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RESEARCH ARTICLE

An amperometric approach towards construction of a cholesterol biosensor

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Abstract

The cholesterol oxidase enzyme breaks down cholesterol into an intermediate cholest-5-en-3-one, which is then converted to cholest-4-en-3-one (Smith and Brooks 1997) . In the present study this mechanism was exploited. The current produced by the release of electrons from the above reaction was used to determine the amount of free cholesterol in the serum sample. The functioning of this amperometric biosensor was checked over a temperature ranging from 20^{O}C to 60^{O}C and a pH range of 5 to 9

Introduction:

Cholesterol level is associated with various pathological conditions like coronary heart disease (CHD), atherosclerosis, cardiovascular diseases, and also in the management of hypercholesterolemia. Amidst the various methods available for estimation of cholesterol; ranging from the simple colorimetric, spectrophotometric; to the more complex and costly chromatographic techniques such as high performance liquid chromatography, gas chromatography/mass spectrometry (GC//MS), the present study focuses on a cost effective approach to develop a cholesterol biosensor. The practice of immobilizing enzymes onto a solid matrix such as multiwalled carbon nanotubes (MWCNts) (Yang., et al2011),octyl-agarose gel (Karube.,et al 1982)and Pt incorporated fullerenes like ZnO nanosphere (Ahmad., et al) had various disadvantages like leaching of immobilized enzymes,low stability to microbial attack, high cost of the support material or the immobilization procedure, vastly reduced enzyme activity, long response time, and low storage stability.

Cholesterol oxidase, a bi-functional FAD-containing microbial enzyme which belongs to the family of oxidoreductases.Cholesterol oxidaseis one of the most widely used enzymes in clinical laboratories as it participates in bile acid biosynthesis. Cholesterol oxidase enzyme is simple, specific, and highly sensitive; its use has become widespread in the determination of serum cholesterol that has direct implications to various severe pathological conditions such as in determining the risk of heart attack and thrombosis.Cholesterol oxidase is an intracellular/extracellular, water soluble enzyme produced by various microorganisms, some of them are Nocardiaerythropolis, Brevibacterium sterolicum, Streptomyces spp.,Pseudomonas spp.(Kumari.,et al 2012). Hence to determine the activity range of cholesterol oxidase in the proposed model, the present study was carried out.

Materials and Methods:

The present study was conducted in DSR Genome Technologies Pvt .Ltd.

Sources of Chemicals:

Cholesterol extra pure was obtained from Nice Chemicals and Triton X 100 from Himedia. Cholesterol oxidase extrapure from Streptomyces sp. was obtained from SRL Mumbai.

The multimeter used in detection of current (μA) Metravi XB-30 was procured from the local market. All other chemicals used were of analytical grade.

Preparation of enzyme solution:
Commercial cholesterol oxidase from Streptomyces sp. (2 mg, containing 25 U) was dissolved in 1.0 ml 0.02 M sodium phosphate buffer, pH 7.0, and stored at 4ºC until use.

Assay of free cholesterol oxidase:
Reaction mixture consisting of 1.0 ml sodium phosphate buffer (0.05 M, pH 7.0) and 0.5 ml cholesterol oxidase was pre- incubated at 37^{O}C for 2 min. The reaction was initiated by adding 0.5 ml cholesterol solution (500 mg/dl). After incubating at 37^{O}C for 10 min, 1.0 ml colour reagent was added and kept at room temperature for 15 min to develop the colour and absorbance was read at 520 nm (A520) against control in a spectrophotometer and the content of H_2O_2 generated in reaction was calculated from standard curve between H_2O_2 concentration and A520 (Pundir., et al 2012). The soluble protein in dissolved enzyme was determined by Lowry method (Lowry., et al 1951).

Preparation of substrate solution:
Cholesterol(50 mg) was dissolved in 1.0 ml of triton X 100 by slowly heating and stirring until solution was clear. Sodium phosphate buffer (0.05 M, pH- 7.0) was added to it to get final concentration of 500 mg/dl. Solutions of different concentrations ranging from 50 to 500 mg/ dl were prepared and stored at 4^{O}C.

Response measurement of the biosensor:
The initial work was carried out using a multimeter. The multimeter was used because of its versatility in measuring resistance, voltage and ampere. The multimeter probes were immersed into 5ml sodium phosphate buffer (0.02 M, pH 7.0) in a beaker. 1ml of dissolved cholesterol solution was added to the beaker and resistance of the resultant solution was checked at 2000k in the multimeter. The reaction was started by adding 0.5ml of enzyme solution.
Based on the current produced during the chemical reaction the number of molecules of cholesterol and consequently the amount of cholesterol present in the sample could be determined. The current was measured at varying concentrations of cholesterol to obtain a full working range.

