholesterol biosensor is attributed to high surface to volume ratio of Co_3O_4 interconnected nanowires and their better electrocatalytic properties. The cobalt oxide nanostructures provided a favourable microenvironment for the immobilization of ChOx, which could enhance the catalytic properties of ChOx molecules. This can be deduced from the Michaelis–Menten constant (K_m). K_m is estimated from Lineweaver-Burke plot and was found to be 0.039 mM, which is much smaller than that obtained at other cholesterol biosensors (see Table I). From the calculations a limit of detection of the presented sensor was found to be 0.035×10^{-7} M and a fast response time of 10 s as shown in Figure 5. The sensing mechanism of developed cholesterol biosensor based on Co_3O_4 interconnected nanowires can be described in two different reaction pathways. One way is attributed to the selective response cholesterol oxidase for cholesterol molecules and second way is the direct interaction of cholesterol molecules with the cholesterol oxidase immobilized on the Co_3O_4 interconnected nanowires (Figure 3 (b)) which speed up the enzyme substrate reaction even better than that obtained in [22]. In reference [22] they demonstrated a sensor based on CuO as a good immobilization matrix for ChOx while here we have used a heterogeneous-catalysis system compromise of Co_3O_4 and ChOx enzyme, we believe that, this system can even acquire improved properties due to a synergistic effect [45]. On the other hand, that might be attributed to impurities and electrostatic formation into the structure upon an addition of a surfactant [46, 47]. During the ChOx catalytic oxidation reaction at the time of measurement results the product [48]:

$$Cholesterol + O_2 \rightarrow 5\text{-}3\text{-}ketosteroid + H_2O_2 \qquad (1)$$

The 5-3-ketosteroid is the intermediate product at the time of reaction and during the isomerization of trans double bond at 5-6 position of steroid ring by intramolecular shifting of proton from 4-6 β sites and a stable product 4-3.ketosteroid is produced as shown below in the chemical reaction.

$$5\text{-}3\text{-}ketosteroid \ (isomerisation) \rightarrow 4\text{-}3\text{-}ketosteroid \qquad (2)$$
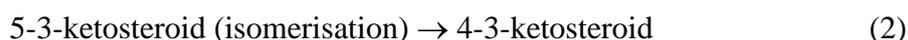
The output potential of the proposed cholesterol biosensor could be assigned to the above reaction mechanism and it creates the charge environment on the surface of ChOx immobilized on the Co_3O_4 interconnected nanowires based electrode. The Co_3O_4 nanostructures themselves have excellent electrocatalytic properties which further improve

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the enzymatic activity of ChOx along with the presence of quick and direct electron transfer between the various available sites of ChOx and the surface of Co_3O_4 nanostructures.

3.3. The study of stability, reproducibility, selectivity and repeatability of proposed cholesterol biosensor based on Co_3O_4 interconnected nanowires.

The stability of cholesterol oxidase immobilized Co_3O_4 interconnected nanowires based cholesterol biosensor was evaluated. The developed cholesterol biosensor exhibits good acceptable storage stability when it was used for 15 days, and the corresponding potential difference values are shown in Figure 6. The relative standard deviation (RSD) of the electrochemical response was found less than 5.0% for sensor electrode. This experiment suggested that the cholesterol biosensor could be used for routine monitoring of cholesterol with same sensitivity and reusability for longer period of time. During these experiments the obtained slopes were compared and it was found that sensor has ability to maintain its 95% sensitivity. This behavior could be assigned to well suited network of interconnected nanowires of Co_3O_4 for ChOx at the time of immobilization and further played a role in enhancing the catalytic properties of ChOx by Co_3O_4 nanostructures. The reproducibility of the proposed cholesterol biosensor was investigated using six individual sensors electrodes in 1×10^{-4} M cholesterol concentration and the results are shown in Figure 7. It can be judged that sensor to sensor response is highly reproducible with a relative standard deviation of 5%. This degree of reproducibility was attributed to the good entrapping of cholesterol oxidase molecules in the interconnected nanowires of Co_3O_4. The selectivity is the main parameter for evaluating the performance of sensor device in the presence of common interferents in human blood serum such as 0.5 mM of ascorbic acid, uric acid, glucose, urea, and Fe (II), respectively. The selectivity coefficient (k_{ij}) value was calculated by the fixed-interference method [44] using formula [49]:

