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PAPER

Mediator free cholesterol biosensor based on self-assembled monolayer platform†

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Self-assembled monolayer (SAM) of 4-aminothiophenol (4-ATP) has been investigated for immobilization of bi-enzymes (ChOx and ChEt) towards development of enzyme biosensors for detection of free and total cholesterol. This enzyme immobilized SAM surface has been characterized by scanning electron microscopy and electrochemical measurements. The results of electrochemical response studies reveal fast enzymatic reaction in phosphate buffer saline solution without using any artificial mediator. This may be attributed to the molecular wire type behavior of short 4-ATP molecule that promotes electron transfer between enzyme and the electrode surface due to its conjugated structure. Interference free estimation of free and total cholesterol has been realized at low operating potential of 0.33 V with range of detection as 25 to 400 mg dl^{-1}, sensitivity of 542.3 nA mM^{-1} (for ChOx/4-ATP/Au) and 886.6 nA mM^{-1} (for ChEt-ChOx/4-ATP/Au) with a response time of 20 s at pH 7.4.

Introduction

The method for controlling orientation of biomolecules to fabricate highly ordered macroscopic architectures with maximum biological activity and long-term stability is considered to be a crucial step in biomolecular electronics. In this context, thin ordered organic films of thickness of a few nanometres such as self-assembled monolayers (SAMs) and Langmuir–Blodgett (LB) films are presently a source of high expectations. SAMs have recently attracted much interest for development of desired biomolecular electronic devices particularly biosensors due to their greater robustness relative to most LB films, and ease of preparation.[1] SAMs are considered important due to their uniqueness in terms of well-ordered structure, ease of preparation, flexibility, reproducibility, organization, molecular control and low non-faradaic currents *etc.*[2] Among the numerous self-assembly systems such as organic amines on platinum,[3] organosilanes on oxide surfaces,[4] self-assembly of organosulfur compounds especially thiols on gold (Au) surfaces have caught much attention.[5,6] The functionalization or chemical modification of these SAMs offers promising possibilities in the area of biosensors.[7] Different biomolecules such as DNA,[8,9] antibodies,[10–13] proteins[13,14] *etc.*, have been immobilized onto SAMs of various organic thiols.

As compared to SAM of alkanethiols, arylthiol SAMs are found to provide better stability as π-stacking of the aromatic rings affords additional stability to the SAMs and decreases the molecular flexibility.[15,16] The delocalized electrons provide high electrical conductivity to these thiols while alkanethiols suffer with flexible nature of carbon chains and poor electrical conductivity.[17–19] Lee *et al.*, have compared the performance of SAM comprising of 4-(2-(4-acetylthio)phenyl)ethynyl) benzoic acid (APBA) with SAM of 11-mercaptoundecanoic acid (MUDA).[12] The *I–V* characteristics have revealed that currents generated due to APBA SAM are higher than that of MUDA SAM because of low gap resistance due to the conjugated backbone structure of APBA molecules. Komura *et al.* have found higher current values with 4-aminothiophenol (4-ATP) as compared to 1-hexadecanethiol SAM.[20] The conjugated structure of aryl or aromatic thiols may thus be of great use towards the fabrication of fast and sensitive electrochemical biosensors.[21] Among the various aromatic thiols, 4-ATP forms a densely packed structure with surface coverage of about 90% onto the Au surface.[20] SAMs of 4-ATP have been utilized for immobilization of DNA,[22] antibodies[23–25] and proteins[21] for biosensor fabrication. Valério *et al.* have prepared 4-ATP SAM based DNA biosensor for detection of cylindrospermopsin producing pyanobacteria.[15] Gupta *et al.* have reported direct electron transfer in laccase immobilized 4-ATP monolayer on Au surface.[26] Wang *et al.*, have fabricated self-assembly of colloidal Au onto SAMs of 4-ATP modified gold electrode, resulting in larger electrode surface area and easier attachment of antibody for detection of hepatitis B by impedance measurements.[24] Matharu *et al.*, have utilized 4-ATP SAM matrix for covalent immobilization of

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anti-apolipoprotein B for surface plasmon resonance based detection of low density lipoprotein.[25] Although SAM of 4-ATP has been used for fabrication of DNA and immunosensor, its utility for the development of enzyme biosensor has not yet been explored. The ability of this short aromatic thiol (~0.7–0.8 nm) to promote electron transfer due to presence of the delocalized electrons in its structure may provide fast electron transfer between the enzyme and the electrode surface leading to fabrication of biosensors without the use of any redox mediator.

