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A Conductometric Cholesterol Sensor Based on the Permeability Effect on BLM and its Application in Clinical Analysis

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Abstract: A new application of liquid membrane bilayers has been explored for the determination of cholesterol in blood serum samples. The method is based on enhancement in the conductance of the lecithin-prostaglandin liquid membrane bilayers due to the formation of hydrophilic pathways by the addition of cholesterol solution. Since enzyme is not used, stability of the sensor is not governed by the stability of the enzyme. The response time of enzyme is less than 60 sec. Effect of applied potential, interference of electroactive species present in the blood samples have also been evaluated to optimize the condition for the determination of cholesterol in blood serum samples. The device can be used for the determination of cholesterol in the concentration range 0.453 mg dL⁻¹- 468.5 mg dL⁻¹ in a solution. The detection limit of the present device is found to be 1.2489 µg dL⁻¹. The relative standard deviation for the liquid membrane bilayer to liquid membrane bilayer reproducibility is found to be 1.4% for the determination of 1.174×10^{-5} M of cholesterol solution (6 replicate bilayer lipid membrane). The device has been tested by estimating free cholesterol in blood serum and it works well.

Key words: Hydrophilic pathways, liquid membrane, lecithin, prostaglandin

Introduction

There is an increased demand for developing fast and sensitive methods for the determination of various analyses present in the whole blood. Biosensors have been considered to be one of the most suitable devices due to selectivity, fast response, reliable and reproducible results. It is a common practice that immobilized enzymes relevant to the species to be assayed are utilized in the fabrication of sensors. No doubt the use of enzymes in the fabrication of sensors has obvious advantages but it has limitations too. The stability of the sensor is mainly controlled by the stability of the enzyme. In this context, design and study of new non-enzymatic Biosensors for the estimation of various analyses of clinical interest such as urea, glucose and cholesterol have received considerable attention (Shoji and Freund, 2001; Bindra and Wilson, 1989; Piletsky *et al.*, 1999).

Cholesterol is an important compound for human beings because it is a precursors of other biological materials such as steroid hormones (Brown, 2000). About 70% of the cholesterol in blood is esterified by fatty acids. The estimation of cholesterol in blood is very important for the diagnosis of malfunctioning of heart; in diseases like arteriosclerosis and cerebral thrombosis (Fredrickson *et al.*, 1972; Nauck *et al.*, 2000). Several cholesterol biosensors including enzymatic and non-enzymatic methods have been reported (Piletsky *et al.*, 1999; Nakaminami *et al.*, 1997; Singh *et al.*, 2004). However, these often have certain difficulties like lack of specificity and selectivity due to the presence of various interfering reactions and the use of unstable and corrosive reagents.

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In the present study a new possibility has been explored which is based on the enhancement in the conductance of liquid membranes generated on hydrophobic supporting membranes. In the earlier studies, it has been shown that in the lecithin-cholesterol liquid membrane bilayers, hydrophilic pathways are generated by the addition of prostaglandins (Srivastava *et al.*, 1991). It is believed that the generation of hydrophilic pathways should manifest itself in the enhancement of electrical conductance of the liquid membrane bilayer separating the two electrolytic solutions. These facts have been exploited for the fabrication of a cholesterol sensor. In the present study we have experimented with the prostaglandin E1 saturated lecithin liquid membrane to fabricate non-enzymatic cholesterol sensors. Since the response time is very short; less than 60 sec and no interfering reactions of electroactive species; the liquid membrane based device described in this paper appears quite attractive.

Materials and Methods

Lecithin (Phosphatidylcholine granular, Across Organic New Jersey, U.S.A.), cholesterol (Sigma, Cat.No.C8667), prostaglandin E₁(Sigma, Cat no. P5515), L-histidine (Aldrich Chem. Co., 15, 168-8), sodium chloride (AnalaR grade), sartorius cellulose acetate membrane (Cat No.11107, Millipore), Ag/AgCl electrodes (BAS, U.S.A.) and bio-analytical voltammograph (BAS, CV-27) were used in the present study. Double-distilled water was used in preparing aqueous solution.

