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Application of octadecanethiol self-assembled monolayer to cholesterol biosensor based on surface plasmon resonance technique

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Abstract

Octadecanethiol (ODT) self-assembled monolayer (SAM) prepared onto gold-coated glass plate has been modified by using nitrene reaction of 1-fluoro-2-nitro-4-azidobenzene (FNAB) that further covalently binds to cholesterol oxidase (ChOx) via thermal reaction. FNAB acts as a bridge (cross-linker) between SAM and ChOx. The ChOx/FNAB/ODT/Au electrode thus fabricated has been characterized using contact angle (CA) measurements, UV–vis spectroscopy, electrochemical techniques and X-ray photoelectron spectroscopy (XPS) technique, respectively. This ChOx/FNAB/ODT/Au bioelectrode has been utilized for estimation of cholesterol in solution using surface plasmon resonance (SPR) technique. This SPR based cholesterol biosensor has linearity from 50 to 500 mg/dl of cholesterol in solution with lower detection limit of 50 mg/dl and shelf life of about 2 months when stored at 4 °C.

Keywords: Cholesterol; Cholesterol oxidase (ChOx); Surface plasmon resonance (SPR); Octadecanethiol (ODT); 1-Fluoro-2-nitro-4-azidobenzene (FNAB)

1. Introduction

Surface plasmon resonance (SPR) technique has recently attracted much interest [1–11]. This is because this technique can be used for the detection of biological molecules, monitoring complex biochemical reactions in real time, resolving the kinetics and mechanism of a biochemical interaction, to probe electrochemically induced chemical changes in surface species and eliminate the need to label reagents. The SPR is a surface-sensitive technique based on the detection of change in the refractive index (RI) [12]. The change in the refractive index is proportional to the change in the mass of the biomolecules bound to an electrode surface [13]. It has recently been shown that SPR technique can be used for estimation of biochemical parameters such as glucose [11], fructose [14], low-density lipoproteins (LDL) [15], etc. in solution.

Cholesterol is an essential structural constituent of cell membranes, provides durability and integrity to the cell architecture. Almost all membranes contain varying level of cholesterol and the level changes during different physiological conditions. Elevated levels of blood cholesterol are associated with atherosclerosis, nephrosis, diabetes milletus (DM), myxedema, jaundice and decreased levels may result in hyperthyroidism, anaemia and maladsorption [15,16]. The determination of cholesterol in blood serum is crucial for the diagnosis and cure of several heart diseases [17]. Compared with biosensors for the estimation of glucose [11,18], only a few biosensors have been developed for cholesterol estimation.

Redox proteins are known to lose their activities at bare electrodes due to strong adsorption [19]. To overcome this problem, the immobilization of desired protein on various matrices such as polymers, membranes, sol–gels, self-assembled monolayer (SAM), etc. can be done by various techniques like physical adsorption [20,21], entrapment [22,23] and covalent linkage [24]. The physical methods of immobilization suffer from several shortcomings such as long incubation time, non-reproducible results due to leaching of biomolecules dur-

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ing washing [25,26] Compared to physical adsorption technique, the covalent method of immobilization is often useful as biomolecules are precious or not affordable in large quantities [27] and covalently coupled proteins on solid support are stable and do not usually undergo solvent mediated desorption [25] also it reduces non-specific binding [26].

Singh et al. [23] reported an amperometric cholesterol biosensor based on the co-immobilization of cholesterol esterase and cholesterol oxidase onto electrochemically prepared polypyrrole films. The sensitivity of the PPY/ChEt/ChOx electrode was 0.15 microampere per millimolar (μA/mM) and it had a shelf life of about 4 weeks. Later, these authors [28] carried out spectrophotometric studies of PPY/ChEt/ChOx electrode as a function of cholesterol palmitate wherein the PPY/ChEt/ChOx electrode had stability for about 18 days. Physical adsorption, physical entrapment and micro encapsulation techniques have been used by Kumar et al. [29] for the co-immobilization of cholesterol oxidase and horseradish peroxidase in a tetraethylorthosilicate (TEOS) sol–gel film. Results revealed that the presence of interferents such as glucose and ascorbic acid has a significant effect on observed anodic current. Later, these authors [30] conducted amperometric measurements on physically immobilized cholesterol oxidase and mediator (potassium ferricyanide) onto a conducting polypyrrole film. Lin and Yang [31] immobilized cholesterol oxidase (ChOx) on the surface of polyacrylonitrile (PAN) hollow fiber with glutaraldehyde. It was shown that this bioelectrode has a poor storage life as immobilized ChOx retains only 53% of its initial activity over a 30-day period.