Cholesterol exists in two forms in blood, free (30%) and esterified (70%) cholesterol. The high concentration of cholesterol in blood causes heart diseases, hypertension, arteriosclerosis, cerebral thrombosis and other disorders, thus estimation of total cholesterol is crucial.[27] Various SAM based electrochemical biosensors have been developed in recent years for estimation of free cholesterol.[28–33] To the best of our knowledge, SAM of a short aromatic thiol has not yet been investigated for fabrication of a cholesterol biosensor.

We report results of studies relating to the application of an aromatic thiol (4-ATP) SAM towards fabrication of enzyme biosensors for estimation of free and total cholesterol. This is done by covalently immobilizing ChOx enzyme and a mixture of ChEt and ChOx enzymes onto SAM of 4-ATP. Attempts have been made to study electrochemical response of ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes towards interference free detection without addition of any artificial redox mediator.

Experimental

Chemicals and reagents

N-hydroxysuccinimide (NHS), *N*-ethyl-*N*-(3-dimethylaminopropyl carbodiimide) (EDC), and 4-ATP were procured from Sigma-Aldrich (USA). Cholesterol esterase (ChEt; EC 3.1.1.13, pseudomonas species), with specific activity of 165 units per milligram (U mg^{-1}), ChOx (EC 1.1.36, from pseudomonas fluorescens) with specific activity of 24 U mg^{-1}, horseradish peroxidase (HRP) (200 U ml^{-1}), cholesterol and cholesteryl oleate (>98%), were purchased from Sigma–Aldrich (USA). All chemicals were of analytical grade and were used without further purification.

Solution preparation

2 mM solution of 4-ATP was prepared in ethanol. Solutions of EDC (0.2 M) and NHS (0.05 M) were prepared in de-ionized water. The solutions of cholesterol, ChOx, ChEt and HRP were freshly prepared in 50 mM phosphate buffer saline (PBS) solution of pH 7.4. A stock solution of cholesteryl oleate was prepared by first dissolving it in Brij by heating along 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 darkness when not in use. Working solutions of cholesteryl oleate were prepared from this stock solution using NaCl solution for further dilution. Stock solution of cholesterol was prepared in 10% Triton X-100 and stored at 4 °C. This stock solution was further diluted to make different concentrations of cholesterol solution. *o*-dianisidine (1%) solution were freshly prepared in de-ionized water.

Preparation of 4-ATP monolayer on Au surface and immobilization of ChOx

Prior to SAM formation, Au plates were treated with piranha solution (H_2SO_4/H_2O_2; 7 : 3) for about 5–10 min followed by rinsing with de-ionized water. The pre-cleaned Au plates were then immersed into a 2mM solution of 4-ATP in ethanol for 18 h at room temperature (25 °C) for SAM formation after which the electrodes were rinsed with ethanol and acetone followed by water to remove any unbound 4-ATP molecules.

The amino group functionality of 4-ATP has been utilized for immobilization of ChOx and mixed ChOx-ChEt enzymes *via* EDC-NHS chemistry[34] for the preparation of ChOx/4-ATP/Au (for free cholesterol detection) and ChEt-ChOx/4-ATP/Au (for total cholesterol detection) bioelectrodes. EDC is a zero-length cross-linker and provides a direct linkage between two molecules (one having –COOH and other with $–NH_2$ group) with no intervening linker or spacer.[25] Most of the other cross-linkers such as glutaraldehyde,[35] glyoxal[36] have been used to attach the desired biomolecule to the matrix. However, these cross-linkers act as a spacer between the biomolecule and the matrix and may affect sensitivity and range of detection. In coupling *via* EDC-NHS, –COOH groups present in enzyme covalently links to amino group of 4-ATP forming a stable amide bond. For this, 0.5 × 1.0 cm^2 area of 4-ATP/Au electrode is dipped in the solution of enzymes (ChOx for free cholesterol estimation and ChOx–ChEt (mixed in 1 : 1) for total cholesterol estimation) containing EDC and NHS and left for 4 h in a humid chamber at room temperature (25 °C). The electrodes are then washed with PBS containing 0.05% tween-20 to remove the unbound enzyme and stored at 4 °C when not in use. The 4-ATP/Au and enzyme modified 4-ATP/Au electrodes have been characterized by scanning electron microscopy (SEM) (Leo 440), electrochemical impedance spectroscopy (EIS) (FRA, Autolab, Eco Chemie, Netherlands) and cyclic voltammetry (CV) (Autolab Potentiostat/Galvanostat, Eco Chemie, Netherlands). The biosensing measurements have been carried out by CV and the enzyme activity and shelf-life studies have been done using UV-vis spectrophotometer (UV-160 A, Shimadzu) measurements.

