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Talanta 69 (2006) 700–705
Talanta
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Tetraethylorthosilicate film modified with protein to fabricate cholesterol biosensor

Arun Kumar [a,*], Ravi Ranjan Pandey [b], Brian Brantley [c]

[a] *Department of Internal Medicine, University of South Florida, Tampa, FL 33612, USA*
[b] *Department of Chemistry, University of Delhi, Delhi 110007, India*
[c] *Nanosense Research and Technology (NRT) Inc., St. Petersburg, FL, USA*

Received 14 October 2004; received in revised form 27 October 2005; accepted 3 November 2005
Available online 2 December 2005

Abstract

Sol–gel derived tetraethylorthosilicate (TEOS) films were prepared by spin coating method on indium tin oxide (ITO) coated glass plate. Hydrophobic interaction method was used to coat the bovine serum albumin film over the surface of tetraethylorthosilicate sol–gel film to minimize cracking, biofouling and to improve the stability of the film. Cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) were immobilized using covalent linkage with bovine albumin serum film to enhance the loading of the enzyme to improve the sensitivity of biosensor. Further ITO–TEOS–BSA–ChOx/HRP film was characterized by UV–vis, FTIR and SEM techniques. The optical response of the ITO–TEOS–BSA–ChOx/HRP biosensor was found to be linear in the range of 2–8 mM for cholesterol concentration with response time approximately 20 s. Amperometric response of ITO–TEOS–BSA–ChOx/HRP was observed to be linear in the range of 2–12 mM of cholesterol concentration with 10-s response time. Michaelis–Menten constant was calculated 21.2 mM .The shelf life of ITO–TEOS–BSA–ChOx/HRP biosensor was approximately about 8 weeks in desiccated conditions at room temperature.

Keywords: Amperometric; Biosensor; Cholesterol sensor; Sol–gel; Covalent coupling

1. Introduction

The researchers to recognize specific molecules with high specificity use biological molecules such as enzymes, antibodies, and nucleic acid. Enzymes have the ability to catalyze many types of chemical reactions at ordinary temperatures with extraordinary specificity and efficacy, and influence the rate of chemical reactions without themselves undergoing any permanent change [1]. The assay of biomolecules such as glucose, urea, cholesterol and lactate play significant role in medicine, biotechnology and food processing industries. Out of these molecules, cholesterol is the most abundant steroid found in the human body and it is an important component of the body tissues. It is widely distributed throughout the human body including cerebral nerves and adrenal glands, moreover, it play vital role as hormone precursor. Cholesterol determination in blood is known to be clinically important for diagnosis of myocardial disorders, arteriosclerosis and cerebral thrombosis. The concentration of cholesterol in blood serum between 3.1 and 6.7 mM is considered to be normal [2,3].

Many types of biochemical tests and sensors are available for the accurate analysis of desired biomolecules, but most of them are either too expensive, too slow and in many cases insensitive and non-specific. In the recent years, applications of sol–gel materials have been remarkably increased in biosensors and enzyme electrode fabrication to enhance the stability and sensitivity of the enzyme [4,5]. The sol–gel technique is particularly advantageous for the development of biosensors due to its number of features such as room temperature processability, control over the surface properties, ability to form the film, monoliths and its chemical inertness towards biomolecules. Silicon alkoxide precursors are the most extensively studied materials for the entrapment of biomolecules as they are inexpensive, an attractive matrix for biochemical reaction due to pores structure and exhibit relative low kinetics [6]. Thus, one can readily prepare silica sol–gel matrix doped with a variety of reagents (i.e. chem-

* Corresponding author. Tel.: +1 8139742476; fax: +1 8139748575.
E-mail address: akumar3@hsc.usf.edu (A. Kumar).