The self-assembled monolayers can be used as immobilization matrices, [19,24,25,32,33] since their biological and organic properties can be easily manipulated via changing functional groups that become compatible for biomolecules. Alkanethiols on gold surfaces have been used for the immobilization of biomolecules such as redox proteins [19,32], enzymes [24,34] and immunoglobulins [25], as they orient these biomolecules without denaturization and facilitate electron transfer between biomolecules and electrode surface. Vidal et al. [17] reported an amperometric cholesterol biosensor based on self-assembled monolayers of propanethiol on the platinum (Pt) surface. The shelf life of this cholesterol biosensor was about 25 days. Later, these authors [35] reported an amperometric cholesterol biosensor based self-assembled monolayer of cystamine on gold. The lifetime of this cholesterol biosensor was about 45–60 days and the electrode preparation involved a lengthy and tedious process. Gobi and Mizutani [36], developed a cholesterol biosensor based on layer by layer nano thin films of ChOx and poly(styrenesulfonate) on covalently immobilized microperoxidase-11 (MP-11) on self-assembled monolayer of mercaptopropionic acid and aminoethanthiol.

It has recently been reported that octadecanethiol (ODT) can be used as a reference material since it forms densely packed monolayer with an average molecular area per molecule for ODT (A_{ODT}) of about 0.2 nanometer square (nm^2) [37]. In this paper, we report the covalent immobilization of cholesterol oxidase onto self-assembled monolayer of ODT using 1-fluoro-2-nitro-4-azidobenzene (FNAB) as a bridge (cross-linker) between CH_3 group of SAM and NH_2 group of ChOx for estimation of cholesterol using SPR technique.

2. Materials and methods

2.1. Chemicals and reagents

Cholesterol oxidase (EC 1.1.36, from pseudomonas fluorescence) with specific activity of 24 units per milligram (U/mg), horseradish peroxidase (HRP, E.C.I. 11.1.7) with a specific activity of 120 U/mg solid were purchased from the Sigma–Aldrich (USA). Octadecanethiol was procured from Merck, India. Gold-coated BK-7 glass plates (24 mm diameter) were made available from Autolab Netherlands. 4-Fluoro-3-nitroaniline was procured from Fluka. All chemicals were of analytical grade and were used without further purification.

2.2. Enzyme solution preparation

The solutions of cholesterol oxidase (24 Unit per milliliter (U/ml)) and horseradish peroxidase (40 U/ml) were freshly prepared in phosphate buffer (50 mM) of pH 7.0 prior to being used. Stock solution of cholesterol was prepared in 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 was freshly prepared in deionized water.

2.3. UV–vis spectrophotometric measurements

The UV–vis experiments were carried out by UV–vis spectrophotometer (model 160A Shimadzu) by measuring the absorbance for the quinoneimine dye (500 nm) produced by the reaction of *o*-dianisidine with hydrogen peroxide in the presence of peroxidase. The following biochemical reaction occurs:

$$\text{cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{4-cholesten-3-one} + H_2O_2 \quad (1)$$

$$H_2O_2 + o\text{-dianisidine}_{(\text{reduced})} \xrightarrow{\text{HRP}} 2H_2O + o\text{-dianisidine}_{(\text{oxidised})} \quad (2)$$

2.4. Cyclic voltammetric response measurements

Cyclic voltammetric studies were carried using potentiostat/galvanostat (Princeton Applied Research Model 273A). ChOx/FNAB/ODT/Au was used as a working electrode, Ag/AgCl as a reference electrode and platinum foil as a counter electrode. Experiments were carried out as a function of pH and substrate concentration using phosphate buffer (50 mM, pH 7.0).

2.5. Contact angle measurements

Contact angle measurements were conducted to check the hydrophilic/hydrophobic nature of the surface before and after modification and the immobilization of enzyme by the Sessile

drop method [38,39] using a drop shape analyzer (DSA 100, DSA/V 1.9) from Kruss Gmbh Hamburg.

2.6. X-ray photoelectron spectroscopy (XPS)

XPS measurements were carried out on a Perkin-Elmer (Phi) Model 1257 system, working at a base pressure of 5×10^{-10} Torr. Aluminium Kα radiation (1483.6 electron volt (eV)) was used as the photon source and the emitted photoelectrons were analyzed by hemispherical analyzer having resolution of 0.1 eV, normal to sample surface. Survey scans were obtained with pass energy of 100 eV.

2.7. Surface plasmon resonance

SPR assays were conducted using an Autolab SPR procured from the Eco Chemie (Netherlands). It has a vibrating mirror to modulate the angle of incidence of the p-polarized light beam (670 nm) Fig. 1. The intensity of reflected light was measured over a range of 4000 millidegrees (m°). In the experiments, the plane face of the prism was coupled to the gold-coated glass via index matching fluid. All samples were carried out at 25 °C.

2.8. Preparation of 1-fluoro-2-nitro-4-azidobenzene

FNAB was prepared from 4-fluoro-3-nitro-aniline by diazotization reaction as reported [40]. $NaNO_2$ solution (4.8 g/12 ml) prepared in water was added drop wise to the clear and cooled solution of 10 g of 4-fluoro-3-nitroaniline, dissolved in a mixture of 65 ml concentrated HCl and 12 ml water. The reaction mixture was continuously stirred at about −20 °C. After addition, reaction mixture was stirred for additional 15 min followed by drop wise addition of sodium azide (NaN_3) (4.4 g/16 ml water) to the reaction mixture. The temperature of the mixture was maintained at around −20 °C and stirred for another 15 min. Yellow product thus formed was filtered and washed in ice-cold water. The obtained product was recrystallized from light petroleum to give needle-shaped, straw-colored crystal of 4-azido-1-fluoro-2-nitrobenzene (FNAB), melting point 52 °C.