Experimental Set-up and Measurements

The present experimental studies have been done in the department of chemistry, IIT, Delhi. Aqueous solutions of lecithin and cholesterol were prepared using the method of Gershfeld and Pagano (1972) as described in earlier publications (Srivastava *et al.*, 1991; 1998, Srivastava and Jakhar, 1982). Aqueous solutions of prostaglandin E₁ was prepared in the usual way as described in earlier publication (Srivastava *et al.*, 1991). Histidine hydrochloride buffer (5 mM) was used to adjust the pH at 7.2. The set-up used is schematically depicted in Fig. 1. which has been well labeled to make it self-explanatory. The perspex glass cell consisted of two compartments A and B, separated by a sartorius cellulose acetate membrane M which acted as a support for the liquid membrane fixed in the middle of the partition wall. The compartments A and B of the cell (Fig. 1) were filled with aqueous solution of lecithin (1.919×10^{-5} M) and prostaglandin E₁ (3×10^{-8} M) prepared in 5 mM histidine hydrochloride buffer (pH = 7.2) along with 0.1 M NaCl solution in compartment A and 0.01M NaCl solution in compartment B and left overnight to equilibrate. The particular composition of

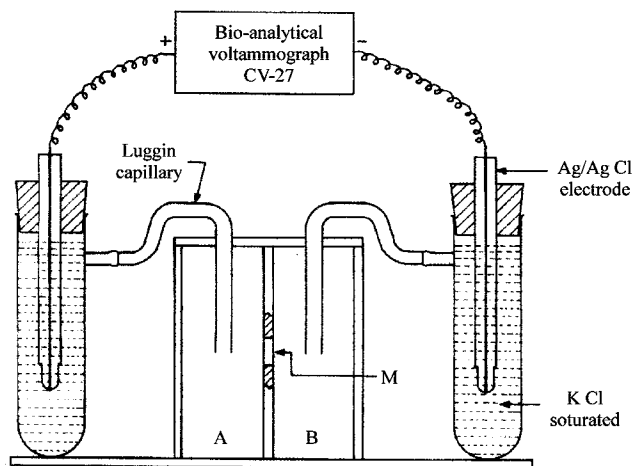


Fig. 1: Schematic representation of the experimental setup. M, supporting membrane, B1 and B2, luggin capillaries and P1, P2 are Ag/AgCl electrode dipped in 3 M KCl solution

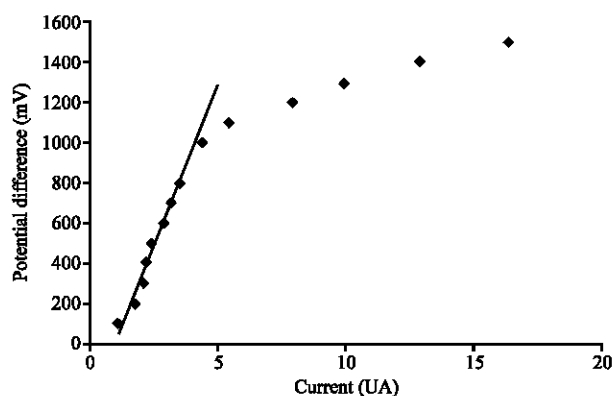


Fig. 2: V-I plot showing range of validity of Ohmic relationship for Prostaglandin saturated lecithin liquid membrane

lecithin-prostaglandin was derived from earlier study (10) where, it was shown that at this composition, the lecithin liquid membrane which was fully developed at the interface was saturated with prostaglandin E1.

Two luggin capillaries, B1 and B2 were then introduced in the compartments A and B respectively which connects the respective compartments of the cell to the two Ag/AgCl electrodes dipped in 3 M KCl solution; care was taken that B1 and B2 were at the fixed distance from the M. To measure the response of the device, known potential differences V , were applied across the Ag/AgCl electrodes and the corresponding currents I , flowing through the circuits were determined using the bioanalytical voltamgraph. The V-I plot shown in the Fig. 2 give the domain of validity of Ohm's law for the liquid membrane.

Known but equal concentration of cholesterol was introduced in both the compartments, A and B and magnetically stirred to ensure the uniformity of concentration. A fixed potential difference, which is well within the range of validity of Ohm's law (Fig. 2), were then applied across the electrodes and current flowing through the circuit was measured. Measurements were repeated with different concentrations of cholesterol to get the calibration curve (I versus cholesterol concentration) at different applied potentials.

To study the effect of interfering endogeneous electroactive species found in blood serum interferences were added on both sides of the membrane at their physiological concentration and responses were measured. To estimate the concentration of free cholesterol in clinical blood samples, 100 μ L of serum was added instead of aqueous solution of cholesterol.

All experiments were carried out at constant temperature using a thermostat set at $37 \pm 0.5^\circ\text{C}$.

