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Zinc oxide nanoparticles-chitosan composite film for cholesterol biosensor

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ABSTRACT

Zinc oxide nanoparticles (NanoZnO) uniformly dispersed in chitosan (CHIT) have been used to fabricate a hybrid nanocomposite film onto indium-tin-oxide (ITO) glass plate. Cholesterol oxidase (ChOx) has been immobilized onto this NanoZnO–CHIT composite film using physiosorption technique. Both NanoZnO-CHIT/ITO electrode and ChOx/NanoZnO-CHIT/ITO bioelectrode have been characterized using Fourier transform-infrared (FTIR), X-ray diffraction (XRD), cyclic voltammetry (CV), scanning electron microscopy (SEM) and electrochemical impedance spectroscopy (EIS) techniques, respectively. The ChOx/NanoZnO-CHIT/ITO bioelectrode exhibits linearity from 5 to 300 mg dl^{-1} of cholesterol with detection limit as 5 mg dl^{-1}, sensitivity as 1.41×10^{-4} A mg dl^{-1} and the value of Michaelis–Menten constant (K_m) as 8.63 mg dl^{-1}. This cholesterol biosensor can be used to estimate cholesterol in serum samples.

1. Introduction

Application of biosensors in clinical diagnostics has recently aroused much interest. Among the various clinical parameters, estimation of cholesterol has been considered very important since its increase is associated with heart diseases, obstructive jaundice, diabetes and nephrosis whereas decreased level of cholesterol is due to anemia, malabsorption wasting syndrome and hypothyroidism etc. [1–4]. Among the various methods of cholesterol estimation, biosensing technique has recently been suggested as vary important [4]. In the fabrication of a cholesterol biosensor, immobilization of an enzyme on an electrode is generally the first step in the fabrication of a biosensor. The selection of an immobilization technique is crucial for the performance of a biosensor and the future progress for development in biosensor design will inevitably focus upon the technology of new materials [5–7] that offer promise to solve the biocompatibility and biofouling problems. However, there is a considerable scope for development of cholesterol biosensor with improved detection limit, sensitivity and shelf-life etc.

Chitosan (CHIT) has been found to be an interesting biopolymer for immobilization of desired biomolecules because of excellent film-forming ability, high permeability, and mechanical strength, nontoxicity, biocompatibility, low cost and easy availability etc. Moreover, chemical modification of the amino groups of CHIT provides hydrophilic environment for the biomolecules [8–9]. Many attempts have been made to improve the properties of CHIT for application to biosensors. Some of the approaches include its structural modification, adjustment of molecular factors and incorporation of metal oxide nanoparticles in the biopolymeric network.

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Nanostructured metal oxides are known to have unique ability to promote faster electron transfer kinetics between electrode and the active site of desired enzyme [10–15]. Among the metal oxide nanoparticles, ZnO nanoparticles have been exploited as a potential material for biosensing, because their unusual properties including high surface area, high catalytic efficiency, nontoxicity, chemical stability and strong adsorption ability (high isoelectric point ~9.5). Nanoporous ZnO greatly enhances the active surface area for strong adsorption of biomolecules [13–15]. Thin films of ZnO nanorods and nanocombs have been investigated for the fabrication of glucose biosensor [14,15]. Electrodeposited nanoporous ZnO film has been used for the interfacing with myoglobin for biosensing applications [16]. Direct electron transfer reactions of microperoxidase have been achieved using ZnO nanoparticles on a pyrolytic graphite electrode for the detection of hydrogen peroxide [17]. A mediator free phenol biosensor has been fabricated based on immobilizing tyrosinase to ZnO nanoparticles [18]. Uric acid has been immobilized on ZnO nanorods for the fabrication of urea biosensor [19].

It is interesting to note that ZnO nanoparticles dispersed in CHIT can be used for immobilization of desired biomolecules [20,21]. In this context, nanocomposite thin film of CHIT and ZnO nanoparticles has been proposed by various workers for fabrication of amperometric immunosensor for human IgG [22], horse raddish peroxidase based biosensor for H_2O_2 detection [23], preparation and mechanical properties of chitosan/CNT composites [24] and tyrosinase biosensor for detection of phenol [18]. However, no attempts have been made towards the development of cholesterol biosensor based on zinc oxide nanoparticles-chitosan composite (NanoZnO-CHIT).

