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A sepiolite modified conducting polymer based biosensor

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ABSTRACT

A conducting polymer modified with sepiolite was utilized in the construction of a highly sensitive and fast amperometric cholesterol biosensor. In this study a monomer; (10,13-bis(2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)dibenzo[*a*,*c*]phenazine (PHED)) was synthesized and then its polymer was coated on a graphite electrode by electropolymerization to obtain a matrix for enzyme immobilization. Cholesterol oxidase was immobilized onto polymer coated electrode by adsorption technique. Sepiolite was introduced for a successful immobilization of the cholesterol oxidase. Immobilized enzyme kinetic parameters (K_M^{app}, I_{max}) were evaluated by Michaelis-Menten kinetics and calculated as 0.031 mM and 6.06 μA, respectively. LOD and sensitivity were estimated as 0.36 μM and 1.64 mA/mMcm^2. Characterization of designed biosensor was done to examine the effect of various factors such as enzyme amount, optimum pH and shelf-life. A novel accurate and inexpensive cholesterol biosensor was developed for the determination of total cholesterol in food samples.

1. Introduction

Enzymes have several applications due to their unique properties such as high specificity, high catalytic efficiency and ability to lower the activation energy. These properties are restricted with some problems like instability and tendency to denaturation. In order to overcome these problems use of immobilized enzyme is the best solution [1]. Immobilization of enzymes has been widely used in various industrial applications and construction of the biosensors. Among the immobilization techniques, adsorption of enzymes on solid supports is suggested to enhance their stability against denaturation. This also enables the easy separation of the enzyme from substrates. Via immobilization of enzyme on a support, structure and activities of enzyme molecules can be changed to achieve a stable and long-life biosensor [2,3]. Conducting polymers (CPs) have been widely used in biosensors as immobilization matrices [4,5] due to their good electrochemical and physical properties [6,7]. Besides polymer layer thickness can be controlled during the electrochemical synthesis [8]. Moreover, enzyme molecules can be easily fixed to the CP coated electrode surface. In addition to use of CPs as immobilization matrices, inorganic host matrices have many advantages such as high surface area, mechanical strength, endurance to chemical inertness and low production cost [2,9].

Cholesterol is a structural component of cell membrane and fundamental for life. Development of reliable methods of quantitative cholesterol determination is very important since high cholesterol level may cause narrowing of the arteries, hypertension and cardiovascular diseases [10,11]. Generally, cholesterol is determined using spectrophotometric methods in both blood and food samples [12]. However such methods are time consuming, high cost due to expensive instrumentation and may contain complicated procedures [13] where an expensive enzyme must be used in each assay. However, biosensors have attracted much attention among the devices for biochemical analyses due to their good selectivity, cheapness, fast response and reproducibility [10]. The main purpose of this study is to constitute a new matrix for the determination of cholesterol. Since immobilization of enzyme molecules in suitable matrices is the main issue for the construction of reliable biosensors; research on new immobilization matrices for biomolecules has attracted much attention in the literature

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[14,15,8,16,17]. In the enzymatic reaction catalyzed by cholesterol oxidase, cholesterol is oxidized to cholest-5-en-3-one by cholesterol oxidase. Consequently, oxygen is used as the final acceptor in enzymatic reaction to form hydrogen peroxide. Amperometric measurement was monitored at -0.7 V versus Ag reference electrode by monitoring the decrease in oxygen level as a result of enzymatic reaction.

