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Cholesterol biosensor based on electrochemically prepared polyaniline conducting polymer film in presence of a nonionic surfactant

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Abstract Cholesterol biosensor has been fabricated by covalently coupling cholesterol oxidase (ChOx) via glutaraldehyde onto electrochemically prepared polyaniline film in presence of TritonX-100 [4-(1,1,3,3-tetramethylbutyl) phenyl polyethylene glycol], a non-ionic surfactant onto indium-tin-oxide (ITO) glass substrate. These ChOx/PANI-TX-100/ITO bioelectrodes have been characterized using Fourier transform infrared (FTIR) spectroscopy, cyclic voltammetry (CV) and scanning electron microscopy (SEM) techniques. The results of response measurements carried out on ChOx/PANI-TX-100/ITO bioelectrodes using amperometric and photometric techniques, reveal detection limit as 5 mg/dl, linearity from 5 to 400 mg/dl of cholesterol and sensitivity as 131 μA/(mg/dl cm^{-2}). These biosensing electrodes are thermally stable up to 65 °C, can be used about 20 times and have a shelf-life of about 10 weeks when stored at 4 °C. Attempts have also been made to utilize the ChOx/PANI-TX-100/ITO bioelectrodes for estimation of free cholesterol concentration in serum samples.

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Introduction

Application of biosensors in clinical diagnostics has recently aroused much interest. This is because biosensors are portable, cost-effective, yield specific information about desired analytes quickly, and can be used by semi-skilled operators. Among the various biochemical metabolites, estimation of cholesterol in blood has been considered very important [1–3]. A high cholesterol level in human blood is related to arteriosclerosis, hypertension, myocardial infarction and many heart related disorders. The clinical analysis of cholesterol is important for the diagnosis and prevention of a large number of disorders with strong positive correlation about total cholesterol to various diseases [4–6]. Various analytical methods such as colorimetric [7] and spectrophotometric have been used for estimation of cholesterol [8–13]. These methods involve complicated procedures and are expensive since high volume of enzyme is needed in each assay [14].

There is considerable interest towards the application of conducting polymers to biosensors. This has been attributed to their many interesting properties such as biocompatibility, redox characteristics and the possibility of direct electron transfer between electrode and active sites of biomolecules.

Conducting polymer based cholesterol biosensors have recently attracted much attention. Singh et al. [15–17] have reported an amperometric cholesterol biosensor based on electrochemically prepared polyaniline (PANI) films. The shelf-life of this cholesterol biosensing electrode has been found to be about 6 weeks. The poly (An-*co*-Py)/ChOx

bioelectrode has been utilized for cholesterol estimation in the range of 1–10 mM with sensitivity as 93.35 μA/mM [18]. Charpentier et al. [10] reported an enzyme electrode based on carbon paste modified by hydroxymethyl ferrocene for estimation of cholesterol. Vidal et al. [19] developed a multi-analyte biosensor based on cholesterol oxidase and glucose oxidase. The detection limit for glucose and cholesterol has been estimated as 6 and 8 μM, respectively. Ram et al. [20] fabricated a cholesterol biosensor by immobilization of cholesterol oxidase and cholesterol esterase on composite of poly (styrene sulphonate) (PSS) deposited onto solid substrate. The linearity of these bioelectrodes has been obtained as 0–1 mM. The use of redox mediator tris (bipyrimidine) osmium (II) bis (hexafluoro phosphate) for fabrication of cholesterol electrode resulted in the linearity from 5 μM to 0.47 mM for free cholesterol and 2 to 1 mM for cholesterol ester [21].

Among the various conducting polymers, polyaniline has attracted much attention due to ease of preparation, high conductivity and good stability in environment. However, efforts are being made to find solutions to the problems relating to processability and poor thermal stability of PANI for application of this electrically conducting polymer in cholesterol biosensor. In this context, PANI has recently being synthesized in presence of nano-sized particles that can be easily dispersed in a polymer matrix or by using an appropriate emulsifier (surfactant) that may enhance its solubility for film formation [15, 16]. Surfactants have been used as additives during the polymerization of aniline such as to effect polymerization by using the emulsion or inverse emulsion pathways for immobilization of desired biomolecules.

In our scheme TX-100 has been introduced in PANI backbone during the electrochemical polymerization of PANI. TX-100 is a nonionic surfactant and has been used in biomedical application. The incorporation of TX-100 in PANI will improve the biocompatibility, better conformation and porous surface morphology in nanodimension to immobilize the ChOx to fabricate cholesterol biosensor. In the present manuscript, electrochemically prepared polyaniline film in presence of TX-100 (non-ionic surfactant) has been utilized for covalent immobilization of ChOx using glutaraldehyde as a cross-linker for application to cholesterol biosensor. ChOx/PANI-TX-100/ITO shows improved sensing characteristics such as high sensitivity, low activation energy, low K_m value and high thermal stability.

Experimental

Chemical and reagents

Aniline (99.5%) and hydrochloric acid (32%) of analytical grade were purchased from Fluka. Nonionic surfactant [TritonX-100 (TX-100), 99%] was procured from Sigma Aldrich, USA. Cholesterol oxidase (EC 1.1.36 from *Pseudomonas fluorescens*) with specific activity of 24 U/mg and horseradish peroxidase (HRP, EC 1.11.1.7) with specific activity of 200 U/mg solid was obtained from Sigma-Aldrich (USA). All other chemicals were of analytical grade and were used without further purification.

Preparation of PANI film in presence of TX-100

Aniline monomer were double distilled and kept under reduced pressure before polymerization. The electrochemical polymerization of PANI films with TX-100 was carried out in the potential range from −200 to +900 mV using chronoamperometry method on indium-tin-oxide (ITO) substrate with three-electrode cell configuration. Platinum foil was used as a counter electrode, Ag/AgCl as a reference electrode and an ITO glass plate as the working electrode. The electrochemical polymerization solution consisted of aniline and TX-100 (1:1) with 1 M HCl, used as supporting electrolyte and dopant. The polymer thin film was rinsed many times with deionized water.