In this manuscript, we report results of the studies carried out on immobilization and characterization of ChOx on NanoZnO-CHIT composite film deposited onto indium-tin-oxide glass using spin coating.

2. Materials and methods

2.1. Materials

Chitosan (Mw 2.4×10^6) zinc acetate dihydrate (98–99.5%), cholesterol oxidase (EC 1.1.36 from *Pseudomonas fluorescens*) with specific activity of 24 U mg^{-1} and horseradish peroxidase (HRP, EC 1.11.1.7) with specific activity of 200 U mg^{-1} solid was purchased from Sigma–Aldrich (USA).

The stock solution of ChOx (1 mg dl^{-1}) of 24 U ml^{-1} and horseradish peroxidase (200 U ml^{-1}) was freshly prepared in phosphate buffer (50 mM) at pH 7. The stock solution of cholesterol was prepared in 10% triton X-100 and was stored at 4 °C. *o*-dianisidine (1%) solution was freshly prepared in deionized water obtained from water purification system (Milli-Q 10 TS).

2.2. Instruments

Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) was used for amperometric measurements as well as electrochemical impedance spectroscopy measurements (0.01–10^5 Hz). These measurements were carried out using a three electrode cell with ChOx/NanoZnO-CHIT/ITO bioelectrode as the working electrode, a platinum (Pt) wire as the counter electrode, and saturated Ag/AgCl electrode as a reference electrode in phosphate buffer saline (PBS, 50 mM, pH 7.0, 0.9 NaCl) containing $[Fe(CN)_6]^{3-/4-}$. The UV–visible spectra were taken on Shimadzu (Model 160 A) and the FTIR spectra were recorded on PerkinElmer, Spectrum BX II spectrophotometer. The morphological studies were performed with scanning electron microscope (LEO-440) and phase identification in the ZnO and NanoZnO-CHIT film was performed with an X-ray diffractometer equipped with a thin-film attachment using Cu Kα radiation (Rigaku).

2.3. Procedure

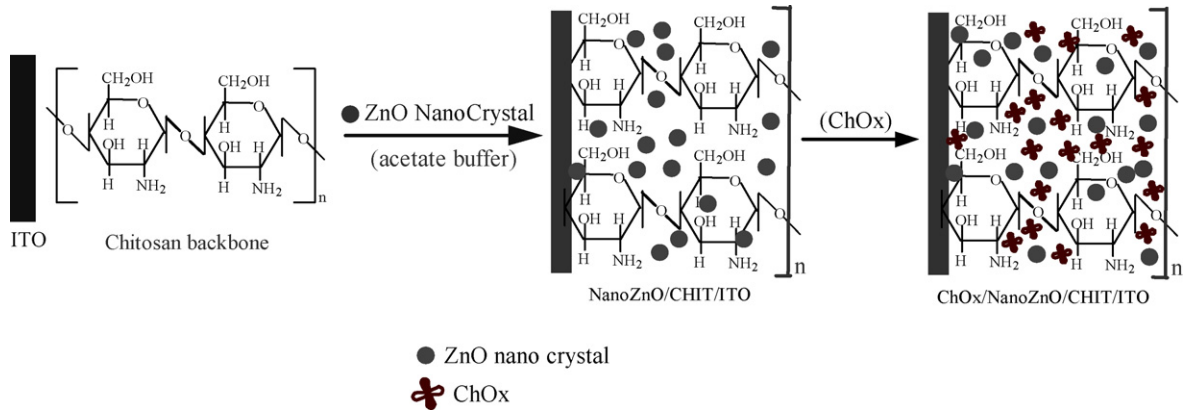
ZnO nanoparticles have been synthesized [25] with zinc acetate dihydrate (in 75 ml of ethanol) and ethanolic solution of lithium hydroxide monohydrate ($LiOH{\cdot}H_2O$) was mixed at room temperature under continuous stirring for 8 h. The nanoparticles were recovered from the colloidal solution by successive precipitation with hexane. 0.5 gm of CHIT flakes was dissolved in 10 ml of 0.05 M acetate buffer solution and was kept overnight at room temperature. 5 mg of ZnO nanoparticles were dispersed into transparent CHIT solution and magnetic stirring for about 30 min at room temperature followed by the sonication for about 1 h. The hybrid nanocomposite film fabricated using 10 μl of NanoZnO-CHIT solution was spread uniformly on to desired ITO substrate (surface area of 0.25 cm^2) using spin coating and was subsequently allowed to dry at room temperature. The NanoZnO-CHIT electrode was washed with deionized water followed by phosphate buffer (50 mM, pH 7.0).