Results and discussion

SEM studies

The morphological features of the surface after several modification steps have been investigated by SEM studies. Fig. 1 shows SEM pictures obtained for the 4-ATP SAM, ChOx, and ChEt-ChOx modified 4-ATP SAM surfaces, respectively. The uniform morphology obtained in 4-ATP/Au [Fig. 1(a)] indicates a well-packed, homogeneous SAM on the Au surface. The 4-ATP/Au surface is seen to be totally covered by bright shining light shade globules like structures after covalent immobilization of ChOx [Fig. 1(b)]. As the immobilization method of ChOx onto SAM surface includes cross-linking *via* EDC-NHS, which is known to be a zero length cross-linker and gets removed during the linking reaction, the globules seen in the SEM image can be assigned to the presence of ChOx molecules. However, during the immobilization process there is possibility that the activated COOH group of one enzyme molecule may react with NH_2 group present on the other enzyme molecule in the enzyme solution that

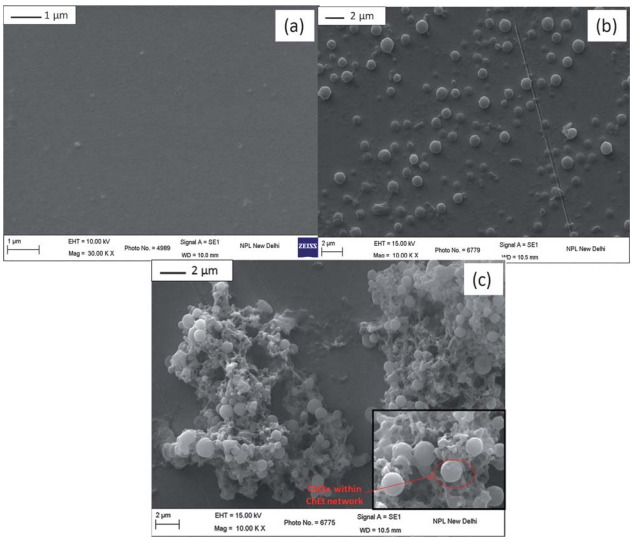

Fig. 1 SEM image of (a) 4-ATP/Au, (b) ChOx/4-ATP/Au and (c) CHEt-ChOx/4-ATP/Au surfaces.

may lead to aggregation as seen in the SEM image of the ChOx/4-ATP/Au surface which shows big globules. Also enzymes are known to undergo agglomeration resulting in the formation of large globules. Thus the observed uniformly distributed globular structures in SEM image of ChOx/4-ATP/Au electrode [Fig. 1(b)], with the size distribution of about 0.3 μm to 1.2 μm, can be attributed to the immobilized ChOx molecules.

Further SEM image of ChEt-ChOx/4-ATP/Au surface reveals network type structures in which embedded globules can be seen indicating immobilization of the two types of enzymes [Fig. 1(c)]. The difference in the morphologies clearly indicates immobilization of two enzymes (ChOx and ChEt) onto 4-ATP monolayer surface.

Electrochemical characterization of 4-ATP/Au, ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au electrodes

4-ATP SAM modified Au and enzyme modified 4-ATP/Au electrodes have been characterized by CV and EIS using a three-electrode cell with platinum (Pt) as counter electrode and Ag/AgCl as reference electrode in PBS solution (50 mM, pH 7.4, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. Fig. 2(a) shows CV response of Au, 4-ATP/Au electrode, ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au in the potential range of −0.8 to 0.8 V at a scan rate of 50 mV s^{-1}. The reduction in oxidation current and the large peak-to-peak potential separation (0.32 mA, 415 mV) of 4-ATP/Au electrode [Fig. 2(a), curve (ii)] as compared to that of blank Au [Fig. 2(a), curve (i)] (0.52 mA, 108 mV) indicates formation of the 4-ATP monolayer. Further, large decrease in currents are obtained after immobilization of ChOx [Fig. 2(a), curve (iii)] and ChOx-ChEt (mixed) enzymes [Fig. 2(a), curve (iv)] onto the surface of 4-ATP monolayer using EDC-NHS as a cross-linker. This may be attributed to the fact that ChOx and ChEt are negatively charged in PBS of pH 7.4 due to their low isoelectric points (~4.5 and ~3.2). Thus the enzyme modified bioelectrodes (ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au) repel the negatively charged $[Fe(CN)_6]^{3-/4-}$ ions present in the solution.