Preparation of solutions

The solutions of cholesterol oxidase (24 U/ml) and horseradish peroxidase (HRP, 200 U/ml) were freshly prepared in phosphate buffer saline (PBS) (50 mM, pH 7.0, 0.9% NaCl) prior to being used. Stock solution of cholesterol was prepared in 10% TX-100 and was stored at 4 °C. This stock solution was further diluted to make different concentrations of cholesterol solution. *o*-Dianisidine (ODA, 1%) solution was prepared freshly in deionized water.

The electrochemical studies carried out on ChOx/PANI-TX-100/ITO bioelectrode for different cholesterol concentration in PBS (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$. The photometric studies of ChOx/PANI-TX-100/ITO bioelectrode as a function of cholesterol concentration (5–400 mg/dl) have been carried out in PBS (50 mM, pH 7.0, 0.9% NaCl) using the UV–vis spectrophotometer. During these experiments ChOx/PANI-TX-100/ITO bioelectrode is dipped in the reaction mixture containing 3.0 ml of PBS (50 mM, pH 7.0), 0.1 ml cholesterol, 0.05 ml HRP, 0.05 ml ODA for about 3 min to allow biochemical reaction to take place.

Immobilization of ChOx onto PANI-TX-100 films

Freshly prepared solution of cholesterol oxidase was prepared in 10% TX-100 and stored at 4 °C. The surface of PANI-TX-100 thin film was immobilized with ChOx by covalent linkage through glutaraldehyde (0.1%) using well-known chemistry. ChOx solution (10 μl) was spread on the

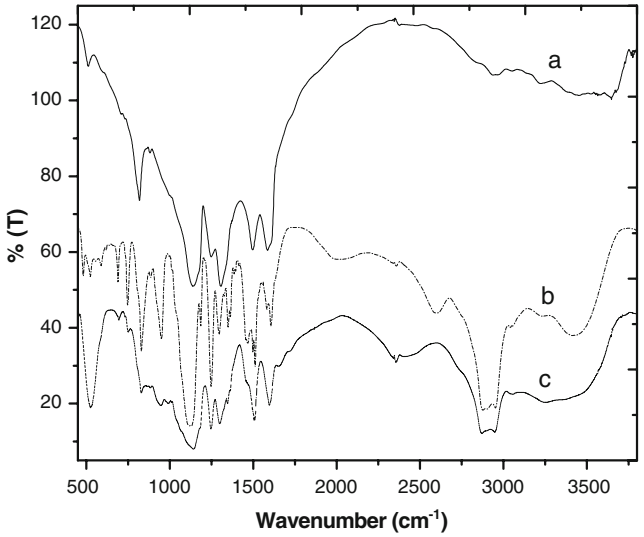


Fig. 1 FTIR spectra of *a* PANI/ITO, *b* PANI-TX-100/ITO electrode and *c* ChOx/PANI-TX-100/ITO bioelectrode

PANI-TX-100 film (containing covalently linked glutaldehyde) for the immobilization and subsequently kept overnight to allow the uniform distribution of enzymes on the surface of PANI-TX-100 matrix. Prior to being used, ChOx/PANI-TX-100/ITO bioelectrodes were rinsed with deionized water to remove any unbound or loosely bound enzyme molecules.

Characterization of PANI-TX-100/ITO electrode ChOx/PANI-TX-100 bioelectrode

Electrochemical polymerization and cyclic voltammetric measurements have been carried out on a Potentiostat/Galvanostat (Princeton Applied Research, 273A). The structural properties of PANI-TX-100/ITO electrodes and ChOx/PANI/TX-100/ITO bioelectrodes have been investigated using Fourier Transform Infrared spectrophotometer (Perkin Elmer) in the frequency range, 400–4,000 cm^{-1}. The surface morphology of thin film has been studied using Scanning Electron Microscopy (SEM), LEO 440 model.

Results and discussion

The FTIR spectra of electrochemically polymerized films of PANI, PANI-TX-100 and ChOx/PANI-TX-100/ITO bioelectrode are shown in Fig. 1. The absorption bands seen at 884 and 816 cm^{-1} (curve a) are attributed to the aromatic ring and out-of-plane C–H deformation vibrations for the 1, 4-disubstituted aromatic ring system [22]. The C–N stretching vibration mode in aromatic amine nitrogen (quinoid system) in doped polyani-