The proposed mechanism for preparation of NanoZnO-CHIT electrode and immobilization of ChOx is shown in Scheme 1. 10 μl solution of freshly prepared ChOx (1 mg dl^{-1}) was spread onto the NanoZnO-CHIT electrodes. The ChOx/NanoZnO-CHIT/ITO bioelectrode was kept undisturbed for about 12 h at 4 °C. Finally, the dry bioelectrode was immersed in 50 mM PBS (pH 7.0) in order to wash out any unbound ChOx from the electrode surface.

The apparent enzyme activity (U cm^{-2}) was estimated using the method based on the difference of absorbance observed before and after the incubation of enzyme-bound electrode [26]. The apparent enzyme activity 5.2×10^{-3} Abs mg^{-1} dl^{-1} was evaluated using Eq. (1):

$$\alpha_{app}^{enz}(\mathrm{U\,cm^{-2}}) = \frac{AV}{\varepsilon_{st}} \quad (1)$$

where *A* is the difference in absorbance before and after incubation, *V* is the total volume (3.08 cm^3), ε is the millimolar extinction coefficient (7.5 for *o*-dianisidine at 500 nm), *t* is the reaction time (min), and *s* is the surface area (0.25 cm^2) of the electrode. For measurement, a solution of 20 μl HRP, 10 μl dye, and 50 μl of 100 mg dl^{-1} cholesterol was diluted by adding 3 ml PBS (pH 7.0) and was kept in a thermostat at 25 °C. The ChOx/NanoZnO-CHIT/ITO bioelectrode was immersed and was incubated for about 3 min. Then, this bioelectrode was removed and the absorbance of the solution was measured at 500 nm using a double beam spectrophotometer to estimate



Scheme 1 – The mechanism for preparation of NanoZnO-CHIT electrode and immobilization of ChOx onto NanoZnO-CHIT nanocomposite.

the concentration of product produced as a result of enzymatic reaction.

3. Results and discussion

3.1. Structural studies

Nano zinc oxide film and the NanoZnO-CHIT/ITO electrode have been characterized using X-ray diffraction (XRD) studies. The various X-ray diffraction peaks (supplementary information) corresponding to reflections $(10\bar{1}0)$, (0002), $(10\bar{1}2)$, $(11\bar{2}0)$, $(10\bar{1}3)$, $(20\bar{2}0)$, $(11\bar{2}2)$ and $(20\bar{1}1)$ indicate the presence of nanosized ZnO (38 nm) in the NanoZnO-CHIT composite film [25,27]. The particle size of ZnO nanoparticle in the NanoZnO-CHIT nanocomposite film has been estimated as about 41 nm.

The FTIR spectra of CHIT/ITO, NanoZnO-CHIT/ITO electrode and ChOx/NanoZnO-CHIT/ITO bioelectrodes have been recorded (Please see supplementary information). The CHIT exhibits characteristic absorption bands of amino saccharide at 3421 cm^{-1} (due to overlapping of –OH and $–NH_2$ stretching), 2811 cm^{-1} ($–CH_2$ stretching) and 1647 cm^{-1} (C–O stretching), while the NanoZnO-CHIT composite film shows additional peak at 492 cm^{-1} corresponding to the ZnO nanoparticle. ChOx/NanoZnO-CHIT/ITO bioelectrode reveals broadening of the peak at 3264 cm^{-1} and 1653 cm^{-1} due to the addition of carbonyl and amino groups confirming the binding of cholesterol oxidase with the NanoZnO-CHIT matrix [3].