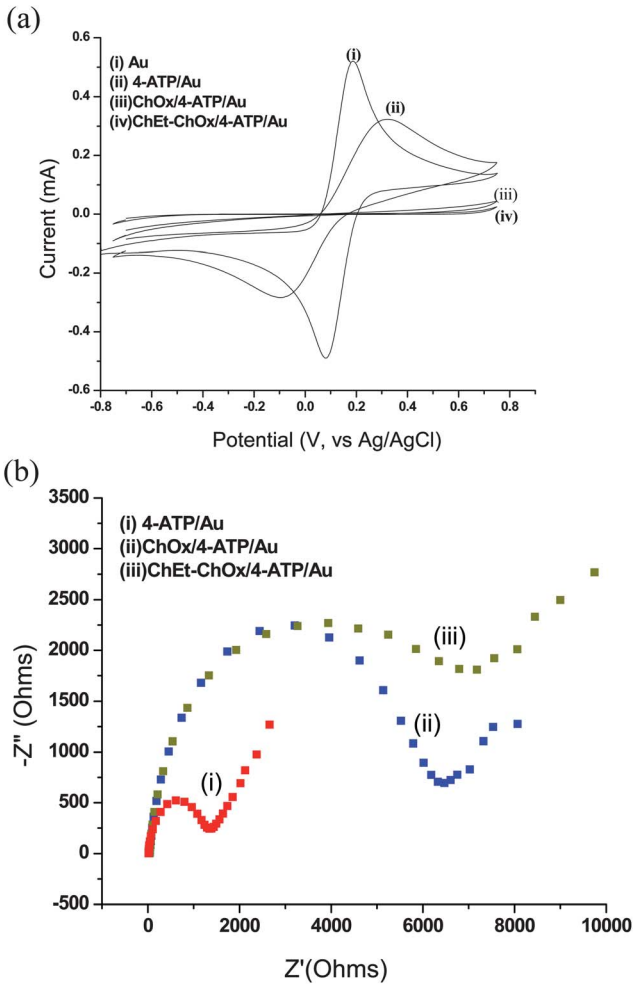

Fig. 2 (a) CV plots of (i) Au electrode, (ii) 4-ATP SAM electrode (iii) ChOx/4-ATP SAM bioelectrode (iv) ChOx/ATP SAM bioelectrode. (b) EIS curves of (i) 4-ATP SAM electrode (ii) ChOx/4-ATP SAM bioelectrode (iii) ChOx/4-ATP SAM bioelectrode.

The surface modification of the electrode at each step has been monitored using EIS [Fig. 2(b)] and the results are found to be complementary to those obtained by the CV studies. The value of charge transfer resistance (R_{ct}) is found to increase from 119 Ω (for bare Au electrode, curve not shown) to 1.32 kΩ after 4-ATP SAM formation [curve (i)]. The R_{ct} values further increases to 6.5 kΩ [curve (ii)] and 7.4 kΩ [curve (iii)] for ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au electrodes, respectively. The surface coverage of 4-ATP SAM on Au and of ChOx and ChEt-ChOx enzymes on 4-ATP/Au surfaces can be calculated using the following Equations:[37]

$$\Theta = 1 - \frac{R_{Au}}{R_{4-ATP}}; \quad \text{(Eq. 1)}$$

$$\Theta = 1 - \frac{R_{4-ATP}}{R_{ChOx}}; \quad \text{(Eq. 2)}$$

$$\Theta = 1 - \frac{R_{4-ATP}}{R_{ChEt-ChOx}} \quad \text{(Eq. 3)}$$

where Θ represents the fraction of occupied binding sites and R_{Au}, R_{4-ATP}, R_{ChOx}, $R_{ChEt-ChOx}$ are charge transfer resistances of

bare Au, 4-ATP/Au, ChOx/4-ATP/Au, ChEt-ChOx/4-ATP/Au electrode, respectively. The values of Θ are found to be 0.91, 0.80 and 0.82 for 4-ATP/Au, ChOx/4-ATP/Au, ChEt-ChOx/4-ATP/Au electrode. The surface coverage 4-ATP SAM is in good agreement with literature.[20] Coverage of immobilized enzymes onto 4-ATP surface suggests more than 80% immobilization.

Detection of free and total cholesterol

The detection of free and total cholesterol have been performed by CV studies (Fig. 3 and 4) with ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes in PBS solution (50 mM, pH 7.4, 0.9% NaCl) in the potential range of −0.8V to 0.9 V at scan rate of 50 mV s^{-1}.