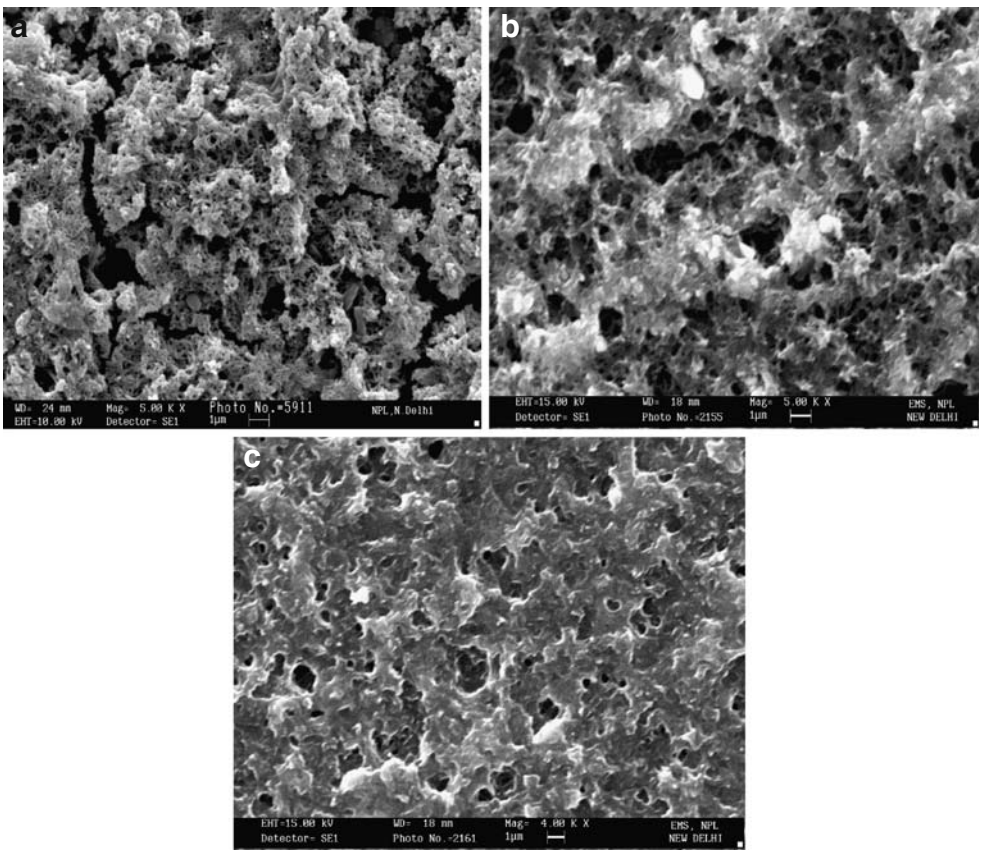


Fig. 2 Scanning microscopy of **a** PANI/ITO electrode, **b** PANI-TX-100/ITO electrode and **c** ChOx/PANI-TX-100/ITO bioelectrode

line is found at 1,297 cm^{-1} corresponding to the oxidation or protonation state. The absorbance peak at 1,237 cm^{-1} is assigned to C–N stretching vibration mode in benzenoid ring system of polyaniline due to the conducted protonated form. The in-plane vibration of C–H bending mode in N=Q=N, Q–N^{+}H–B or B–N^{+}H–B (where Q = quinoid and B = benzenoid) is observed at 1,156 cm^{-1} in the FTIR spectra. The presence of this absorption band is due to the polymerization of PANI i.e., polar structure of the conducting protonated form. The infrared spectrum of PANI-TX-100 consists of the absorption bands of TX-100 at 1,600, 1,460, 1,351, 1,298, 1,246, 1,184, 1,124 and 953 cm^{-1} [23]. The absorption bands observed at 1,570 and 1,469 cm^{-1} in PANI-TX-100 are assigned to the non-symmetric vibration mode of C=C in quinoid and benzenoid ring system in polyaniline, respectively.

Infrared spectra of ChOx-PANI-TX-100 bioelectrode consists of the absorption bands of PANI-TX-100 along with the characteristic bands of ChOx at 3,257, 1,720, 1,663 and 1,511 cm^{-1} (curve c). These modes assigned to the amide bonds [24] confirm the immobilization of ChOx on PANI-TX-100 thin film. It may be noted that the broad mode at 3,251 cm^{-1} may be assigned to the covalent bonding of ChOx.

Surface morphology studies of PANI-TX-100/ITO electrode and ChOx/PANI-TX-100/ITO bioelectrode

The surface morphologies of PANI/ITO, PANI-TX-100/ITO electrode and ChOx/PANI-TX-100/ITO bioelectrodes have been investigated using Scanning Electron Microscopy (SEM) (Fig. 2a–c), respectively. SEM of PANI film shows porous, rough structure (image a) wherein, a new regular cage like fibrillar surface morphology obtained after the incorporation of TX-100 in PANI matrix (image b). This might be due the interfacial polymerization of aniline in the vicinity of the micelle–water interface that produces fibrillar polymer growth [25]. After the immobilization of ChOx, cage like morphology of PANI-TX-100 has been changes into another regular form resulting due to coverage of available active cites on PANI-TX-100 film surface by ChOx (image c). It may be noted that the affinity of ChOx is very strong with TX-100 and its incorporation in PANI matrices may prevent the leakage of enzyme. The rough surface morphology has added advantage and ChOx are expected to adsorb strongly on the surface of PANI-TX-100. The observed change in the morphology of PANI by the incorporation of TX-100 may provide effective confirmation for the immobilization of ChOx.

