

Biosensor for total cholesterol estimation using *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane self-assembled monolayer

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Abstract Cholesterol oxidase (ChOx), cholesterol esterase (ChEt), and horseradish peroxidase (HRP) have been co-immobilized covalently on a self-assembled monolayer (SAM) of *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPTS) deposited on an indium–tin–oxide (ITO) glass surface. These enzyme-modified (ChOx-ChEt-HRP/AEAPTS/ITO) biosensing electrodes have been used to estimate cholesteryl oleate from 10 to 500 mg dL⁻¹. The sensitivity, K_m value, and shelf-life of these ChEt-ChOx-HRP/AEAPTS/ITO biosensing electrodes have been found to be 124 nA mg⁻¹ dL, 95.098 mg dL⁻¹ (1.46 mmol L⁻¹), and ten weeks, respectively. The ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes have been used to estimate total cholesterol in serum samples.

Keywords Cholesterol oleate · *N*-(2-Aminoethyl)-3-aminopropyltrimethoxysilane (AEAPTS) · Self-assembled monolayer (SAM) · Cyclic voltammetry · Biosensor

Introduction

There is an urgent need for the estimation of biochemical analytes such as glucose, galactose, uric acid, urea, and cholesterol in biological samples to monitor human health. Among these, estimation of total cholesterol has acquired the maximum attention since total cholesterol in serum is an indicator of abnormality in lipid metabolism and its abnormal level is associated with coronary artery disease, diabetes mellitus, hypothyroidism, anemia, and wasting syndromes, etc. In the past few years, biosensors have shown promise to provide a rapid and convenient alternative to conventional analytical methods to monitor these substances for various applications [1–6]. Efforts to develop a biosensor to estimate total cholesterol in routine tests are continuing due to the prevalence of cardiovascular diseases as a major threat around the world.

A crucial step in biosensor development is the immobilization of desired biomolecules on an electrode surface, because they rapidly lose their biological activity in an external environment. For fabricating a cholesterol biosensor, several matrices such as conducting polymers [7], nano-materials [8, 9], sol–gel films [10], and self-assembled monolayers [11], etc., have been used. Also, a number of immobilizing techniques such as the layer-by-layer technique [12], physical adsorption [13], hydrogel or sol–gel entrapment [14, 15], electro-polymerization entrapment [16], cross-linking [17], and covalent attachment [18, 19] have been attempted to stabilize biomolecules on the electrode surface. Among these, covalent immobilization of desired enzyme/s on the surface of an electrode via a self-assembled monolayer (SAM) is very attractive. The use of SAMs, ultra-thin and rugged organic films of controlled

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thickness, for anchoring enzymes has a number of advantages, such as high reproducibility, high degree of ordered molecular level control (e.g. distribution on the surface), and closeness to the surface [20, 21]. SAMs can be further modified into a chemically or biologically reactive surface via coupling of different materials to functional end groups of SAM [4, 22, 23]. Also, the covalent immobilization of biomolecules is known to satisfy various requisites, such as reproducibility, durability, and stability of the bio-electrode surface against variations in pH, temperature, ionic strength, and chemical nature of the microenvironment [24]. This immobilization method provides uniform, dense, oriented localization and prevents leaching resulting in higher enzymatic activity with low mass transfer resistance of the biomolecules.

Self-assembled monolayers consisting of different functional groups can be formed on metal and metal oxide surfaces. This can be achieved by using well-known metal–sulfur chemistry and silanization of metal oxide surfaces [25]. Silanes containing different terminal functional groups such as $-\text{NH}_2$ (amino), $-\text{COOH}$ (carboxyl), etc., can easily bind to oxide surfaces such as indium–tin–oxide (ITO) glass, silicon, etc., forming a thin organic self-assembled monolayer (SAM) [26–30]. These groups can be utilized for covalent immobilization of enzymes on a two-dimensional network of silanes. It has recently been shown that a self assembled monolayer of *N*-(2-aminoethyl)-3-aminopropyl-

trimethoxysilane (AEAPTS) can be used for estimation of free cholesterol via covalently bound ChOx [31].