3.2. Scanning electron microscopy studies

The surface morphologies of CHIT/ITO electrode, NanoZnO-CHIT/ITO electrode and ChOx/NanoZnO-CHIT/ITO bioelectrode have been investigated using SEM (Fig. 1). CHIT/ITO electrode shows homogenous surface (Fig. 1a), whereas the NanoZnO/CHIT/ITO electrode has uniform granular porous morphology attributed to the homogeneous dispersion of ZnO nanoparticles in CHIT (Fig. 1b) network. On immobilization of ChOx, the granular porous morphology of NanoZnO-CHIT changes into the regular form due to the electrostatic interaction between NanoZnO-CHIT with ChOx (Fig. 1c).

3.3. Electrochemical impedance measurements

Fig. 2 shows electrochemical impedance spectra (EIS) of (a) CHIT/ITO electrode (b) NanoZnO-CHIT/ITO electrode and (c) ChOx/NanoZnO-CHIT/ITO bioelectrode, respectively. The charge transfer process in ChOx/NanoZnO-CHIT/ITO bioelectrode has been studied by monitoring charge transfer resistance (R_{CT}) at the electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) (R_{CT}) depends on the dielectric and insulating features at the electrode/electrolyte interface. The R_{CT} values for the CHIT/ITO, NanoZnO-CHIT/ITO and ChOx/NanoZnO-CHIT/ITO electrodes have been obtained as 6.68×10^2, 4.47×10^2 and 9.38×10^2 Ω, respectively. The electron transfer via redox couple is hindered by the presence of cholesterol oxidase on the

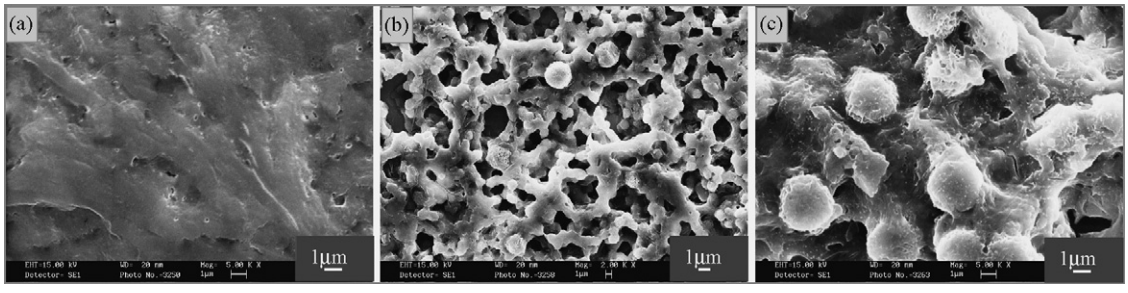


Fig. 1 – Scanning electron microscopy of (a) CHIT/ITO electrode (b) NanoZnO-CHIT/ITO electrode (c) ChOx/NanoZnO-CHIT/ITO bioelectrode.

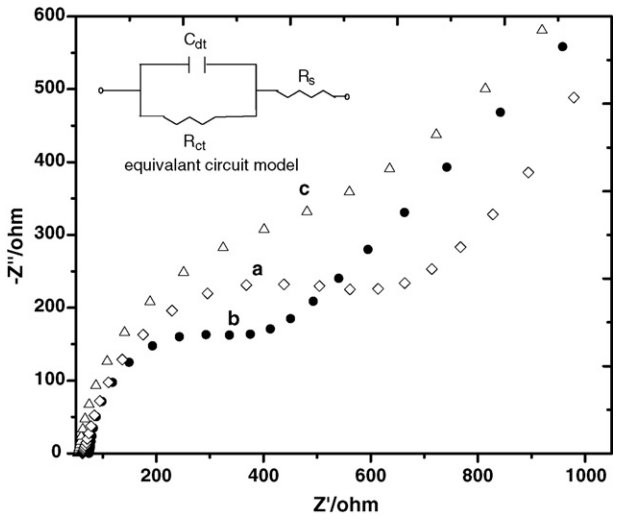


Fig. 2 – Impedance spectra of (a) CHIT/ITO electrode (b) NanoZnO-CHIT/ITO electrode and (c) ChOx/NanoZnO-CHIT/ITO bioelectrode in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$ (5 mM).

electrode surface. The increased R_{CT} value of ChOx/NanoZnO-CHIT/ITO electrode is due to the immobilization of ChOx onto NanoZnO-CHIT/ITO surface.

