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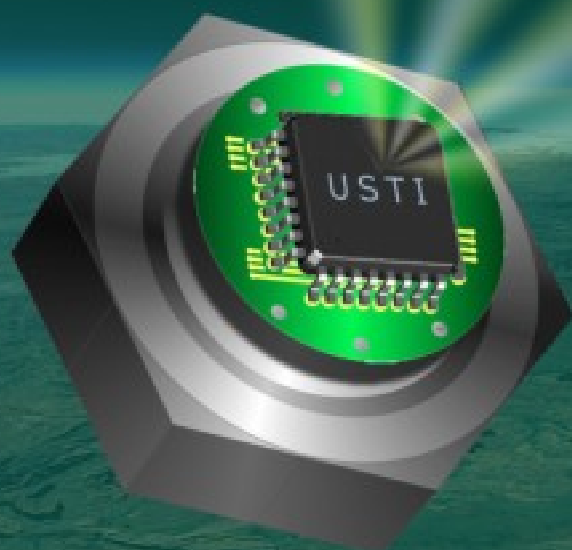

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Cholesterol Biosensor Based on Polyvinyl Formal Membrane Bound Cholesterol Esterase and Oxidase

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Abstract: Cholesterol biosensor was fabricated by co-immobilizing cholesterol esterase and cholesterol oxidase in polyvinyl formal membrane and by mounting over the sensing part of oxygen electrode. The immobilized enzymes membrane was characterized by atomic force microscopy. The biosensor showed the linearity for 100-450 mg dl^{-1} cholesterol acetate. The minimum detection limit of the cholesterol was 50 mg dl^{-1}. The effects of temperature, pH and the stability of immobilized enzymes were also studied. A good correlation was found among cholesterol values obtained by colorimetric, Enzo-kit and auto analyzer method. The shelf-life of the biosensor was more than three month at 4ºC and enzyme membrane can be re-used 20 times without any significant loss in enzyme activity. *Copyright © 2007 IFSA.*

Keywords: Cholesterol biosensor, Cholesterol esterase, Cholesterol oxidase, PVA membrane

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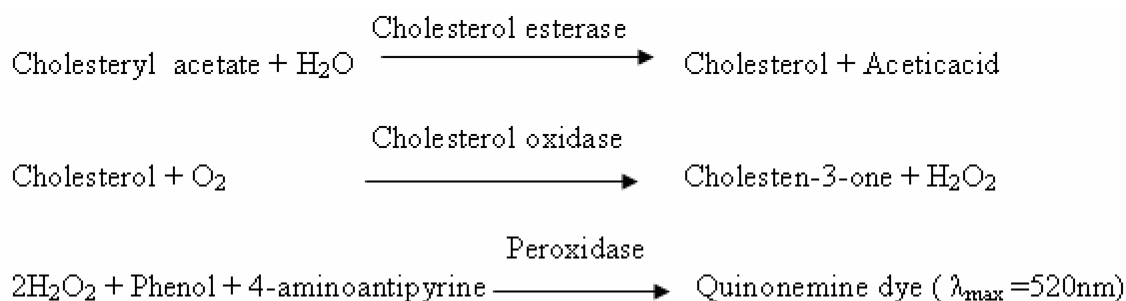
1. Introduction

The estimation of blood cholesterol concentration is one of the most widely performed assays in biochemistry. Elevated serum cholesterol is supposed to be a risk factor in the development of arteriosclerosis and myocardial infarction. The normal level of cholesterol in blood/serum is 150-250 mg dl^{-1}. Cholesterol in blood is predominantly esterified with fattyacids and associated with lipoproteins. Among the various methods available for determination of total cholesterol [1, 2], enzymatic methods employing cholesterol esterase (CE), cholesterol oxidase (COD) and peroxidase (POD) is simple, sensitive and specific therefore suitable for routine analysis [3, 4]. It is based on the following chemical reactions:



The enzyme kits are expensive for routine clinical analysis. Therefore, the high cost of enzyme as well as time consumption in analysis restricts the use of this method for routine estimation of cholesterol in patients samples. The immobilization of enzymes on suitable insoluble support provides their reuse and thus reduces the cost of the analysis. Cholesterol esterase (CE) and cholesterol oxidase (COD) have been immobilized/co-immobilized onto various supports such as alkylamine glass [5, 6] silica gel and controlled pore glass [7, 8] through glutaraldehyde coupling, octyl agarose gel [9], formvar [10], COD and peroxidase (POD) co-immobilized in a sol–gel film through physical adsorption and microencapsulation [11] and on octadecanethiol monolayer in SPR [12]. All these three enzymes (CE COD and POD) have been also co-immobilized onto alkylamine glass beads [13]. A co-immobilized system is, due to the shorter diffusional distance and the restricted diffusion out to the surroundings, more sensitive towards low concentration of substrate for the enzyme than a corresponding system with the enzymes separately immobilized. In diagnosis, quick and sensitive methods are in demand for the determination of various blood analytes.