In this manuscript we report results of studies carried out on a tri-enzyme (ChOx, ChEt, HRP)-modified AEAPTS SAM, on an ITO electrode, to realize a cholesterol biosensor for estimation of total cholesterol in a real sample.

Materials and methods

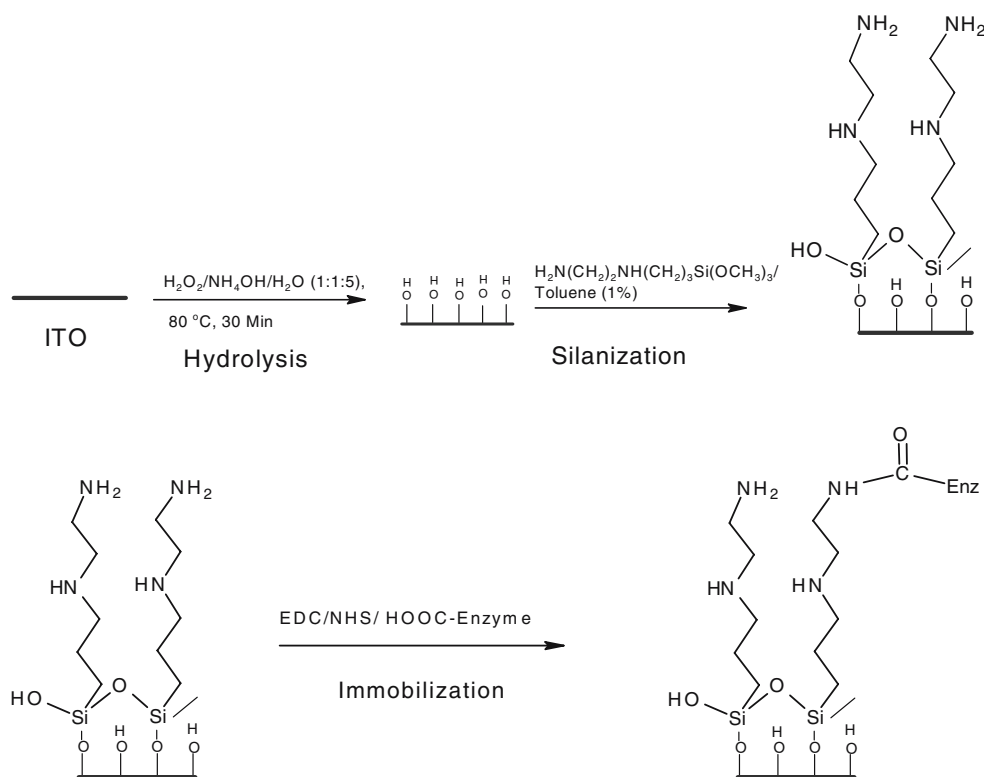
Chemicals and reagents

Cholesterol esterase (ChEt; EC 3.1.1.13, *Pseudomonas species*), specific activity 165 Units per milligram (U mg^{-1}), cholesterol oxidase (ChOx; EC 1.1.3.6, from *Pseudomonas fluorescens*), specific activity 24 U mg^{-1} , horseradish peroxidase (HRP, E.C.1.11.1.7), specific activity 200 U mg^{-1} , Brij (polydocanol), and cholesteryl oleate (>98%), were purchased from Sigma–Aldrich (USA). *N*-(2-Aminoethyl)-3-aminopropyltrimethoxysilane was procured from Merck, India. All other chemicals were of analytical grade and were used without further purification.