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doi:10.1016/j.talanta.2005.11.003

ical and biochemical recognition elements) and can tune the characteristics of the final product by adjusting the processing conditions (pH, precursor ratios, etc.). Further, sol–gel materials are also used for the development of ceramic films for mechanical, electrical and optical applications [7]. Apart from the optical and electronics applications, sol–gel materials have many interesting micro-structural properties like tunable porosity, large pore volume and grain size. Moreover, the relative rate of hydrolysis and condensation, as well as pore size and extent of branching of the gel in the precursor solution prior to film formation, controls its final properties. Another advantage of the sol–gel matrix is the large number of interaction sites due to large surface area [8].

Researchers have used various techniques to develop sol–gel based cholesterol biosensors to meet the ever-growing sensor demand for clinical and industrial applications. A sol–gel based biosensor for cholesterol, glucose and galactose was developed by using a composite membrane of sol–gel film and electrochemically generated poly (1,2-diaminobenzene) film to improve the selectivity of the enzyme sensors [9]. In another approach, cholesterol oxidase and horseradish peroxidase was immobilized on sol–gel film by using various techniques like physical absorption, entrapment and sandwich configuration [5]. However, all of these approaches have the problem of stability, sensitivity and uneven surface due to developments of cracks in sol–gel derived films that cause insufficient loading of enzyme over the film [10].

Protein adsorption over the sol–gel film is a significant part of the present research. We are focused much attention mainly on biocompatibility of various interfaces exits in enzyme electrode fabrication. The stability of the enzyme electrode when protein interacts at the solid–liquid interface is essential for a number of medical and biochemical applications [11]. In the present approach, sol–gel film was coated over ITO glass plate using spin coating technique and bovine serum albumin film attached with it by ionic and hydrophobic interaction techniques. Further, after coating the bovine serum albumin film over the electrode surface cholesterol oxidase and horseradish peroxidase were covalently linked with it using glutraldehyde. Present approach was selected to improve the sensitivity and stability of the cholesterol biosensor. The modified electrodes were characterized with UV–vis, FTIR and scanning electron microscope (SEM) technique to confirm the modification of electrode. Response of the modified enzyme electrode was studied optically as well as amperometrically.

2. Experimental

2.1. Material

The material used in this investigation and their sources are as follows: the hydrochloric acid (HCl), tetraethylorthosilicate (TEOS), HNO_3, dichloromethyl, trichloroethylene, phenol, 4-aminoatipyrine, ethanol are obtained from (Aldrich, USA). All the biochemicals used such as cholesterol oxidase (E.C.1.1.3.6), horseradish peroxidase (E.C.1.11.1.7) and bovine serum albumin (EG/EC number 2329362) mol. wt. ∼66 kDa are procured from (Sigma–Aldrich Corp., St. Louis, MO, USA). Other chemicals are used of analytical grade. All the aqueous solutions were prepared in Millipore water (RO10TS) with resistivity levels of 18.2 MΩ cm at 25 °C.

2.2. Apparatus

The spectroscopic studies of ITO–TEOS–BSA–ChOx/HRP films carried out with UV–vis (Shimadzu Model 160A) spectrophotometer and Fourier transforms infrared (FTIR) (Nicolet 510P) spectrophotometer. Scanning electron microscopic (SEM, LEO 440, UK) studies were performed by using SEI mode. The sample fixed with silver paste on the sample holder to obtain the required scans. All the electrochemical experiments were performed with electrochemical potentiostat interface (Model SI 1286) with three-electrode configuration. ITO–TEOS–BSA–ChOx/HRP used as working electrode Ag/AgCl, as a reference electrode and a platinum wire as a counter electrode.

2.3. Preparation of stock solution of cholesterol

Stock solution of cholesterol prepared by dissolving 3.86 g of cholesterol in12.8 mL of propan-2-ol and mixed with 3.85 mL of Triton X-100. The solution stirred gently with constant speed to obtain a clear solution after that solution made up to 100 mL at 35 °C. Stock solution, further, diluted using Millipore water according to make different standards of cholesterol solution for measurements [12].