Most of the amperometric cholesterol biosensors utilize ChOx, a flavin enzyme, as a bio-recognition element for free cholesterol detection.[38] The biochemical reaction includes the catalysis reaction of ChOx that converts free cholesterol into cholest-4-en-3-one. In this oxidative reaction, the FAD active sites of ChOx get reduced to $FADH_2$ and H_2O_2 is produced during the re-oxidation of ChOx. H_2O_2 is further oxidized during the CV scan and results in increased oxidation peak current. However, most (~70%) of cholesterol in the human blood is esterified with long chain fatty acids. Since cholesterol esters do not function as substrates for ChOx, biosensors based on bi-enzymes (ChEt and ChOx) have been utilized for the purpose. The ChEt enzyme catalyses hydrolysis of cholesterol esters to free cholesterol that further gets converted into cholest-4-en-3-one by ChOx resulting in the production of H_2O_2.

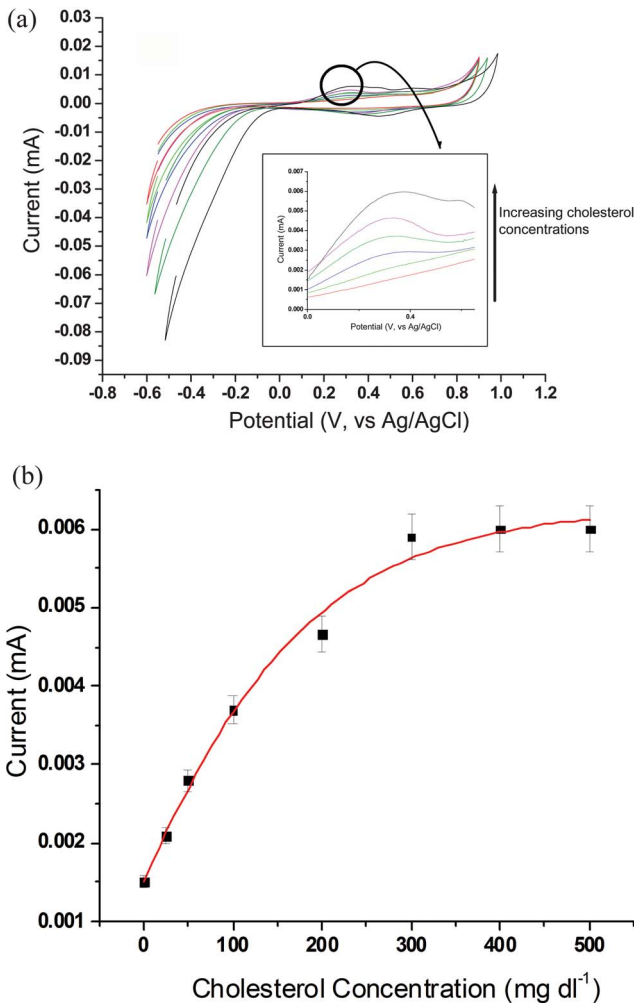

Fig. 3 (a) CV plots of ChOx/4-ATP/Au bioelectrode in PBS as a function of cholesterol concentration. (b) Linear calibration plots of current obtained for ChOx/4-ATP/Au bioelectrode as a function of cholesterol concentrations.