Solution preparation

Cholesterol oxidase (24 U mL^{-1}) and horseradish peroxidase (200 U mL^{-1}) solutions were freshly prepared in phosphate

Scheme 1 Schematic of covalent immobilization of enzymes onto AEAPTS/ITO surface using EDC/NHS



buffer (50 mmol L⁻¹), pH 7.0. Cholesterol esterase solution was prepared in phosphate buffer (50 mmol L⁻¹), pH 7.4, immediately before use. A stock solution of cholesteryl oleate (600 mg dL⁻¹) was prepared by first dissolving cholesteryl oleate in 10% Brij, by heating with stirring until it became clear and colorless, followed by addition of 1% hot NaCl solution to obtain the desired concentration. The prepared solution was stored at 4 °C in the dark when not in use. Working solutions of cholesteryl oleate were prepared from this stock solution using NaCl solution for further dilution. 4-Aminoantipyrine (1%) solution was freshly prepared in de-ionized water containing 2% 4-hydroxysulfonic acid.

Preparation of SAM of AEAPTS and Modification of SAM using ChEt, ChOx and HRP

SAM of AEAPTS on pre-cleaned ITO plates was prepared as reported elsewhere [31]. In brief, ITO plates were immersed in a solution of 1:1:5 (v/v) H₂O₂–NH₄OH–H₂O for about 30 min at 80 °C for hydrolysis after which they were rinsed thoroughly with de-ionized water and dried. The hydrolyzed ITO plates were immersed in a 1% (v/v) solution of AEAPTS in toluene overnight at room temperature for silanization. After the coupling reaction, the electrodes were rinsed with toluene and water to remove the physically absorbed silanes from the ITO surface. AEAPTS/ITO electrodes were then dried under a stream of nitrogen. For co-immobilization of ChEt, ChOx and HRP using EDC/NHS on to AEAPTS/ITO electrodes, the optimized ratio of enzyme solution (1.5 U ChEt, 0.12 U ChOx, and 0.2 U HRP) containing 13 μmol EDC and 3.5 μmol NHS was poured onto a 1 cm² area of ITO and kept for about 4 h in a humid chamber at room temperature for binding of the desired enzymes (Scheme 1). The ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode thus formed was washed thoroughly with phosphate buffer (50 mmol L⁻¹, pH 7.0) containing 0.9% NaCl and 0.05% Tween 20 to remove any unbound enzymes and was stored at 4 °C when not in use.

Characterization

These tri-enzyme-modified bio-electrodes were characterized using contact-angle (CA), atomic force microscopy (AFM), Fourier-transform infra-red (FT-IR), electrochemical impedance, cyclic voltammetry (CV), and UV–visible techniques. The hydrolyzed ITO, AEAPTS-silanized ITO, and ChEt-ChOx-HRP/AEAPTS/ITO electrodes were characterized for hydrophobic–hydrophilic character by contact-angle measurement using a drop shape analyzer (DSA 100, DSA/V 1.9) from Kruss, Hamburg. Atomic force microscopic (Veeco DICP2 atomic force microscope loaded with

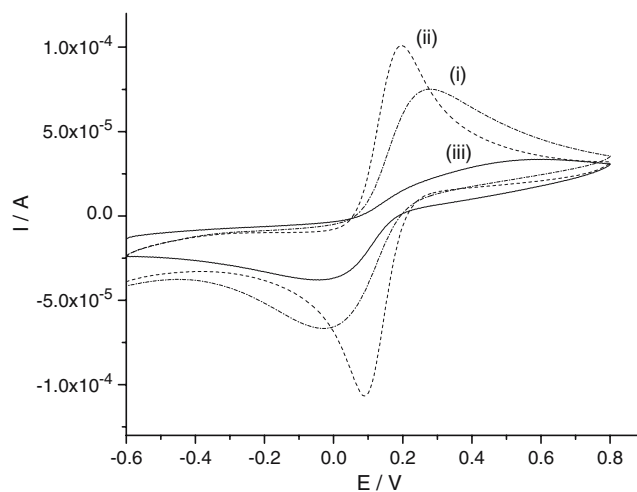


Fig. 1 Cyclic voltammetric response of (i) hydrolyzed ITO, (ii) AEAPTS/ITO electrode, and (iii) ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode

