

# Amperometric Determination of Serum Cholesterol with Pencil Graphite Rod

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## Abstract

A cholesterol oxidase from *Streptomyces sp.* was immobilized onto pencil graphite rod and employed for amperometric determination of serum cholesterol. The method has the advantage over earlier amperometric methods that it requires low potential to generate electrons from  $H_2O_2$ , which does not allow ionization of serum substances. The optimum working conditions of amperometric determination were pH 6.8,  $25^\circ C$  and 30 s. The current measured was in proportion to cholesterol concentration ranging from  $1.29 \times 10^{-3}$  to  $10.33 \times 10^{-3}$  M. Minimum detection limit of the method was  $0.09 \times 10^{-3}$  M. Mean analytical recovery of added cholesterol (100 mg/dl and 200 mg/dl) in serum was 85.0% & 90.0% respectively. Within batch and between batch coefficients of variations were 1.59% & 4.15% respectively. A good correlation ( $r = 0.99$ ) was obtained between serum cholesterol values by standard enzymic colorimetric method and the present method. No interference by metabolites was observed in the method. The enzyme electrode was reused 200 times over a period of 25 days, when stored at  $4^\circ C$ .

**Keywords:** Cholesterol, Cholesterol Oxidase, Pencil Graphite, Enzyme Electrode, Serum, Amperometric Biosensor

## 1. Introduction

Cholesterol an important steroid in human body plays a vital role as a precursor to various hormones. Cholesterol determination in blood is known to be clinically important for diagnosis of various diseases like cardiac disorders, atherosclerosis, nephritis, diabetes mellitus, myxodema, obstructive jaundice and cerebral thrombosis [1]. Among various methods available for cholesterol determination, biosensors are comparatively simpler, rapid, sensitive and specific [2-6]. Various amperometric cholesterol biosensors have been reported, employing cholesterol esterase, cholesterol oxidase and peroxidase immobilized onto nylon mesh over a platinum electrode [7], octyl-agarose gel activated with cyanogen bromide and placed in a reactor [8], pyrrole membrane through electropolymerization and coupled with FIA for  $H_2O_2$  analysis [9], carbon paste electrode modified with hydroxymethyl ferrocene and hydrogen peroxide [10], poly (2-hydroxyethyl methacrylate) (p(HEMA))/polypyrrole membrane [11], graphite-teflon composite matrix with incorporated potassium ferrocyanide [12], layer of silica sol-gel matrix on the top of Prussian blue-modified glassy carbon electrode [13], photosensitive polymer on

ultra-thin dialysis membrane [14], conducting polypyrrole (PPY) films using electrochemical entrapment technique [15], porous silicon [16], poly(vinylferrocenium) film [17]. Due to high electrochemical reactivity, good mechanical rigidity, low cost and ease of modification, renewal and miniaturization, pencil graphite electrode (PGE) received attention of workers for its use in working electrode of biosensor [18]. Furthermore, PGE has a large active electrode surface area and therefore able to detect low concentrations and/or volume of the analyte [19]. The aim of this work was to develop a simple cholesterol biosensor based on PGE bound cholesterol oxidase. The electrode is better in the economic sense and a small amount of material used; hence it seems to be a better electrode than "high tech" electrodes described previously [20].

## 2. Experimental

4-Amino-phenazone/4-aminoantipyrene, horseradish peroxidase from Sigma Aldrich, USA, Triton X-100, Cholesterol, Cholesterol oxidase from *Streptomyces sp.* (500 units/10mg) from SRL, Mumbai. 'HB' lead pencil was from local market. The kit of enzymic colorimetric

method for cholesterol determination was from Erba Transasia, Daman, India. All other chemicals were of analytical reagent grade.

### 2.1. Assay of Free Cholesterol Oxidase

Assay of free cholesterol oxidase was carried out in a 15 ml test tube wrapped with black paper according to Alain *et al.* (1974) with modification. The reaction mixture, consisting of 1.8 ml sodium phosphate buffer (0.05 M, pH 7.0) containing 0.4% Triton X-100, 0.1 ml of cholesterol solution (10mM) and 0.1 ml of cholesterol oxidase solution (13 Units) incubated for 5 min at 37°C. Color reagent (1.0 ml) was added and kept at 37°C for 10 min to develop the colour.  $A_{520}$  was read and the content of  $H_2O_2$  was extrapolated from standard curve between  $H_2O_2$  concentration and  $A_{520}$ . Color reagent consisted 50 mg 4-aminophenazone, 100 mg phenol and 1 mg horseradish peroxidase per 100 ml 0.4 M sodium phosphate buffer (pH 7.0). It was stored in amber colored bottle at 4°C & prepared fresh after one week. One enzyme unit is defined as the amount of enzyme required to generate 1.0 nmol of  $H_2O_2$  per min per ml.

