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Silk Mat as Bio-matrix for the Immobilization of Cholesterol Oxidase

Urmila Saxena · Pranab Goswami

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Abstract Cholesterol oxidase (ChOx) was covalently immobilized onto the woven silk fiber (silk mat) produced by *Antheraea assamensis*. The immobilization was done using N-ethyl-N'-(3-dimethylaminopropyl) carbodimide and N-hydroxysuccinimide ligand chemistry. The attachment of ChOx to the silk mat was demonstrated by scanning electron microscopy and activity study. The kinetic studies of the immobilized ChOx were performed by using a biological oxygen monitor. The enzyme loading was found to be 0.046 U cm^{-2} of silk mat and the enzyme loading efficiency of the silk mat was estimated to be 70%. Remarkably high storage and operational stability ($t_{1/2}$ of initial activities) corresponding to 13 months and 25 numbers of assay (for a period of 6 h), respectively, of the fabricated ChOx electrode were demonstrated.

Keywords Cholesterol · Cholesterol oxidase · Silk mat · Immobilization

Introduction

Role of enzymes in clinical diagnosis, food industries, and environmental monitoring has been known for several years [1–3]. However, the high cost of enzymes makes their use less economic. This can be overcome through immobilization of enzymes which is a promising way for enhancing the enzyme reuse and stability and reducing the overall cost of biocatalytic processes [4]. Enzyme biosensors based on immobilized enzymes on a suitable solid matrix are rapidly gaining interest in the field of analytical technology [4, 5]. The matrix and method used for immobilization of enzymes on the solid support are considered as the most critical factors that govern the efficiency of the fabricated biosensor. Different matrix compositions and techniques have been used to achieve enzyme immobilization over the years [6]. However, frequent loss of enzyme activity and stability resulting from the immobilization of the enzymes on the matrix is posing challenge in

U. Saxena · P. Goswami (✉)
Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
e-mail: pgoswami@iitg.ernet.in

developing commercially viable biosensors. Hence, the biosensor research is currently focusing upon developing new materials that offer promise to solve the problem of impairment of enzyme activity and stability upon immobilization [7, 8].

One possible and effective way of improving the stability and activity of the immobilized enzyme is to incorporate the enzyme into suitable biomaterial matrix that is more biocompatible. In this perspective, silk protein matrix can be chosen as an ideal bio-platform for enzyme immobilization as the backbone of silk is mainly protein fiber [9, 10], so it is expected to be biocompatible with enzymes. The intricate lattice network of stable and water-insoluble protein fibers of silk has high surface area which is useful for various applications. Enzymes can be physically or chemically entrapped in the three-dimensional interpenetrating network of the silk fiber that may provide a biocompatible microenvironment around the enzyme. Moreover, silk is inexpensive, inert, non-toxic, readily available, and can be obtained in a number of forms. There are some reports where silk protein-based materials (mainly silk fibroin) have found application as solid supports for potentially expensive and commercially important enzymes and organometallic catalysts including glucose oxidase, L-asparginase, lipase, peroxidase, etc. [11–15]. Demura and Asakura et al. first reported using a glucose oxidase-immobilized silk fibroin membrane sensor in a steady-state analysis system [16]. Since then there are many other reports on the glucose oxidase-immobilized silk fibroin membrane in biosensor applications [17, 18]. The effectiveness and biocompatibility of the support matrix, however, varies with the kind of enzymes and therefore the immobilization needs to be studied independently for different enzyme systems for their tailored applications.

The clinical determination of cholesterol is very important and for this purpose, cholesterol oxidase has been immobilized on a variety of support matrices including polymeric membranes, hydrogels, sol-gel films, etc. for the development of various cholesterol biosensors [19–29]. Until now, there are no reports on the use of silk as the immobilization support for cholesterol oxidase (ChOx). We aim to exploit woven silk fiber (silk mat) as the support matrix for the immobilization of ChOx. The ChOx was covalently immobilized onto the surface of woven silk mat and the response characteristics of the immobilized enzyme were studied and reported in this paper.