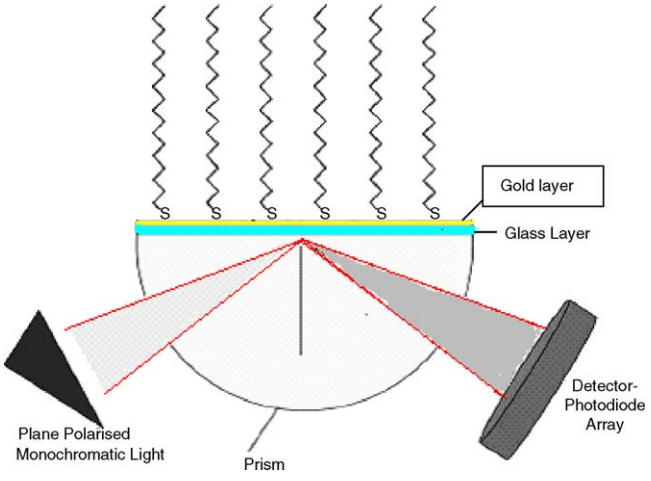


Fig. 1. Schematic of surface plasmon resonance (SPR) set-up.

2.9. Preparation and modification of self-assembled monolayer of octadecanethiol on gold plate

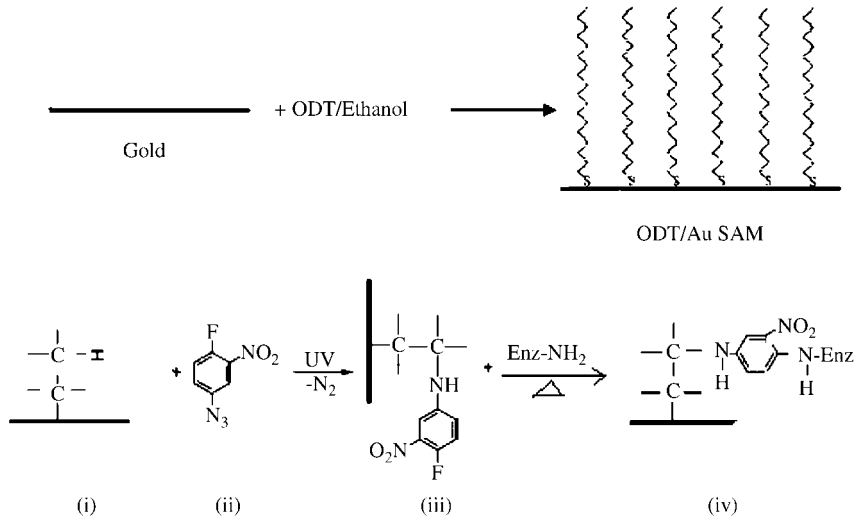
Prior to the formation of SAM the gold electrodes were rinsed with deionized water and ultra-sonicated in absolute ethanol and then in deionized water for around 10 min. The pre-cleaned gold plate was then immersed in 1 mM octadecanethiol/ethanol solution for 2, 4, 6 and 16 h after which these were sonicated in water–ethanol (1:1) solution for about 10 min followed by sonication in water and finally rinsed with deionized water and dried [41]. The contact angle measurements were undertaken to optimize the time required for good quality ODT self-assembled monolayer formation. It was seen that 4 h were sufficient to obtain good quality self-assembled monolayer of ODT on gold surface. The SAM (4 h) of ODT thus prepared was modified using FNAB. For modification of self-assembled monolayer of ODT on gold surface, FNAB solution (5 mg FNAB in 100 μl of ethanol) was spread onto the entire plate (24 mm in diameter) and then air-dried in dark after which plate was exposed to sunlight [42] for nitrene reaction between azide (N_3) group of FNAB and C–H group of SAM on gold. After about 30 min of exposure the plate was washed with ethanol after which the plate was used for the covalent immobilization of ChOx.

2.10. Immobilization of cholesterol oxidase on FNAB/ODT/Au plate

Cholesterol oxidase (24 U/ml) in phosphate buffer (50 mM, pH 7.0) was prepared as a stock solution. From this stock solution, different dilutions were prepared and binding was recorded using SPR, the optimum was found when diluted solution of 8 μl ChOx per 100 μl phosphate buffer was used. For covalent immobilization the ChOx solution in phosphate buffer was taken in vertical cuvettes (approximately 8.5 mm^2 area) of SPR set-up over the FNAB/ODT/Au plate. The enzyme solution was then kept at 37 °C for 3000 s and the immobilization was recorded by recording the SPR signal. The electrode (ChOx/FNAB/ODT/Au) thus formed was washed thoroughly with phosphate buffer (50 mM, pH 7.0) containing 0.85% NaCl and 0.05% Tween 20 to remove any unbound enzyme and stored at 4 °C when not in use.