SPM Lab analysis software) and FTIR (Perkin–Elmer Model Spectrum BX) measurements were used for microstructural investigations and functional group characterization. Electrochemical studies (electrochemical impedance and cyclic voltammetry) were carried out using a three-electrode system with platinum (Pt) foil as counter-electrode and Ag/AgCl as reference electrode in phosphate buffer saline (PBS) solution (50 mmol L⁻¹, pH 7.3, 0.9% NaCl) containing a mixture of 0.5 mmol L⁻¹ Fe(CN)₆³⁻ and 0.5 mmol L⁻¹ Fe(CN)₆⁴⁻ (0.5 mmol L⁻¹ Fe(CN)₆^{3-/4-}) as redox probe, using an Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands). UV–visible experiments were conducted to study the activity of the tri-enzyme-modified bio-electrode (ChEt-ChOx-HRP/AEAPTS/ITO) using a UV–visible spectrophotometer (Model 160A, Shimadzu) and 4-aminoantipyrine dye (1%) in de-ionized water containing 4-hydroxysulfonic acid (2%). All reactions were carried out in triplicate at 30 °C. For the UV–visible measurements the electrode was dipped in 3 mL PBS solution containing 20 μL dye solution and 100 μL of substrate (cholesteryl oleate) and is left for about 5 min. Differences between the initial and final values of absorbance at 305 nm after 5 min incubation of the substrate were plotted.

Results and discussion

Results from CA, AFM, FT-IR, and electrochemical impedance studies (Electronic Supplementary Material S1–S4) carried out on hydrolyzed ITO, AEAPTS SAM, and tri-enzyme (ChOx, ChEt, HRP)-modified AEAPTS SAM were found to be similar to those reported elsewhere [31] confirming AEAPTS SAM formation and its modification by the three enzymes.