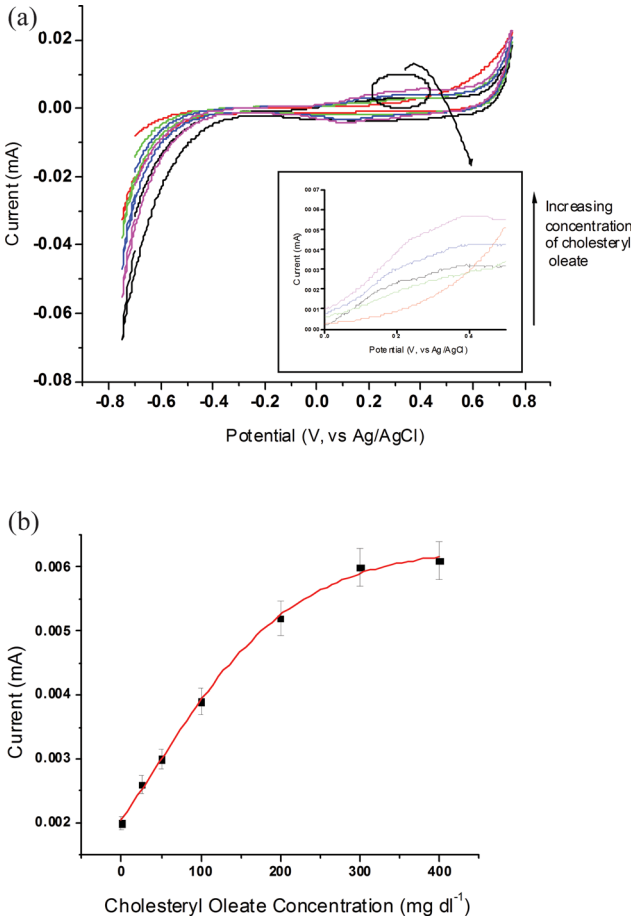

Fig. 4 (a) CV plots of ChEt-ChOx/4-ATP/Au bioelectrode in PBS as a function of cholesteryl oleate concentration. (b) Linear calibration plots of current obtained for ChEt-ChOx/4-ATP/Au bioelectrode as a function of cholesteryl oleate concentrations.

The CV measurements have been carried out to study the response of ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes towards cholesterol and cholesteryl oleate, respectively. CV scans have been taken after the incubation of each bioelectrode (ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au) for 20 s in PBS solution containing cholesterol [Fig. 3(a)] and cholesteryl oleate [Fig. 4(a)], respectively. In the present case the peak current seen around 0.33 V is found to increase with increasing analyte concentration [cholesterol Fig. 3(a), cholesteryl oleate Fig. 4(a)] Fig. 3(b) and Fig. 4(b) show plots of increase in peak current for ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au electrodes as a function of cholesterol and cholesteryl oleate concentration, respectively. It has been reported that the 4-ATP gives a redox at about 0.32 V in acidic solution due to the

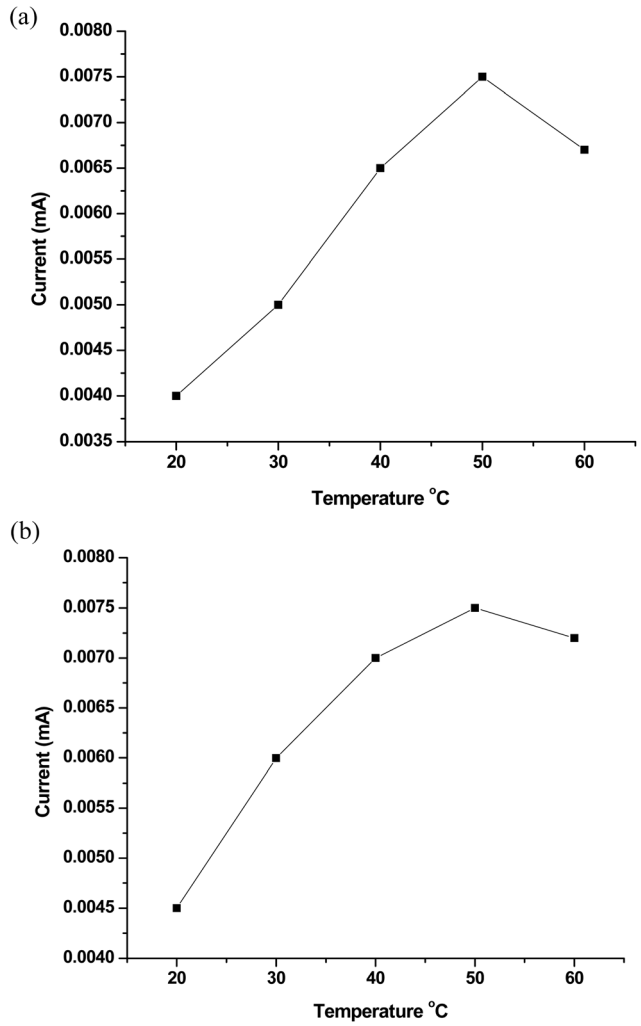

Fig. 5 (a) A plot of peak current for ChOx/4-ATP/Au bioelectrode as a function of temperature. (b) A plot of peak current for ChEt-ChOx/4-ATP/Au bioelectrode as a function of temperature.

presence of quinonediimine moieties[39,40] and these voltammetric features suppress at pH $\geq$ 6. However, in the present case we have observed an increase in peak current with increasing analyte concentration at 0.33 V in PBS of almost neutral pH. The possible reason for this observed behavior in the present case can be assigned to decomposition of H_2O_2, produced in the enzymatic reaction, giving H^+ ions during the CV scan. These H^+ ions are taken up by 4-ATP that further form the quinonediimine moiety. To verify this mechanism a control experiment has been performed in which cyclic voltammogram of 4-ATP modified Au plate is recorded in PBS containing fixed concentration of H_2O_2. A similar peak around 0.3 V is observed indicating the formation of quinonediimine.