3. Results and discussion

Surface plasmon resonance is an optoelectronic phenomenon through which real-time data of the binding of ligands [43], e.g. glucose [18], fructose [14], low-density protein [15] to their respective target can be recorded. SPR can be used for the detection of desired analytes in complex biological media with high specificity and sensitivity. The shift in the SPR angle is used to measure refractive index (RI) change at a surface. This shift in the SPR angle is known to be sensitive to the metal complex permittivity and the change in electron concentration at or near the metal surface [18,44]. Keeping the other parameter constant except changing the concentration of analyte the variation in the refractive index is directly proportional to the amount of the analyte bound to a surface [15].



Scheme 1. Schematic of photochemical activation of an inert surface for immobilization of enzyme. Solid surface having C–H group (i) 1-fluoro-2-nitro-4-azidobenzene, (ii) product of photochemical reaction of (i) and (ii) in sunlight produces an activated support, having a labile fluoro group (iii). Enzyme (ChOx) reacts with activated surface resulting in the immobilization of enzyme (ChOx) (iv).

3.1. Modification of octadecanethiol self-assembled monolayer and covalent immobilization of cholesterol oxidase

For covalent immobilization of cholesterol oxidase, octadecanethiol SAM was modified by 1-fluoro-2-nitro-4-azidobenzene. 1-Fluoro-2-nitro-4-azidobenzene is known to activate a matrix containing C–H bond under sunlight/UV through its azide group [42,45]. On its exposure to sunlight it produces highly reactive nitrene that gets inserted into C–H bond (Scheme 1). After modification of the matrix with FNAB the enzyme (cholesterol oxidase) was linked to FNAB covalently by thermal reaction at 37–40 °C [45,46]. The binding of the enzyme (cholesterol oxidase) was recorded using SPR (Fig. 2). In SPR curve the first phase of 120 s shows the base line after which ChOx solution was added and kept at 37 °C for 3000 s for binding to the FNAB modified SAM of ODT on gold plate, followed by washing with buffer. The increase in SPR angle with time shows gradual binding of ChOx. The difference observed in SPR angle (546 millidegrees) before and after cholesterol oxidase binding corresponds to 4.55 ng/mm^2 of ChOx (a change of 120 millidegrees corresponds to 1 ng/mm^2 binding) on FNAB/ODT/Au plate. These results suggest that 38.7 ng of cholesterol oxidase is immobilized over an area (8.5 mm^2) made by cuvette on FNAB/ODT/Au plate.

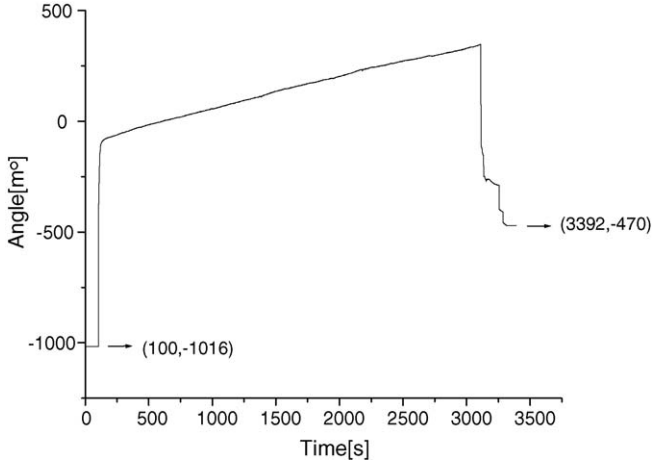


Fig. 2. SPR curve for covalent immobilization of cholesterol oxidase onto FNAB modified octadecane SAM on gold surface.

Scheme 1 depicts the principle of photochemical activation of SAM and covalent immobilization of ChOx onto the activated surface via thermal reaction. The ODT SAM on gold surface (i) was treated with FNAB (ii) where the azido group of FNAB on sunlight irradiation gets transformed into nitrene, a highly reactive specie, gets inserted into the C–H bond of ODT molecules on gold surface forming a covalent bond and the thermally active fluoro group of FNAB remains available for covalent binding with ChOx. The active surface (iii) binds with the enzyme on incubation of enzyme-buffer solution at 37 °C via displacement of its fluoro group by the amino group of enzyme resulting into covalent immobilization of enzyme (iv).

3.2. Contact angle studies

To investigate ODT SAM formation, FNAB modification and ChOx immobilization, the contact angle measurements were carried out using Sessile drop method. The change in the value of the contact angle reveals the hydrophobic/hydrophilic character of the surface, which in turn can be related to ODT SAM formation, FNAB modification and ChOx immobilization on the gold surface.

