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A novel non-enzymatic sensing probe for detection of cholesterol in solution

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ABSTRACT

In this work we have established that a new Schiff base (Z)-2-((pyridin-2-yl) methyleneamino) benzenethiol (2PMAB) prepared by reaction of 2-aminobenzenethiol with picolinaldehyde is a good electrode modifying agent to sense cholesterol. Glassy Carbon electrode surface has been modified with styrene film impregnated with 2PMAB. Ag^+ ion has been attached to the S atoms of 2PMAB on the electrode surface resulting in modified electrode of the type GC/styrene/2PMABAg. Cholesterol imparts a significant change of 0.048V in redox potential of the modified electrode. For the GC/styrene/2PMABAg electrode the response time is 3 s, detection limit (3σ) $1.99X10^{-5}$ M; sensitivity 0.0722 V/mM and linear range is $3.96X10^{-5}$M to $37.03X10^{-5}$M. Moreover, fluorescence study of the compound is also done in presence of the soft metal Ag and analyte cholesterol which indicates a significant "turn off" (420nm) of the fluorescence intensity of 2PMAB in presence of Ag and "turn on" at a new wavelength (520nm) in presence of cholesterol when excited at 350nm.

Key words: electrochemical, fluorescent, cholesterol, silver, redox potential

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INTRODUCTION

Biosensors have recently gained much attention in the field of health care for the management of various important analytes in a biological system. The area achieved tremendous progress from the time when the first Clark electrode for measurement of glucose was realized. Advances in the biosensor design are appearing at a high rate as these devices play increasingly important roles in our daily lives. The increasing incidences of cardiovascular diseases and cardiac arrest are major cause of death of human's world over. One of the most important reasons is hypercholesterolemia, i.e. increased concentration of cholesterol in blood.

Cholesterol and its fatty acid esters are one of the main constituents for the human beings as they are the components of nerve and brain cells [1] and are the precursors for other biological materials, such as bile acid and steroid hormones [2]. Abnormalities of cholesterol levels in blood are symptoms of several diseases, such as hypertension, coronary heart disease, arteriosclerosis, brain thrombosis, lipid metabolism dysfunction and myocardial infarction [3]. Various methods have been reported for the analysis of cholesterol in serum including colorimetric [4] spectrophotometric [5] and high performance liquid chromatography (HPLC) [6]. But most of these methods often present certain disadvantages, such as lack of specificity and selectivity due to interfering reactions and use of unstable and corrosive reagents [7]; inspiring scientists to develop electrochemical biosensors for cholesterol.

Electrochemical biosensors based on electron transfer between an electrode and immobilized cholesterol oxidase are especially promising because of their practical advantages such as operational simplicity, low fabrication cost and suitable for real time detection [8-10]. In most of the electrochemical biosensors for cholesterol, cholesterol oxidase (COx) is immobilized on a suitable matrix such as conducting polymers [11], carbon nanotubes (CNTs) [12],

nanoparticles (NPs) [13, 14], sol–gel/hydrogels [15,16] and self-assembled monolayer (SAM) [17,18]. COx is a flavin-adenine-dinucleotide (FAD) containing flavoenzyme that catalyses the dehydrogenation of C (3)-OH of the cholestan system in presence of di-oxygen, into 4-cholesten-3-one and hydrogen peroxide [19]. The enzymatic reactions in the use of cholesterol oxidase (COx) as a receptor can be described as follows:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{COx}} \text{Cholest-4-en-3-one} + H_2O_2 \qquad \text{---------- (1)}$$

$$H_2O_2 \xrightarrow{\text{E}} O_2 + 2H^+ + 2e^- \qquad \text{---------- (2)}$$

The electro oxidation current of hydrogen peroxide is detected after applying a suitable potential to the system.

The main problem with enzyme based biosensors is their less than desirable performance and high cost [20]. Moreover in amperometric detection the overestimation of the response current due to interferences is one of the major problems. Piletsky and his colleagues proposed cholesterol sensing method based on molecular imprinting technique such as hexadecylmercaptan as film medium on gold electrode surface and potassium ferricyanide as mediator [21]. Ji-Lai Gang et al. reported poly (2-marcaptobenzimidazole) imprinted gold electrode using potassium ferricyanide as mediator [22]. In molecular imprinting method, the mediator molecules come in contact with the electrode surface through the channels created by the template molecule, cholesterol. The redox current of mediator decreases on addition of cholesterol into the electrolytic solution as they can block the channels. Hence, molecular imprinting method is an indirect one and estimation of cholesterol is based on decrease in redox currents.