A number of amperometric cholesterol biosensor have been developed employing cholesterol esterase, cholesterol oxidase and peroxidase immobilized onto octyl agarose gel [9], pyrole membrane [14], nylon mesh [15], screen printed strip [16], carbon paste electrode [17], polypyrrole film [18-21], graphite-teflon matrix [22], dialysis membrane [23], conducting polypyrrole films [19,20] and polyanniline films [24, 25]. Carbon has proved to be the best matrix for fabrication of enzyme electrode [26]. Biosensors are most suitable devices due to simple, sensitive, quick results. The importance of biosensors has increased considerably during the past decade, as it combine the specificity of the biological systems with the advantage of electrochemical transduction [27]. Therefore, the present work was aimed to immobilize CE and COD in polyvinyl formal membrane to develop cholesterol biosensor for estimation of total cholesterol in blood.

2. Materials and method

2.1. Chemicals

Cholesterol esterase, cholesterol oxidase, peroxidase and glutaraldehyde (25% solution) were from M/s Fluka Chemicals, Germany. Polyvinyl formal (Commercial name Formvar), TritonX-100, Cholesteryl acetate and 4-aminoantipyrine were from M/s Sigma Chemical Co., USA. Cholesterol kit was from M/S Miles India Ltd., Baroda India. All other chemicals used were of analytical reagent grade. Response measurements were conducted by using dissolved oxygen analyzer, Germany. Atomic force microscopy was carried out by using Nanoscope workstation II Digital, USA.

2.2. Preparation of Cholesteryl Acetate Solution

Cholesteryl acetate was used as a substrate for cholesterol esterase. Cholesteryl acetate (50mg dl^{-1}) was initially dissolved in 1.0 ml of Triton X-100 by heating and stirring and final concentration of 50 mg dl^{-1} was prepared by dissolving in 50 mM sodium phosphate buffer, pH 7.0. Solutions of different

concentrations of cholesteryl acetate (50-700 mg dl^{-1}) were prepared as described above and stored at 4°C until use.

2.3. Preparation of Dye Reagent

The dye reagent was prepared according to Bias *et al.* [28]. It contained 50 mg 4-aminoantipyrine, 100 mg phenol per 100 ml of 0.4 M sodium phosphate buffer, pH 7.0. The colour reagent can be stored in brown bottle at 4 °C .The colour reagent can not be used older than a week.

2.4. Co-immobilization of Cholesterol Esterase and Cholesterol Oxidase in Membrane

Cholesterol esterase (10 units, lyophilized powder) and cholesterol oxidase (15units, lyophilized powder) were dissolved in 1 ml of 50 mM phosphate buffer, pH 7.0. Polyvinyl formal (40 mg) was dissolved in 1 ml of chloroform/ethylene dichloride mixture (1:1 v/v) in a tube. The enzyme solution and polyvinyl formal solution were mixed quickly and spread evenly on to a clean glass plate at room temperature (25°C) for 1 h. A thin membrane was formed on glass surface. The cholesterol esterase and oxidase immobilized in polyvinyl formal membrane (11X5 cm^2) was then carefully scrapped from the glass plate and incubated with 0.5% glutaraldehyde a cross-linking agent for 30 min at room temperature. The membrane was washed several times with distilled water to remove unbound enzymes and excess glutaraldehyde. The immobilized enzyme membrane was stored at 4°C till further use. The dissolved oxygen analyzer contains a single electrode to which the immobilized enzyme PVF membrane is attached at the sensing tip of the electrode by o-ring. The PVF membrane with and without immobilized enzymes was studied by atomic force microscopy.