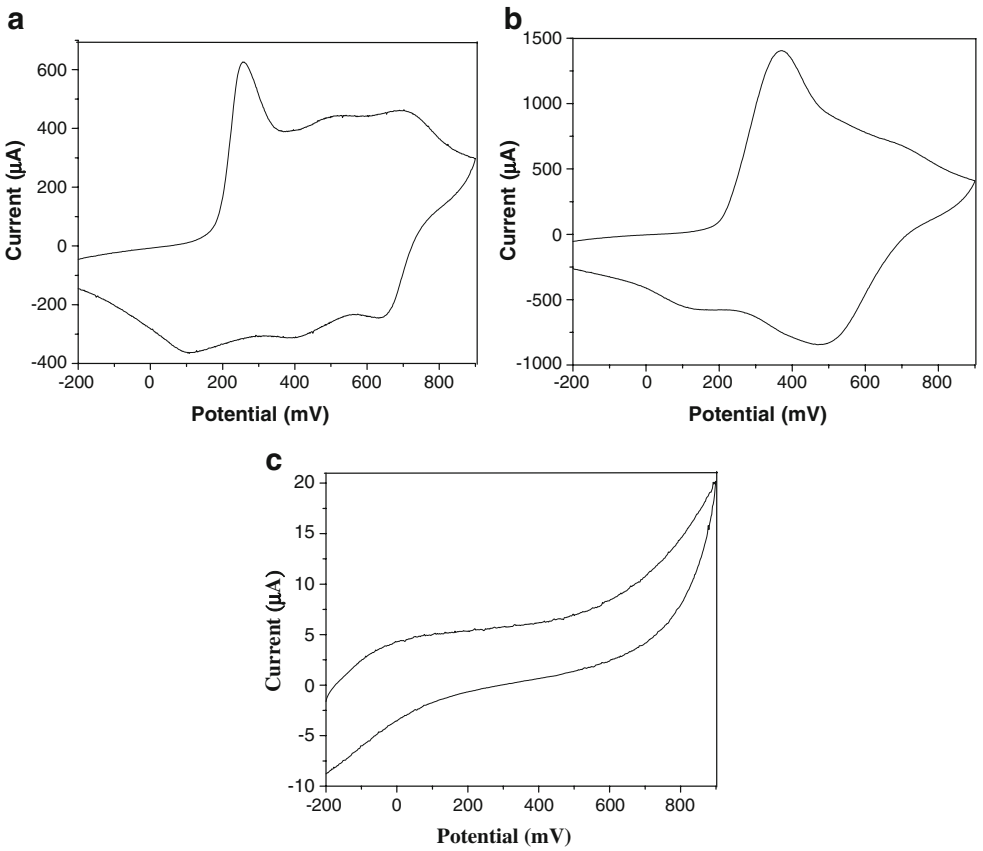


Fig. 3 Cyclic voltammograms of **a** PANI-HCl/ITO, **b** PANI/TX-100/ITO, **c** ChOx/PANI-TX-100/ITO bioelectrode in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) at scan rate 50 mV s^{-1}

Mechanism of covalent immobilization of ChOx onto PANI-TX-100 matrix

Glutaraldehyde is a bifunctional compound mainly used as a cross-linker for the chemical modifications of proteins and polymers. This bifunctional compound links covalently to the amine groups of ChOx creating a structure more stable than that attained by the physical aggregation. This type of bonding reinforces the compact tertiary structures resulting in protein stabilization against pH inactivation for immobilizing the respective enzymes. In the electrochemical polymerization of PANI-TX-100, a large number of free NH_2 groups available at the terminal of PANI unit, which bind with the NH_2 groups of ChOx with glutaldehyde as a cross-linker. And the C=O group of glutaldehyde binds with the NH_2 group via condensation reaction with the elimination of water molecules resulting in covalent linkage of ChOx with PANI.

Cyclic voltammetric studies

Figure 3 shows the cyclic voltammograms of (a) PANI-HCl/ITO, (b) PANI-TX-100/ITO and (c) ChOx/PANI-TX-100/ITO bioelectrodes recorded at scan rate of 50 mV s^{-1} in the range −200 to +900 mV using PBS (50 mM, pH 7.0, 0.9% NaCl). In PANI-HCl, the redox couple occurred at approximately +200 mV is assigned to the transition from the reduced leucoemealdine (LE) state to partially oxidized emeraldine state (EM) and the redox couple at +800 mV corresponding to the transition from LE to pernigraniline (PE) state, is accompanied by the oxidation of aniline monomer. A less intense redox couple observed at +500 mV is generally assigned to the redox reaction of *p*-benzoquinone. A shift in the redox peak towards high potentials with high magnitude of the current PANI-TX-100 (curve b) corresponds to PANI-HCl. The observed shift may be due to electrostatic interactions between TX-100 and PANI revealing that TX-100 increases the active surface area resulting in the electron transfer kinetics between medium and electrode. The ChOx/PANI-TX-100/ITO bioelectrode shows a broad hump (Fig. 3c) that may be assigned to insulating characteristics of ChOx protein. The immobilization of ChOx onto PANI-TX-100 blocks the charge carriers of PANI-TX-100 matrix resulting in slow redox process in ChOx/PANI-TX-100/ITO bioelectrode during the bio-electrochemical reaction.

Sensing characteristics of ChOx/PANI-TX-100/ITO bioelectrodes

Electrochemical studies

It can be seen (Fig. 4) that no defined peak observed in CV of ChOx/PANI-TX-100/ITO in PBS at pH 7. The electrochemical studies have been out on ChOx/PANI-TX-100/ITO