Materials and Methods

Reagents and Stock Solutions

ChOx (EC 1.1.3.6 from *Pseudomonas fluorescens*, 2.4 U mg^{-1}), N-ethyl-N'-(3-dimethylaminopropyl carbodiimide) (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (USA). Cholesterol was purchased from Tokyo Chemical Industries (Tokyo, Japan). Triton-X-100, urea, and uric acid were procured from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India). All other chemicals were of analytical grade and used without further purification. Deionized water from the Millipore water purification system was used throughout the experiments. Woven Muga silk fiber (silk mat) produced by *Antheraea assamensis* was purchased from Sualkuchi (Silk village of Assam, India).

The stock solution of ChOx (10 mg mL^{-1}) was freshly prepared in 50 mM potassium phosphate buffer (pH 7.0). Cholesterol stock (20 mM) was prepared in 10% Triton-X-100 solution and stored at 4°C. This stock solution was further diluted with potassium phosphate buffer (50 mM, pH 7) to make different concentrations of the cholesterol. The final concentration of the Triton-X-100 in the reaction mixture was not more than 0.05% so that it does not have any effect on enzyme activity.

Apparatus

A biological oxygen monitor (BOM; 5300A, YSI, USA) with Clark-type polarographic oxygen probes (5331A, YSI, USA) and a thermostat water bath with magnetic stirrer (5301B, YSI, USA) were employed for carrying out ChOx activity studies. It was attached to a chart recorder (Kipp & Zonen, USA) used for real-time display and recording of the experimental results. Scanning electron microscopy (SEM) of silk mat was carried out with a scanning electron microscope (Leo 1430vp, Germany).

Enzyme Immobilization on Silk Mat

A silk mat was washed thoroughly with deionized water and then dried at room temperature under laminar hood. A circular piece of diameter 15 mm was cut from this dried silk mat and dipped for 2 h in a solution of EDC (0.4 M) and NHS (0.1 M) prepared freshly in 50 mM potassium phosphate buffer (pH 6.5). The membrane was then washed thoroughly with 50 mM potassium phosphate buffer (pH 7.0). After placing it on a clean glass slide, 5 μL of ChOx solution (10 mg mL^{-1}) was dropped on its surface and then dried at ambient conditions for 24 h. For removing any loosely bound enzyme from the ChOx-immobilized silk mat, it was immersed in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.2% Triton-X-100 and gently vortexed for 15 min. The membrane was then taken out and stored at 4°C until use.

Determination of Enzyme Loading Efficiency of the Silk Mat

The amount of protein that is leached out in the wash solution was estimated following Bradford method using BSA as standard [30]. The amount of protein retained in the ChOx-immobilized silk mat was calculated from the protein mass balance among the amount that is initially added to the membrane during immobilization and the amount that is leached out. The enzyme loading on the silk mat was determined in terms of number of enzyme units retained per unit surface area of the silk mat and the enzyme loading efficiency of the silk mat was determined in terms of percent protein immobilized.

Scanning Electron Microscopy of the Silk Mat

The unmodified and ChOx-immobilized silk mat were placed onto round copper grids of about 10 mm diameter, coated with a thin layer of gold using a spray gun, and then examined under scanning electron microscope using the following setting conditions: 15 KeV EHT, 50 μm aperture, 1,000× magnification.

ChOx Activity Measurements

Cholesterol oxidase catalyzes the conversion of cholesterol into cholest-4en-3one and hydrogen peroxide according to Eq. 1:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholest}-4\text{en}-3\text{one} + H_2O_2 \quad (1)$$

The cholesterol oxidase activity can be measured by monitoring the rate of oxygen depletion in the presence of a specific amount of cholesterol. Keeping in view of the highly porous morphology of the silk mat to be free from any diffusion limitation, the activity

studies of this ChOx-immobilized silk mat were carried out using BOM. Figure 1 is showing the detailed scheme of the measurement system of the immobilized ChOx activity using the BOM. The ChOx-immobilized silk mat was mounted on top of the oxygen probe with the help of an O-ring. Air-saturated potassium (3 mL) phosphate buffer (50 mM, pH 7.5) was taken in the reaction chamber and kept under stirring condition. The oxygen probe assembled with enzyme modified silk mat was dipped into it and after it got stabilized, assay was started by injecting standard cholesterol stock solution into the phosphate buffer. The enzyme activity was determined by observing the O_2 depletion rate processed and recorded by the oxygen monitor and chart recorder. Once the assay is complete, the ChOx-immobilized silk mat was removed from the oxygen probe, washed several times with phosphate buffer (50 mM, pH 7.0), and stored at 4°C.