As far as the knowledge of the author no such methods have been developed where cholesterol comes in direct contact with the electrode surface. Moreover, use of Osteryoung Square Wave Voltammetric (OSWV) and fluorescence technique to detect cholesterol is also not found in the literature. The aim of the study is to develop a cholesterol biosensor without COx as a catalyst and redox current as a sensing signal. While significant developments have been made in cholesterol sensing, we present herein a simple and highly sensitive dual indicator system that can detect cholesterol electrochemically as well as by fluorescence by utilizing voltametric and fluorogenic "turn-off" and "turn-on" signals. In this paper we report that cholesterol imparts a significant change in redox potential of a Schiff base (Z)-2-((pyridin-2-yl)methyleneamino)benzenethiol (2PMAB) (prepared by reaction of 2-aminobenzenethiol with picolinaldehyde) in presence of soft metal like silver (Ag). This shift in redox potential can be calculated with the help of square wave voltammetric technique and could be utilized in estimation of cholesterol in solution. Moreover, fluorescence study of the compound is also done in presence of the soft metal Ag and analyte cholesterol which indicates a significant "turn off" (420nm) of the fluorescence intensity of 2PMAB in presence of Ag and "turn on" at a new wavelength (520nm) in presence of cholesterol when excited at 350nm.

EXPERIMENTAL SECTION

Reagents and general conditions

All reagents were purchased from Loba Chemie and were used without any further purification. Double distilled water (prepared by using Rieviera quartz double distillation apparatus) was used for all electrochemical experiments. Phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of 0.05M NaCl and 0.05M NaH_2PO_4-Na_2HPO_4, and then adjusting the pH with 0.05M H_3PO_4 or 0.05M NaOH.

^{1}H-NMR spectra were measured using a Bruker Ultrashield 300 MHz NMR spectrometer. Chemical shifts were expressed in ppm (in $CDCl_3$, with TMS as internal standard) and coupling constants (J) in Hz. Fluorescence experiments were performed on a Hitachi F-2500 spectrophotometer at room temperature. FTIR data were measured on a KBr pallet, using a Perkin Elmer spectrophotometer (RX1).

Electrochemistry

CHI 600B Electrochemical Analyzer (USA) with a three electrode cell assembly was used for electrochemical studies. Electrochemical experiments were carried out under a blanket of Nitrogen gas after passing the gas through the solution for 10 minutes. The electrode potential values were reported with respect to the normal hydrogen electrode, NHE. The working electrode is Glassy Carbon (GC) disc, reference electrode is Ag-AgCl (3M NaCl) and Sodium Nitrate (0.1M) is the supporting electrolyte. In the Osteryoung Square Wave Voltammetry (OSWV) experiments, the square wave amplitude is 25 mV, the frequency is 15 Hz and the potential height for base stair case wave front is 4 mV. Prior to every experiment the GC electrode was polished firmly on micro- cloth using fine 0.05 μM alumina powders followed by sonication for 2-3 minutes in Millipore water and then rinsed thoroughly with water [23].

Synthesis of 2PMAB

(Z)-2-((pyridin-2-yl)methyleneamino)benzenethiol (2PMAB) was prepared by stirring a mixture of 1 mmol (0.125 g or 0.4 ml) 2-aminobenzenethiol with 1 mmol (0.107g) picolinaldehyde in 10 mL methanol for 2 hours. Pale yellow crystalline compound was obtained which was filtered and recrystallized from methanol.