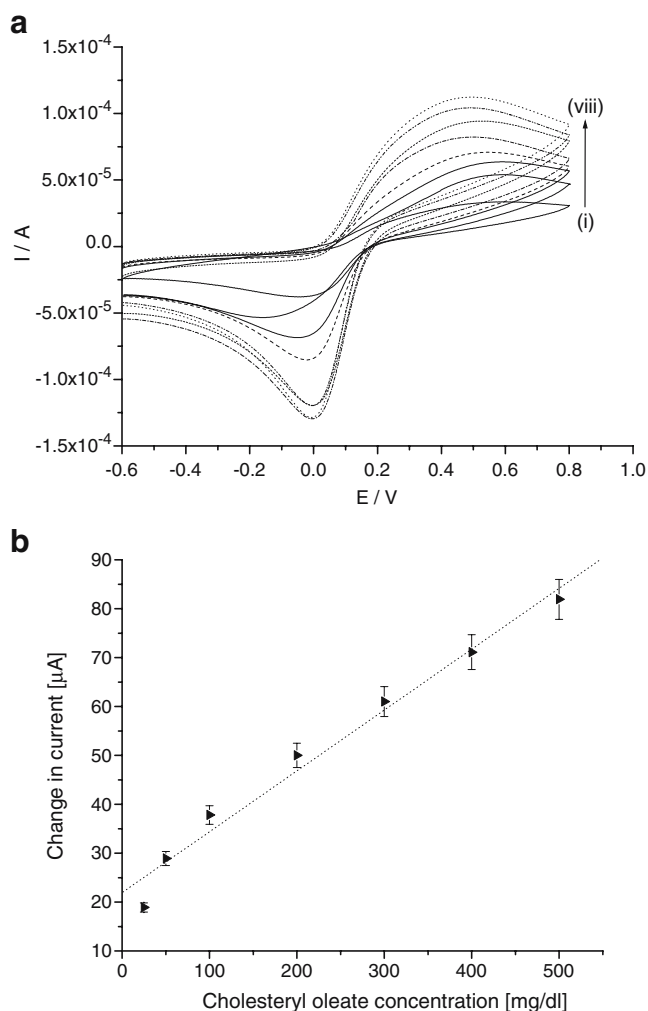


Fig. 2 (a) Cyclic voltammetric response curve of ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode as function of cholesteryl oleate concentrations of (i) 0 mg dL⁻¹, (ii) 25 mg dL⁻¹, (iii) 50 mg dL⁻¹, (iv) 100 mg dL⁻¹, (v) 200 mg dL⁻¹, (vi) 300 mg dL⁻¹, (vii) 400 mg dL⁻¹, and (viii) 500 mg dL⁻¹. (b) Linear regression curve of ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode

Cyclic voltammetric studies of hydrolyzed ITO, AEAPTS/ITO, and ChEt-ChOx-HRP/AEAPTS/ITO electrodes

Figure 1 shows the cyclic voltammograms obtained from (i) hydrolyzed ITO, (ii) AEAPTS/ITO, and (iii) ChEt-ChOx-HRP/AEAPTS/ITO electrodes. The increase in the oxidation current of the AEAPTS/ITO (curve ii) on gold plate compared to that of the hydrolyzed ITO electrode (curve i) indicates formation of the AEAPTS SAM. The increase in the value of current with improved reversibility of the system is attributed to the favorable redox behavior of $\text{Fe}(\text{CN})_6^{3-/4-}$ on the positively charged polarized AEAPTS SAM under experimental conditions. The presence of positive charge on the surface attracts the negatively charged redox probe towards the electrode surface and facilitates redox reaction at the surface. Further, the decrease in the

magnitude of the oxidation current in the cyclic voltammogram of the tri-enzyme-modified AEAPTS/ITO electrode (curve iii), due to the hindrance caused by the macromolecular structure and non-conducting nature of enzymes, confirms the immobilization of enzymes.

Response studies of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode

To study the activity of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode cyclic voltammetric (CV) experiments were conducted after incubation of the bio-electrode for 60 s in PBS-redox probe buffer solution containing cholesteryl oleate to produce H_2O_2 . H_2O_2 was oxidized by use of a cyclic voltammetric scan in the range -0.6 to 0.8 V (Fig. 2a) and the resulting increase in current at 0.5 V was recorded.

Figure 2a shows the current recorded in the cyclic voltammogram of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode as a function of cholesteryl oleate concentration wherein the oxidation peak observed around 0.5 V corresponds to the oxidation of H_2O_2 arising due to the enzymatic reaction between cholesteryl oleate and ChOx-ChEt. The increase in the value of oxidation current with increasing cholesteryl oleate concentration indicates an increase in the concentration of H_2O_2 . It can be seen from the linear regression curve of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode (Fig. 2b) that this electrode can be used to estimate cholesteryl oleate from 25 to 500 mg dL⁻¹. The sensitivity of the multi-enzyme bio-electrode calculated from the slope of curve was found to be 124 nA mg⁻¹ dL. The results from triplicate experiments, indicated by the error bars, reveal reproducibility within 4%.

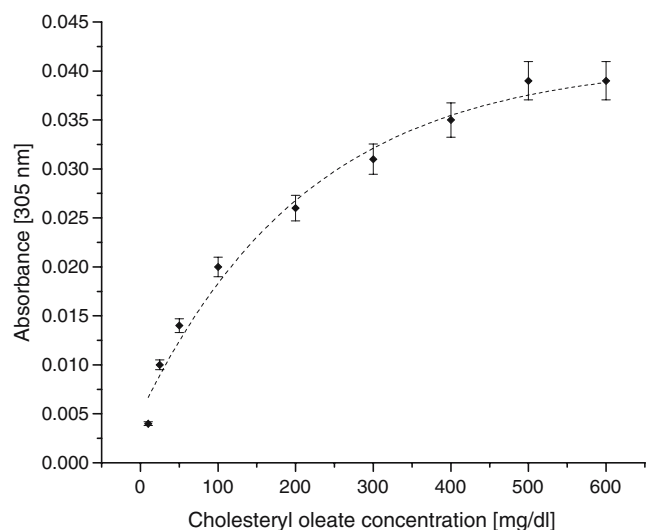


Fig. 3 Variation of UV-visible absorbance as a function of cholesteryl oleate concentration