Results and Discussion

It has been shown (Srivastava *et al.*, 1991; 1998; Srivastava and Jakhar, 1982) that when two compartments separated by a hydrophobic supporting membrane are each filled with the aqueous solutions of lecithin-cholesterol mixture of concentration 1.919×10^{-5} M with respect to lecithin and 1.175×10^{-6} M with respect to cholesterol, a bilayer of liquid membrane consisting of lecithin and cholesterol is formed within the voids of the supporting membrane. The formation of liquid membrane is based on Kesting's hypothesis (Kesting *et al.*, 1968), when a surfactant is added to an aqueous phase, the surfactant layer which forms spontaneously at the interface acts as a liquid membrane. As the concentration of surfactant is increased, the interface gets progressively covered with the surfactant

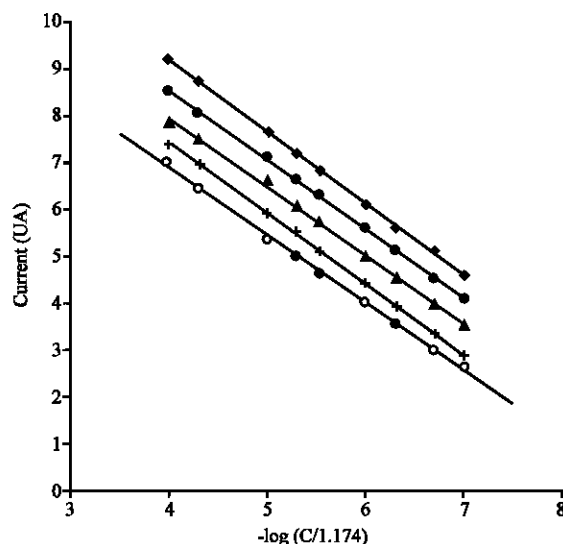


Fig. 3: Calibration curve showing the variation of current I (μA) with different concentration of Cholesterol solution at different potential differences. (■) at 700 mV, (●) at 600 mV, (▲) at 500 mV, (×) at 400 mV, (○) at 300 mV. Test points (●) at 300 mV

layer liquid membrane and at the critical micelle concentration of the surfactant, it is completely covered. The observations made on the lecithin cholesterol liquid membrane bilayers generated (Srivastava *et al.*, 1991) forms the basis of the present study.

In the lecithin-cholesterol liquid membrane bilayer when prostaglandin E1 is incorporated by adding its aqueous solution in the two compartments, hydrophilic pathways are generated in the bilayers. It was found that the presence of both cholesterol and prostaglandin in both compartments was essential for the full generation of hydrophilic pathways; in fact it was demonstrated (Srivastava *et al.*, 1991) that cholesterol in association with prostaglandin drills the hydrophilic pathways in the liquid membrane bilayers.

Prompted by these observations we experimented with prostaglandin saturated lecithin liquid membrane bilayers to develop membrane permeability based cholesterol sensors.

This observation implies that if cholesterol is added on the two sides of the lecithin liquid membrane bilayer saturated with prostaglandin, the electrical conductance of the bilayer should increase due to the formation of hydrophilic pathways; the increase in conductance is likely to vary with the concentration of cholesterol added.

Effect of Applied Potential

To know the range of validity of Ohmic relationship, known potential differences, V were applied across the bilayer membrane using Ag/AgCl electrodes and the corresponding currents, I flowing through the circuits were determined using the bioanalytical voltamograph. The V - I plot shown in the Fig. 2 gives the domain of validity of Ohm's law for the liquid membrane upto around 850 mV. Therefore, 850 mV is the maximum applied potential in the present device.

Characteristic Response of the Liquid Membrane Bilayer

Electrical potential difference of 100, 200, 300, 400, 500, 600, 700 mV were applied on the prostaglandin saturated lecithin liquid membrane across the two Ag/AgCl electrodes against cholesterol concentration introduced in the two compartments A and B and calibration curves were obtained by plotting the current flowing in the circuits by applying electrical potential difference lower than 850 mV as shown in Fig. 3. The value of current (I) increased upon increasing the concentration of