Table 1 shows the contact angle data of bare Au, ODT/Au, FNAB/ODT/Au and ChOx/FNAB/ODT/Au electrodes respectively. It can be seen that the value of the contact angle increases dramatically within about 2 h whereafter it stabilizes, indicating that the ODT molecules are coated on the Au surface. It appears that 4 h are enough for the ODT SAM formation on gold surface (Table 1). We selected 4 h optimize SAM/Au, to be modified with

Table 1
Variation in the contact angle of Au, ODT/Au, FNAB/ODT/Au and ChOx/FNAB/ODT/Au as a function of time

S. No.	Bare gold (°)	Au/SAM				Au/SAM/FNAB	Au/SAM/FNAB/ChOx
		2 h	4 h	6 h	16 h	4 h	4 h
1	89.53	113.27	117.91	118.23	118.29	106.80	62.00
2	90.03	113.68	118.03	118.50	118.50	107.18	62.18
3	89.63	112.93	117.71	117.97	117.98	108.21	61.90
4	90.31	114.00	117.48	118.17	118.20	105.75	62.25

FNAB (FNAB/ODT/Au) and ChOx (ChOx/FNAB/ODT/Au). The contact angle measurements were carried out on these electrodes to confirm the presence of FNAB and ChOx, respectively. The decrease in the value of the contact angle from 118° to 107° after modification with FNAB can be attributed to the covalent linkage of FNAB to the hydrophobic CH_3 group on ODT/Au. The value of the contact angle obtained as 107° reveals that the surface is still highly hydrophobic due to the presence of FNAB. Finally, on reaction with enzyme, the observed decrease in value of the contact angle from 107° to 62° shows the hydrophilic nature of the surface confirming the immobilization of cholesterol oxidase onto the FNAB/ODT/Au surface.

3.3. X-ray photoelectron spectroscopy studies

The surface of ODT/Au, FNAB/ODT/Au and ChOx/FNAB/ODT/Au were characterized using XPS technique (data not shown). In XPS spectra of ODT SAM two peaks at 164 and 284 electron volts (eV) for sulfur and carbon were obtained confirming the ODT SAM formation. The XPS spectra obtained for FNAB/ODT/Au 537 and 687 eV peaks correspond to O 1s of oxygen and F 1s of fluorine and two additional peaks in the range of 400–408 eV correspond to N 1s. Peaks observed in 400–408 eV range were resolved by taking selected range of spectra for FNAB/ODT/Au. The 400 eV peak was assigned to N 1s of NH group and the 407 eV peak to N 1s of NO_2 group present in FNAB. These results confirmed the covalent binding of FNAB to the ODT self-assembled monolayer on the gold plate.

Finally the XPS spectra of ChOx/FNAB/ODT/Au was recorded and it was observed that the height of N-H peak increased and that of the fluorine peak disappeared suggesting the covalent binding of enzyme (ChOx) to benzene ring of the FNAB due replacement of fluorine group. The XPS data also confirm the mechanism given in Scheme 1.

3.4. UV–vis studies

The UV–vis measurements were carried out to check the ChOx activity of the ChOx/FNAB/ODT/Au plate at various intervals of time (0, 2, 7, 14, 30, 45 and 60 days). The ChOx/FNAB/ODT/Au plates were kept at 4 °C prior to being used. The percent residual activity was measured by assigning 100% activity observed in the initial stage (0 day). The storage stability of the immobilized cholesterol oxidase was found to be satisfactory giving around 80–85% of original activity measured at zero time.

3.5. Cyclic voltammetry studies

Fig. 3 shows the cyclic voltammogram (CV) of ODT/Au (ii) wherein no redox peak current is observed in comparison to the bare gold electrode (i) in the potential range of 0–1.0 V in phosphate buffer (50 mM, pH 7.0). The significant drop in background current may be due to the stable modified monolayer of octadecanethiol, formed by S–Au bond on the surface of gold electrode that reduces the electron transfer rate between the surface of the electrode and the buffer. However, in the case of ChOx/ODT/Au electrode, the electro-active group of ChOx is present in much closer vicinity of the ODT/Au modified electrode and provides redox active groups leading to faster kinetics of ChOx redox on the ODT/Au SAM modified electrode as shown in Fig. 3(iii).

3.6. The effect of pH on ChOx/FANB/ODT/Au electrode

The effect of pH on the cyclic voltammetric response of ChOx/FANB/ODT/Au electrode at different pH (6.5–8.0) in phosphate buffer (50 mM) was studied. The cyclic voltammetric response of ChOx/FANB/ODT/Au electrode as a function of pH (6.5–8.0) in phosphate buffer (50 mM) has been shown in Fig. 4. It was observed that the peak current varied with the pH value in the range 6.5–8.0 and the optimum current was obtained at pH 7.0. Thus, all the experiments were conducted at pH 7.0.