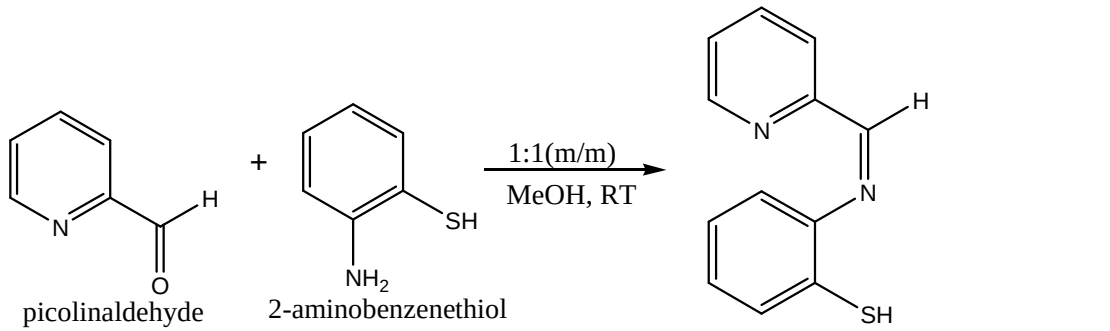
FTIR spectrum shows peaks corresponding to –C=N– stretching at 1589 cm^{-1}. The assignments of the peaks have been made from reported literature [24].

^1H nmr spetrum of the compound was recorded in $CDCl_3$. The peak at 1.7 ppm(s) may be assigned to aromatic C-SH; peak at 7.3 ppm(s) to H-C=N- proton; and the multiplates to aromatic protons.

Reaction scheme of 2PMAB

Scheme 1

Compound	Yield	Solubility
2PMAB	86%	MeOH



Electrode modification

0.1 g of 2PMAB was dissolved in 10 mL dichloromethane and 1.0 μL of the solution was placed on the tip of a pre-cleaned GC electrode surface using a Hamilton micro-syringe. The electrode was dried under nitrogen environment for 5 minutes. 1.0 μL of a styrene solution (prepared by dissolving 0.1 g styrene in 10 mL of dichloromethane) was dropped over the above modified electrode and again dried under the blanket of nitrogen for 10 minutes. The modified electrode is now designated as GC/styrene/2PMAB henceforth in this paper.

RESULTS AND DISCUSSION

Electrochemistry of the compound

Cyclic voltammogram of GC/styrene/2PMAB electrode was recorded in PBS at pH 7.0 and at scan rate 0.1 $Vs^{-1.}$ A pair of redox peaks with redox potential value -0.451 V ± 0.005 V (vs Ag-AgCl) and peak separation (ΔE_p) 0.104 V was observed. The redox peak currents were found to increase linearly with scan rates (not shown). This linear increase in current confirmed the electrochemical reversibility of 2PMAB. The ratio of cathodic to anodic peak current, I_{pc}/I_{pa} is almost unity (1.31).

An approximate estimation of the surface coverage of the electrode was made by adopting the method used by Sharp et al [25]. According to this method, the peak current is related to the surface concentration of the electroactive species, Γ, by the following equation:

$$Ip = n^2F^2 A\Gamma v/4RT$$

Where n represents the number of electrons involved in the reaction, A the geometric surface area (0.09 cm^2) of the electrode, Γ (mol m^{-2}) the surface coverage and v the scan rate and other symbols have their usual meanings. For the anodic peak current at scan rate 0.1 Vs^{-1}, the calculated surface concentration of 2PMAB is 3.02 X 10^{-6} mol m^{-2}.

It is well known that the sensitivity of SWV of absorbed species is proportional to the degree of reversibility of the electrochemical reaction [26, 27]. Since the redox couple of 2PMAB (not shown) showed a more reversible behaviour at the GC/styrene/2PMAB electrode, a clear advantage of using the electrode in SW mode with respect to

the sensitivity of cholesterol detections is expected. The SWV of the GC/styrene/2PMAB electrode in phosphate buffer solution (PBS) at pH=7.0 is shown in Fig.1 (curve a). The redox potential was found to be -0.380 V vs. Ag-AgCl.