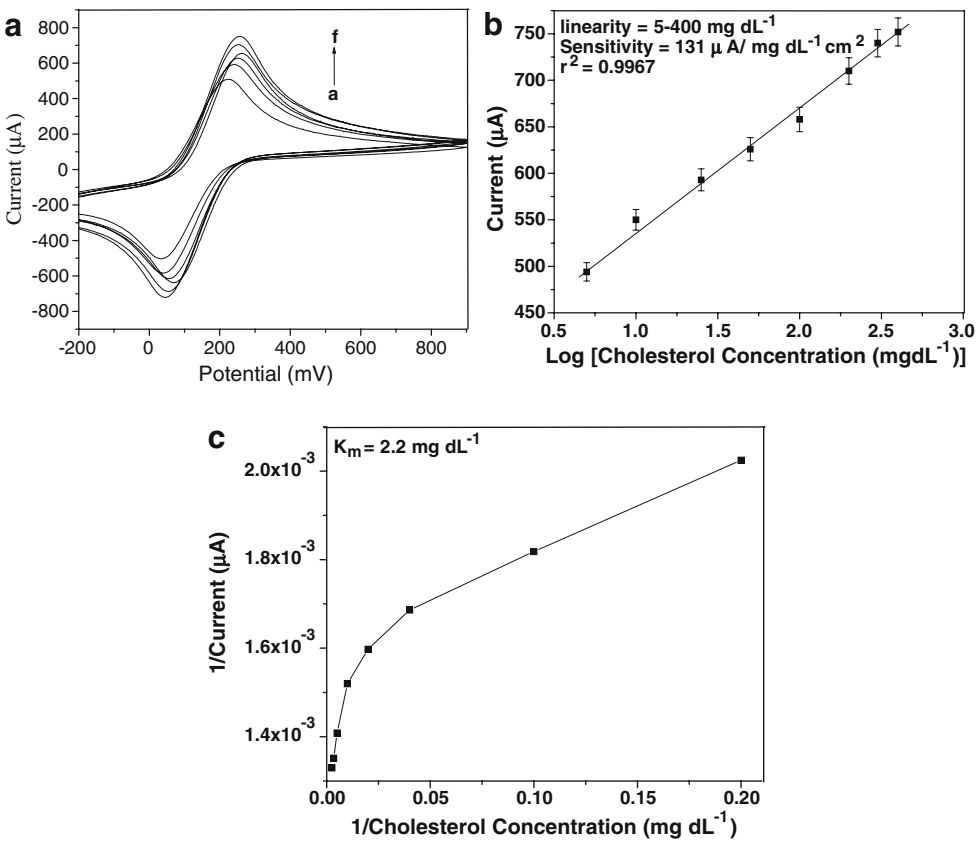
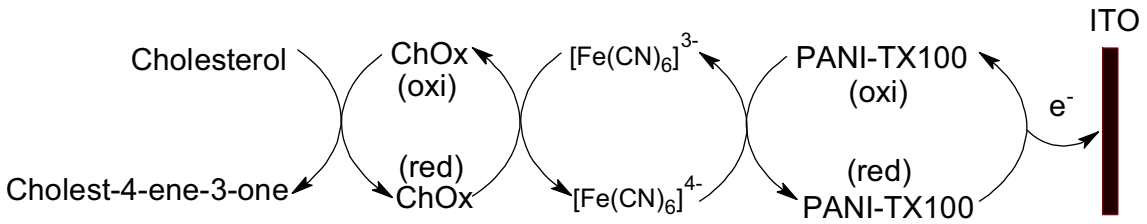


Fig. 4 **a** Amperometric studies of ChOx/PANI-TX-100/ITO bioelectrode as a function of cholesterol concentration using phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing $[Fe(CN)_6]^{3-/4-}$ at scan rate of 50 mV s^{-1}. **b** Current response curve (current magnitude vs log of cholesterol concentration) as a function of cholesterol concentration, **c** Lineweaver–Burke plot between reciprocal of cholesterol concentration and current magnitude



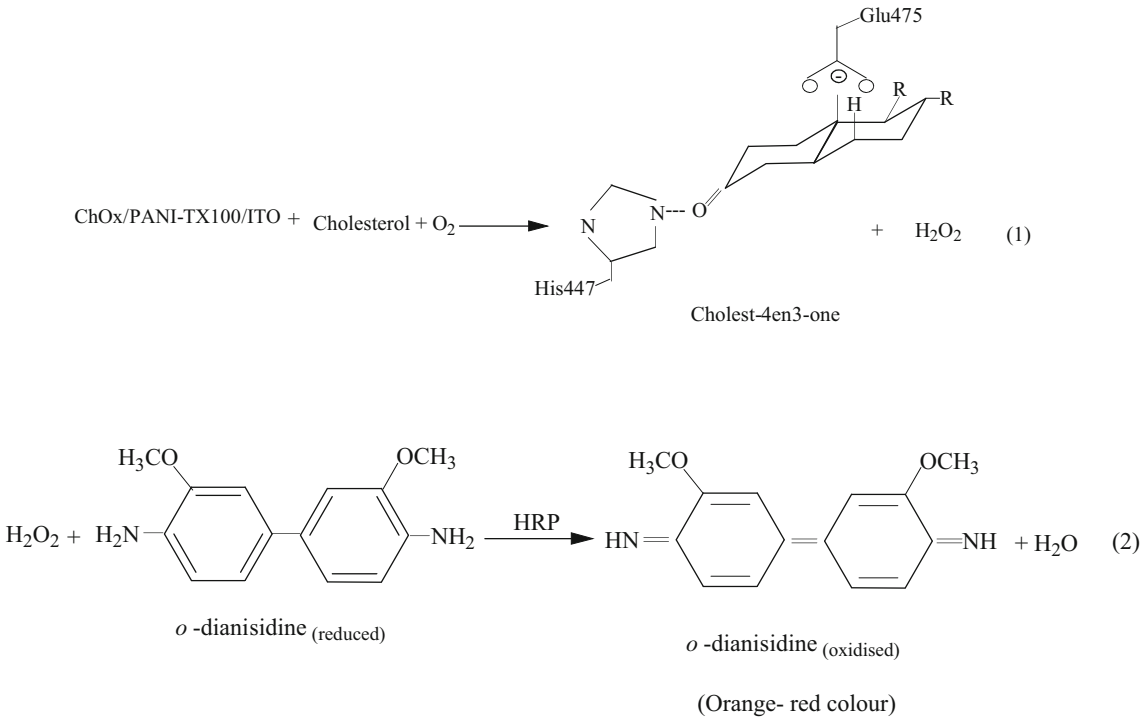
Scheme 1 Proposed biomedical reaction at ChOx/PANI-TX-100/ITO bioelectrode during cholesterol detection

bioelectrode for different cholesterol concentration in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl) containing [Fe $(CN)_6]^{3-/4-}$ are shown in Fig. 4. When $[Fe(CN)_6]^{3-/4-}$ is added onto electrode, it should be reduced by the enzyme and the oxidation current due to re-oxidation at the electrode should increase. The oxidation peak at around 0.222 V is due to the oxidation of PANI-TX-100 is at higher potential compared to that of PANI (0.2 V). However, there is no oxidation peak related to the generation of H_2O_2 (usually obtained at 0.05 V). This may be due to the well aligned and close pack network of PANI-TX-100 that act as a good electron accepter compare to the molecular oxygen. During the reoxidation of ChOx after enzymatic reaction, the well-aligned PANI chains accept electrons from the reduced enzyme (via Fe (III)/Fe(IV) conversion), thereby causing an increase in the oxidation current of PANI in CV measurements (Fig. 4).