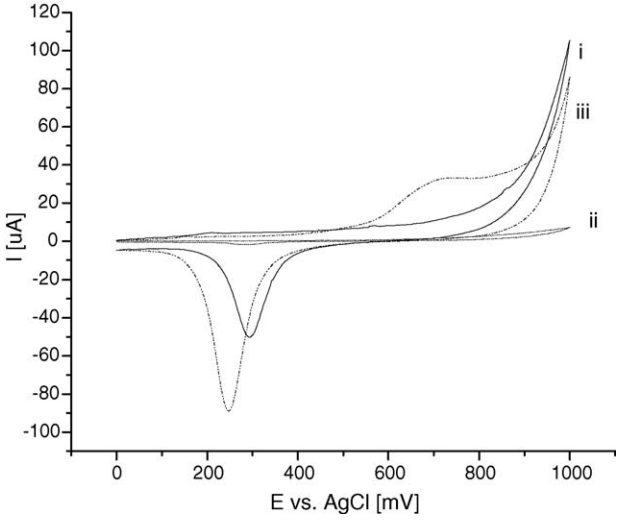


Fig. 3. Cyclic voltammograms of bare Au (i), SAM of octadecanethiol on Au plate (ii) ChOx/FANB/ODT/Au electrode (iii) in phosphate buffer (50 mM, pH 7.0) at a scan rate of 20 mV s^{-1}.

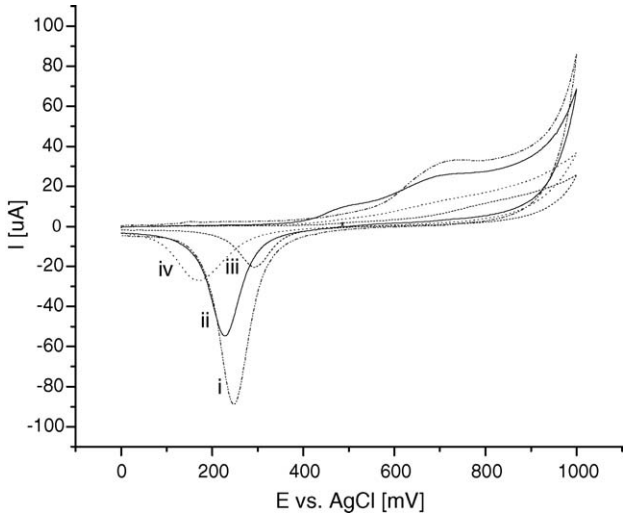


Fig. 4. CV of ChOx/FANB/ODT/Au electrode at different pH in phosphate buffer (50 mM); i: 7.0; ii: 7.5; iii: 6.5; iv: 8.0.

3.7. Surface plasmon resonance studies

SPR technique is known to provide quantitative information that can be used to estimate active concentration of biomolecules in solution. Fig. 5 shows the SPR signal obtained as a function of cholesterol concentrations (50–500 mg/dl). It is clear from the change in SPR angle versus time graph that SPR signal increases with the increase of cholesterol concentration confirming the interaction of ChOx with cholesterol. During experiments, the first phase in 120 s represents the base line after which cholesterol solution is added for association reaction to take place in the second phase of 300 s. It was observed that after addition of cholesterol, SPR signal increases rapidly in the first few seconds [1]. This is attributed to the change in plasmon frequency and hence the changes in the bulk refractive index [47]. This is followed by the gradual increase that represents the attachment of cholesterol onto active sites of immobilized ChOx on the surface and the increase in electron density due to redox reaction between cholesterol and ChOx on the ChOx/FNAB/ODT/Au electrode (association phase). Soon after the completion of association phase the remaining analyte in SPR cuvette was discarded and the buffer was introduced, this resulted in the sharp drop in the SPR signal indicating the change in the bulk refractive index (dissociation phase). Since similar conditions were taken before and after association phase so the difference between the SPR signal represents the amount of bound cholesterol with the cholesterol oxidase on ChOx/FNAB/ODT/Au electrode [1]. The removal of the remaining bound substrate and the regeneration of the enzyme catalytic site was done in the regeneration phase where the SPR signal returned to the base line. In the control experiment buffer was added in place of cholesterol during association phase and a straight line was observed showing no binding as can be seen in all figures for SPR studies. Each experiment was done at least three times and found to be almost similar with error of around 5–7% (Fig. 6). For quantification the shift in the SPR angle change was plotted as a function cholesterol concentration, straight line was obtained representing the linearity of 50–500 mg/dl of cholesterol (Figs. 7 and 8) corresponding to data obtained from Figs. 5 and 6, respectively.

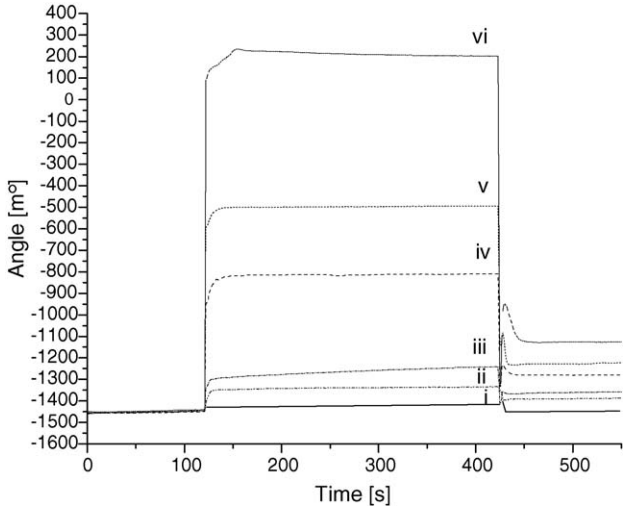


Fig. 5. SPR signal as a function of different cholesterol concentration [(i) phosphate buffer; (ii) 50 mg/dl; (iii) 100 mg/dl; (iv) 200 mg/dl; (v) 300 mg/dl; (vi) 500 mg/dl].