Table 1 Comparison of data obtained by use of our electrode with those obtained by use of an auto-analyzer for total cholesterol measurement in serum

Sample no.	Standard auto-analyzer value (mg dL ⁻¹)	Value obtained by use of our electrode (mg dL ⁻¹)	Recovery on addition of 100 mg dL ⁻¹ cholesteryl oleate and measurement
1	103	108	210
2	152	143	239
3	200	188	285
4	173	177	270
5	180	170	269
6	132	127	230
7	142	144	250

UV-visible response studies of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode

Figure 3 shows the UV response of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode as a function of cholesteryl oleate concentration. It is clear from the figure that the electrode can be used to estimate cholesteryl oleate from 10 to 500 mg dL⁻¹. The results from triplicate experiments (4% error) indicate the reproducibility of the system. Further, the efficiency of immobilization was determined by UV-visible studies and has been estimated to be 13%. The value reveals that 13% of enzymes used for immobilization undergo binding. The immobilization procedure used is found to result in a reproducible bio-electrode, as indicated by the results from triplicate experiments. Utilizing the UV response curve, the enzyme-substrate kinetic parameter (Michaelis-Menten constant, K_m) was estimated from the Lineweaver-Burke plot, i.e. the graph of inverse absorption against inverse cholesteryl oleate concentration (Electronic Supplementary Material S5). Depending on the immobilization matrix and on the immobilization conditions [7, 32, 33] the value of K_m reveals the affinity of the enzyme for its substrate. The value of K_m for ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes was found to be 95.098 mg dL⁻¹ (1.46 mmol L⁻¹).

Effect of pH and interferents on the ChEt-ChOx-HRP/AEAPTS/ITO electrode

The crucial conformation and electrostatic state at the active site of the enzyme, which are important to enzyme activity, depend on the pH of the system. To find the optimum pH, the activity of the tri-enzyme-modified bio-electrode was investigated in the pH range 6.0–8.0 at 30 °C. The behavior of ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode at differ-

ent pH (Electronic Supplementary Material S6) indicates the electrode is most active between pH 7.0 and 7.5 (blood pH). Thus all the experiments were carried out at pH 7.3.

To investigate the effect of interferents present in serum samples on total cholesterol measurement, a large number of experiments were carried out. Serum samples with a known value of total cholesterol were tested to investigate the effect of proteins and other moieties present in serum (Table 1). All serum samples with a known value of cholesterol were collected from Dr Thakur's Pathological Laboratory, New Delhi, India. An RA50 auto-analyzer from Bayer was also used for cholesterol estimation. The auto-analyzer measurements, based on the absorbance of color produced with reagent, were recorded after 5 min incubation of the sample at 37 °C.

Table 1 shows a comparison of ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode data with those obtained by use of the auto-analyzer. Further, to these samples, a standard solution of cholesteryl oleate was added and 98–102% recovery was obtained (Table 1). A large number of serum samples of humans having cholesterol values similar to those indicated in Table 1 were tested and it was found that ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes have the potential for realization of a total cholesterol biosensor. A paired *t*-test was performed on the cholesterol values estimated with the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes and by use of the auto-analyzer for 20 serum samples. The calculated paired *t*-value for probability (*p*) of 0.05 was found to be 1.51. This *t*-value is lower than the tabulated value of 2.09 indicating that at the 0.05 level the two mean values are not significantly different, i.e. there is no significant difference between the values obtained with ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes and with the auto-analyzer.