Optimizing the working conditions:
The pH of the sodium phosphate buffer (0.02M) was varied between 5 to 9 and the response was measured. To determine optimum temperature, the temperature was increased from 20^{O}C to 60^{O}C at an interval of 5 minutes. To determine the response time, the current was measured up to 80s at an interval of 5s. To study effect of cholesterol concentration, the concentration was varied from 50 to 500 mg/dl.Michaelis-Menten constant (Km) and maximum current (Imax) were calculated from Lineweaver-burk (Lb) plot.

Cholesterol estimation from biological samples:
Blood samples were procured from Centre for Genetic Studies (West Bengal University of Technology) and 1 ml of serum was collected by centrifuging it at 1500xg for 10 minutes at 4^{O}C.This study protocol was approved by the institute's ethics committee.The serum was added to 1.0 ml of 0.02 M sodium phosphate buffer at pH 7.0The free cholesterol was estimated by performing the enzyme–substrate reaction and the current (μA) was measured and concentration of cholesterol was extrapolated from the standard curve between cholesterol solution (mg/dl) Vs electrical response in μA. No sample calculation was done.

Results and Discussions:

Amperometric detection:
Cholesterol solutions ranging from 50 to 500 mg/dl were made and amperometric measurements were made after 0.5ml of cholesterol oxidase was added. The probes were immersed into the solutions and the displayed ampere was noted against the concentration of the solution. The same procedure was repeated with biological sample (serum).

Table 1:Table showing cholesterol concentration and corresponding ampere recorded at 60s [V]

mg/dl [S]	**μA at (60s) [V]**	**1/S**	**1/V**	**$\frac{Vmax*[S]}{Km+[S]}$**

0	000.00	0.0000	0.00000000	000.00
50	93502.50	0.0200	0.00001069	9090.90
60	112203.00	0.0167	0.00000891	0169.49
70	150613.50	0.0143	0.00000664	11111.11
80	128247.00	0.0125	0.00000780	11940.3
90	135754.50	0.0111	0.00000737	12676.06
100	157433.26	0.0100	0.00000635	13333.33
110	171824.95	0.0091	0.00000582	13924.05
120	194324.95	0.0083	0.00000515	14457.83
130	196552.50	0.0077	0.00000509	14942.53
140	213418.99	0.0071	0.00000469	15384.62
150	216000.65	0.0067	0.00000463	15789.47
160	232565.25	0.0063	0.00000430	16161.62
170	264525.00	0.0059	0.00000378	16504.85
180	281407.50	0.0056	0.00000355	16822.43
190	299047.50	0.0053	0.00000334	17117.12
200	336931.50	0.0050	0.00000297	17391.30
210	358999.80	0.0048	0.00000279	17647.06
220	374152.50	0.0045	0.00000267	17886.18
230	388335.00	0.0043	0.00000258	18110.24
240	404400.00	0.0042	0.00000247	18320.61
250	427102.50	0.0040	0.00000234	18518.52
260	438097.50	0.0038	0.00000228	18705.04
270	440403.05	0.0037	0.00000227	18881.12
280	474765.65	0.0036	0.00000211	19047.62
290	481125.00	0.0034	0.00000208	19205.30
300	498000.00	0.0033	0.00000201	19354.84
310	507352.50	0.0032	0.00000197	19496.86
320	508851.75	0.0031	0.00000197	19631.90
330	503550.00	0.0030	0.00000199	19760.48
340	505402.50	0.0029	0.00000198	19883.04
350	507255.00	0.0029	0.00000197	20000.00
360	509100.00	0.0028	0.00000196	20111.73
370	510952.50	0.0027	0.00000196	20218.58
380	512797.50	0.0026	0.00000195	20320.86
390	514650.00	0.0026	0.00000194	20418.85
400	516502.50	0.0025	0.00000194	20512.82
410	518347.50	0.0024	0.00000193	20603.02
420	519450.34	0.0024	0.00000193	20689.66
430	519053.16	0.0023	0.00000193	20772.95
440	531397.50	0.0023	0.00000188	20853.08
450	521857.40	0.0022	0.00000192	20930.23
460	505103.14	0.0022	0.00000198	21004.57

470	514500.00	0.0021	0.00000194	21076.23
480	523800.00	0.0021	0.00000191	21145.37
490	524977.50	0.0020	0.00000190	21212.12
500	542497.50	0.0020	0.00000184	21276.60