2.5. Assay of Co-immobilized Enzymes in Membrane

The assay of co-immobilized cholesterol esterase and cholesterol oxidase enzyme was carried out as described by Allain *et al* [1] with modifications. The reaction mixture contained 1.9 ml 50 mM sodium phosphate buffer, pH 7.0 containing 0.8 units peroxidase , 0.1 ml cholesteryl acetate solution (200 mg dl^{-1}) and 1cm^2 co-immobilized enzyme membrane. Colour reagent (1.0 ml) was added and incubated at 37 °C for 10 min under constant stirring. The absorbance of the developed colour was read at 520 nm and the content of H_2O_2 generated in the reaction was calculated from standard curve between A_{520} vs. H_2O_2.

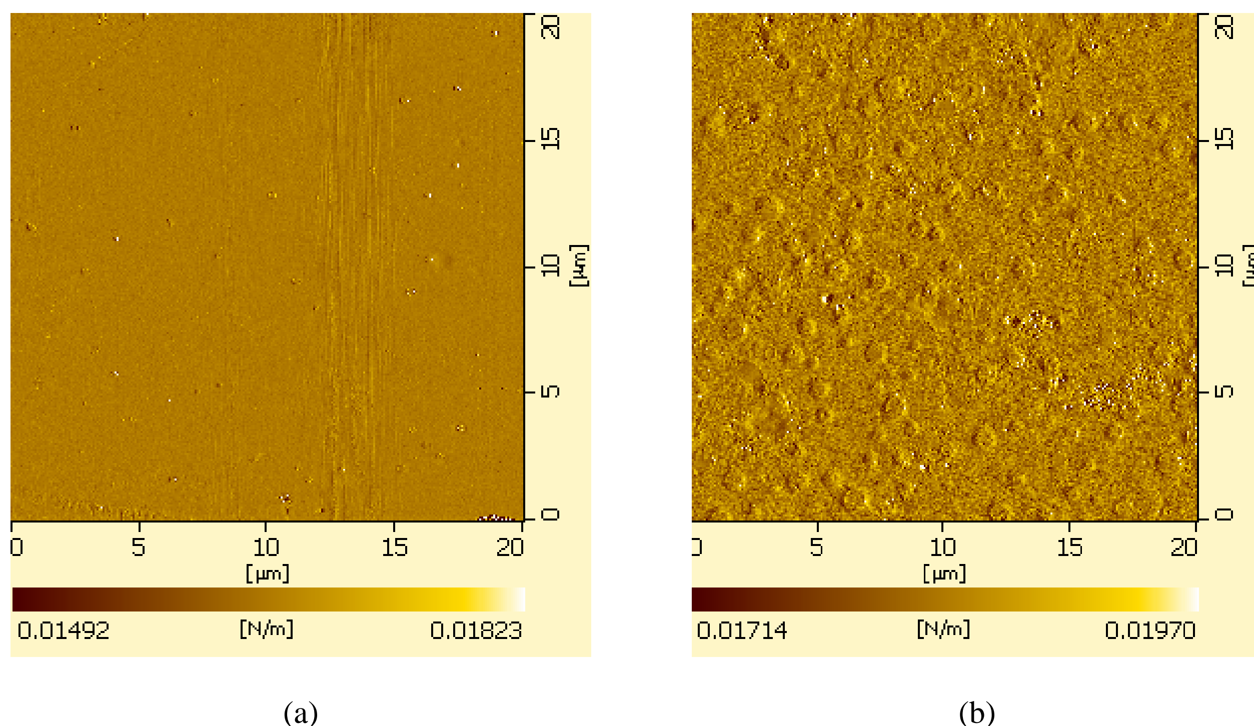
3. Results and Discussion

3.1. Activity of Cholesterol Esterase and Cholesterol Oxidase in PVF Membrane

The enzyme membrane was washed several times to observe leaching out cholesterol esterase and cholesterol oxidase. After several washings it was found that membrane retained 90-95 % activity of the enzymes.

3.2. Atomic Force Microscopy of the Membrane

Atomic force micrographs (AFM) of polyvinyl formal and enzymes immobilized in polyvinyl formal membranes are shown in Fig. 1 (a) and (b), respectively. The AFM of polyvinyl formal membrane without entrapped enzymes (Fig. 1a) indicates uniform polymeric layer, whereas, globular structures due to entrapment of enzymes are seen in Fig. 1b. This suggests that the enzyme molecules are uniformally distributed and packed in the PVF membrane.