### 2.2. Immobilization of Enzyme onto Pencil Graphite Rod

The wooden cover of a lead pencil was removed from its both the ends with a sharp blade upto 2 cm height. The one end of pencil graphite rod (0.15 diameter and 2 cm long) was dipped into 60% HCl at room temperature for 24 h and then washed thoroughly with 0.05 M sodium phosphate buffer (pH 7.4). It was dipped again into 70%  $HNO_3$ . After keeping it for 24h at room temperature, the pencil rod was washed thoroughly with the same buffer and then put into 0.2% enzyme solution. After keeping it at 4°C for 8h, the rod was taken off and washed thoroughly with the reaction buffer & tested for cholesterol oxidase activity. The residual enzyme solution was tested for activity and protein by Lowry method. The pencil graphite rod containing immobilized enzyme acted as working electrode (PGE).

### 2.3. Construction and Response Measurement of Amperometric Cholesterol Biosensor

An amperometric cholesterol biosensor was constructed by connecting pencil graphite electrode (PGE) as working electrode, silver/silver chloride (Ag/AgCl) as reference electrode and Cu wire as auxiliary electrode through electrometer/high resistance meter (Keithley 6517A, Japan). To test the activity of this biosensor, the electrode system was immersed into 1.8 ml 0.02 M sodium phosphate solution pH 7.0 and 0.2 ml of cholesterol (12.9 mM) and polarized at a potential in the range 0-0.4

V versus Ag/AgCl. The current was maximum at 0.1 V. Hence in the subsequent amperometric studies; the sensor was polarized at 0.1 V to generate current. The electrochemical reactions involved in response measurement are given in **Figure 1**.

### 2.4. Optimization of Cholesterol Biosensor

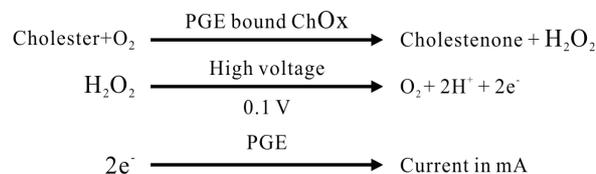
The optimal working conditions of cholesterol biosensor were studied in terms of the current (mA) generated. To study optimum pH, the pH of reaction buffer was varied in the range pH 6.2 to pH 7.8 using the 0.02 M sodium phosphate buffer. Similarly for optimum temperature, the reaction mixture was incubated at temperature ranging from 20 to 50°C at an interval of 5°C. Time course was studied by incubating reaction mixture for different time ranging from 5 to 40 s at an interval of 5 s. To study effect of substrate concentration, the concentrations of cholesterol was varied from 1.29 to 12.9 mM.  $K_m$  (Michaelis Menten constant) and  $I_{max}$  (maximum current) were calculated from L.B. plot.

### 2.5. Electrochemical Determination of Cholesterol in Serum

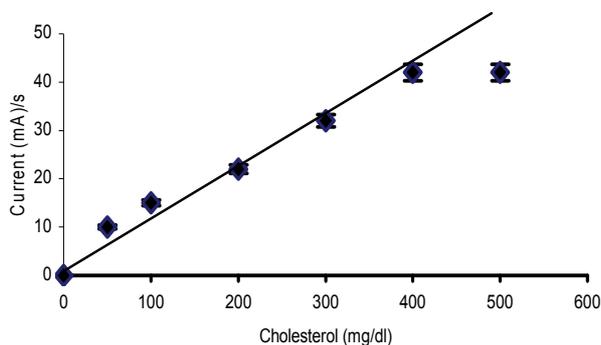
Blood samples (1.0 ml each) from apparently healthy male and female (10 each) and diseased persons (suffering from coronary heart diseases, hypertension and atherosclerosis) were collected from local Pt BD Sharma Post Graduate Institute of Medical Sciences, Rohtak, and centrifuged at 5000 rpm for 5 min and their supernatant (serum) was collected. Cholesterol content in serum was determined by the present biosensor in the similar manner as described for its response measurement, under its optimal working conditions except that cholesterol was replaced by serum. The current (mA) was measured and the amount of cholesterol in serum extrapolated from standard curve between cholesterol concentrations and current (in mA) prepared under optimal working conditions (**Figure 2**).

### 2.6. Evaluation of Cholesterol Biosensor

The biosensor was evaluated by studying analytical re-



**Figure 1.** Chemical reactions involved in generation of electron in amperometric cholesterol biosensor based on pencil graphite electrode (PGE) bound cholesterol oxidase (ChOx).