3.4. *Cyclic voltammetry studies*

Fig. 4a–c shows various cyclic voltammogrammes obtained for CHIT/ITO electrode, NanoZnO-CHIT/ITO electrode and ChOx/NanoZnO-CHIT/ITO bioelectrode in PBS (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$ (5 mM) recorded at different scan rates (10–100 mV s^{-1}). It can be seen that the anodic potential (Fig. 3a–c) shifts towards positive side and the cathodic peak potential shifts in the reverse direction. Besides this, the redox peak currents are proportional to the square root of scan rate, $\upsilon_{1/2}$ (inset of Fig. 3), indicating a diffusion electron-transfer process. it can be seen (Fig. 3b) that the redox potential of NanoZnO-CHIT electrode shifts towards the higher side than that of pure CHIT film due to the incorporation of ZnO nanoparticles. It appears that the NanoZnO-CHIT nanocomposite electrode provides a biocompatible environment to the ChOx enzyme, and ZnO nanoparticles act as an electron mediator resulting in a accelerated electron transfer between ChOx and electrode [28].

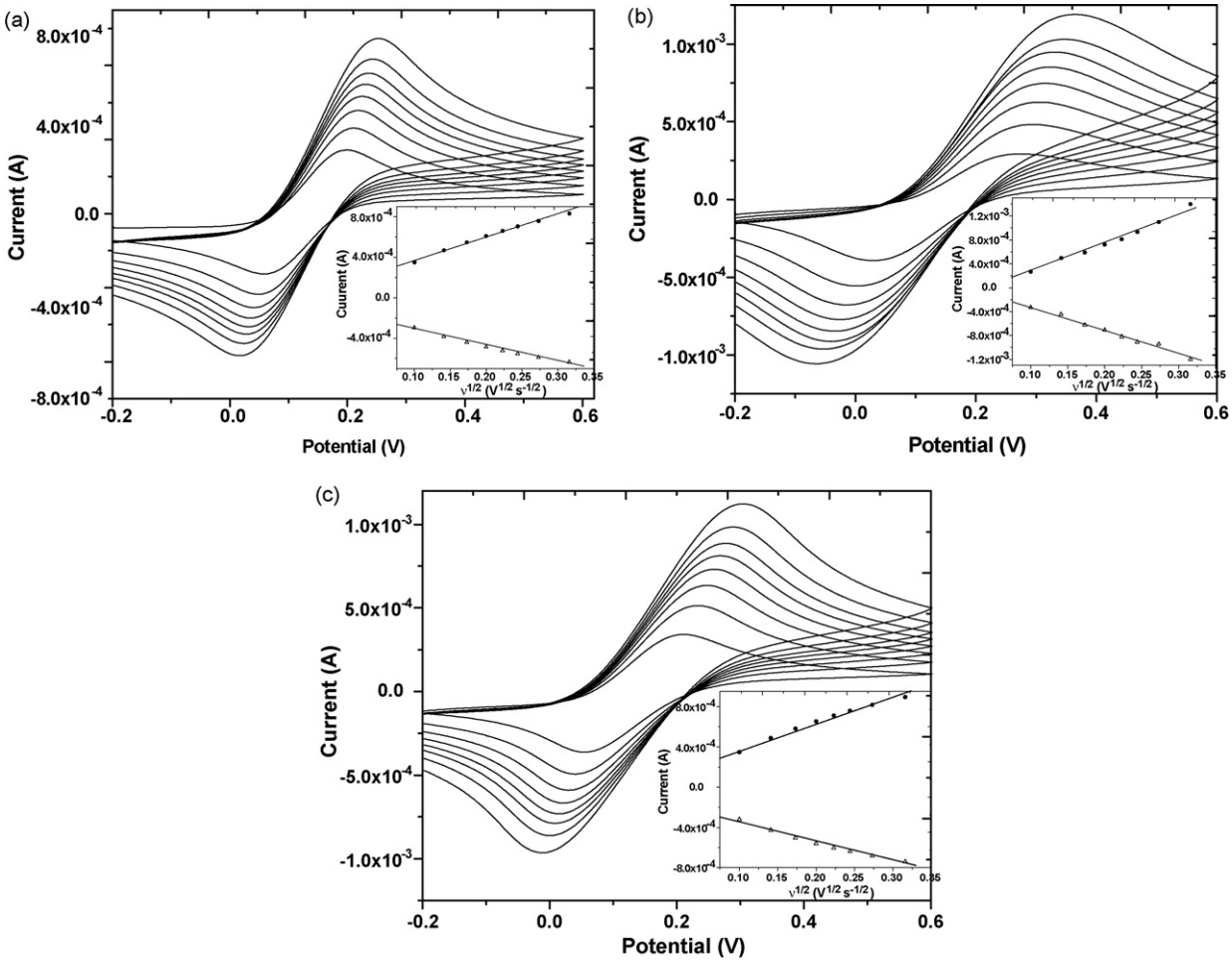


Fig. 3 – Cyclic voltamogrammes of (a) CHIT/ITO electrode (b) NanoZnO-CHIT/ITO electrode and (c) ChOx/NanoZnO-CHIT/ITO bioelectrode in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$ (5 mM), at 10–100 mV s^{-1} (from inner to outer). Inset: plots of peak currents versus square root of scan rate.

The surface concentration of the NanoZnO/CHIT film can be estimated from the plot of current versus potential (CV) using Brown-Anson model that is based on the following equation [29]

$$I_p = \frac{n^2F^2I^*AV}{4RT} \quad (2)$$