(a) (b)

Fig.1. Atomic force microscopy pictures of (A) a polyvinyl formal membrane and (B) a cholesterol esterase and cholesterol oxidase immobilized polyvinyl formal membrane.

3.3. Response Measurements

The response (dissolved oxygen mg l^{-1} depletion) of the immobilized enzymes in a PVF membrane attached to an oxygen electrode (cholesterol sensor) at different concentrations (50-700 mg dl^{-1}) of cholesterol acetate in a 50 mM phosphate buffer, pH 7.0 was studied. It showed hyperbolic curve and linearity was from 100 to 400 mg dl^{-1} after which a limiting value of response was obtained. The minimum detection limit is 50 mg dl^{-1} which is comparable to those reported earlier using immobilized enzyme onto alkylamine glass beads for measurement of total cholesterol [13], nylon mesh immobilized enzymes [29] and enzyme electrode [15].The linearity of the biosensor is similar to the cholesterol oleate and other cholesterol esters [17, 19, 25] After measuring the response, the membrane was washed thoroughly with water and subsequently dried. The same electrode was used for testing the reusability and the response was compared with a fresh membrane. It was observed that the membrane may be used 20 times, with 5-10 % loss in response (data not shown). Beyond this, there was severe loss (40-50 %).

3.4. Effect of Temperature and pH

Thermal stability and effect of pH on immobilized enzymes in PVF membrane were investigated at 200 mg dl^{-1} cholesterol acetate in a 50 mM phosphate buffer of pH 7.0 by response (dissolved oxygen mg l^{-1} depletion) measurements (Fig.2). The enzyme membrane was kept at the desired temperature for 5 min. Fig. 3 shows the result of the immobilized enzymes (cholesterol esterase and cholesterol oxidase) response measurements as a function of temperature at 200 mg dl^{-1} cholesterol acetate solution in a 50 mM phosphate buffer of pH 7.0 It was observed that response increased up to 40°C and then it decreases drastically. Therefore, the immobilized enzyme membrane is used below 40°C to prevent inactivation of the immobilized cholesterol esterase and cholesterol oxidase enzymes. The optimum temperature of cholesterol biosensor was comparable to the other cholesterol biosensor based on conducting polyaniline films 38 ^{o}C [24], polypyrrole film 40 ^{o}C [20] and polyaniline films 48 ^{o}C [25].

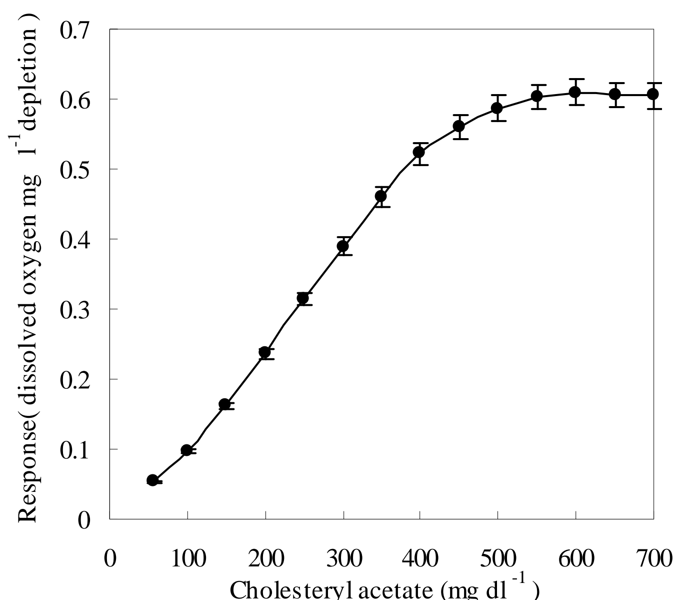


Fig.2. Response of the immobilized enzymes in PVF membrane at different concentrations of cholesteryl acetate in 50 mM phosphate buffer, pH 7.0.Five readings were taken at each measurement.
readings were taken at each measurement.