In this study immobilization of cholesterol oxidase (ChOx) was performed via adsorption technique within a conducting polymer of 10,13-bis(2,3-dihydrothieno[3,4-*b*][1,4]dioxin-5-yl)dibenzo[*a*,*c*]phenazine (PHED) and sepiolite which is a clay mineral deserving many diverse applications due to its structural characteristics compared to other clays [18]. This technique is enhanced via strong π–π stacking of aromatic groups in the structures of polymer backbone and enzyme molecules to stabilize tertiary structure of proteins. Conducting polymer of PHED (PPHED) was coated by electropolymerization onto graphite electrode. Use of this conducting polymer improved the electron transfer during the enzymatic reactions, increased sensitivity and reduced detection limit. ChOx and sepiolite mixture was immobilized onto the polymer coated graphite electrode to construct the amperometric cholesterol biosensor. It was reported that sepiolite is non-toxic and biocompatible [19,20]. Thus, sepiolite was used for the stable immobilization of the ChOx. In enzyme immobilization it is crucial to fix enzyme molecules on biosensor surfaces for a long time. For this purpose, generally, covalent binding is used between enzyme molecules and support. In order to achieve these bindings, the support should bear functional groups. Since PPHED do not have functional pendant groups in its structure for the link between enzyme molecules and support, sepiolite was used to fix enzyme molecules to polymer coated electrode surface by adsorption. Sepiolite ($Mg_2H_2(SiO_3)_3xH_2O$) is a naturally occurring clay-like mineral exhibiting microfibrous morphology which provides high surface area and extended porous volume. Therefore, these properties make sepiolite a good candidate as a support for immobilization of materials via adsorption [21]. Due to the large surface area of sepiolite and its needle shaped fiber structure, polymers can interact with sepiolite via penetration through its structural channels [22,23]. Moreover, reinforcing capability of sepiolite was enhanced with polymeric matrices [22]. Thus, the introduction of sepiolite to PPHED based biosensor is expected to enhance both the mechanical properties and bioactivity of PPHED. Hence, efficient, highly sensitive and fast response biosensor was successfully fabricated in this work. Conducting polymer enables the efficient immobilization and charge transfer on the transducer surface in biosensor system while, with its sandwich-like structure and channels sepiolite enhances the enzyme immobilization and facilitates the adsorption of enzyme molecules on the electrode surface. A representative preparation for the proposed biosensor was depicted in Scheme 1. The biosensor was characterized by scanning electron microscopy (SEM). Optimization and characterization studies were done and practical application of modified electrode was achieved via determining total cholesterol in food samples.

2. Materials and methods

2.1. Materials

Cholesterol oxidase (E.C.1.1.3.6) (26.4 U/mg protein) from *Pseudomonas fluorescens*, cholesterol, Triton-X 100, tetrabutylammonium hexafluorophosphate ($TBAPF_6$), sepiolite ($Mg_2H_2(SiO_3)_3xH_2O$) and glutaraldehyde for enzyme immobilization were purchased from Sigma–Aldrich and used with no further purification. Dichloromethane (DCM) was purchased from Merck (Darmstadt, Germany). A solution of cholesterol (0.005 M) was freshly prepared by dissolving cholesterol in 1% (v/v) Triton-X 100 in 2-propanol (Merck). This mixture provides solubility and stability of cholesterol in aqueous solutions at room temperature. To obtain a clear solution it was then diluted with 50 mM PBS (pH 7.0) consisting of 0.025 M Na_2HPO_4 (Fisher Scientific Company) and 0.025 M NaH_2PO_4 (Fisher Scientific Company) and distilled water. The chemicals used in the synthesis of the monomer were purchased from Sigma–Aldrich. All other chemicals were analytical grade. Cholesterol oxidase colorimetric kit was obtained from Human GmbH-65205 (Wiesbaden-Germany).

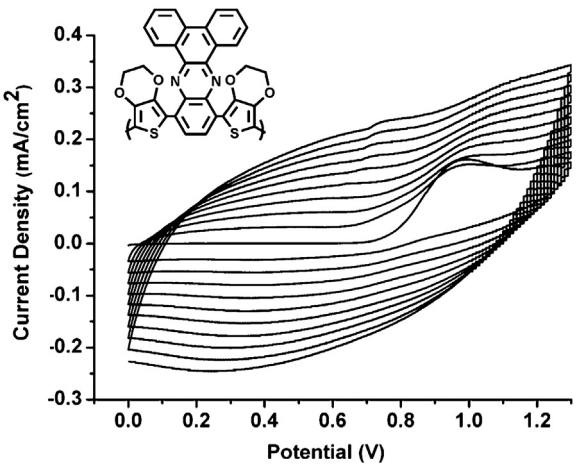


Fig. 1. Repeated potential-scan electropolymerization of PHED in 0.1 M $TBAPF_6$/DCM on the graphite electrode (10 cycles).