where n is the number of electrons transferred which is 1 in the case of NanoZnO/CHIT, F is the Faraday constant (96,584 C mol^{-1}), I^* is the surface concentration (mol cm^2) obtained for the NanoZnO/CHIT electrode film, A is the surface area of the electrode (0.25 cm^2), V is the scan rate (30 mV s^{-1}), R is the gas constant (8.314 J^{-1} mol K), and T is the absolute temperature (298 K). The value of the surface concentration of the NanoZnO/CHIT electrode has been found to be 9.02×10^{-8} mol cm^2.

3.5. *Electrochemical response studies of ChOx/NanoZnO-CHIT/ITO bioelectrode*

Electrochemical response studies have been carried out on ChOx/NanoZnO-CHIT/ITO bioelectrode for different cholesterol concentrations in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$ (Fig. 4). Scheme 2 shows the biochemical reaction between ChOx and cholesterol.

The oxidation peak seen at 0.3 V corresponding to the oxidation of H_2O_2 arises due to the enzymatic reaction between cholesterol oxidase and cholesterol. The increase in the value of the oxidation current with increasing cholesterol concentration results in the increased concentration of H_2O_2 during the enzymatic reaction. The inset in Fig. 4 shows the variation of oxidation current obtained for the ChOx/NanoZnO-CHIT/ITO bioelectrode indicating linearity as 5–300 mg dl^{-1}.

3.6. *Photometric response studies*

The photometric studies of ChOx/NanoZnO-CHIT/ITO bioelectrode have been carried out at 500 nm as a function of cholesterol concentration (5–300 mg dl^{-1}) in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing the *o*-dianisidine and horseradish peroxidase. Fig. 5 shows increase in the absorbance with increase in cholesterol concentration. The