The current is found to increase in the range from 25 mg dl^{-1} to 400 mg dl^{-1} of cholesterol and cholesteryl oleate concentration for both ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes respectively [Fig. 3(b) and Fig. 4(b)]. The sensitivities of ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes are found to be 542.3 nA mM^{-1} and 886.6 nA mM^{-1}, respectively, in the linear region. The prepared bioelectrode shows response time of 20 s. The fast response may be attributed to the fast electron transfer characteristics of well-aligned 4-ATP molecules having conjugated structure that acts as a molecular wire facilitating electron transfer between the enzyme and the electrode.[15,26] Thus enzyme modified 4-ATP SAM matrix is capable of detecting free and total cholesterol in PBS without any artificial mediator. However, SAM of long chain aliphatic thiols may interrupt interfacial electron transfer as the electron tunneling through a long chain diminishes electron transfer rate exponentially with the monolayer thickness.[41] Therefore, biosensors based on these SAMs utilize artificial mediators for enhancing the electron transfer rate.

Effect of interferents

The observed cholesterol detection at lower potential (0.33 V) by 4-ATP SAM based bioelectrode may reduce the effect of various interferents such as ascorbic acid, uric acid, bilirubin and acetaminophen that are usually present in biological samples and get oxidized near 0.6 V.[38] The effect of interferents [uric acid (0.1 mM), ascorbic acid (0.05 mM), lactic acid (0.5 mM), and urea (1 mM)] on free and total cholesterol sensing has been tested by taking CV scans of ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes in solution containing a 1 : 1 volume ratio of analyte (200 mg dl^{-1} or 5.17 mM) and the desired interferent. However, no significant change is observed in sensing of the free and total cholesterol. Hence, the ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes show almost interference free detection of free and total cholesterol, respectively.

Many redox mediators have been used by several researchers to enhance the electron transfer and to detect cholesterol at lower potentials to avoid co-oxidation of several interferents.[38,42] However in the present case the 4-ATP based SAM matrix facilitates the electron transfer rate without any redox mediator.

Calculation of the Michaelis–Menten parameters

The value of Michaelis-Menton kinetic parameters K_m^{app} and V_{max} which determine the affinity of the enzyme for the substrate and maximum rate of enzyme reaction,[43–45] respectively, have been calculated using a Lineweaver–Burke plot, *i.e.*, a graph between inverse of substrate concentration and inverse of corresponding current obtained. In the Lineweaver–Burke plot inverse of X-axis intercept gives the value of K_m^{app} and inverse of Y-axis intercept gives the value of V_{max}. The values of K_m^{app} for ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes from the Lineweaver–Burke plot have been found to be as 1.34 mM and 1.06 mM with V_{max} of 6.20 μA and 7.06 μA, respectively. The obtained values of K_m^{app} in the present case are found to be relatively lower than the values reported in literature for other matrices.[46–49] This indicates better affinity of enzymes, immobilized onto 4-ATP SAM, for cholesterol.

Effect of temperature

CV experiments have been carried out to estimate the thermal stability of ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes. The value of peak current, for a fixed cholesterol and cholesteryl oleate concentration (200 mg dl^{-1}), is found to increase with increasing temperature up to ~50 °C, whereafter it

Table 1 Characteristics of the 4-ATP SAM based free and total cholesterol biosensors along with other monolayer based electrochemical cholesterol biosensors reported in literature