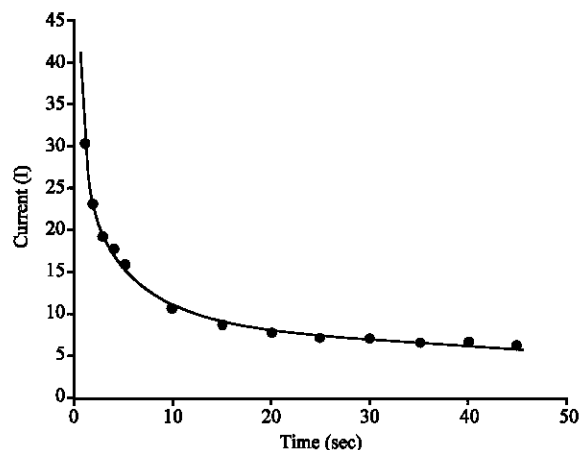


Fig. 4: Response time graph (current I vs. time) in one particular case (1.174×10^{-5})

Table 1: Effect of interferences on the response measurements when 1.175×10^{-5} M cholesterol added on both sides of the device

Interferents (concentrations in mM)	Response before addition (μ A)	Response after addition (μ A)
Glucose	3.57	3.600
Lactate	3.57	3.610
Uric acid	3.57	3.670
Urea	3.57	3.590
Ascorbic acid	3.57	3.64

cholesterol at all applied potential. For constructing the calibration curves, we could not take higher concentrations of cholesterol due to its limited solubility in aqueous medium. To check the validity of the calibration curves, the concentration of a few samples of known strengths within the range of the calibration curves were estimated using the present device. The experimental test points do fall on the calibration curve. The experimental test points are shown in case of 300 mV applied potential in Fig. 3. The response curve obtained, on the injection of 1.174×10^{-4} M cholesterol solution in the two compartment of the cell containing prostaglandin saturated lecithin liquid membrane is shown in Fig. 4. The response time was found to be less than 60 sec for all the concentration of cholesterol at different potentials, from 100 to 700 mV. The quick response time suggests that the present device can possibly be used for sensing cholesterol in aqueous media.

Interferences

To decide whether the method could be applicable to the real samples or not, the interference effect of various endogenous oxidisable substances found in blood serum was studied at the optimum working condition. A definite amount of interferences (at physiological concentration) were introduced into the test solution before adding cholesterol solution and biosensor response was measured. Most of the interferences showed higher response between 400-700 mV. Therefore, the operational potential chosen for the present device is 300 mV. As can be seen from Table 1, these interfering species have negligible effect on the biosensor response at 300 mV because the working potential chosen for the device is less than the potential at which they showed interfering effects.

Clinical Analysis of Cholesterol in Blood Serum

The concentrations of free cholesterol in clinical blood samples were estimated using the present device. The serum was appropriately diluted to bring the concentration of cholesterol within the range

Table 2: Determination of cholesterol concentration in blood serum samples

Sample no.	Concentration of free cholesterol ^a (from clinical method) (mg dL ⁻¹)	Concentration of free cholesterol ^b (from present method) (mg dL ⁻¹)
1.	43.00	42.4±0.81
2.	52.10	50.78±1.13
3.	46.00	43.20±1.20
4.	60.80	56.20±1.28
5.	70.95	68.02±0.98
6.	65.50	60.60±0.92

^aThe analysis using enzymatic colorimetric method by the pathological laboratory. ^b Results obtained using the present method. Values reported as average of 10 repeats±standard deviation

of the calibration curve. The results obtained using the present method compare favourably well with the results obtained by the usual clinical method (Table 2).

Storage, Stability and Application

The stability of the sensor is mainly determined by the stability of the enzyme used in the device. Since the present device doesn't make any use of enzyme, its stability is free from this limitation. The solutions of lecithin and prostaglandin E1 prepared in histidine-hydrochloride buffer (pH = 7.2) and stored in a refrigerator, remain usable in the device for more than a week. After about 15 days, the solutions particularly the aqueous solutions of lecithin developed turbidity, most probably due to microbial growth and then it became unusable in the device. If sterile conditions are maintained the solutions should remain usable for much longer time.

Conclusions

The present device utilizes the phenomenon of increased conductance due to the formation of hydrophilic pathways by cholesterol in association with prostaglandin. It shows very quick response time i.e., 60 sec and considerable stability. There is negligible effect of electroactive interferences on the sensing performance. The biosensor works at a appreciably low potential of 0.3V and exhibit linear response with cholesterol concentration in the range 0.453-468.5 mg dL⁻¹. And it shows temperature tolerance between 25-40°C. The sensitivity of the method has been found to be 1.54. Therefore, it appears quite attractive for cholesterol estimation.

Studies pertaining to the longer shelf life of the device and estimation of total cholesterol are under progress.

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