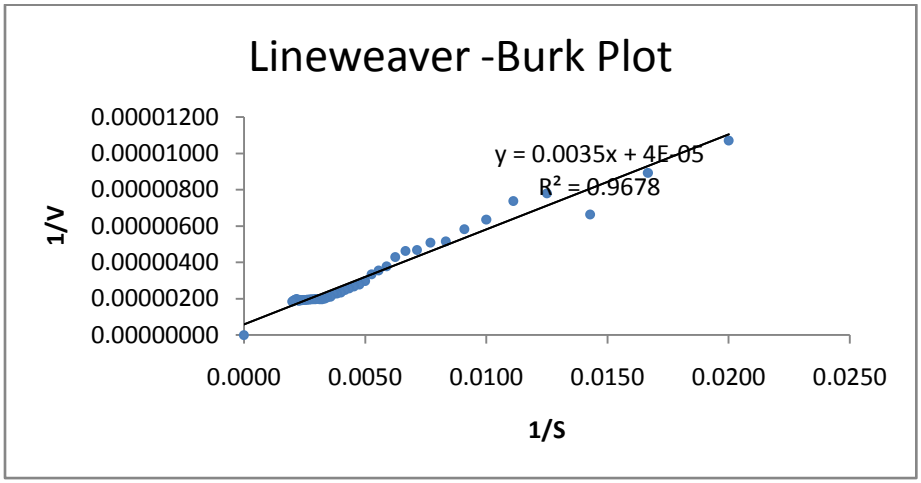


Figure 1: Graph showing 1/[V] vs 1/[S].

Calculation of Vmax:

From the lineweaverburk plot the Y intercept (c) was found to be $4*10^{-5}$. Imax was thus calculated as 0.02A and Km was found to be 87.5mM.These values were used for plotting the MichaelisMenten graph for the enzyme catalyzed reaction.

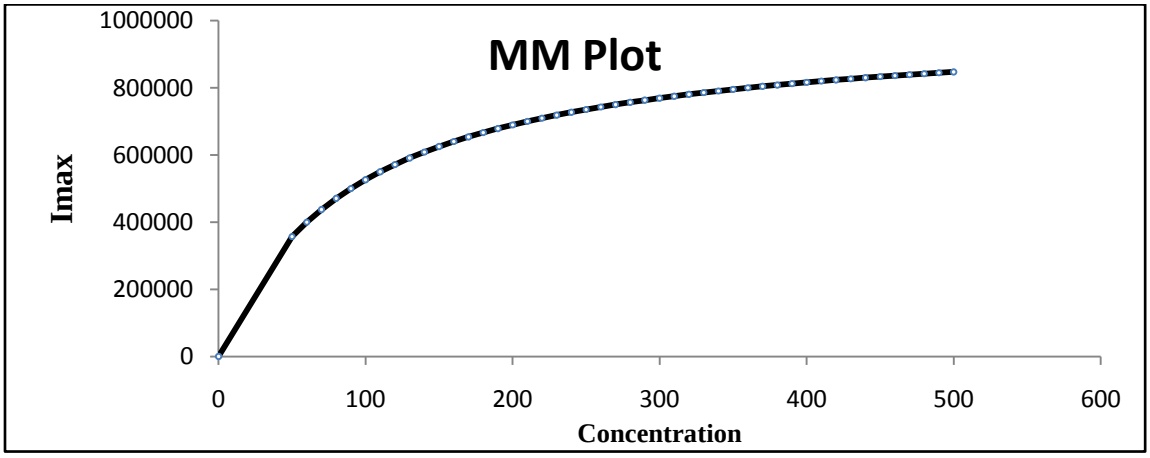


Figure 2:MichaelisMenten plot showing concentration Vs Imax of the enzyme catalyzed reaction.

Accuracy:

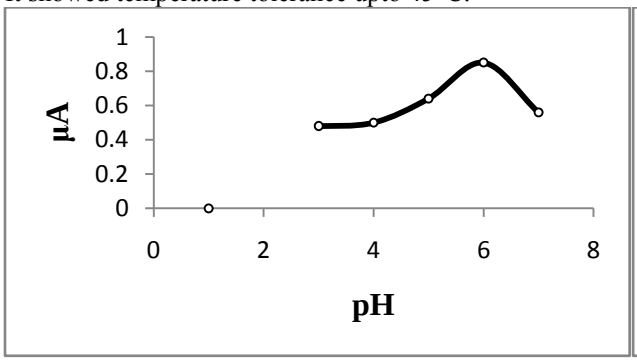
In order to determine the accuracy of the biosensor, the values of cholesterol in serum were determined by commercial enzymatic colorimetric method and compared with those obtained by present methods.The values obtained by these two methods matched with each other and showed a good correlation (r= 0.96) with the following regression equation: Y(0.0035x + 4E-05). The values obtained by both the methods were also in agreement with the

earlier study with pencil graphite rod (r=0.99), cellulose acetate membrane (0.990) and chitosan- modified glassy carbon electrode (r=0.99).