Results and Discussion

Characterization of the Immobilization of Cholesterol Oxidase on Silk Mat

ChOx was covalently immobilized onto the silk mat via formation of amide bond between -COOH group of silk mat protein and -NH_2 group of ChOx using EDC as the coupling agent and NHS as activator [31, 32]. Figure 2 is showing the chemistry of covalent coupling of ChOx to the surface of silk mat. EDC activates the -COOH functional groups present in the silk protein to form an unstable *O*-acyl intermediate. NHS converts this unstable derivative to a stable amine-reactive ester. -NH_2 group of the ChOx acts as a nucleophile and attacks the amine-reactive ester to form an amide link between silk and ChOx.

The attachment of ChOx to the silk mat was demonstrated by SEM. Figure 3a, b shows the SEM images of silk mat before and after immobilization of the enzyme, respectively.

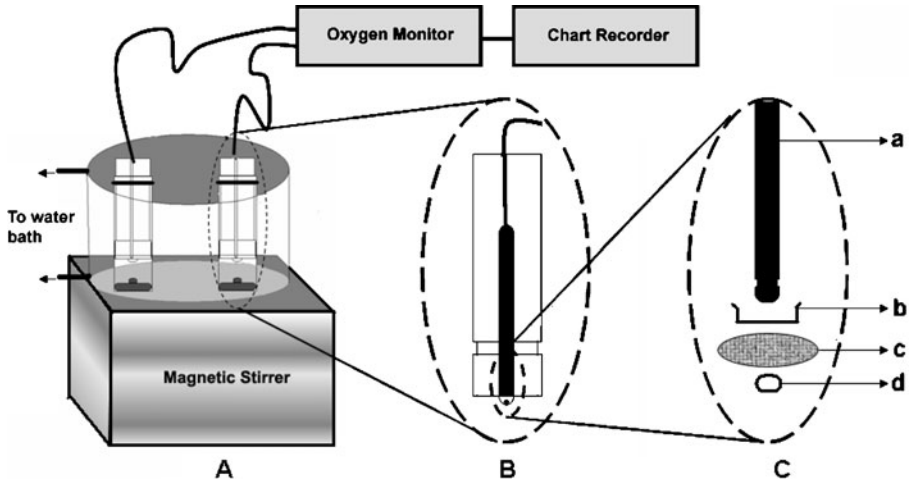

Fig. 1 **A** Schematic diagram for the measurement of silk mat-immobilized ChOx activity using a biological oxygen monitor consisting of a thermostat water bath, a magnetic stirrer, and oxygen probes connected with the oxygen monitor and a chart recorder. **B** Oxygen probe with adapter. **C** Exploded diagram of the assembly of ChOx-immobilized silk mat with the oxygen electrode: *a* oxygen electrode body, *b* teflon membrane, *c* ChOx-immobilized silk mat, *d* O-ring

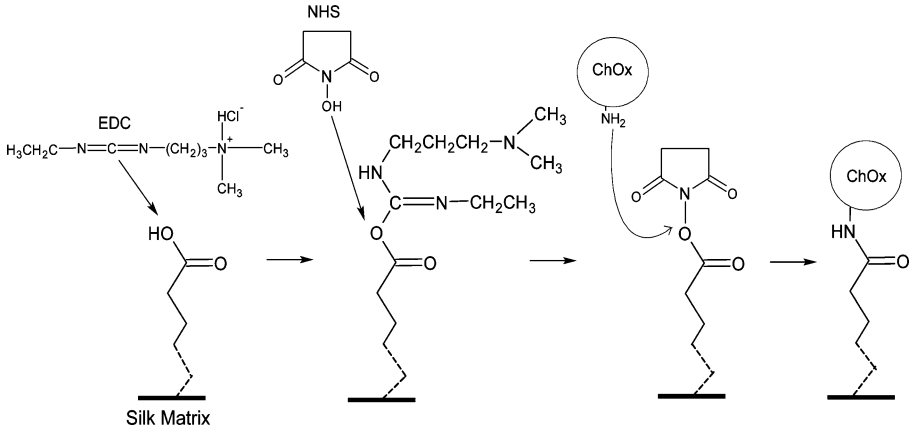

Fig. 2 Mechanism of covalent immobilization of ChOx to the surface of silk mat

Silk mat shows a network-like fibrous morphology (Fig. 3a) which provides large effective surface area to load large amount of enzyme. Short irregular globules or aggregates of enzyme are distributed randomly on the protein fibers of the ChOx-immobilized silk mat (Fig. 3b), whereas such attachment are void on the unmodified silk mat (Fig. 3a). The highly hydrophobic nature of silk [23] and rapid cross-linking reaction adapted for this immobilization approach are attributed to the observed aggregation of the enzyme molecules on the silk surface.