The mechanism for enzymatic reaction and catalytic action of PANI has been shown in Scheme 1.

It can be seen from the linear regression curve (magnitude of the current vs log of cholesterol concentration) of the ChOx/PANI-TX-100/ITO bioelectrode (Fig. 4b) that the ChOx/PANI-TX-100/ITO bioelectrode can be used to estimate cholesterol from 5 to 400 mg/dl. The sensitivity of the ChOx/Glu/PANI-TX-100/ITO bioelectrode calculated from the slope of curve has been found to be 131 μA/mg/dl cm^{-2}. The correlation coefficient and standard deviation from the linear regression analysis for the bioelectrode have been found to be 0.98 and 1.41, respectively.

The value of the Michaelis–Menten constant (K_m) has been estimated using the Lineweaver–Burke plot i.e. a plot between inverse of cholesterol concentration and is the inverse of absorption (Fig. 4c). The Michaelis–Menten constant (K_m), indicating the affinity of enzyme for



Scheme 2 Biochemical reaction between the cholesterol and ChOx/PANI-TX-100/ITO bioelectrode in PBS (50 mM, pH 7.0, 0.9% NaCl), *o*-dianisidine and HRP

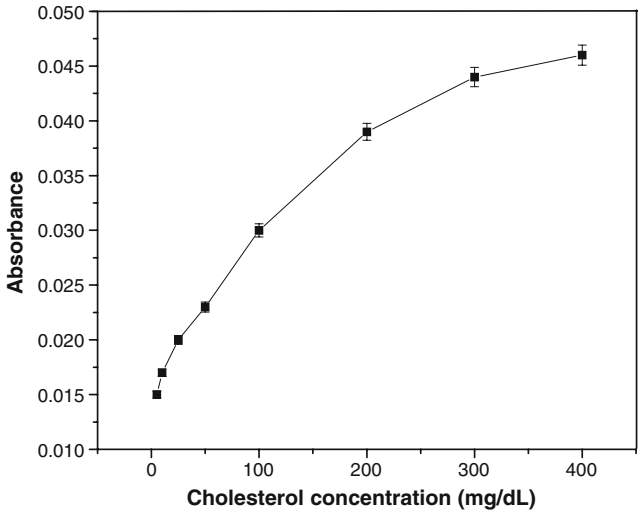


Fig. 5 Photometric response of ChOx/PANI-TX-100 bioelectrode as a function of cholesterol concentration (5–400 mg/dl) in PBS (50 mM, pH 7.0, 0.9% NaCl)

substrate, has been calculated as 2.2 mg/dl for cholesterol. The low K_m value of ChOx/PANI-TX-100/ITO bioelectrode indicates strong affinity of immobilized ChOx towards cholesterol. This result can be assigned to the uniform distribution of ChOx molecules retain its native characteristics on to the PANI-TX-100.

Photometric studies of ChOx/PANI-TX-100/ITO bioelectrode

The photometric studies of ChOx/PANI-TX-100/ITO bioelectrode as a function of cholesterol concentration (5–400 mg/dl) have been carried out in PBS (50 mM, pH 7.0, 0.9% NaCl) using the UV–vis spectrophotometer. During these experiments, ChOx/PANI-TX-100/ITO bioelectrode is dipped in the reaction mixture containing 3.0 ml of PBS (50 mM, pH 7.0, 0.9% NaCl), 0.1 ml cholesterol, 0.05 ml HRP, 0.05 ml ODA for about 3 min to allow biochemical reaction to take place. The biochemical reaction takes place during the incubation time and absorbance is monitored at