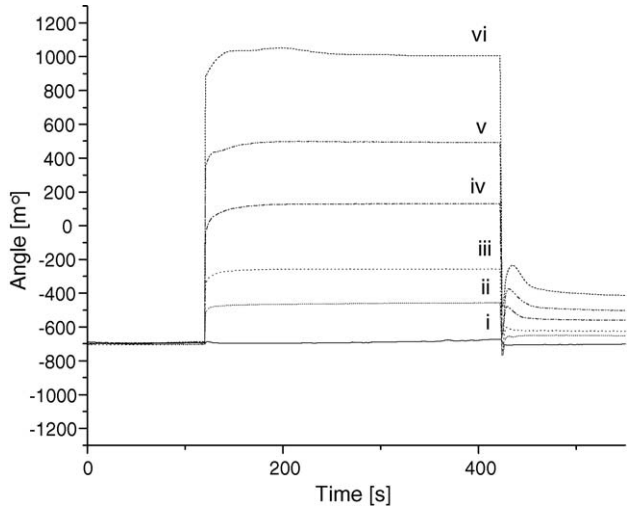


Fig. 6. Variation in SPR signal as a function of different cholesterol concentration [(i) phosphate buffer; (ii) 50 mg/dl; (iii) 100 mg/dl; (iv) 200 mg/dl; (v) 300 mg/dl; (vi) 500 mg/dl] in another freshly prepared ChOx/FNAB/ODT/Au electrode.

In other sets of experiments, different cholesterol concentrations were tested in set (A) the FNAB modified ODT self-assembled monolayer without immobilizing cholesterol oxidase and set (B) the cholesterol oxidase physically bound onto the SAM without FNAB modification under same conditions used for covalent immobilization of ChOx. Fig. 9 represents set (A) where one can see that there is no difference between the values of angle before and after the association phase, indicating that there is no binding or we can say substrate binding is specific for its enzyme, the same can be seen from Fig. 10 representing set (B), the very small change in angle values may be attributed to small amount of physical binding of ChOx.

Table 2
The characteristics of SAM based cholesterol biosensor along with those reported in literature

S. No.	Immobilization matrix	Sensing element	Method of immobilization	Response time	Linearity	Detection limit	Transducer used	Shelf life	Reusability	References
1	Polycation/alkylthiol layer	1-Stearoyl-2-oleoyl phosphatidyserine (SOPS)/cholesterol	Adsorption		–	–	–	–	–	[48]
2	Cystamine (SAM)	Cholesterol oxidase	Covalently	3 s	0.07–1.25 mM		Flow-injection	5 days	–	[35]
3	Pt/SAM/Prussian-Blue (PB)	Cholesterol oxidase	Entrapment	–	0.35 mM	8 μM	Amperometric	25 days	–	[17]
4	Alkanethiol	Cholesterol oxidase and microperoxidase	Covalently	<20 s	0.2–3.0 mM	–	–	–		[36]
5	Modified ODT	Cholesterol oxidase	Covalently	On-line	50–500 mg/dl	50 mg/dl	Optical	2 months	<15 times	Present work

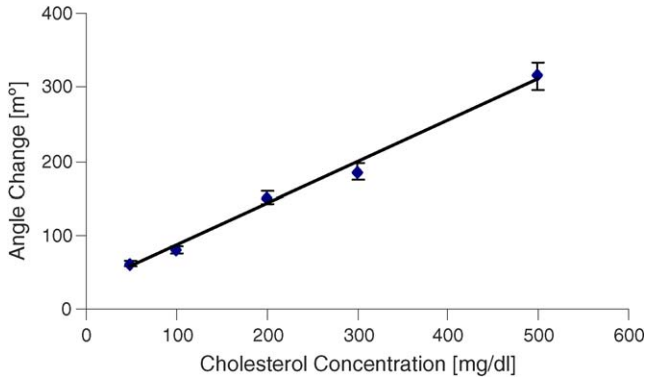


Fig. 7. The variation in SPR angle with change in concentration of cholesterol for data obtained from Fig. 5.