The enzyme loading on the silk mat was found to be 0.046 U cm^{-2} of silk mat and the enzyme loading efficiency was determined to be 70%. The optimum enzyme loading resulted from the cross-linking reaction could effectively facilitated the reaction between the substrate and the enzyme that resulted good response of the biosensor as shown later. To determine the apparent Mchaelis–Menten constant $\left(K_m^{app}\right)$ and maximum rate of reaction (V_{max}) of the immobilized ChOx, the activity assay was done with different concentrations (20–90 μM) of cholesterol at optimum conditions of 30°C temperature and pH 7.5. Using the Lineweaver–Burk plot, the K_m^{app} and V_{max} were determined to be 259.89 μM and 11.614 μmol O_2 consumed per minute, respectively. The specificity constant (K_{cat}/K_m) which is the measure of how efficiently an enzyme converts a substrate into product was determined to be 1.3 $(\mu M)^{-1}$ s^{-1}. The activity assay of free ChOx in solution was also done in the same conditions as that for the immobilized enzyme. For the free enzyme, the K_m and V_{max} were determined to be 212.31 μM and 19.305 μmol O_2 consumed per minute. The higher K_m value and a lower V_{max} value observed in case of immobilized ChOx is due to either the conformational changes of the enzyme, which results in a lower possibility of forming an enzyme-substrate complex or a less accessibility of the substrate to the active sites of the immobilized enzyme.

Effects of pH and Temperature on the Response of the Immobilized ChOx

Effect of pH

The effect of pH on the immobilized ChOx activity was investigated by subjecting it to 50 μM cholesterol standards in various pH (4.0–10.0) buffer solutions. The results (Fig. 4a) showed that the activity increased with increasing pH value from 4 to 7.5 and then declined

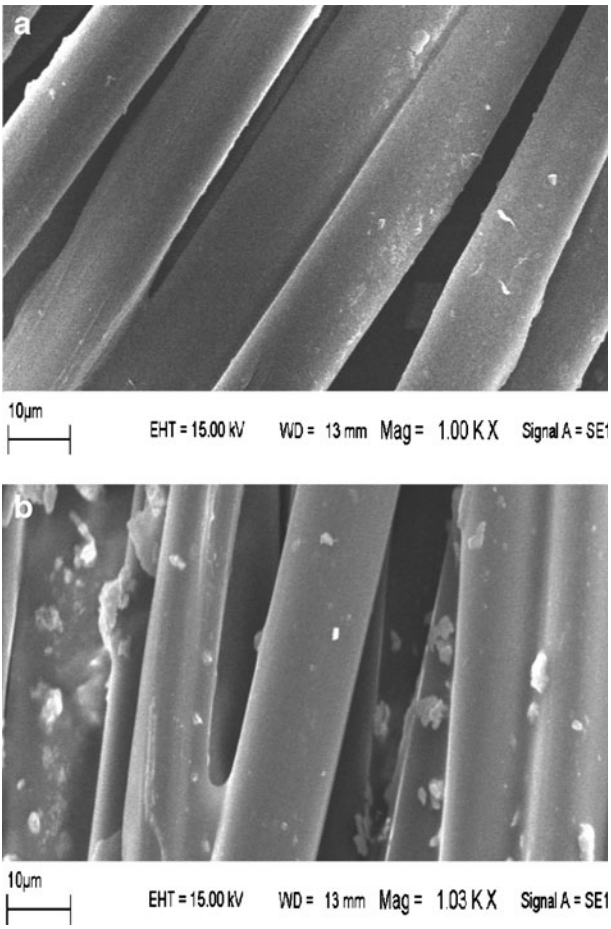

Fig. 3 Scanning electron micrographs of the silk mat: **a** fresh silk mat treated with EDC and NHS and **b** silk mat after immobilization of ChOx

as pH increases further. The optimum activity of immobilized enzyme was achieved at pH 7.5. Consequently, 50 mM potassium phosphate buffer with pH 7.5 was chosen for all the remaining studies.