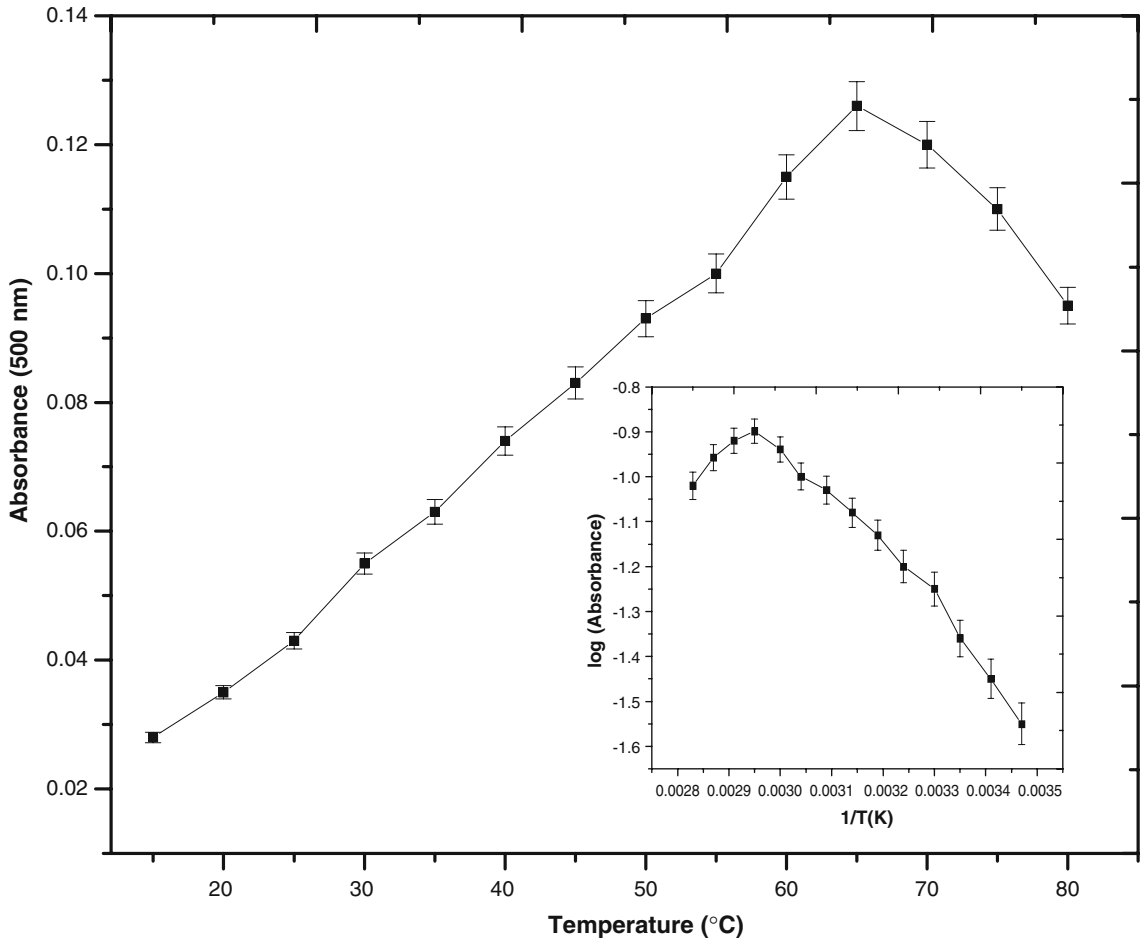


Fig. 6 Temperature study of ChOx/PANI-TX-100/ITO bioelectrode from 10–80 °C. *Inset:* Arrhenius plot for the effect of temperature on the response of ChOx/PANI-TX-100/ITO bioelectrode in the reaction mixture of PBS (50 mM, pH 7.0, 0.9% NaCl) and HRP and *o*-dianisidine and cholesterol 100 mg/dl

Table 1 Effect of interferents on to ChOx/PANI-TX-100/ITO bioelectrode in phosphate buffer (50 mM, pH 7.0, 0.9% NaCl)

Bioelectrode	Cholesterol concentration (100 mg/dl)	Cholesterol + urea (100 mg/dl+2 mM)	Cholesterol + uric acid (100 mg/dl+0.2 mM)	Cholesterol + glucose (100 mg/dl+5 mM)	Cholesterol + ascorbic acid (100 mg/dl+0.05 mM)
ChOx/PANI-TX-100/ ITO bioelectrode	0.029	0.024	0.024	0.029	0.027

500 nm. All reactions have been carried out in triplicate sets at room temperature (Scheme 2).

In a biochemical reaction, the ChOx/PANI-TX-100/ITO bioelectrode dip in cholesterol solution produced choleste-4en-3one and hydrogen peroxide (H_2O_2). H_2O_2 then oxidise *o*-dianisidine resulting change in color in the presence of HRP. The photometric technique wherein the enzymatic reaction results in increase in the intensity of orange red color (*o*-dianisidine$_{oxi}$) produced with increasing concentration of cholesterol via oxidation of *o*-dianisidine.

Figure 5 shows the photometric response of ChOx/PANI-TX-100/ITO bioelectrode as a function of cholesterol concentration. The linear relationship is observed in the cholesterol concentration range from 5 to 200 mg/dl. When cholesterol concentration is larger than 200 mg/dl the absorbance value gets saturated this may be due to restriction of the enzymatic reaction involved. The lower detection limit is obtained as 5 mg/dl.

The thermal stability of ChOx/PANI-TX-100/ITO bioelectrode has been investigated by measuring absorbance at temperatures ranging from 10 to 80 °C in PBS (50 mM, pH 7.0, 0.9% NaCl) at 500 nm. A fresh enzyme electrode is used to monitor the change in absorbance at different temperatures. The absorbance value increases on increases temperature to 65 °C and absorbance value deceases on further increasing the temperature (Fig. 6). The higher thermal stability of these ChOx/PANI-TX-100/ITO bioelectrodes can be attributed to the strong covalent binding of ChOx with to PANI-TX-100/ITO electrode. It appears that TX-100 enhances the thermal stability of ChOx/PANI-TX-100/ITO bioelectrode. The inset in Fig. 6 shows the variation of log (absorbance) as a function of reciprocal temperature (Arrhenius plot). The activation energy of ChOx/PANI-TX-100/ITO bioelectrode 22.96 kJ mol^{-1} has been obtained using the following equation:

$$\frac{d(\log k)}{d\,T} = \frac{\mathrm{Ea}}{2.303\,RT^2}$$

where, Ea is the activation energy; R is gas constant; k is the reaction constant at room temperature and T is the absolute temperature. The observed Ea value is smaller than the reported value for PANI based cholesterol biosensor [14, 17] indicating that the ChOx/PANI-TX-100 bioelectrode possesses higher enzyme (cholesterol oxidase) activity revealing higher affinity to cholesterol [26].

Effect of interferents on response of ChOx/PANI-TX-100/ITO bioelectrode

The performance of the ChOx/PANI-TX-100/ITO bioelectrode has been studied for various interferents such as ascorbic acid (0.05 mM), uric acid (0.2 mM), glucose (5.0 mM) and urea (2.0 mM) using UV–vis studies. Table 1 shows the effect of interferents on ChOx/PANI-TX-100/ITO during the estimation of cholesterol. This indicates that ChOx/PANI-TX-100/ITO bioelectrode during the cholesterol measurements in clinical sample does not interfere with the presence of interferents like urea, uric acid, glucose and ascorbic acid. This high selectivity can be attributed to the strong bonding of ChOx with the PANI-TX-100 matrix and high affinity of immobilized ChOx with free cholesterol.