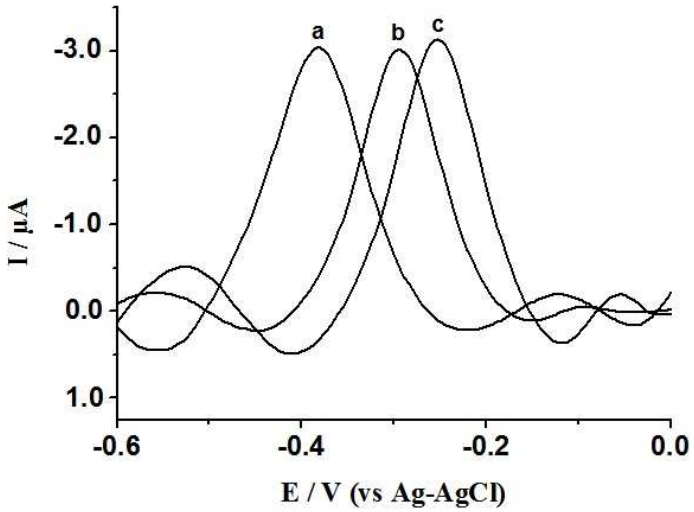


Fig .1: Square wave voltammogram of GC/styrene/2PMAB (a), GC/styrene/2PMABAg (b) and GC/styrene/2PMABAg in presence of 0.37 mM cholesterol (c). SWV was recorded in PBS at pH 7 against 3M NaCl, Ag/AgCl as reference

When Ag^+ ion was added gradually into the electrolytic solution the redox potential was found to undergo anodic shift till the redox potential becomes -0.290 V. This value of redox potential was obtained when final concentration of Ag^+ ion in the electrolytic medium was $5.35x10^{-4}$ M. Hence, addition of Ag^+ ion into the electrolytic medium caused an anodic shift of 0.090 V to the modified electrode (Fig.1, curve b). Ag^+ ions coordinated to the S atom of 2PMAB molecule coated onto the electrode surface. This was evident from the fact that the colour of the modified electrode surface was yellow before addition of Ag^+ which became white after addition of Ag^+ into the electrolytic medium. This white coating, observed by naked eye, must be of Ag on electrode surface. The modified electrode attained a new composition GC/styrene/2PMABAg.

Co-ordination of 2PMAB to Ag^+ through S resulted in an excess in electron density on Ag^+ as Ag^+ has $3d^{10}$ configurations. Donation of electron density from S atom to Ag^+ lowers the electron density on the S atom as well as on the benzene ring of the 2PMAB. This lowering in electron density lowers the oxidation tendency of the ring which results a positive shift in redox potential of 2PMABAg as compared to that of 2PMAB.

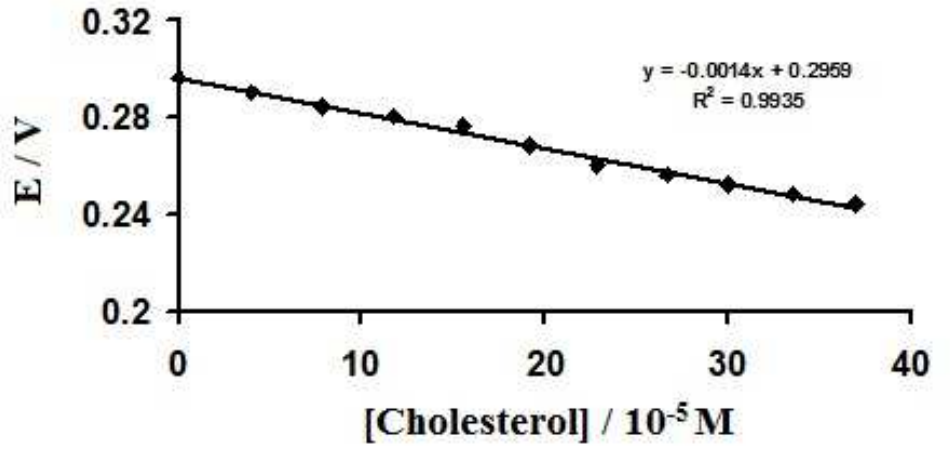
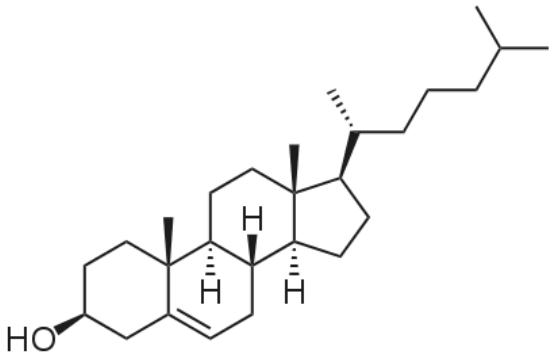


Fig .2: Effect of cholesterol concentration on the redox potential of GC/styrene/2PMABAg in PBS at pH 7