Effect of Temperature

The other most critical parameter affecting the enzyme activity is the working temperature as it affects both the kinetics of the enzymatic reactions as well as the performance of the oxygen probe. The effect of temperature on the activity of the immobilized enzyme was studied by carrying out enzyme activity assay with a 50 μM cholesterol solution over the temperature range from 25°C to 45°C. From Fig. 4b it can be noted that the oxygen depletion rate was increased with increasing temperature from 25°C to 40°C but it decreased sharply as the temperature increases further to 45°C. This decrease in enzymatic activity may be attributed to the partial denaturation of the ChOx and low solubility of oxygen in water at higher temperatures. The temperature above 45°C could not be studied

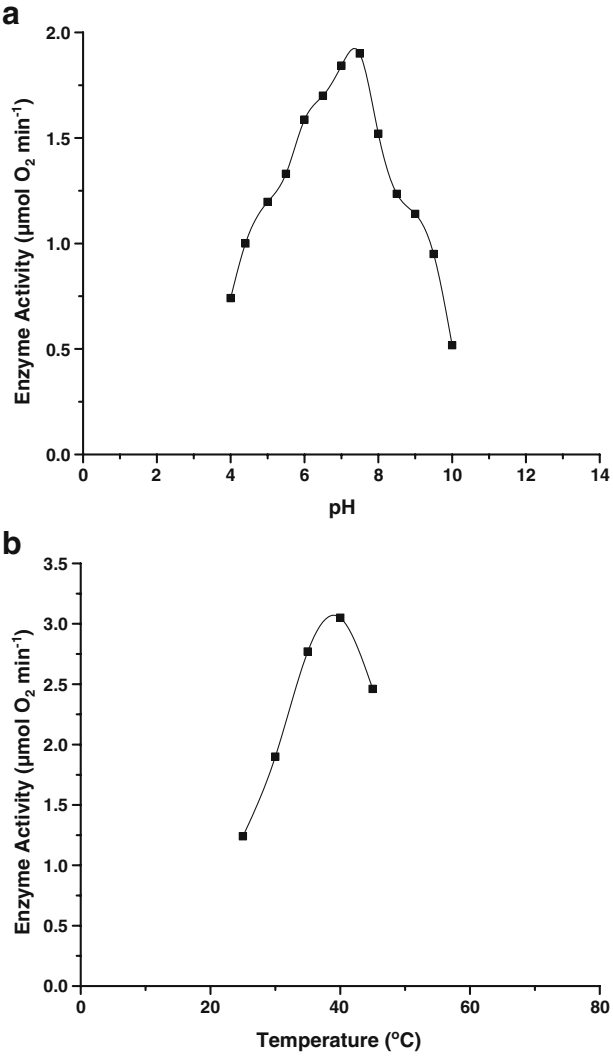

Fig. 4 Activity of immobilized enzyme as a function of **a** pH and **b** temperature

because of limitations of the commercial oxygen sensor. Although the response of the biosensor was highest at 40°C, further experiments were carried out at 30°C to prevent enzyme denaturation after repeated use and thus prolong the lifetime of the immobilized ChOx.

Reproducibility, Operational Stability, and Storage Stability

The reproducibility of the immobilization procedure was studied by exposing five different ChOx-immobilized silk mat prepared nominally in the same way to a 50 μM cholesterol standard under optimum conditions (50 mM potassium phosphate buffer, pH 7.5, and temp 30°C). The results revealed that the relative standard deviation of immobilized ChOx

activity is 4.5±0.43% (n=5) which demonstrates a high reproducibility of the immobilization procedure

The operational stability was examined by subjecting a freshly prepared ChOx-immobilized silk mat at optimal working conditions to a 50 μM cholesterol standard and assessing its activity successively for 27 times for a period of 6 h. After each measurement the ChOx-immobilized silk mat was washed with a pH 7.0 phosphate buffer. Our results (Fig. 5a) demonstrated that the ChOx-immobilized silk mat maintained its initial activity till the fourth successive measurement and retained ~50% of its initial activity at the end of 25 measurements.