Analyte detected	Monolayer matrix	Recognition element and method of immobilization	Transduction method and detection potential	Sensitivity (S), response time (RT), shelf life (SL) and reusability (Re)	Reference
Free cholesterol	1-Hexadecanethiol (SAM)/Au	Molecular imprinted layer; Template	Cyclic voltammetry at 0.3V using $K_3Fe(CN)_6$ as redox mediator	(RT): 300 s	51
Free cholesterol	AuNps/cysteamine (SAM)/Au	ChOx; Physical adsorption	Cyclic voltammetry with $K_4Fe(CN)_6/K_3Fe(CN)_6$ as redox mediator	(RT): 15 min	52
Total cholesterol	3-Aminopropyl-modified controlled-pore glass/rotating disk	ChOx, ChEt, HRP; Cross-linking *via* Glutaraldehyde	Amperometry at −0.15 V *vs.* with TBC as mediator	(S): 69.49 nA mM^{-1} (SL): 25days (RT): 2 min	42
Total cholesterol	Streptavidin/biotin/thioctic acid (SAM)/Au nanowires	ChOx, ChEt; covalent *via* Biotin-avidin	Square wave voltammetry, detection at 0.6 V	(S): 69 nA mM^{-1} (SL): 10 days (Re): 20 times	31
Free cholesterol	3-Mercaptopropionic acid/Au	ChOx; covalent *via* EDC/NHS	Ampero. at 0.35 V *vs.* Ag/AgCl	(RT): 30 s after 2 min incubation (SL) = about 2 days	29
Total cholesterol	Dithiobis-(succinimidyl propionate)/Au	ChOx, ChEt; covalent	Differential pulse voltammetry at 0.5 V with $K_4Fe(CN)_6/K_3Fe(CN)_6$ as redox mediator	(SL): 50 days	30
Free cholesterol	Octadecyltrimethyl ammonium(ODTA)-prussian blue (PB) Hybrid LB on Au	ChOx; electrostatically attached in LB trough	Amperometric at 0.0 V, prussian blue as redox probe	(RT) = 20 s; (S) = 1.6 μA $mM\ L^{-1}\ cm^{-2}$	53
Free cholesterol	polyaniline LB films on ITO	ChOx covalent	Linear sweep voltammetry at 0.2 V without any redox mediator	(S): 88.9 $nAmg^{-1}$ dl (SL): 10 weeks (RT): 60 s	54
Free cholesterol	3-Aminopropyltriethoxysilane/Pt/Porous silicon	ChOx; cross linking *via* glutaraldehyde	Amperometric at.0.6 V	(S): 0.2656 μA mM^{-1} (SL): 120 days	55
Free cholesterol	11-amino-1-undecanethiol on Au	ChOx; cross-linking *via* glutaraldehyde	Cyclic voltammetry at 0.3 V with $K_3Fe(CN)_6$ as redox probe	(SL): 10 weeks	28
Free and total cholesterol	4-ATP SAM on Au	ChOx, ChEt-ChOx; covalent binding *via* EDC-NHS	Cyclic voltammetry at 0.33 V in PBS	(S): 542.3 nA mM^{-1}, 886.6 nA mM^{-1} (SL): 4 months (RT): 20 s (Re): 20 times	Present Work

shows a decrease, indicating denaturing of the enzyme at temperature >50 °C [Fig. 5(a) and Fig. 5(b)]. The observed results show that 4-ATP SAM based cholesterol sensor is extremely stable at temperatures more than normal body temperature (37 °C). The higher thermal stability of the ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes can be attributed to the stable SAM on Au surface and stable covalent binding of enzyme that perhaps prevent the unfolding of the protein structure and reduce the dissociation of various oligomeric proteins to subunits.[50]

The sensing responses of prepared ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes along with their thermal stabilities and shelf-lives have been studied by UV-visible spectrophotometry (supplementary data†). The photometric studies reveal that 4-ATP SAM based cholesterol sensor is extremely stable at temperatures more than normal body temperature (37 °C) and is stable for about 4 months with more than 90% of the initial activity.

Table 1 shows characteristics of the prepared ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au biosensors along with other monolayer based electrochemical cholesterol biosensors reported in literature. It can be seen that the present 4-ATP monolayer provides an excellent immobilization matrix for ChOx and mixed ChEt-ChOx enzymes. The prepared ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes can detect cholesterol in PBS solution at relatively low potential with fast response and long shelf-life. Thus better performance based biosensors can be fabricated using 4-ATP even without any artificial mediator. These attributes make this short aromatic thiol a good contender for development of the desired electrochemical biosensor.

Conclusions

An enzymatic biosensor for the detection of free and total cholesterol has been fabricated using SAM of 4-ATP. The ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au bioelectrodes can be used for detection of free and total cholesterol in the range of 25 to 400 mg dl^{-1}. The fabricated biosensor shows good sensitivity of 542.3 nA mM^{-1} (for ChOx/4-ATP/Au) and 886.6 nA mM^{-1} (for ChEt-ChOx/4-ATP/Au) with response time of 20 s. The low K_m^{app} (1.34 mM and 1.06 mM) value and high thermal stability (up to 50 °C) and long shelf life (4 months) indicate ChOx/4-ATP/Au and ChEt-ChOx/4-ATP/Au to be promising matrices for detection of cholesterol. The well-aligned uniformly packed monolayer film

with conjugated structure provides an interesting platform for interference free detection of cholesterol at low potential (0.33 V) without using any artificial mediator. Efforts should be made to utilize this 4-ATP SAM based bioelectrode to detect total cholesterol in real samples.

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