Effect of addition of cholesterol into the electrolytic solution on the redox potential of GC/styrene/2PMABAg electrode is shown in Fig.1 (curve c). A net anodic shift of 0.048V can be clearly observed when cholesterol interacts with GC/styrene/2PMABAg electrode. Linear calibration plot for the shifts in redox potential of

GC/styrene/2PMABAg electrode are obtained over $3.96X10^{-5}$ to $37.03X10^{-5}$M concentration of cholesterol in the electrolytic medium. The slopes (V/M) and correlation coefficients are found to be -0.0014 and 0.9935 respectively (Fig 2). The sensitivity was calculated to be 0.0722 V/mM for GC/styrene/2PMABAg electrode. The detection limit (3σ) found was $1.99X10^{-5}$ M for the GC/styrene/2PMABAg electrode. As there is not any reported voltammetric sensors for cholesterol based on redox potential versus cholesterol concentration, it is not possible to make a comparative study.

A response time of 3 s was obtained after each addition of cholesterol. The response time of the biosensor was defined as the time after analyte addition for the biosensor response to reach 95% of its final value. The response time in this study is better than the methods for immobilization of COx in carbon nanotube-chitosan-platinum composite (8s) [28], chitosan hybrid composite (13s) [29], poly(2-hydroxyethyl methacrylate) polypyrrole composite film (30s) [30], layer-by-layer assembling polymer films (30–40s) [31], poly(1,2-diaminobenzene) film (51s) [32], and silicic sol–gel matrix (60s) [33].



Scheme 2: Structure of cholesterol

The most favourable site in cholesterol to be targeted by the modified electrode for interaction is its lone double bond (Scheme 2). Ag is prone to form complex containing double bond. The association of Ag with cholesterol will enhance electron density on them resulting again decrease in electron density on 2PMAB and induces electropositivity on that compound. The impact is a further anodic shift in redox potential of the modified electrode on interaction with cholesterol.

Fluorescence study of the compound

1:1 (v/v) acetonitrile : water solution of 2PMAB in PBS at pH7 shows fluorescence emission in the range 300 nm to 650 nm with λ_{max} at 420 nm when excited with 350 nm radiation.

The fluorescence titration reaction of 1:1 (v/v) acetonitrile : water solution of 2PMAB by aqueous solution of silver(I) ions ($5 X 10^{-3}$ M), shows a steady and smooth decrease in fluorescence intensity that saturates at 1.0 equivalent (Fig.3).

The plot of I/Io Versus concentration of Ag ion is shown in Fig.4 (where I is the fluorescence intensity at particular $[Ag^+]$ and I_o is the initial fluorescence intensity of the ligand and I_{max} is the fluorescence intensity of the ligand at maximum $[Ag^+]$). The decrease in fluorescence intensity may be attributed to redistribution of electron density upon Ag^+ ion binding to -S- (soft-soft interaction).

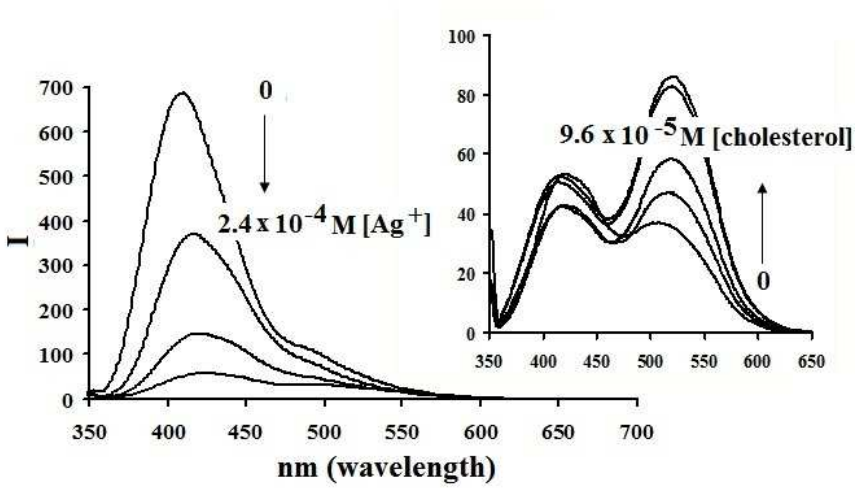


Fig .3: Changes in fluorescence intensity of the ligand when 1:1 acetonitrile:water (v/v) solution of the ligand is titrated with aqueous Ag(I) solution (Fig .3 inset Changes in fluorescence intensity upon addition of cholesterol to the Ag(I) saturated 1:1 acetonitrile:water (v/v) solution of the ligand) when excited at 350nm