**Figure 2. Standard curve of cholesterol by biosensor based on pencil graphite rod bound cholesterol oxidase.**

covery, precision and correlation. The effect of various metals and metabolites found in blood, such as uric acid, cholesterol, ascorbic acid, bilirubin, glucose, pyruate and glutathione were studied at their physiological concentration.

### 3. Results and Discussions

#### 3.1. Immobilization of Cholesterol Oxidase onto PG Rod and Construction of Amperometric Cholesterol Biosensor

Commercial cholesterol oxidase from *Streptomyces* species was immobilized on PGE through chemisorption (Figure 3). The HCl treatment of PGE forms a monolayer which helps in electrostatic interaction of negatively charged cholesterol oxidase at pH 7.0. Nevertheless treatment of graphite with  $\text{HNO}_3$  makes it highly porous to provide the large surface area for adsorption or chemical reaction [21]. The chemisorption is better than physisorption for immobilization of enzyme which is characterized by weak Van der Waals forces. A method is described for construction of amperometric cholesterol biosensor based on this PGE bound with cholesterol oxidase. The biosensor showed optimum response at low voltage *i.e.* 0.1 V had advantage that it does not allow the ionization of number of serum substances which get ionized at high voltage and interfere in current measurement [9].

#### 3.2. Optimization of Cholesterol Biosensor

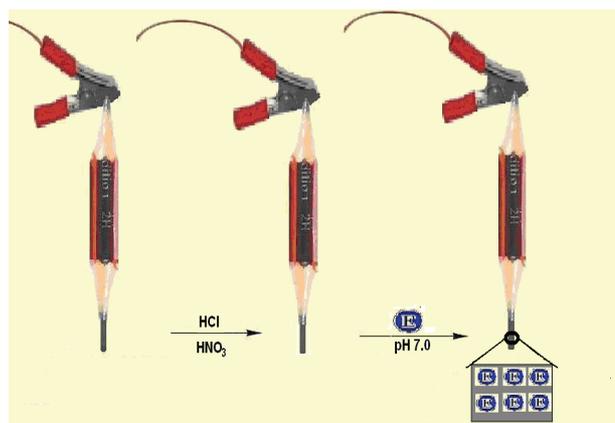
The optimum response for pencil graphite electrode was at pH 6.8 (Figure 4), which is comparable to earlier reports pH 7.0 [8,9,15,24] and pH 7.5 [22]. The PGE showed optimum response at 40°C (Figure 5), which is higher than that of free enzyme in presence of free cholesterol esterase and peroxidase (30°C). The increase in optimum temperature might be due to change in conformation of enzyme after immobilization or due to steric hindrance. PGE response was increased from 5 to 30 s

after which it became stable (Figure 6). Therefore in all subsequent assays, the electrometer reading was recorded at 30 s. A hyperbolic relationship was observed between electrode response (current in mA/s) and cholesterol concentration up to a final concentration of  $12.9 \times 10^{-3}$  M, which is similar to earlier cholesterol biosensor, but higher than  $1 \times 10^{-3}$  M to  $8 \times 10^{-3}$  M for cholesterol ester [15], and  $8 \times 10^{-3}$  M for cholesterol [22]. Lineweaver-Burk plot between the reciprocals of cholesterol concentration and response of PGE working electrode was linear.  $K_m$  (Michaelis constant) for cholesterol was  $7.38 \times 10^{-3}$  M (Figure 7) which is lower than that for earlier cholesterol biosensor ( $21.2 \times 10^{-3}$  M) [23] and  $19.6 \times 10^{-3}$  M [9]. This might be due to the hydrophobic forces of pencil graphite, which facilitate the cholesterol binding with the graphite bound enzyme.  $I_{max}$  was 62.5 mA/s.

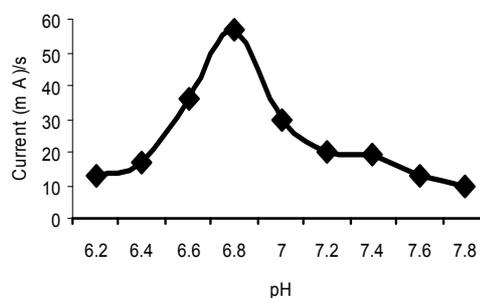
### 3.3. Evaluation of Cholesterol Biosensor

#### 3.3.1. Linearity

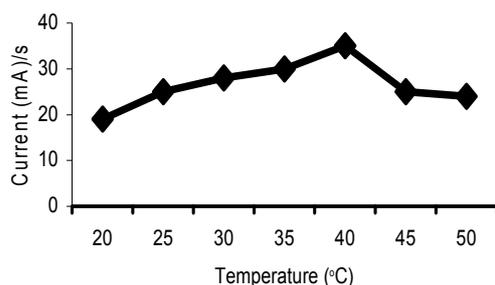
A linear relationship was obtained between cholesterol concentrations ranging from  $1.29 \times 10^{-3}$  M to  $10.3 \times 10^{-3}$  M and current (mA) measured (Figure 2).