2.4. Preparation of sol–gel film

The modified sol–gel film was prepared by mixing 4.5 mL TEOS, 1.4 mL H_2O and 0.1 M HCl 100 μL in a glass vial. The mixture was stirred regularly until a clear solution was obtained. This solution was treated as stock solution and diluted in 1:1 ratio with H_2O (Millipore). Prior to sol–gel film casting, the ITO glass pates were treated with 5% HNO_3 and subsequently washed several times with Millipore water and, after that, with ethanol. The casting solution prepared by mixing 0.5 mL of stock solution and 1 mL of Millipore water containing in a separate glass vial. The films cast over the glass plates by spin coating techniques as reported earlier [13].

2.5. Procedure for bovine serum albumin film coating over the sol–gel film

Spin coated sol–gel film over ITO glass plate dried at 100 °C overnight. After cooling the film at room temperature, it washed with Millipore water and dried it with a hot air blower. The film obtained was treated with solution of di-chlorodimethyl silane and trichloroethylene in 1:1 ratio (100 μL each) in order to obtain strong hydrophobic surface over the sol–gel film. The film was kept for drying in an oven at 35 °C. After 1 h, the film was removed from the oven and brought to room temperature. The sol–gel film, then, incubated with bovine serum albumin solution (25%) in sodium phosphate buffer (0.1 M, pH 7.2) for 24 h at

room temperature. The surface was washed with Millipore water several times and used for enzyme immobilization [14].

2.6. Covalent coupling of cholesterol oxidase and horseradish peroxidase with BSA film

Bovine serum albumin (BSA) film coated over the surface of sol–gel derived tetraethylorthosilicate was exploited for immobilization of cholesterol oxidase and horseradish peroxidase. About 200 μL phosphate buffer (0.1 M, pH 7.2) containing 25 IU of cholesterol oxidase, 10 IU of horseradish peroxidase and 50 μL 5% glutaraldehyde solution was spread over gently over the film. The immobilized enzymes containing sol–gel–BSA film was kept for drying at room temperature for 6 h followed by a washing with Millipore water to be used for further investigations.

2.7. Assay for enzyme activity

A volume of 0.05 cm^3 of 6 mM cholesterol solution dissolved in propan-2-ol and a volume of 3 cm^3 of phosphate buffer (0.1 M, pH 7.2) were mixed and kept in a thermostat at 35 °C. The ITO–TEOS–BSA–ChOx/HRP films was immersed in the solution and incubated for 3 min. The plate was, then, removed and absorbance of the solution was measured at 240 nm using a double beam spectrophotometer to determine the cholestenone produced due to the interaction of enzymes with cholesterol. The apparent enzyme activity (U cm^{-2}) was determined by using Eq. (1). The procedure is based on the difference in absorbance before and after incubation of the enzyme-coated glass:

$$a_{\text{apparent enzyme activity}}\ (\text{U cm}^{-2}) = \frac{AV}{\varepsilon t s} \quad (1)$$

where A is the difference in absorbance before and after incubation; V the total volume, i.e. 3.05 cm^3; ε the millimolar extinction coefficient of cholestenone (12.2); t the reaction time (min) and s is the surface area in (cm^2) [5]. One unit of enzyme activity (U cm^{-2}) is defined as the production of 1 μmol of cholestenone per minute. The working electrode immersed in fresh 1 mL Millipore water for over night and tested next day for any leakage of enzyme ChOx using spectrophotometer. There was no leaching observed for ChOx covalently linked with BSA coated sol–gel film.

3. Results and discussion

Sol–gel derived TEOS films thicker then 1.5 μm was found to be brittle and showed a tendency to crack when heated. The cracking at critical thickness may be attributed to the effect of biaxial stress of such film. However, the film having less than 0.15 μm displays non-uniform surface and variable alone the edges [15]. Uniform immobilization of enzymes on such surface is not possible. To overcome these problems, the sol-film coated with bovine serum albumin to fabricate stable and sensitive electrode with improved shelf life. Moreover, the three dimensional structure of bovine serum albumin stabilized by 17 di-nucleotide bridges. Further, due to stable 17 di-nucleotide structure of bovine serum albumin, change in the pH of solution and temperature do not have any effect onto the measurements and provide more stability to enzyme electrode [16]. The scheme of immobilization of enzyme is shown in Figs. 1 and 2.