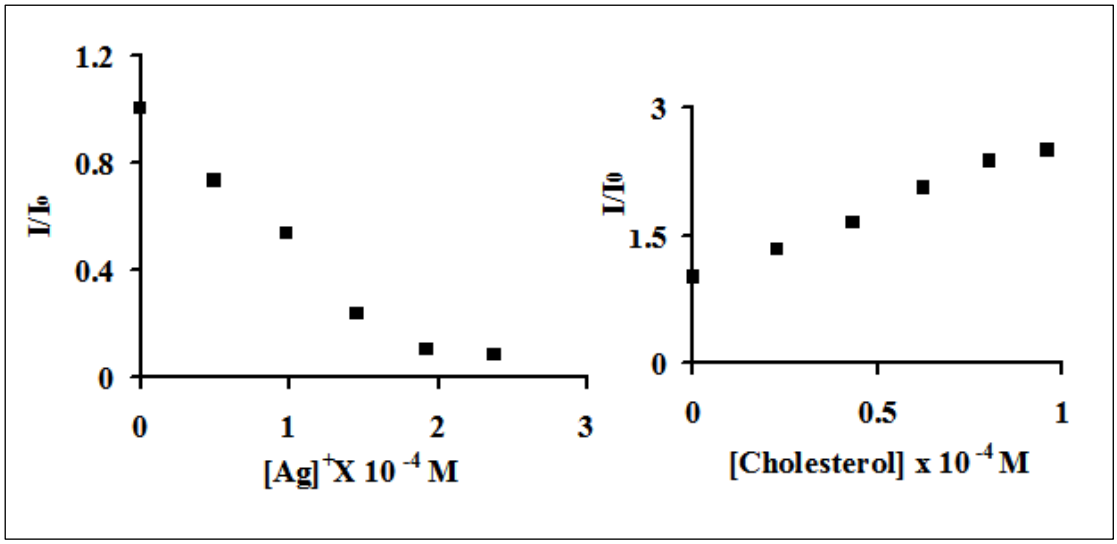


Fig .4: Plot of I/I_o Versus concentration of Ag(I) and plot of I/I_o Versus concentration of cholesterol

To calculate the binding constant and the stoichiometry of binding [34], $\log\{(I-I_o)/(I_{max}-I)\}$ was potted against $\log[Ag^+]$ (Fig. 5) and found to be linear. A least squares fitting of data yielded the slope as 1.2±0.02 indicating a 1:1 binding between 2PMAB and Ag^+. The binding constant was calculated to be $\log\beta=5.67$.

The fluorescence titration reaction of 1:1 (v/v) acetonitrile:water solution of 2PMAB saturated with aqueous solution of silver(I) ions (5 X 10^{-3} M), by aqueous cholesterol solution(5 X 10^{-3} M), gave rise to a new peak at wavelength 520 nm which showed a smooth and steady increase in fluorescence intensity (Fig.3 inset) with increase in cholesterol concentration whereas the peak at wavelength 420nm remains almost constant. The plot of I/I_o Versus concentration of cholesterol is shown in Fig.4. The change in fluorescence intensity may be attributed redistribution of electron density due to Ag ion binding to cholesterol aromatic –C=C- bond.