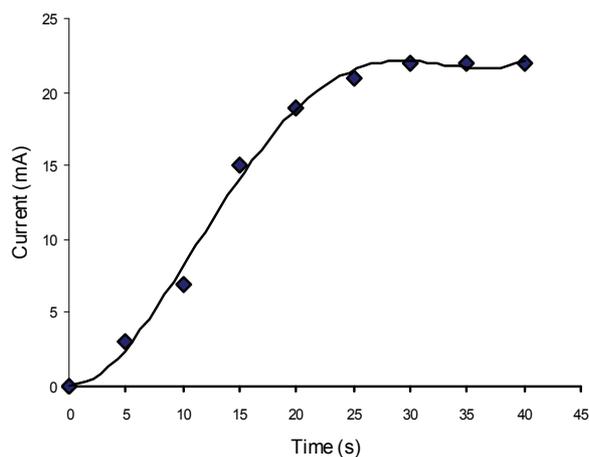
**Figure 3. Chemisorption immobilization of cholesterol oxidase onto pencil graphite rod.**



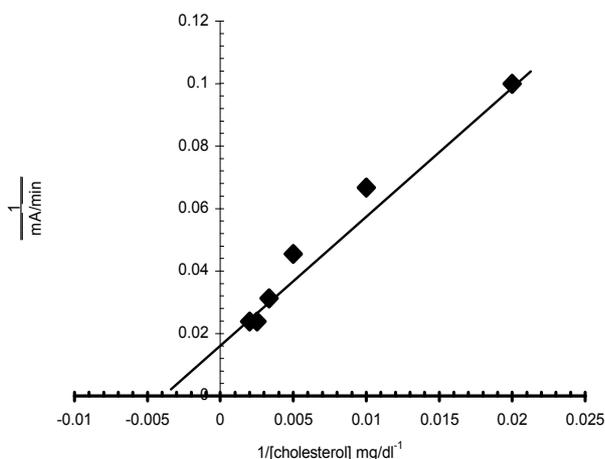
**Figure 4. Effect of pH on the response of cholesterol biosensor based on pencil graphite rod bound cholesterol oxidase. Standard assay conditions were used except for the pH that was varied from pH 6.2-7.8.**



**Figure 5.** Effect of incubation temperature on the response of cholesterol biosensor based on pencil graphite rod bound cholesterol oxidase. Standard assay conditions were used except for the incubation temperature which was varied from 15–45°C.



**Figure 6.** Effect of time of incubation on the response of cholesterol biosensor based on pencil graphite bound cholesterol oxidase.



**Figure 7.** A Lineweaver-Burk plot for cholesterol biosensor based on cholesterol oxidase immobilized onto pencil graphite rod.

### 3.3.2. Minimum Detection Limit

The minimum detection limit of the present amperometric biosensor was  $0.09 \times 10^{-3}$  M, which is almost 3 times lower than paleographic method employing soluble enzymes ( $0.32 \times 10^{-3}$  M) [24], but higher than those methods employing silica gel bound enzyme ( $0.003 \times 10^{-3}$  M) [25] and amperometric cholesterol biosensor ( $0.064 \times 10^{-3}$  M) [9].

### 3.3.3. Analytical Recovery

In order to check the accuracy of the method, the analytical recovery of added cholesterol in the serum samples was determined. The mean analytical recovery of added cholesterol (100 mg/dl and 200 mg/dl) in serum was 85% (100 mg/dl) and 90% (200 mg/dl) (Table 1), which is comparable with colorimetric method employing alkyl amine glass bound enzyme (95–102%) [26], amperometric method (95–101% recovery) [7], enzymic fluorometric method (103–104%) for added cholesterol concentration of 150 mg/dl and 50 mg/dl [26].