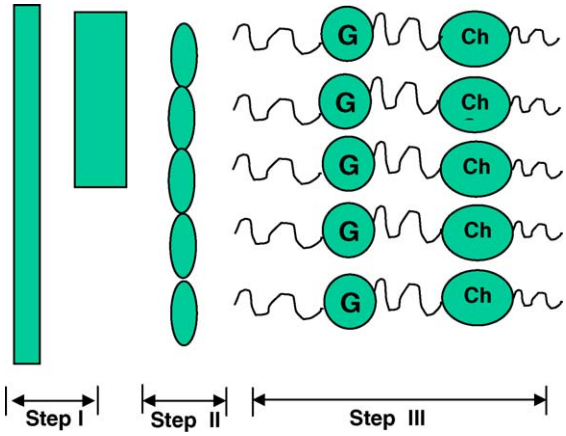

Fig. 1. Schematic diagram of immobilization of cholesterol oxidase over bovine albumin coated tetraethylorthosilicate film (TEOS).

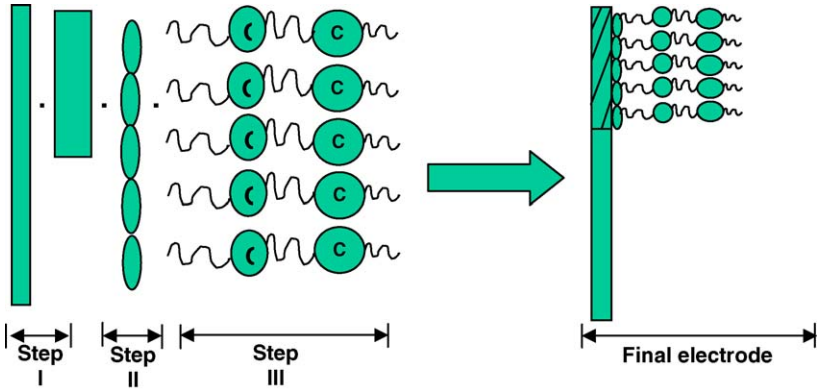

Fig. 2. Schematic diagram of final electrode configuration.

Fig. 3. SEM microphotograph of bovine serum coated on tetraethylorthosilicate film.

3.1. SEM investigation of bovine serum albumin coated sol–gel film

Scanning electron microscopy studies of ITO–TEOS–BSA–ChOx/HRP film show smooth surfaces and non-fragility (Fig. 3). Further, the low-resolution microphotograph of BSA–sol–gel–ChOx/HRP film shows clustered structure of covalently immobilized cholesterol oxidase and horseradish peroxidase. In high-resolution microphotography of the film, the attachment of the ChOx and HRP is clearly visible (Fig. 4). These investigations confirm the increase in loading of enzyme by covalent linkage.

3.2. Spectroscopic characterization of the sol–gel–BSA film

The immobilized cholesterol oxidase and horseradish peroxidase with covalent linkage further characterized with UV–vis and Fourier transform infrared spectroscopy (FTIR) spectrophotometery. The prominent peak in the region 2800 and 3000 cm^{-1} are due to N–H linkage and free amide N–H stretching, respectively. The broad peak at 1400–1600 cm^{-1} confirms the presence of SiO_4 (Fig. 5). When the ITO–TEOS–BSA–ChOx/HRP sensor