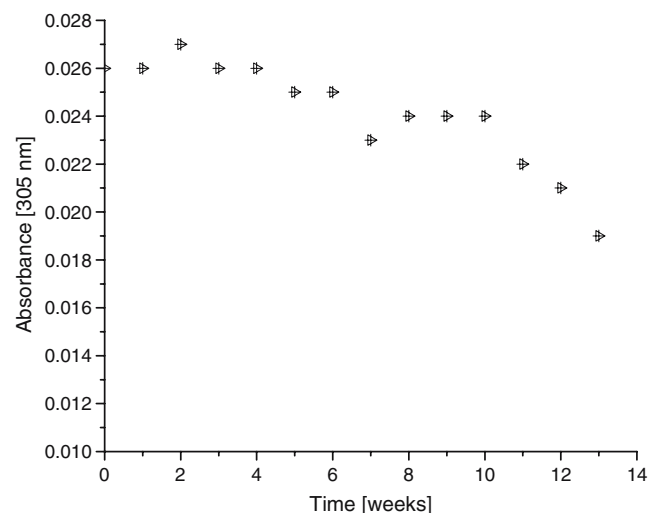
**Fig. 4** Stability curve for ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode with time

Table 2 Characteristics of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode and those reported in literature

No.	Immobilization matrix	Sensing element	Method of Immobilization	Detection range	Transducer used	K_m	Sensitivity	Shelf life	Ref.
1	Iron nanoparticles	ChOx	Covalent	1.3–5.2 mmol L ⁻¹	Spectrophotometric	2.08 mmol L ⁻¹ (free ChOx) and 0.45 mmol L ⁻¹ (bound ChOx)		15 days	[8]
2	Screen printed rhodium-graphite	Cytochrome P450cc on gold nanoparticles	Drop-cast	0.01–0.07 mmol L ⁻¹	Amperometric		0.13 μ A μ mol ⁻¹ L		[9]
3	Tetraethylorthosilicate sol-gel	ChEt, ChOx	Covalent	12–780 mg dL ⁻¹ (0.18–12 M)	Spectrophotometric	130 mg dL ⁻¹ (2 mmol L ⁻¹)	5.4 $\times 10^{-5}$ Abs (mg dL ⁻¹) ⁻¹	1 month	[10]
4	Modified ODT	Cholesterol oxidase (ChOx)	Cross-linking	50–500 mg dL ⁻¹ (1.29–12.93 mmol L ⁻¹)	Optical			2 months	[11]
5	Silicic sol-gel	ChOx	Entrapment	0.001–0.08 mmol L ⁻¹	Amperometric		0.329 A mol ⁻¹ L	35 days	[14]
6	Polypyrrole (PPy)/ITO	ChEt, ChOx	Entrapment	1–8 mmol L ⁻¹	Amperometric	9.8 mmol L ⁻¹	0.15 μ A mmol ⁻¹ L	4 weeks	[16]
7	3-Mercaptopropionic acid	ChOx	Covalent	50–200 mg dL ⁻¹ (1.29–5.17 mmol L ⁻¹)	Amperometric			4 days	[18]
8	Tributylmethyl phosphonium chloride polymer	ChOx/HRP	Physical entrapment	0.07–0.27 mmol L ⁻¹	Amperometric (flow injection)	6 mmol L ⁻¹		7 days	[35]
9	Polyaniline (PANI)	ChOx	Electrochemical doping	0.05–0.5 mmol L ⁻¹		2.27 and 3.26 mmol L ⁻¹		11 days	[36]
10	Tetraethylorthosilicate (sol-gel)	ChOx/HRP	Covalent	2–12 mmol L ⁻¹	Amperometric	21.2 mmol L ⁻¹		8 weeks	[37]
11	Polydiaminonaphthalene/Pt disc	ChOx, ChEt	Entrapment	Up to 0.8 mmol L ⁻¹	Amperometric	1.45 mmol L ⁻¹	0.39 nA mmol ⁻¹ L		[38]
12	PPy/Pt disk	ChOx, ChEt	Entrapment	1–8 mmol L ⁻¹	Spectrophotometric			1 month	[39]
13	PANI/ITO	ChOx, ChEt	Cross-linking	50–500 mg dL ⁻¹ (0.768–7.68 mmol L ⁻¹)	Amperometric		7.5 $\times 10^{-4}$ nA mg ⁻¹ dL	6 weeks	[40]
14	PANI/ITO	ChOx, ChEt, HRP	Cross-linking	100–400 mg dL ⁻¹ (1.536–6.144 mmol L ⁻¹)	Spectrophotometric	75 mg dL ⁻¹ (1.15 mmol L ⁻¹)		6 weeks	[41]
15	Streptavidin/biotin/thiolic acid/Au nanowires	ChOx, ChEt	Covalent	1–6 mmol L ⁻¹	Electrochemical		69 nA mmol ⁻¹ L	10 days	[42]
16	N-(2-aminoethyl)-3-aminopropyltrimethoxysilane	ChEt, ChOx, HRP	Covalent	10–500 mg dL ⁻¹ (0.153–7.68 mmol L ⁻¹)	Electrochemical (CV) and spectrophotometric	95.098 mg dL ⁻¹ (1.46 mmol L ⁻¹)	124 nA mg ⁻¹ dL	10 weeks	This work