Estimation of free cholesterol in serum samples

Cholesterol is found in blood samples as 30% in free and 70% in ester form in the biological media. In the present

Table 2 Determination of free cholesterol concentration in blood serum samples

Samples	Free cholesterol (mg/dl)	Average (mg/dl)	RSD (%)	Total cholesterol concentration in mg/dl (auto analyzer)
1	49.5, 48.0, 47.8	48.6	10.04	156
2	62.4, 61.2, 60.9	61.5	2.90	208
3	69.9, 69.0, 68.5	69.1	1.10	233

Table 3 Characteristics of ChOx/PANI-TX-100/ITO bioelectrode cholesterol biosensing electrode along with those reported in literature

Electrode	Immobilization technique	Sensing element	Linearity	Transducer used	Shelf-life	Sensitivity	Reference
Polypyrrole/PTS	Electrochemical entrapment	Cholesterol esterase, cholesterol oxidase	1–8 mM	Amperometric	28 days	0.15 μA/mM	[17]
Poly *N*-methyl pyrrole	Electrochemical entrapment and physical adsorption	Cholesterol oxidase	2–12 mM	Amperometric	60 days	0.205 mA(mM cm^2)$^{-1}$, 0.245 mA(mM cm^2)$^{-1}$, 0.45 mA(mM cm^2)$^{-1}$	[18]
Pt Prussian Blue polypyrrole	Entrapment	Cholesterol oxidase, chytochrome P450seck 201E	0.025–0.35 mM	Amperometric	25 days	441 nA mM^{-1}	[19]
Poly(styrene sulfonate) + poly (ethylene imine)	Adsorption	Cholesterol oxidase	0–1 mM	Amperometric	–	–	[20]
Poly(vinylferrocenium)	Electrostatic	Cholesterol oxidase	0.1–0.5 mM	Amperometric	14 days	140 μA M^{-1} m^{-2}	[27]
Polyaniline	Covalent linkage	Cholesterol oxidase, cholesterol esterase	50–500 mg/dl	Amperometric	42 days	7.5×10^{-4} nA/mg/dl	[28]
Polypyrrole	Electrochemical Entrapment	Cholestrol oxidase, ferrocene monocarboxylic acid	0.3 mM	Amperometric	10 days	–	[29]
3-Amino propyl-modified controlled -pore glass	Cross-linker	Cholesterol esterase, cholesterol oxidase, peroxidasse	1.2 μM–1 mM	Amperometric	25 days	–	[30]
Hydrogel	Cross-linking (glutaldehyde) and entrapment (agarose)	Flavocytochrome P450 sec	~300 μM (cross-link), 150 μM agarose)	Amperometric	–	13.8 nA μM^{-1} (glutaldehyde), 6.9 nA μM^{-1} (agarose)	[31]
Pt/Polyaniline/ChOx	Electrochemical entrapment	Cholesterol oxidase	0.01–0.1 mM	Amperometric	11 days	199.6 μA/M	[32]
Diaminonaphthalene isomers: Poly (1,5-DAN), poly (1,8-DAN)	Entrapment	Cholesterol oxidase	0.3 mM	Amperometric	10 days	19.58 nA mM^{-1}, 35.51 nA mM^{-1}	[33]
Polymeric film (TBMPC)	Physical entrapment	Cholesterol oxidase, horseradish peroxidase	0.04–0.27 mM	Amperometric (flow-injection)	–	–	[34]
PANI-TX-100/ITO	Covalent linkage	Cholesterol oxidase	5–400 mg/dl	Amperometric	70 days	131 μA/mg/dl	Present work

work free cholesterol has been estimated in serum by photometric method using the ChOx/PANI-TX-100/ITO bioelectrode employing cholesterol oxidase, peroxidase, *o*-dianisidine in the reaction media using PBS (50 mM, 0.9% NaCl, pH 7.0) at 500 nm. Fresh serum samples were obtained from a local hospital. The value obtained by Auto analyzer is the total cholesterol in serum sample while the developed ChOx/PANI-TX-100/ITO bioelectrode detect only free cholesterol (30%) in serum sample. The cholesterol concentration in serum sample is calculated using ChOx/PANI-TX-100/ITO bioelectrode that is nearby 30% of total cholesterol (Table 2). This shows the value obtained using ChOx/PANI-TX-100/ITO bioelectrode is in good agreement with that of obtained using Auto analyzer.

Shelf life

The stability of ChOx/PANI-TX-100/ITO bioelectrode has been investigated by observing the photometric response at various intervals of time (0, 2, 7, 14, 30, 45, 60 and 70 days). The ChOx/PANI-TX-100/ITO bioelectrodes are kept at 4 °C prior to being used. The value of the absorbance obtained at different time intervals are compared with those obtained in the initial stage (0 day). On the basis of UV–vis absorbance measurements for given concentration of cholesterol, the activity of the immobilized cholesterol oxidase is found to be more than 85% after about 70 days suggesting the stability of electrode for about 10 weeks. Table 3 show the results of the present studies obtained using ChOx/PANI-TX-100/ITO along with those reported in literature.

Conclusions

Cholesterol oxidase has been covalently linked to electrochemically prepared polyaniline film in presence of non-ionic surfactants Triton X-100, [4-(1,1,3,3-tetramethylbutyl) phenyl polyethylene glycol] using glutaraldehyde on to indium-tin-oxide (ITO) glass substrate. These ChOx/PANI-TX-100/ITO bioelectrodes are found to be thermally stable up to 65 °C and can be used to estimate cholesterol from 5 to 400 mg/dl. The ChOx/PANI-TX-100/ITO bioelectrodes has been used to estimate free cholesterol concentration in serum samples. Attempts should be made to utilize these electrodes for estimation of total cholesterol.

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