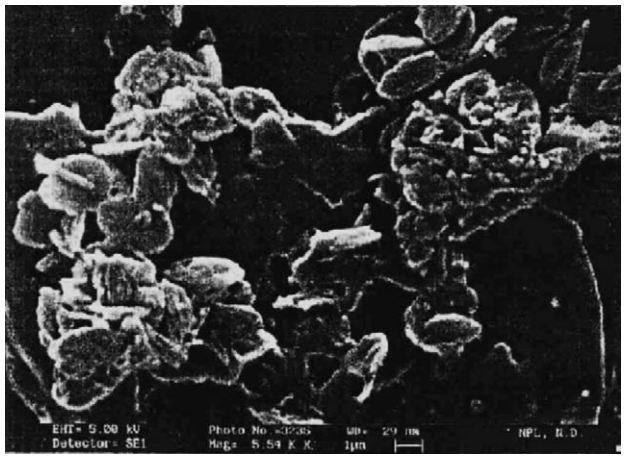

Fig. 4. SEM microphotograph of cholesterol oxidase (ChOx) covalently coupled with bovine serum albumin (BSA) coated tetraethylorthosilicate film (TEOS).

immersed in the phosphate buffer solution of 5 mM cholesterol the increasing absorbance observed at 240 nm. This confirms that the covalently linked cholesterol oxidase and horseradish are in active form [17].

3.3. Optical response studies

The optical response of the sol–gel–BSA film is based on the intensity of the color of quinoneimine dye produced during the reaction of 4-aminoantipyrine and phenol with the H_2O_2, which is produced by cholesterol oxidase during its interaction with cholesterol in the presence of horseradish peroxidase [18]:

$$\text{cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{cholest-4en-3one} + H_2O_2 \quad (2)$$

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{HRP}} (p\text{-benzoquinone monoimino})\text{phenazone} + 4H_2O \quad (3)$$

Fig. 6 shows UV–vis spectra and linear spectrophotometric response of the new sol–gel–BSA film when exposed to 2–8 mM cholesterol in the presence of 4-aminoantipyrine and phenol

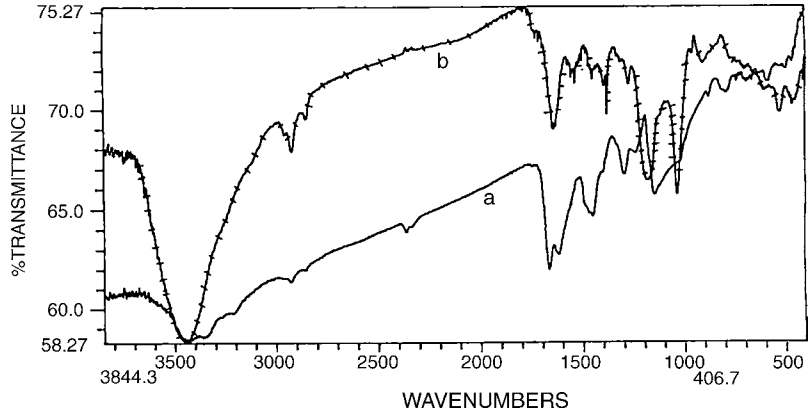

Fig. 5. FTIR spectra of: (a) bovine albumin coated tetraethylorthosilicate (TEOS) film and (b) covalently coupled cholesterol oxidase with bovine serum (BSA) coated TEOS film.

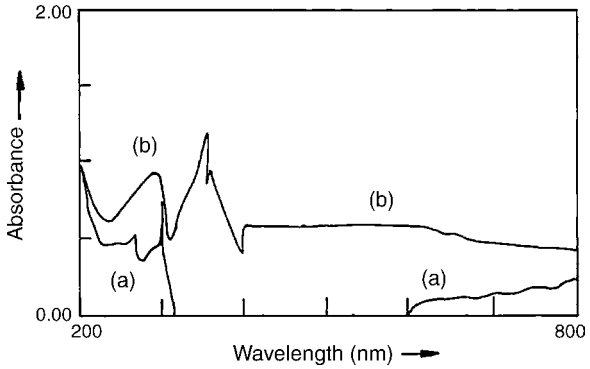

Fig. 6. UV–vis spectra obtained on exposure to varying concentrations of cholesterol solution with identified amount of dye (4-aminoantipyrine) for TEOS–BSA–ChOx film.