Optimization of biosensor:

The biosensor showed optimum response within 60 seconds at pH 6.0.

It showed temperature tolerance upto 45^{O}C.



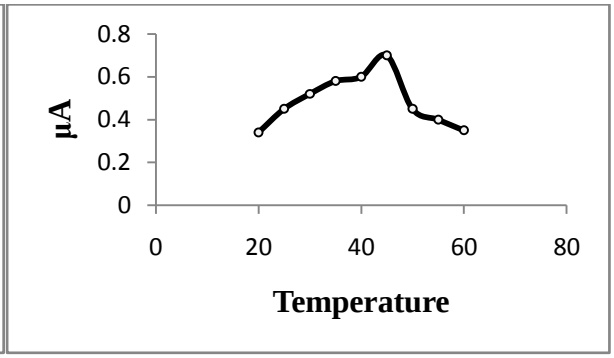


Figure 3:Graph showing optimum pH. Figure4:Graph showing optimum temperature.

Linearity:

There was a linear relationship between current (µA) and cholesterol concentration ranging from 2.78mM(50mg/dl) to 12.22mM(220mg/dl) in the reaction mixture, which is comparable to earlier amperometric biosensors, using cholesterol oxidase, co-immobilized with peroxidase on polyaniline film (1-12.9 mM), and was found higher than but higher than those immobilized on carbon paste electrode modified with hydroxymethyl ferrocene and hydrogen peroxide (0.15 mM)[11], pencil graphite rod (1.29to 10.3 mM), epoxy resin medium (1 to 8 mM), electro polymerized pyrrole (8 µM).

Conclusion:

An approach that describes a simple redox mechanism utilized to determine serum cholesterol from blood samples with no loss of enzyme efficiency. The biosensor showed optimum activity within 60 seconds at a pH of 6 and exhibited a temperature tolerance up to 45^{O}C.It showed linearity with cholesterol concentration between 2.78mM to12.22mM and good accuracy.

References:

1. Ahmad M, Caofeng P, Lin G, Zeeshan N, Jing Z.(2010). Highly sensitive amperometric cholesterol biosensor based on Pt-incorporated fullerene-like ZnO nanospheres. J PhysChem C; 114 : 243-50.
2. Andrew G. Smith and Charles J.W. Brooks (1997).The Substrate Specificity and Stereochemistry, Reversibility and Inhibition of the 3-Oxo Steroid AM-A5-Isomerase Component of Cholesterol Oxidase.Biochem. J.167:121-129.
3. Changzhi Z, Wan L, Jiang L, Wang Q, Jiao K. Developed a highly sensitive and selective cholesterol biosensor based on direct electron transfer of hemoglobin (2008).Anal Biochem; 383 : 25-30.
4. Chauhan N, Narang J, Pundir CS. Amperometric determination of serum cholesterol with pencil graphite rod (2010). Am J Anal Chem; 2 : 41-6.
5. Hooda V, Pundir CS. (2008). Preparation of Pt based amperometric cholesterol biosensor using cellulose acetate membrane. J ScientIndust Res; 67 : 299-306.
6. Jeng-You Yang, Ying Li, Shen-Ming Chen, Kuo-Chiang Lin (2011).Fabrication of a cholesterol biosensor based on cholesterol oxidase and multiwall carbon nanotube hybrid composites. Int. J. Electrochem. Sci. 6:2223 - 2234
7. Karube I, Hera K, Matsuoka H, Suzuki S (1982). Amperometricdetermination of total cholesterol in serum with use of immobilized cholesterol esterase and cholesterol oxidase. Anal ChimActa.139 : 127-32.
8. KumariLata, KanwarShamsher S. (2012).Cholesterol Oxidase and Its Applications.Advances in Microbiology. 2: 49-65.
9. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J BiolChem 1951; 193 : 265-75.
10. PundirC.S,NarangJagriti, ChauhanNidhi, Sharma Renu, Sharma P. (2012).An amperometric cholesterol biosensor based on epoxy resin membrane bound cholesterol oxidase. Indian J Med Res. 6: 633-640.

11. Sean b, Dyer NS, Anthony G. Amperometric determination of cholesterol in serum using a biosensor of cholesterol oxidase contained within a polypyrrole-hydrogel membrane.(2001) Anal Chm Acta; 448 : 27-36.
12. Vidal JC, Garcia E, castillo JR. Development of a platinized and ferrocene- mediated cholesterol amperometric biosensor based on electropolymerization of polypyrrole in a flow system. Anal Sci 2002; 18 : 537-41.