2.2. Apparatus

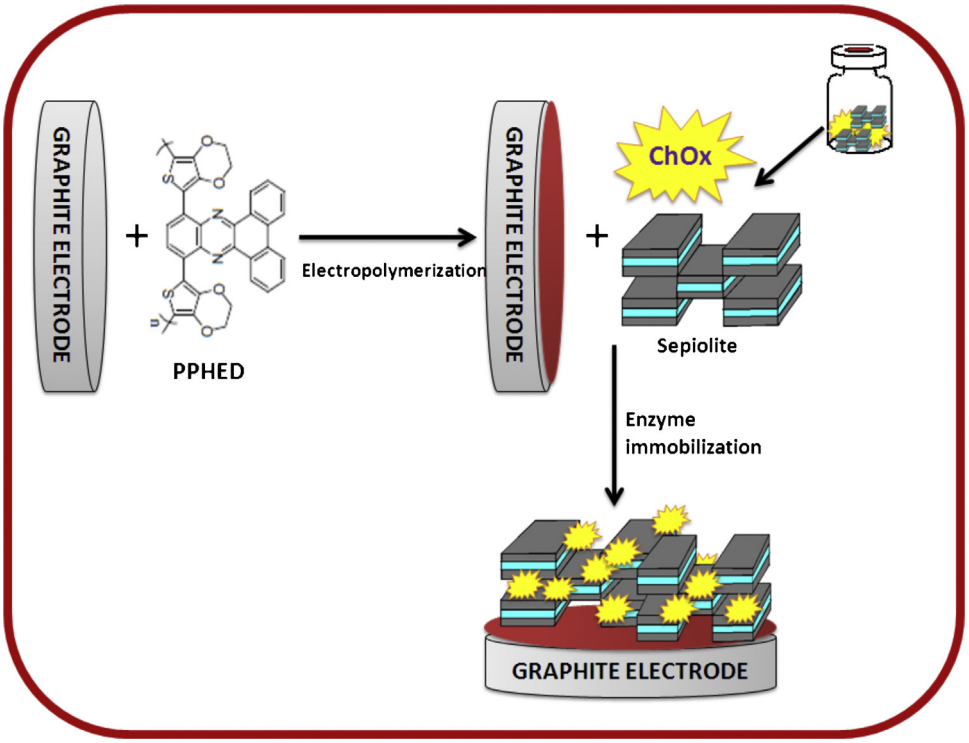
Electrochemical and amperometric measurements were performed using Ivium CompactStat (The Netherlands) potentiostat in a reaction cell equipped with three electrodes consisting of graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode, platinum wire as the counter electrode and a Ag wire as the pseudo reference electrode. Measurement of amperometric analyses were calculated as average of four measurements and standard derivations were given as ±SD. In order to perform the surface imaging of the constructed cholesterol electrode, scanning electron microscope (SEM) (JEOL JSM-6400 model) was used.

2.3. Synthesis of 10,13-bis(2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)dibenzo[a,c]phenazine (PHED)

Synthesis and characterization of the monomer, PHED was prepared according to a previously described method [24]. After the synthesis of 10,13-dibromodibenzo[*a*,*c*]phenazine, via Stille coupling reaction with tributyl(2,3-dihydrothino[3,4-*b*][1,4]dioxin-5-yl)stannane, PHED was synthesized successfully.

2.4. Preparation of poly(10,13-bis(2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)dibenzo[a,c]phenazine) (PPHED) film by electropolymerization

A graphite electrode was polished on an emery paper and rinsed several times with distilled water. PHED was polymerized electrochemically on graphite electrode (area 0.07 cm^2) using 0.1 M $TBAPF_6$ as the supporting electrolyte in DCM with repeated scan intervals between 0.0 and 1.30 V versus Ag wire pseudo-reference electrode with a scan rate of 100 mV s^{-1} for 10 cycles (Fig. 1). After



Scheme 1. The schematic representation of construction of the proposed biosensor.

electropolymerization was completed, polymer coated surface of the electrode was rinsed with distilled water to remove possible organic impurities. 10 cycle polymerization refers to 0.1 coul/cm^2 where the thickness of the polymer is 180 nm.