$$a_{i,\min} = k_{ij}a_j^{z_i/z_j}$$

where: $a_{i,min}$ is the detection limit for the cholesterol molecule, all the interferents concentration (a_j) are fixed (0.5 mM), and the calculated K_{ij} values are found to be and 1.2 x10^{-4}, 1.2 x10^{-4}, 7 x 10^{-6}, and 7 x 10^{-6} , and for ascorbic acid, glucose, uric acid, , and Fe (II) respectively. The result shows a high degree of selectivity towards the detection of cholesterol even in more than the physiological concentrations of each interferent. The high selectivity of

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presented cholesterol biosensor might be due to immobilized cholesterol oxidase towards the selective sensing of cholesterol molecules.

Table I describe some characteristics of the ChOx/Co$_3$O$_4$ nanowires/Au cholesterol sensor along with some of those reported in the literature for other enzymatic nanostructured metal oxide electrodes. As can be seen there is a variety of materials used for cholesterol sensing. We have used potentiometric measurements which is easy to use, reliable, accurate, and inexpensive technique in addition to its suitability for biological systems for the analytical determination of cholesterol.

Conclusions

The surfactant assisted Co$_3$O$_4$ interconnected nanowires are synthesized on a gold coated glass substrate by hydrothermal method and cholesterol oxidase was entrapped in the interconnected nanowires of cobalt oxide for the chemical sensing of cholesterol molecules. Potentiometric measurements were used for the quantification of cholesterol molecules in the phosphate buffer solution of pH 7.3. The sensor device exhibited a sensitivity of −94.031 mV/decade and a fast response time of 10 s. The low limit of detection for cholesterol was found to be 5×10^{-8} M. The presented cholesterol biosensor is highly selective towards the detection of cholesterol concentrations and showed no response to common interferents. The high sensitivity and excellent selectivity are combined results of heavy loading of cholesterol oxidase in the interconnected nanowires of Co$_3$O$_4$ and enhanced catalytic properties of cholesterol oxidase on the sensitive surface of cobalt oxide nanostructures.

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Figure captions

Figure 1**:** XRD spectrum of Co_3O_4 interconnected nanowires.

Figure 2: SEM images of Co_3O_4 interconnected nanowires at different magnifications.

Figure 3: Schematic diagram of (a) an experimental setup of two electrodes system, and (b) the arrangement of the potentiometric cholesterol biosensor.

Figure 4: Calibration curve for the cholesterol biosensor.

Figure 5: Response time curve for the fabricated sensor device.

Figure 6: Stability graph for the fabricated biosensor.

Figure 7**:** Reproducibility curve for the sensor to sensor response.