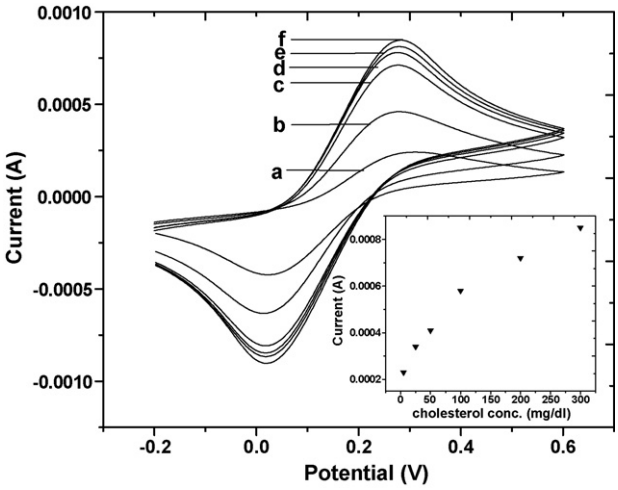
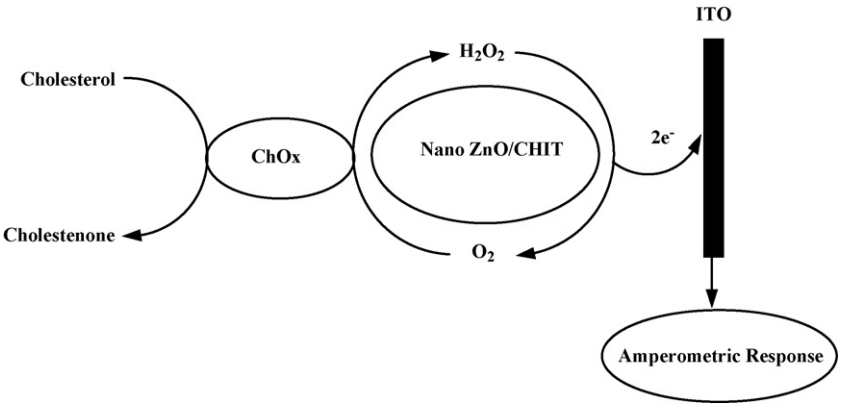


Fig. 4 – Amperometric response studies of ChOx/NanoZnO-CHIT/ITO bioelectrode as a function of cholesterol concentration from (5–300 mg dl^{-1}) using phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$ at scan rate of 30 mV s^{-1}. Inset: variation of amperometric current obtained as a function of cholesterol concentration.

linearity of ChOx/NanoZnO-CHIT/ITO bioelectrode is obtained as 5 to 300 mg dl^{-1} and the detection limit as 5 mg dl^{-1} with sensitivity as 1.41×10^{-4} A mg dl^{-1}.

The value of the Michaelis–Menten constant (K_m), that gives an indication of the enzyme-substrate kinetics, can be obtained from the photometric version of the Lineweaver–Burke plot [30]. The K_m value for this cholesterol biosensing electrode calculated, using the graph between inverse of absorbance and inverse of cholesterol has been found to be 8.63 mg dl^{-1} indicating high affinity for cholesterol.

The thermal stability of ChOx/NanoZnO-CHIT/ITO bioelectrode has been studied by measuring the absorbance at temperature ranging from 10 to 60 °C in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) at 500 nm. The absorbance increases with increasing temperature and becomes constant at 60 °C. The higher thermal stability of the ChOx/NanoZnO-CHIT/ITO bioelectrode can be attributed to the strong covalent



Scheme 2 – Biochemical reaction at the ChOx/NanoZnO-CHIT/ITO bioelectrode.

Table 1 – Effect of interferents on to ChOx/NanoZnO-CHIT/ITO bioelectrode in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl)

Substrate/ interferent	Cholesterol	Cholesterol + glucose	Cholesterol + ascorbic acid	Cholesterol + uric acid	Cholesterol + urea
Absorbance	0.012	0.012	0.013	0.011	0.014
Error		0	−0.001	+0.001	−0.002

Table 2 – Determination of free cholesterol concentration in blood serum samples

Samples (mg dl^{-1})	Free cholesterol (mg dl^{-1})	Average (mg dl^{-1})	RSD (%)	Total cholesterol concentration (auto analyzer)
1	48.3, 49.5, 50.0, 52.1	49.97	3.05	165
2	64.5, 62.2, 61.5, 65.3	63.37	1.79	215
3	69.2, 70.1, 72.3, 73.6	71.30	1.59	240

Table 3 – Summary of the various metal oxide nanoparticles and metal mixed biopolymer based cholesterol biosensor reported in the literature, along with their important parameters

S. No.	Electrode	Sensitivity	Response time (s)	Linear range	K_m values	Shelf life	Reference
1	ZnO nanoparticles	–	15	25–400 mg dl^{-1}	2.1 mM	75 days	[13]
2	Ni/K_3Fe(CN)6/CNT	–	<20	0.005–3 mM	–	1 month	[31]
3	ChOx/NanoPt/CNT	–	<20	4.0×10^{-6}–1.0×10^{-4} mol l^{-1}	–	6 weeks	[32]
4	ChOx/CS/MWCNT	1.55 μA mM^{-1}	<20	4.0×10^{-6} to 7.0×10^{-4}	0.24 mM	50 days	[33]
5	ChOx/NanoZnO-CHIT	1.41×10^{-4} A mg dl^{-1}	15	05–300 mg dl^{-1}	8.63 mg dl^{-1}	85 days	Present work

binding of ChOx with NanoZnO-CHIT/ITO bioelectrode. The value of the activation energy calculated from the Arrhenius plot has been obtained as 17.4 kJ mol^{-1}.