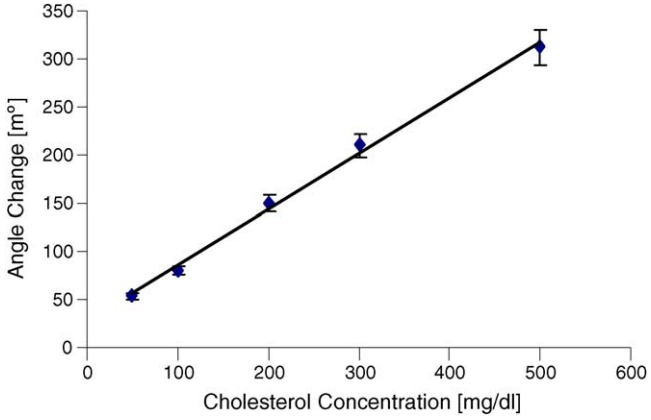


Fig. 8. Linearity curve for variation in SPR angle with cholesterol concentration for data obtained from Fig. 6.

3.8. Effect of interferents

The presence of interferents such as urea, glucose, ascorbic acid uric acid, etc. in blood sample affects the measurement of cholesterol. SPR signal of solution containing (1:1) ratio of

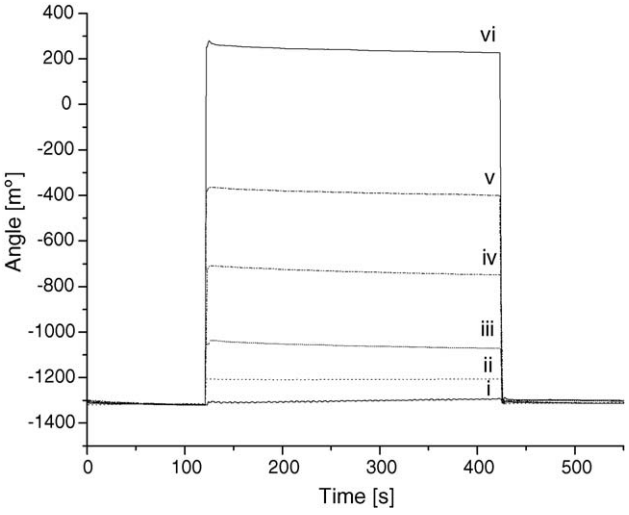


Fig. 9. SPR signal obtained at different cholesterol concentration for set (A) [(i) phosphate buffer; (ii) 50 mg/dl; (iii) 100 mg/dl; (iv) 200 mg/dl; (v) 300 mg/dl; (vi) 500 mg/dl].

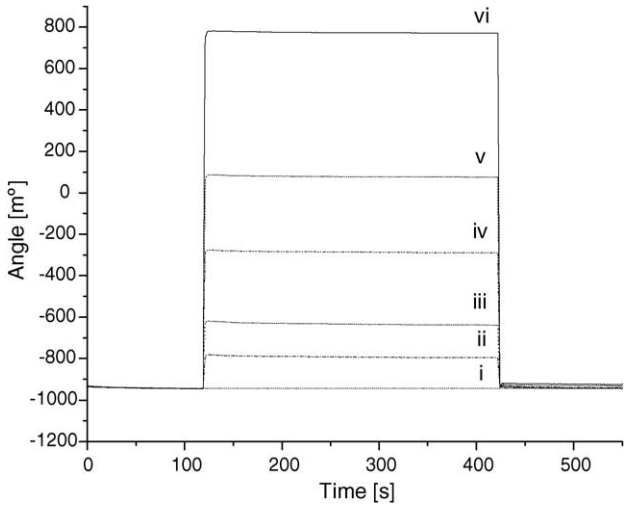


Fig. 10. Variation in SPR signal obtained at different cholesterol concentration [(i) phosphate buffer; (ii) 50 mg/dl; (iii) 100 mg/dl; (iv) 200 mg/dl; (v) 300 mg/dl; (vi) 500 mg/dl] for set (B).

cholesterol (200 mg/dl) and interferents such as ascorbic acid (5 mM), uric acid (5 mM), sodium azide (5 mM), glucose (5 mM) and urea (5 mM) were recorded (data not shown). The experiment was carried out in similar conditions as were for different cholesterol concentration without interferents. The change in SPR angle was found in the permissible range of error, i.e. less than 7%. It indicates that SPR technique is very specific and interferents have not much effect on the cholesterol measurement.

Table 2 shows the characteristics of cholesterol biosensor based on cholesterol oxidase covalently immobilized onto modified self-assembled monolayer of octadecanethiol on gold plate along with those reported in the literature. It can be seen that the ChOx/FANB/ODT/Au biosensor based on SPR has improved stability and linearity for cholesterol measurements.

4. Conclusion

Cholesterol oxidase has been covalently immobilized onto 1-fluoro-2-nitro-4-azidobenzene/octadecanethiol/Au electrode. It has been shown that surface plasmon resonance technique can be used to estimate cholesterol using ChOx/FANB/ODT/Au self-assembled monolayer. This cholesterol biosensor has improved the detection limit (50 mg/dl) and response time (550 s). The presence of interferents such as glucose, urea and ascorbic acid does not alter the observed amperometric response. Efforts are in progress to use this ChOx/FANB/ODT/Au self-assembled monolayer based biosensor for estimation of cholesterol in serum and blood samples and to improve the stability beyond 2 months.

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