Thermal stability and shelf life of ChEt-ChOx-HRP/AEAPTS/ITO electrodes

The effect of temperature on the reaction kinetics of the immobilized enzymes was investigated in the range 15 to 55 °C (Electronic Supplementary Material S7). It was observed that the value of absorbance increases with increasing temperature up to 50 °C. The higher thermal stability of the ChEt-ChOx-HRP/AEAPTS/ITO electrode can be attributed to the covalent binding that, perhaps, prevents the unfolding of the protein structure and reduces the dissociation of various oligomeric proteins to subunits [34].

To investigate the shelf-life of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode, UV–visible experiments were carried out at regular intervals of a week for about 3 months. The ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes were kept at 4 °C prior to being used. For activity measurements, a temperature of 30 °C and a cholesteryl oleate concentration of 200 mg dL⁻¹ were used. The observed values of absorbance for activity at regular intervals of time showed that ChEt-ChOx-HRP/AEAPTS/ITO bio-electrodes can be used for about 10 weeks (Fig. 4).

Table 2 summarizes the characteristics of the ChEt-ChOx-HRP/AEAPTS/ITO bio-electrode and those reported in the literature. Table 2 clearly indicates that the nature of the matrix not only affects the catalytic property of the enzymes but also plays an important role in the storage stability and the range over which bound enzyme shows a linear response. The advantage of the AEAPTS/ITO electrode as matrix over other matrices for enzyme immobilization is clearly visible from Table 2, as it shows linearity over a broad range of 0.153–7.68 mmol L⁻¹ for total cholesterol along with a relatively low value of K_m , and an improved shelf-life of about 10 weeks.

Conclusions

It has been demonstrated that tri-enzyme (ChOx, ChEt, HRP)-modified two-dimensional self-assembled monolayers of *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane onto ITO using EDC/NHS chemistry can be fabricated for estimation of total cholesterol. *N*-(2-Aminoethyl)-3-aminopropyltrimethoxysilane SAM provides a high surface area to the biomolecules at the molecular level, making the system suitable for binding multi-enzymes without denaturation. The stability of the bond between the specific functional group of enzymes and AEAPTS SAM on the ITO electrode surface results in increased thermal stability of the bio-electrode up to about 50 °C. The enhanced enzyme activity, reflected in a smaller K_m value of 1.46 mmol L⁻¹ and the observed stability of 10 weeks when stored at 4 °C

suggests the potential for development of a commercial cholesterol biosensor based on the tri-enzyme-modified AEAPTS SAM on an ITO surface. Efforts are currently being made to improve the stability beyond 10 weeks.

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