Table 1: Characteristics of $ChOx/Co_3O_4$ nanowires/Au cholesterol sensor along with those reported in some literature for other enzymatic nanostructured metal oxide electrodes.
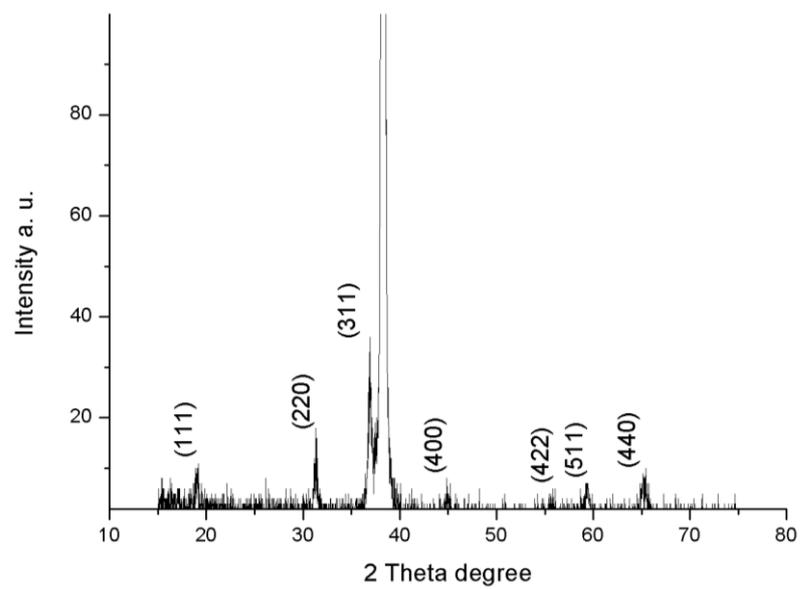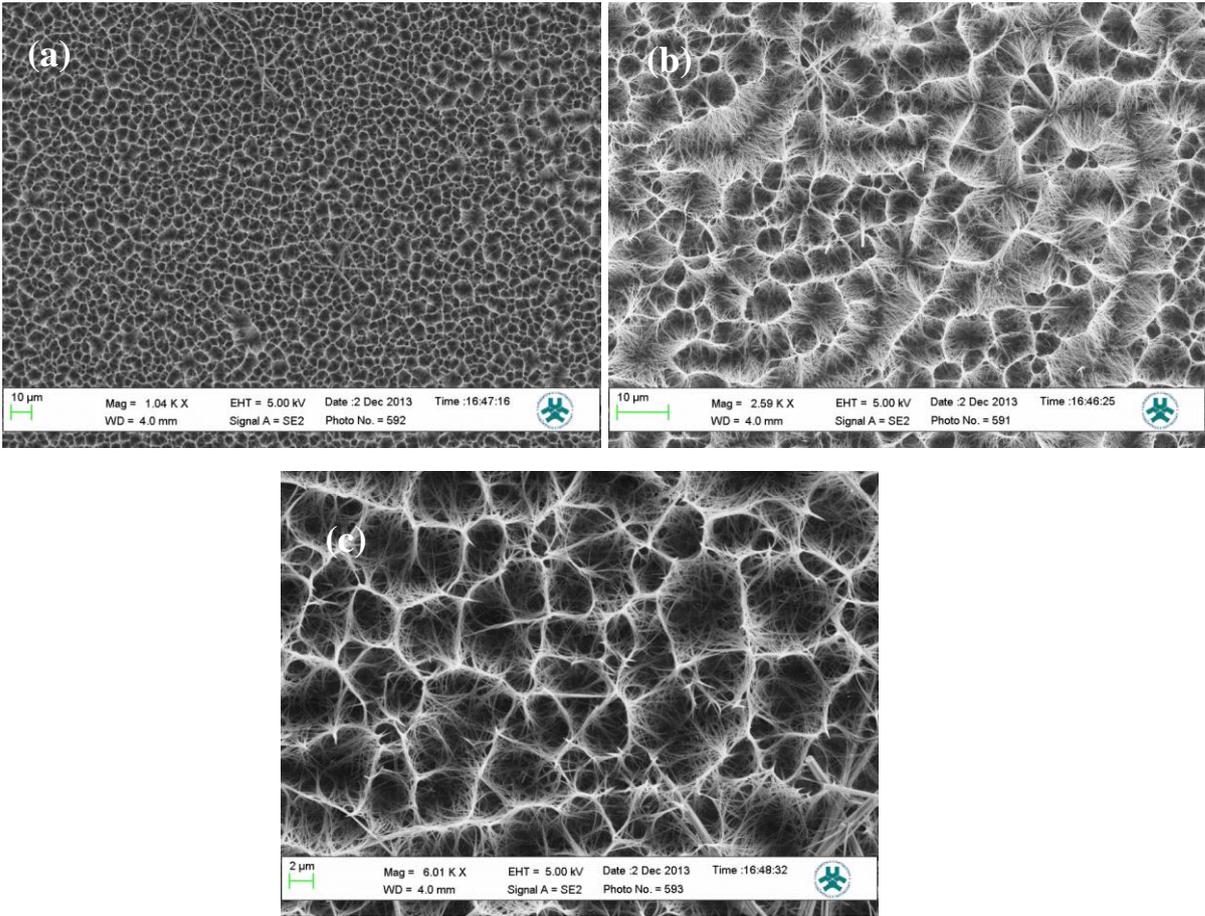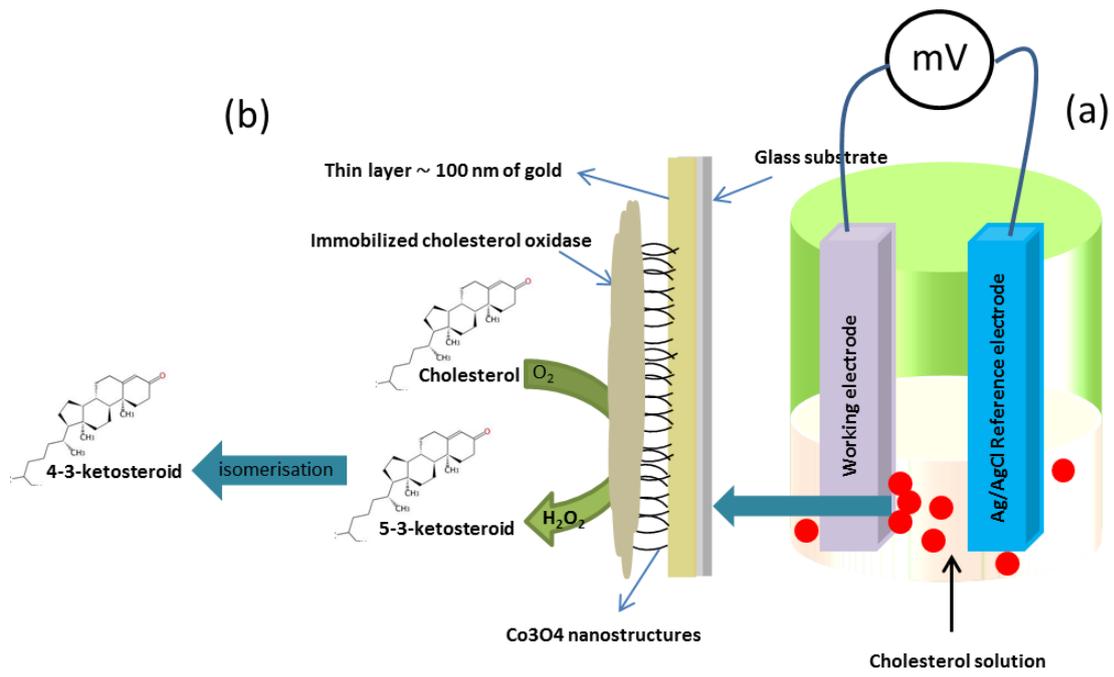
Fig. 1:

Fig.2:

Fig. 3:

(b)

(a)

mV

Glass substrate

Thin layer ~ 100 nm of gold

Immobilized cholesterol oxidase

Cholesterol O₂

4-3-ketosteroid   isomerisation

5-3-ketosteroid   H₂O₂

Co3O4 nanostructures

Working electrode

Ag/AgCl Reference electrode
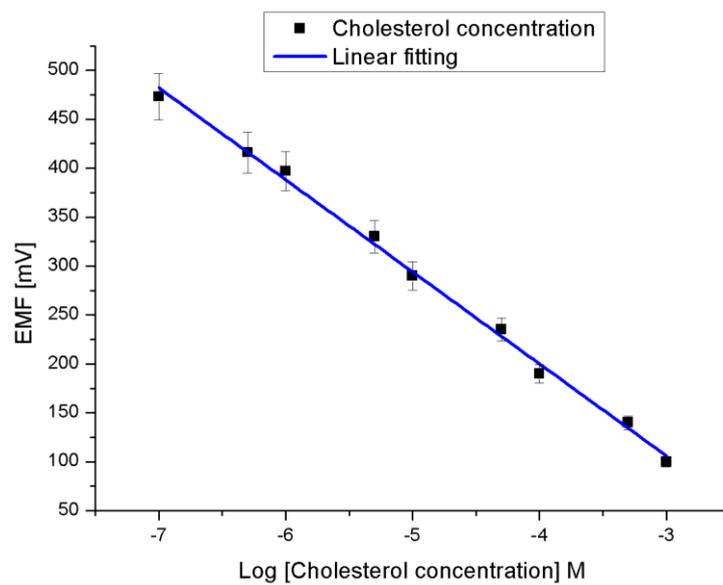
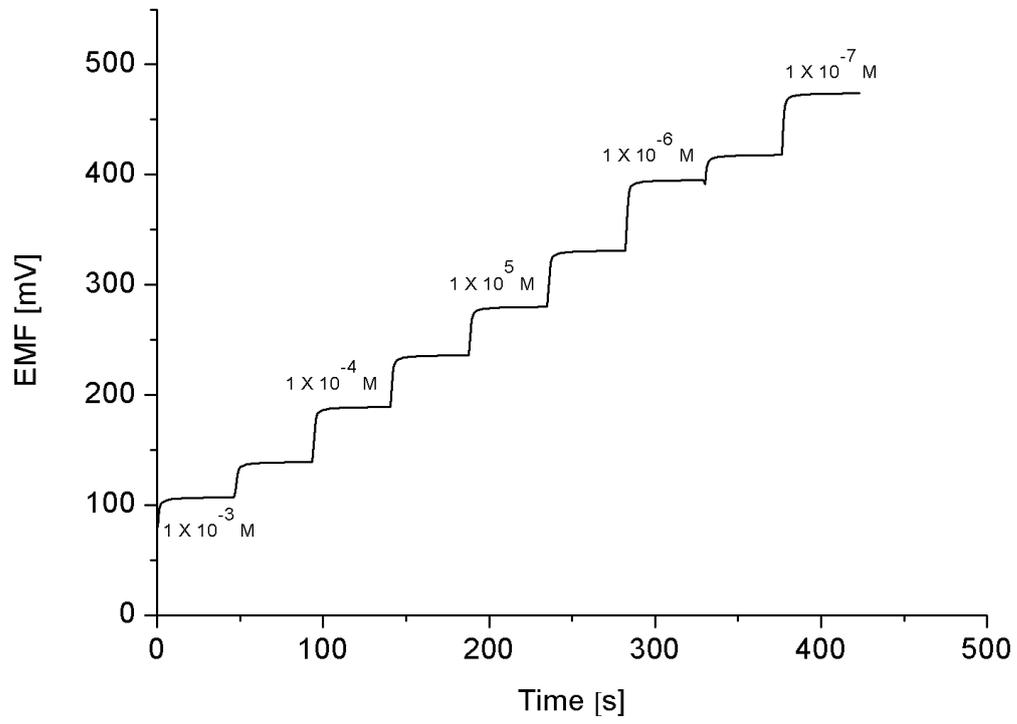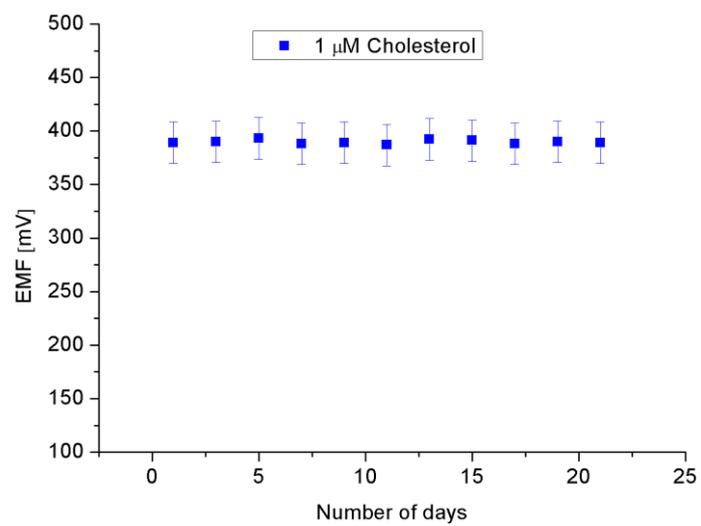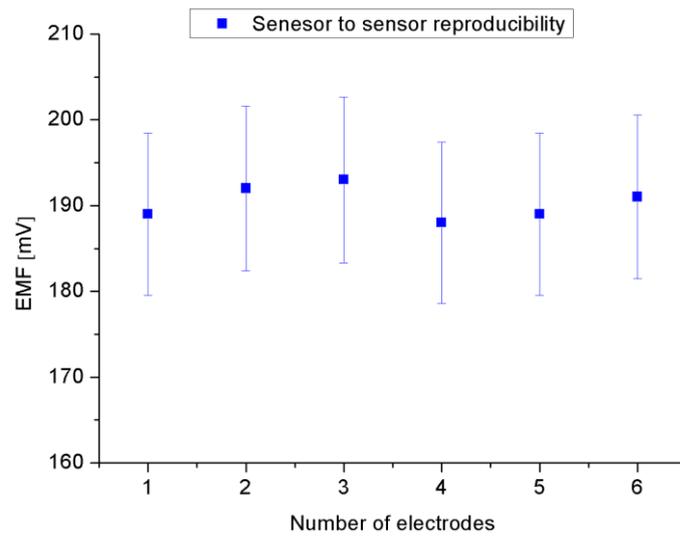Cholesterol solution