2.5. Biosensor preparation

The electrochemically prepared polymer was constructed on bare graphite electrode. 3.0 mg sepiolite was dissolved into 5 mL of 50 mM sodium phosphate buffer solution (PBS) pH 7.0 in a beaker and stirred at room temperature overnight. 1.5 mg (39 U) of cholesterol oxidase was dissolved in 50 mM PBS pH 7.0. 10 μL of ChOx and sepiolite mixture was spread over polymer coated graphite electrode. After that, glutaraldehyde was also spread over the electrode as the crosslinking agent (prepared in 50 mM PBS pH 7.0) and then left to dry for 3 h at room temperature. Glutaraldehyde was used as the protein cross-linking agent and has undoubtedly found a wide application in enzyme immobilization technology [25]. The enzyme electrode was rinsed with distilled water to remove unbound enzyme molecules and it was stored at 4 °C for overnight. Hence, this modified graphite electrode was successfully achieved.

2.6. Amperometric biosensor measurements

Amperometric studies were performed in a reaction cell containing 10 mL phosphate buffer solution (50 mM, pH 7.0) under mild stirring. After each measurement buffer solution was refreshed and electrodes were washed with distilled water and kept in phosphate buffer solution (50 mM, pH 7.0) for 3 min. Unless the buffer is refreshed, there may be accumulation of excess substrate in the bulk solution close to the electrode diffusion layer. In higher concentrations, this may cause substrate inhibition effect on the biosensor. Moreover, this can bring the faster enzyme saturation and the linear range may be shortened. Hence, the buffer was refreshed for each measurement. 60 measurements of cholesterol were carried out although not consecutively. Decrease in oxygen level as a result of enzymatic reaction was monitored at −0.7 V versus Ag reference electrode in phosphate buffer. When the background current reached a steady state, freshly prepared substrate (cholesterol) was added to the medium; the current change was recorded as biosensor response. All experiments were carried out at ambient conditions. The reaction medium (buffer solution) was refreshed before the measurements. During the experiments, substrate solution was stored in dark at room temperature.

3. Results and discussion

3.1. Optimization studies

The proposed cholesterol biosensor was optimized in order to improve the interactions between the enzyme and layers of working electrode including sepiolite and conducting polymer. Polymer layer thickness, amount of the enzyme, sepiolite, the crosslinker and also pH of the biosensor environment are very important parameters in the construction of the biosensor.

It was observed that when a biosensor with the pristine conducting polymer was constructed it is hard to fix the enzyme molecules on the polymer coat due to the incompatibility of the enzyme with the hydrophobic polymer pendant groups. It was noted that without sepiolite, the enzyme molecules leached from the electrode surface due to the incompatibility of the enzyme molecules and polymer. Therefore, it was even impossible to keep the enzyme molecules on the electrode surface hence; no amperometric response could be recorded. Various solid matrices such as sepiolite, bentonite and chitosan have been considered for enzyme immobilization in order to construct biosensors [1].

On the other hand, some researchers reported that conducting and electrochemically generated polymers were used as immobilization matrices. By using these organic polymers, properties of constructed biosensors were improved [6,26]. Besides, due to the presence of aromatic and highly conjugated structure of PPHED,