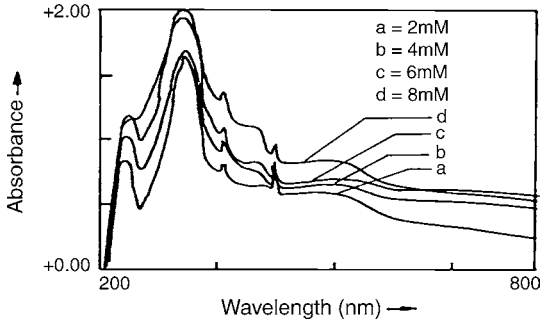

Fig. 7. Photometric response curve of different cholesterol concentration (1–8 mM) when exposed to fixed amount of 4-aminoantipyrine (dye).

(Fig. 7). The slow and low range linear response observed in sol–gel–BSA enzyme film, which may be attributed to an increase in opaqueness of the film due to formation of quinoneimine dye during the course of reactions, as revealed by microscopic examination (Fig. 8). And change in absorbance versus time profile of new sol–gel–BSA film is shown in Fig. 9.

3.4. Amperometric response studies

The three-electrode cell configuration has been used for the amperometric measurements of cholesterol in phosphate buffer (pH 7.2 and 0.1 M) by polarizing the working electrode ITO–TEOS–BSA–ChOx/HRP at 0.75 V versus Ag/AgCl

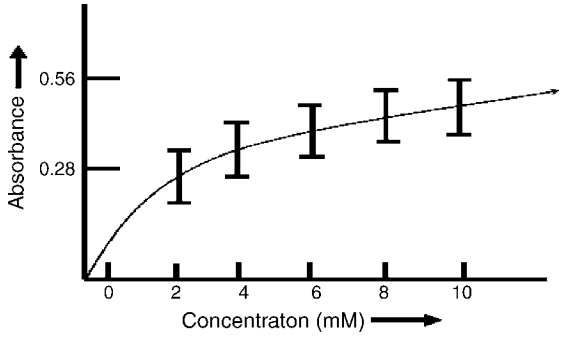

Fig. 8. Photometric calibration (λ 400 nm) curve on exposure to varying concentration of cholesterol and identified amount of dye (4-aminoantipyrine) for TEOS–BSA–ChOx film.

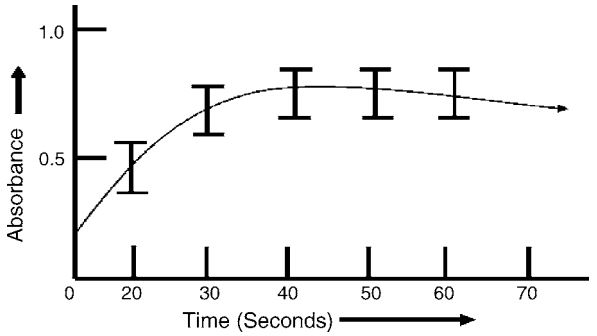

Fig. 9. Change in absorbance at 400 nm vs. time profile for covalently coupled ChOx with BSA (bovine serum albumin) coated sol–gel film (TEOS) on exposure to dye (4-aminoantipyrine) for 4 mM cholesterol concentration.

and using amperometric calibration for H_2O_2. The current was monitored every 5 s after exposing the sol–gel–BSA enzyme electrode to different concentrations of cholesterol solutions. Prior to actual response measurements, the contents of the cell were gently stirred for approximately 3 s. Fig. 10 shows a typical calibration curve obtained with ITO–TEOS–BSA–ChOx/HRP sensor for 2–12 mM cholesterol. A maximum current of 70 μA was obtained for 12 mM cholesterol further increase in cholesterol concentration resulted no significance change in the current. The response time of the electrode was found to be10 s.