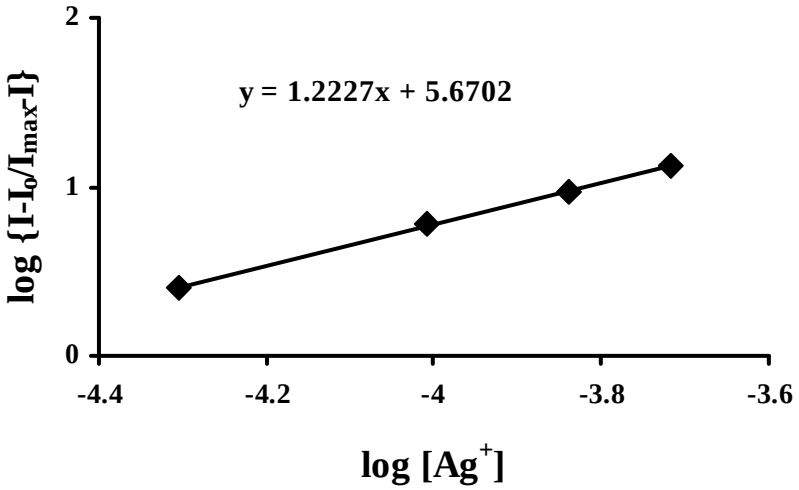


Fig .5: Plot for binding constant from fluorescence showing 1:1 metal:ligand binding of silver ion with the ligand

To calculate the binding constant and the stoichiometry of binding, $\log\{(I-I_o)/(I_{max}-I)\}$ was potted against log[cholesterol] (Fig. 6) and found to be linear. A least squares fitting of data yielded the slope as 1.02 ±0.02 indicating a 1:1 binding between 2PMAB-Ag and cholesterol. The binding constant was calculated to be logβ=4.7.

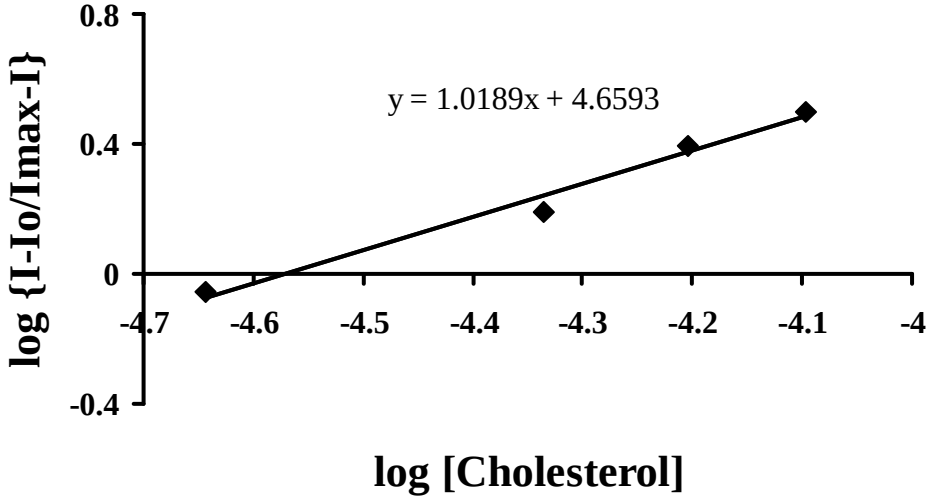


Fig .6: Plot for binding constant from fluorescence showing cholesterol binding to silver ion already bounded to the ligand

Interference studies

Interference by ascorbic acid, uric acid and glucose are generally studied for voltammetric sensors for cholesterol [35, 36]. We too studied the effect of these three compounds on the cyclic voltammetric response of the modified electrode GC/styrene/2PMABAg. The redox potential and redox currents were not affected by any one of the interfering compounds at their 10^{-2} M concentration. There was no any enhancement of fluorescent peak at 520nm was observed upon addition of these three compounds to the Ag(I) ion saturated ligand solution.

CONCLUSION

In summary, we have modified a GC electrode surface with a Schiff base (Z)-2-((pyridin-2-yl) methyleneamino) benzenethiol (2PMAB) and developed a new modified electrode GC/styrene/2PMABAg. The redox potential of this modified electrode is affected by cholesterol present in the solution. The modified electrode has very low response time of 3 s, detection limit $1.99X10^{-5}$ M; sensitivity 0.0722 V/mM and linear range $3.96X10^{-5}$M to $37.03X10^{-5}$M.

Fluorescence study of the 2PMAB also established the cholesterol sensitivity of the ligand. On binding to Ag(I) the peak obtained at 420nm of the ligand when excited at wavelength 350nm decreases whereas the Ag(I) ion saturated ligand solution showed a increase in fluorescence intensity at a new wavelength 520nm upon addition of cholesterol to the Ag bounded ligand.

Hence the new Schiff base synthesized can be utilized as electrochemical and fluorescence cholesterol sensor.

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