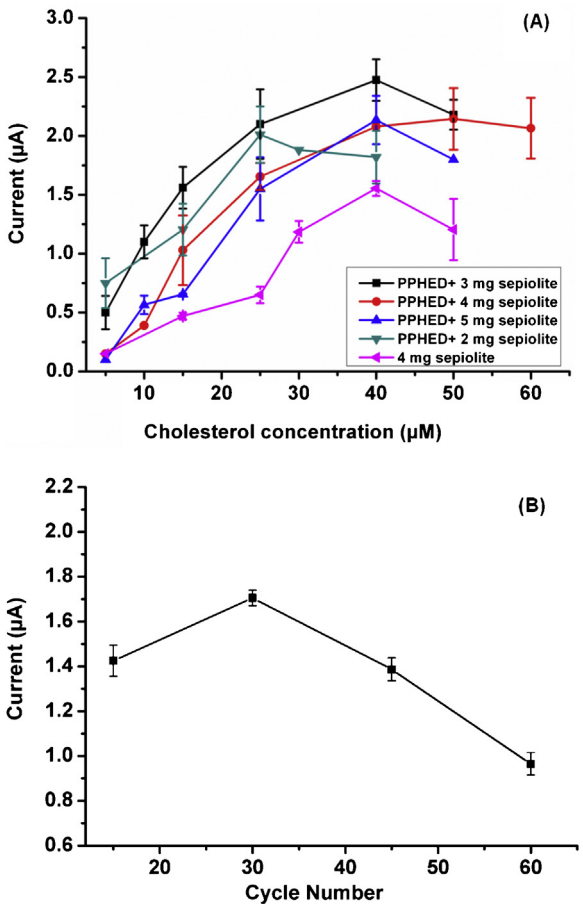


Fig. 2. (A) Effect of sepiolite amount (in phosphate buffer, 50 mM, pH 7.0, 25 °C, −0.7 V), (B) effect of cycle number (in phosphate buffer, 50 mM, pH 7.0, 25 μM cholesterol, 25 °C, −0.7 V). Error bars show standard deviation (SD) of three measurements.

tertiary structure of enzyme molecule is stabilized and enhanced via strong π–π stacking of aromatic groups in the structures of polymer backbone. Also, electrostatic and hydrophobic interactions, both from different protein residues and the involved particular surfaces, may contribute for good interaction between enzyme molecule and the sepiolite [27]. Combination of organic polymer and sepiolite improved the stability of enzyme molecules and well immobilization. This is confirmed by preparing a biosensor consisting only sepiolite as the support which came up with even worse response (Fig. 2).

Constructed biosensor was optimized with respect to sepiolite, enzyme amount and glutaraldehyde, pH and conducting polymer thickness. Since the sensitivity and stability of the biosensor change with the medium conditions, it is important to define optimum conditions for modified biosensor. The optimum electrode shows the best responses.

First, the amount of sepiolite in enzyme solution was optimized. For this optimization study, amperometric responses of the biosensors with different sepiolite amounts were investigated. In these experiments, amount of enzyme, crosslinker amount and scan number during polymerization (30 cycles) in biosensor construction were kept constant. Four different biosensors with polymer coated electrode were prepared and calibration curves for cholesterol were obtained. While increasing sepiolite amount, surface characteristics of biosensors were changed. Since sepiolite as a clay mineral is highly porous in nature, enzyme molecules may be trapped in sepiolite channels and three dimensional structures of the immobilized enzyme molecules were fixed. Hence, the amount of sepiolite improves the success of the immobilization and performance of the biosensor. With lower amounts of sepiolite, enzyme molecules could not be fixed properly onto PPHED coated electrode which resulted in lower responses. Accordingly, low concentrations of analyte could not be detected with these biosensors. Fig. 2(A) shows that the optimum sepiolite amount was found as 3 mg/5 mL in PBS (pH 7.0). Satisfactory immobilization of the enzyme molecules were achieved with the biosensor containing sepiolite amount of 3 mg. Hence, the best results were recorded with this sepiolite amount and this was kept as is in following studies.

The polymer thickness was optimized by adjusting the scan number during electropolymerization. When the thickness of polymer film increases, the stability and fine structure of a polymer film destroyed. On the contrary, very thin films may be unable to protect the enzyme molecule from the environmental effects and deteriorate the immobilization matrix characteristics. In order to investigate the effect of polymer layer thickness, polymerization was achieved on the graphite electrode with different scan numbers (15, 30, 45 and 60 scans) (Fig. 2(B)). The best responses were obtained by 30 cycle electropolymerization.

The effect of the enzyme amount on the amperometric response was also investigated in the presence of 25 μM cholesterol. For this optimization, five different biosensors were constructed with five different amounts of ChOx ranging from 21 U to 42 U. ChOx enzymes were immobilized on the modified electrode surface and optimum amount of enzyme was found as 1.5 mg. As illustrated in Fig. 3(A), an efficient interaction with enzyme molecules and sepiolite modified polymeric layer was not observed without the optimum amount of enzyme. Moreover, it was observed that with the excess loading of enzyme molecules, the enzyme layer removed from the sepiolite and polymeric layer. In addition, enzyme layer gets thicker which may bring diffusion problems and slow electron transfer on the electrode.