3.5. Substrate kinetics investigation with respect to change in pH and temperature

A Michaelis–Menten Eq. (4) explains the relationship between the reaction rate and substrate concentration:

$$V_0 = V_{\max}\frac{S}{K_m + S} \tag{4}$$

where V_0 is the initial rate of reaction, $V_{\max}$ the maximum rate of reaction, S the solution concentration and K_m is the Michaelis–Menten constant. The K_m value characterizes the affinity between the substrate and the enzyme. A low K_m value reflects high affinity. At low substrate concentrations, the reaction rate is directly proportional to the substrate concentration

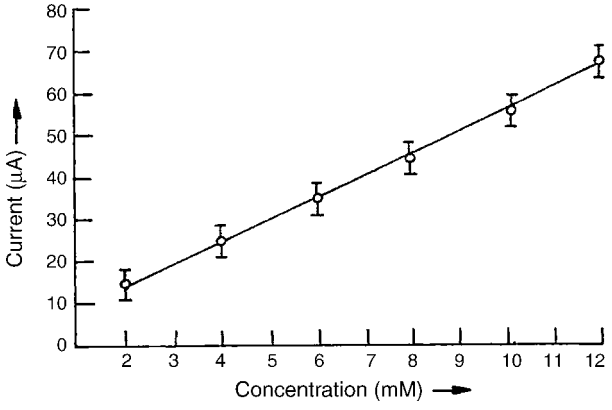

Fig. 10. Amerometric response curve of the ITO–TEOS–BSA–ChOx film when exposed to different concentration of cholesterol (1–12 mM).

and belongs to first-order reaction; at high concentrations, the reaction displays zero order and no longer depends on the substrate concentration, only on enzyme activity. K_m values can be calculated from Lineweaver–Bruke chart by plotting the reciprocal values of V_0 and S on the X- and Y-axis, respectively. A Lineweaver–Bruke Eq. (5) is as follows:

$$\frac{1}{V_0} = \frac{1}{V_{max}}\left(1 + \frac{K_m}{S}\right) \quad (5)$$

The resulting plot gives K_m and V_{max} at the intercepts of the curve with the abscissa and the ordinates, respectively. The K_m value was obtained by extrapolation of Lineweaver–Burke plot. The K_m values for zero-order reactions were calculated at change in pH of the solution and change in temperature of the solution by exposing the enzyme electrode TEOS–BSA–ChOx/HRP–ITO for 14 mM of cholesterol concentration. Further, our investigation shows that that the sol–gel–BSA enzyme electrode responds at pH 6.8 and temperature 35 °C and its K_m value is 21.2.

3.6. Studies on shelf life

The ITO–TEOS–BSA–ChOx/HRP sensors tested for stability under the same operating conditions as used for amperometric response for 8 weeks at room temperature and observed 1.5–2.5% observed degradation. We are, now, in the process of further improvements of the shelf like.

4. Conclusions

In the present investigations, we have demonstrated that bovine serum albumin coating over the tetraethylorthosilicate film improved cracking of sol–gel film over electrode surface. Moreover, sensitivity of the sensor increased due to more loading of cholesterol oxidase and horseradish peroxidase. The loading of enzyme increased as enzymes covalently linked with bovine serum albumin film. The covalent linkage reduced the interatomic distance, which facilitates the electrons to jump over to the other side of the interfacing surfaces, which may enhance the sensitivity of the sensor. These modifications results in more linear amperometric response of electrode. This approach can used for in vivo biosensor fabricating due to its more biocompatibility. Further, electrochemical measurements performed at low potential minimize the risk of many interfering species, which oxidize at higher potential.

Acknowledgements

Author Ravi Ranjan Pandey likes to thank head of the Department of Chemistry, University of Delhi, India, for valuable cooperation during the course of above studies. Author Arun Kumar likes to thank Nanosense Research and Technology (NRT) Inc., St. Petersburg, FL, USA, for many valuable suggestions.

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