Fig. 4:

Fig. 5:

Fig. 6:

Fig. 7:

Table I:

Working electrode	Technique	Linearity [M]	LOD [M]	Km [M]	Sensitivity	Response time [s]
ChOx/ Iron nanoparticales [17]	Spectrophotometric	1.3×10^{-3} – 5.2×10^{-3}	-	0.45×10^{-3}	-	-
ChOx / ZnO nanoporous thin film /Au [18]	Electrochemical	0.65×10^{-3} – 10.34×10^{-3}	-	2.1×10^{-3}	-	-
ChOx/ cobalt oxide nanomaterials/ Glassy Carbon [19]	Flow injection analysis	4.2×10^{-6} – 50×10^{-6}	4.2×10^{-6}	0.49	43.5 nA mM^{-1} cm^{-2}	15
ChOx/ chitosan-tin oxide nanobiocomposite/ ITO [20]	Amperomtric	0.26×10^{-3} – 10.36×10^{-3}	0.13×10^{-3}	3.8×10^{-3}	34.7 mA/mg dL^{-1} cm^2	5
ChOx/Fe$_3$O$_4$ nanoparticles [21]	Amperomtric	1.3×10^{-3} – 5.2×10^{-3}	0.5×10^{-3}	0.45×10^{-3}	-	50
ChOx/ CuO nanowires/Au [22]	Potentiomtirc	5×10^{-6} – 5×10^{-3}	1×10^{-6}	-	33.88 mV/decade	10
ChOx/ Co$_3$O$_4$ nanowires/Au [this work]	Potentiomtirc	1×10^{-7} – 1×10^{-3}	0.035×10^{-7}	0.039×10^{-3}	-94.031 mV/decade	10