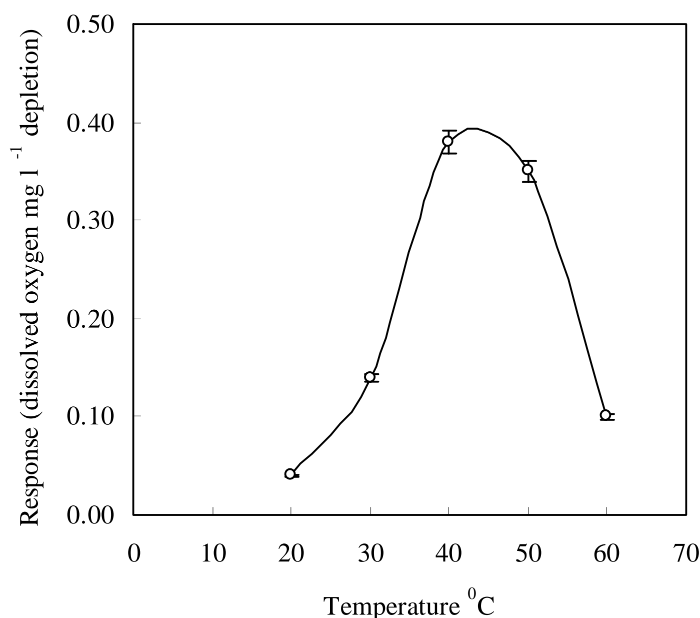


Fig.3. Response of the immobilized enzymes in PVF membrane at different temperatures in the presence of 200 mg dl^{-1} cholesterol acetate in a 50 mM phosphate buffer of pH 7.0. The enzyme membrane was kept at the desired temperature for 5 min. Five readings were taken at each measurement.

The activity of immobilized enzymes are highly influenced by the change in pH. Cholesterol acetate solutions (200mg dl^{-1}) were made in 50mM citrate buffer (pH 4.0 – 5.5) and in 50 mM phosphate buffers (pH 6.0 – 8.0) and response measurements were taken at room temperature (25^0C) using immobilized enzymes in PVF membrane (Fig.4). The response increased gradually from pH 4.0 to pH 7.0 and after that decreased sharply, indicating that the optimum pH 7.0 can be used for total cholesterol estimation. The optimum pH 7.0 is similar to the earlier reports of cholesterol biosensors [17, 19, 25] but more than pH 6.5 [24].

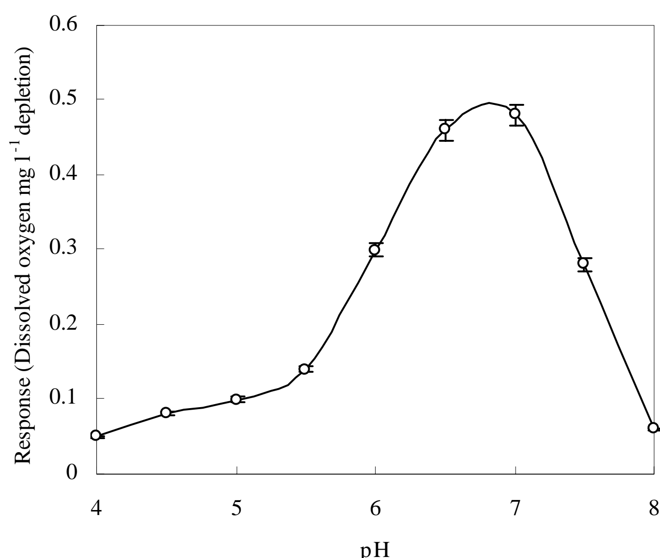


Fig.4. Response of the immobilized enzymes in PVF membrane at different pH of 50 mM Citrate buffer (pH 4.0 – 5.5) and 50 mM phosphate buffers (pH 6.0 – 8.0) containing 200 mg dl^{-1} cholesterol acetate. The response was measured at room temperature (25°C). Five readings were taken at each measurement.

3.5 Shelf Life

Enzymes immobilized in PVF membrane was tested for stability under the same operating conditions as for response measurements (dissolved oxygen mg l^{-1} depletion). The response of the enzyme membrane attached with an oxygen electrode was measured once in 10 days. The enzyme membrane was stored at 4 ºC when not in use. Fig. 5 shows the response of the enzyme membrane on different days of storage. It showed that the response of the membrane was almost same for 20 days after that decreases gradually. This may be due to partial decay in the activity of the immobilized enzymes cholesterol esterase and cholesterol oxidase. The shelf life of the immobilized enzymes in PVF was found to be more than three months. The shelf life of the biosensor is more than amperometric biosensors of conducting polymers [19, 24, 25].