### 3.3.4. Precision

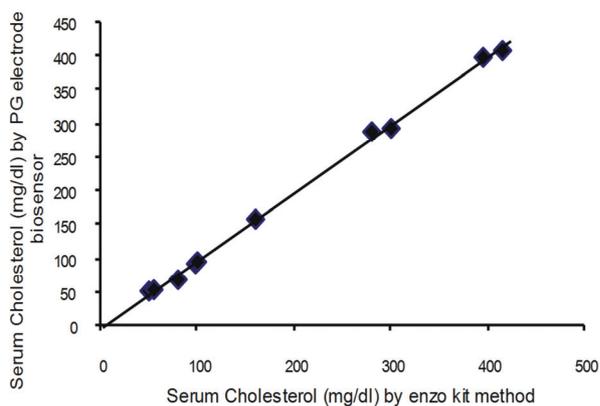
To check the reproducibility and reliability of the methods, the cholesterol content of the sample in one run (Within batch) and after storage at  $-20^\circ\text{C}$  for one week (Between batch) were determined. The results showed that the cholesterol value of these determination agreed with each other and within batch and between batch coefficient of variation (CV) were 1.59% & 4.15% (Table 2), which is quite close to earlier reports such as colorimetric, electrochemical method [26] employing alkyl amine glass bound enzyme (1.6% for intrabatch and 3.2% for interbatch), amperometric method using silica gel bound enzyme ( $< 1.5\%$  for all samples) [7] and flow injection method employing controlled pore glass bound cholesterol esterase and cholesterol oxidase (within day  $< 1.0$  and between day  $< 2.5\%$ ) [27], measuring cholesterol after precipitation with phosphotungstic acid/ $\text{MgCl}_2$  (within day 5.0% and between day 8.2%) [28] and amperometric detection of cholesterol (within day 2%–between day 4%) [9]. The low coefficient of variation values indicated the accuracy, reproducibility and reliability of the method.

### 3.3.5. Accuracy

In order to know the accuracy of present method, the level of cholesterol in 10 serum samples was determined by standard enzymic colorimetric method with modification and compared with those obtained by present method. The serum cholesterol values obtained by standard enzymes colorimetric method (x) agreed with the present biosensor (y) with a good correlation ( $r = 0.99$ ) (Figure 8).

### 3.3.6. Effect of metal ions and metal salts

The effect of some metal salts such as KCl,  $\text{MgCl}_2$ , NaCl,  $\text{CaSO}_4$ ,  $\text{CuSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,



**Figure 8.** Correlation between serum cholesterol value as determined by Enzo kit method employing free enzymes (x axis) and present biosensor method (y-axis) based on pencil graphite rod bound cholesterol oxidase.

**Table 1.** Analytical recovery of added cholesterol in serum by biosensor based on pencil graphite electrode.

Cholesterol added (mg/dl)	Cholesterol found (mg/dl)	% Recovery
Nil	150	-
100	235	85
200	330	90

Serum cholesterol was measured by cholesterol biosensor as described in text. It was measured again after adding cholesterol into serum at 100 mg/dl & 200 mg/dl. % Recovery was calculated. Values are mean of six serum samples.

**Table 2.** Precision measurement of serum cholesterol by a cholesterol biosensor based on pencil graphite electrode (PGE).

Total number of samples (n = 6)	Mean Cholesterol (mg/dl)	% CV
6 (within assay)	54.25	1.59
6 (between assay)Ψ	54.2	4.15

Cholesterol was measured in six serum samples six times on the same day (Within assay) and after one week storage at  $-20^{\circ}\text{C}$  (Between assays) by cholesterol biosensor based on pencil graphite rod bound cholesterol oxidase. % Coefficient of variation (CV) was calculated.

each at a final concentration of 1.0 mM was tested on the response of the working electrode. Only  $\text{Mg}^{2+}$  caused slight stimulation, while rest metals had practically no effect.

### 3.3.7. Effect of Serum Metabolites

To study interference by serum metabolites glucose, uric acid, ascorbic acid, acetone and bilirubin were added into the reaction mixture at their normal physiological concentration before addition of cholesterol. The results showed that there was practically no interference in presence of these metabolites. Earlier uric acid and ascorbic acid, at 0.6 V caused significant increase in the value of current [29], which were attributed to the fact

that at high potentials for both uric acid & ascorbic acid got oxidized contributing to oxidation current. Some interference of endogenous electro reactive species like uric acid, glucose had been reported when their concentration was higher than their normal physiological concentrations [15].

### 3.4. Storage Stability and Reusability

The PG electrode lost 50% of its initial activity after its regular use for 200 times over a period of 25 days, when stored in 0.05 M sodium phosphate buffer, pH 7.0 at  $4^{\circ}\text{C}$ .

## 4. Conclusions

A method is described for immobilization of cholesterol oxidase onto pencil graphite (PG) rod and its use in construction of a simple amperometric cholesterol biosensor. The biosensor had an advantage that it worked at low potential and thus had no interference by serum substances. The sensor was evaluated.

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