In order to detect the effect of glutaraldehyde (GA) used in biosensor construction, four different biosensors containing different amounts of GA (0.5, 1.0, 1.5 and 2.5% in PBS (pH 7.0)) were prepared. GA was used as a bifunctional cross-linking agent in sensor construction to improve the 3D structure of the enzyme molecules. Hence, enzyme molecules can be easily fixed to the electrode surface by the help of GA. The best responses were obtained with 1% GA.

Moreover, bioactivity is affected by the pH of the medium; hence the pH optimization was investigated using 50 mM phosphate buffer solution in a range of pH 6.0–7.5 and 50 mM sodium bicarbonate buffer at pH 8.0 in the presence of substrate (25 μM). For all the experiments, concentration of phosphate buffer concentration was used as 50 mM [15]. The experiments were performed with freshly prepared enzyme electrodes. Fig. 3(B) displays the effect of pH on amperometric response current of the biosensor. At high acidic or basic conditions enzyme molecules lose their activity. The optimum enzyme activity was found at pH 7.0.

3.2. Characterization

Surface morphologies of the polymer coated graphite electrode, sepiolite modified graphite electrode and ChOx immobilized graphite electrode were determined by SEM. In Fig. 4(A), the SEM image of the sepiolite showed typical fibrous shape contrary to the ones for other common clays. In Fig. 4(B–C), the image was taken after 30 cycles of electropolymerization on graphite. The surface morphology of the polymer film shows the porous, cauliflower and homogenous structure of the conducting polymer. Also, it was clearly shown that polymer was spread over the electrode surface homogeneously. The morphology of the surface changed drastically after immobilization of ChOx in Fig. 4(D). This change can be due to the fact that enzyme molecules hold on the surface of the

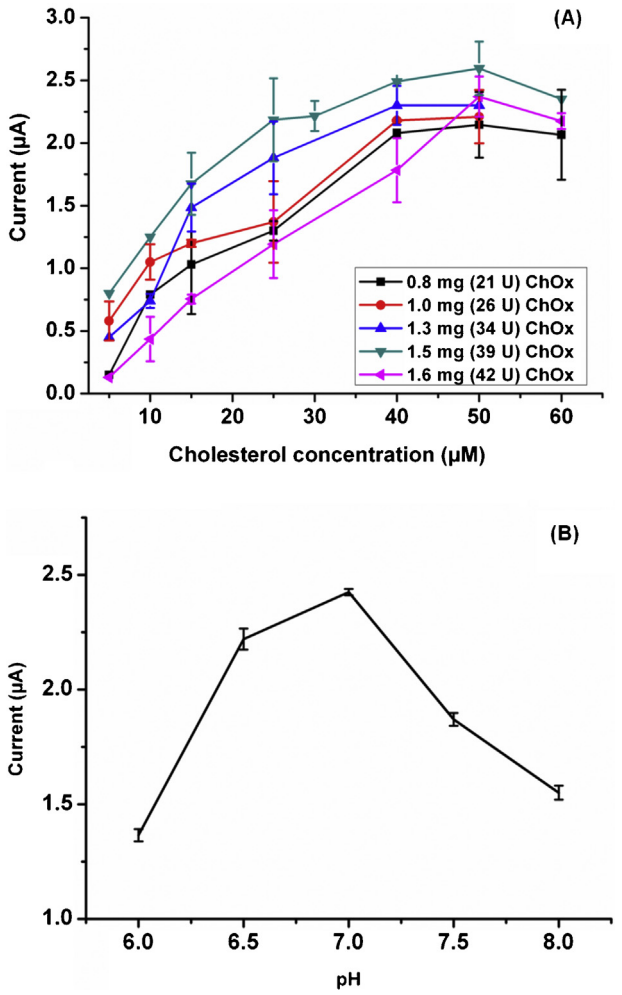


Fig. 3. (A) Effect of loaded enzyme amount (in phosphate buffer, 50 mM, pH 7.0, 25 °C, −0.7 V), (B) effect of pH (50 mM sodium phosphate buffer at pH 6.0; 6.5, 7.0; 7.5 and 50 mM sodium bicarbonate buffer at pH 8.0, 25 μM cholesterol, 25 °C, −0.7 V). Error bars show standard deviation (SD) of three measurements.

sepiolite. In addition penetration of enzyme molecules through the cavities of sepiolite may form electrostatic interactions between enzyme and PPHED. Moreover, it can be derived from the figure that the 3D structure of enzyme molecules were protected during the successful immobilization. This means that the enzyme was well-immobilized onto the modified electrode surface.