3.6. Role of Interferents on Response

Different interferents which are mostly present in blood such as ascorbic acid (15µmole l^{-1}), glucose (80mg dl^{-1}), uric acid (30mg dl^{-1}), EDTA (1mmol dl^{-1}), acetone (20mg dl^{-1}) and bilirubin (2.2mg dl^{-1}) were tested with immobilized enzymes in PVF membrane fabricated cholesterol biosensor. These substances were added at their physiological normal level in the presence of 200mg dl^{-1} cholesterol acetate solution. There was no significant effect of these interferents on the performance of the enzymes PVF membrane based cholesterol biosensor.

3.7. Estimation of Total Cholesterol in Serum Samples

The total cholesterol in serum sample of healthy person was estimated by different available methods such as Colorimetric [3], Cholesterol test kits (Enzo-kit) [30] and Biochemistry analyzer [31] and its comparison was made by PVF based biosensor (Fig.6). The result suggests the PVF based biosensor works satisfactory and the value of total cholesterol in serum sample is comparable by other methods. The total cholesterol value was also found in the range of other methods.

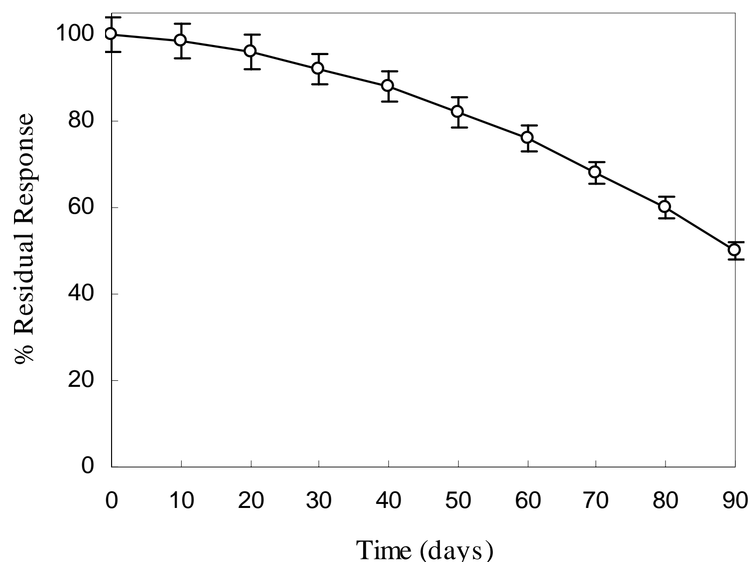


Fig.5. Stability of the immobilized enzymes in PVF membrane on storage at 4ºC. Enzymes stability was monitored at 10 days interval using 200 mg dl^{-1} cholesterol acetate in 50 mM phosphate buffer, pH 7.0. Five

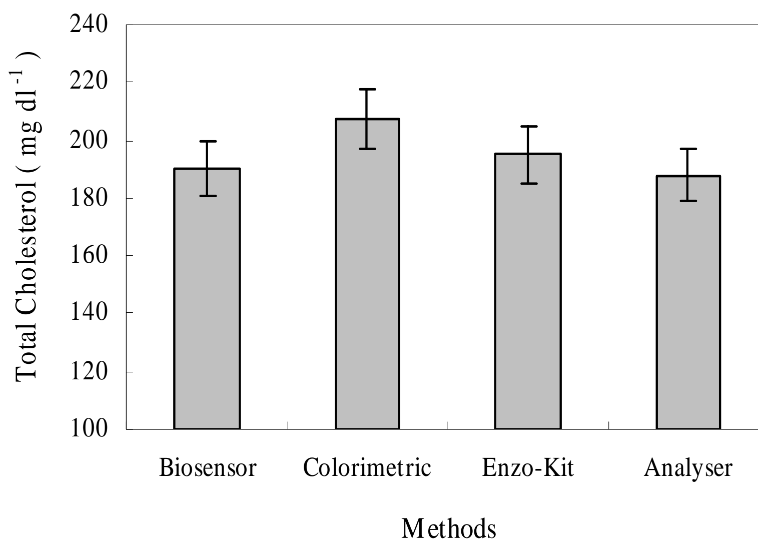


Fig.6. Estimation of total cholesterol by different methods. The serum of normal person was taken and total cholesterol was estimated by different methods and compared with PVF based biosensor. Five readings were taken at each measurement.

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