The operational stability of the ChOx-immobilized silk mat was also determined with intermittent storage. For this purpose, activities of two different ChOx-immobilized silk mats prepared in the similar manner was assessed with 50 μM cholesterol periodically at 3

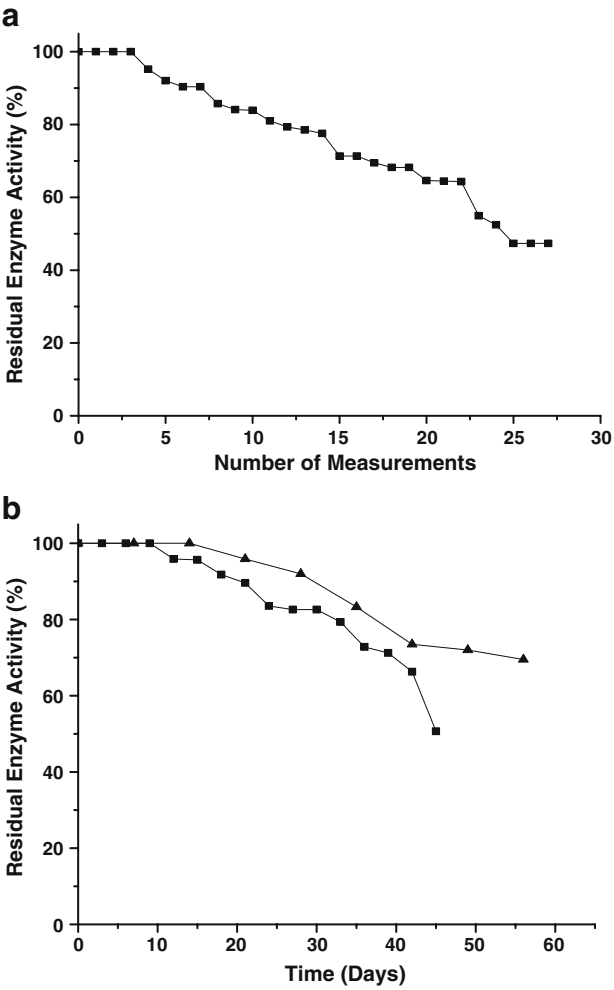

Fig. 5 **a** Operational stability of the ChOx-immobilized silk mat. **b** Operational stability of the ChOx-immobilized silk mat with different intermittent storage time; *filled square*—3 days and *filled triangle*—7 days

and 7 days intervals for 45 and 60 days, respectively. When not in use, the ChOx-immobilized silk mat was removed from the tip of the oxygen electrode, washed with 50 mM phosphate buffer (pH 7), and stored at 4°C. It was observed that when the measurements were made at 3 days interval, the immobilized ChOx could retain ~50% of its original activity after 45 days of storage and when the same carried out with 7 days interval, ~70% of its original activity was retained till 60 days (Fig. 5b). This indicates that the stability of the fabricated ChOx-immobilized silk mat for repeated use is governed by both the number of measurements made and the storage time. The results of our stability tests are comparable to those observed with some other matrices used for the immobilization of ChOx. For instance, when ChOx was immobilized in zinc-oxide nanoparticle–chitosan composite film for the development of cholesterol biosensor, the decrease in the value of amperometric current has been found to be about 25% up to about 8 weeks [33]. The stability observed in our case is better than that observed with tetraethylorthosilicate (TEOS) sol-gel films as an immobilization support for cholesterol oxidase where after 5 weeks, TEOS sol-gel enzyme films exhibit about 65% of the enzyme activity [34]. The half life ($t_{1/2}$ of initial activities) of the immobilized ChOx when stored in a closed container at 4°C without being used was nearly 13 months.

Conclusions

The present study demonstrates the silk mat as a suitable immobilizing matrix for cholesterol oxidase. The fibrous and porous morphology of the silk mat provide an ideal microenvironment to the immobilized ChOx that resulted good analytical performance with high stability, sensitivity, reproducibility, and good selectivity for cholesterol. In the present study, conventional biological oxygen monitor is used for carrying out the activity studies. In the future, the silk mat-immobilized cholesterol oxidase may be used in the fabrication of cholesterol biosensor by assembling it with other modern electrochemical or optical transducers. Since, the silk mat is a commercially available low cost material, this may be a promising biocompatible biomaterial which could be useful for the future development of biosensors.

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