The effect of interferents (ascorbic acid, uric acid, glucose, lactic acid and urea) on the cholesterol measurements has been investigated by taking the solution containing (1:1) ratio of cholesterol (100 mg dl^{-1}) and interferents such as glucose (5 mM), ascorbic acid (0.05 mM), uric acid (0.1 mM), lactic acid (0.5 mM) and urea (1 mM). The results (Table 1) indicate negligible effect of these interferents on the photometric response of ChOx/NanoZnO-CHIT/ITO electrode.

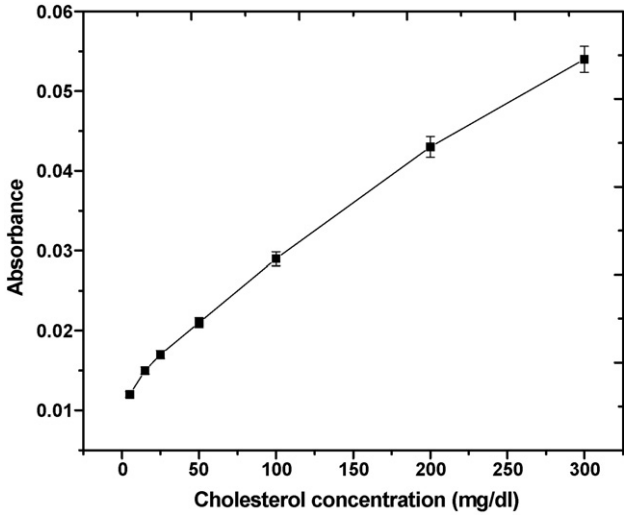


Fig. 5 – Photometric response of ChOx/NanoZnO-CHIT/ITO bioelectrode as a function of cholesterol concentration (5–300 mg dl^{-1}) in PBS.

The response time of the ChOx/NanoZnO-CHIT/ITO bioelectrode has been determined as 15 s. The shelf-life of the ChOx/NanoZnO-CHIT/ITO bioelectrode measured after an interval of 6 days has been estimated as 85 days. The decrease in the value of the amperomteric current has been found to be about 25% up to about 8 weeks where after the current decreases sharply resulting in about 75% loss in about 12 weeks (data not shown).

3.7. *Response to serum samples*

Attempts have been made to estimate free cholesterol (30%) using ChOx/NanoZnO-CHIT/ITO bioelectrode in serum samples obtained from a clinic located in Delhi. During these experiments, the ChOx/NanoZnO-CHIT/ITO bioelectrode dipped into the reaction mixture containing the peroxidase, o-dianisidine in PBS and absorbance have been recorded at 500 nm. It can be seen (Table 2) that the results are in reasonable agreement with those obtained using the ChOx/NanoZnO-CHIT/ITO bioelectrode. Table 3 shows the results of the present studies obtained using ChOx/NanoZnO-CHIT/ITO bioelectrode along with those reported in literature.

4. Conclusions

Nanocomposite thin film of ZnO nanoparticle and CHIT has been fabricated to immobilize ChOx via electrostatic interaction for the estimation cholesterol. It is found that granular porous morphology of NanoZnO-CHIT provides a better biocompatible environment for the enzyme. ChOx/NanoZnO-CHIT/ITO bioelectrode has been used for estimation of cholesterol in solution as well as in blood serum samples

by photometric and amperometric techniques, respectively. ChOx/NanoZnO-CHIT/ITO bioelectrode has linearity from 5 to 300 mg dl^{-1} and shelf life of 85 days. Efforts should be made to utilize ChOx/NanoZnO-CHIT/ITO bioelectrode to detect other metabolites such as urea, glucose, uric acid including toxicants etc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2008.04.010.

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