Table 1
Some characteristic of the proposed biosensor.

Parameter	
Linear range	0.0–40.0 μM
K_M^{app}	0.031 mM
I_{max}	6.06 μA
LOD	0.36 μM
Sensitivity	1.64 mA/mMcm^2

3.3. Analytical properties, repeatability and storage stability of the cholesterol biosensor

After all optimizations, kinetic parameters (K_M^{app}, I_{max}), from the Lineweaver–Burk plot and sensitivity were shown in Table 1. Calibration curve for cholesterol was plotted according to the responses of biosensor for different amounts of cholesterol (Fig. 5). The biosensor showed a very low limits of detection (LOD) value which was calculated using S/N (signal-to-noise ratio) = 3 criterion. K_M is the indication for affinity of enzyme molecule to substrate. In this study, it was found that K_M^{app} value is 0.031 mM for sepiolite modified ChOx biosensor. The K_M value of immobilized enzyme is lower than the free enzyme. This may be related to steric effect of the active site by the sepiolite and PPHED and conformational changes in the enzyme molecules. K_M^{app} is extremely smaller than the ones reported in earlier studies (Table 2). This shows the effectiveness and success of the immobilization matrix, sensitivity of the biosensor and applicability in other operations. In recent years, several studies related to conducting polymer based cholesterol biosensors reported in literature are summarized in Table 2.

As seen from Fig. 5, the biosensor shows fast response to substrate. This confirms the efficiency of the immobilization matrix. Moreover, it is proved that electron transfer through the matrix and the transducer is very fast which brings the high sensitivity and low detection limit (Table 3).

The lifetime of the modified electrode was determined by measuring the amperometric response during 28 days. During the experiments, activity loss of 15% was observed on the 28th day. For a cholesterol biosensor, this long period shows the reliability and superiority of the constructed biosensor. The biosensor was kept at +4 °C when not in use.

For the investigation of interference effects of some substances, such as ascorbic acid, urea and glucose were tested. Ascorbic acid, urea and glucose solutions (1 mM–0.1 M) were injected to reaction cell containing 50 mM PBS (pH 7.0). Under the experimental conditions, no interference effect was detected in the sample applications. Hence, the constructed biosensor was applied in various applications.

Table 2
Various studies from the literature involving cholesterol oxidase.

Conducting polymer	Immob. tech.	K_M/V_{max} or I_{max}	Linear range	Ref.
Poly(3,4-ethylenedioxypyrrole)	Entrapment	3.4 mM/ 34 μAcm^{-2}	NR	[28]
Poly(3,4-ethylenedioxythiophene)	Entrapment	1.3 mM/17.9 μAcm^{-2}	NR	[29]
Polypyrrole(Ppy)	Entrapment	9.8 mM/NR	1.0–8.0 mM	[30]
Poly(3-thiopheneacetic acid)	Covalent binding	NR	0.0–8.0 mM	[31]
P(NMPY)-PTS	Entrapment	4.35 mM/NR	2.0–12.0 mM	[32]
P(AAm-co-AA)/PEI	Covalent binding	2.72 mM/0.908 mM min^{-1}	NR	[3]
Au electrode	Adsorption	2.94 mM/0.66 μA	Up to 2.1 mM	[33]
ZnO/Au electrode	Adsorption	2.1 mM/NR	0.65–10.34 mM	[34]
Gold nanoprticle/carbon nanotube	Adsorption	0.29 mM/NR	0.01–5.0 mM	[35]
Pt/Au/ZnO nanorods	Adsorption	1.84 mM/NR	0.1–759.3 μM	[36]
PPHED-sepiolite	Adsorption	0.031 mM/6.06 μA	1.0–40.0 μM	This work

NR: not reported.

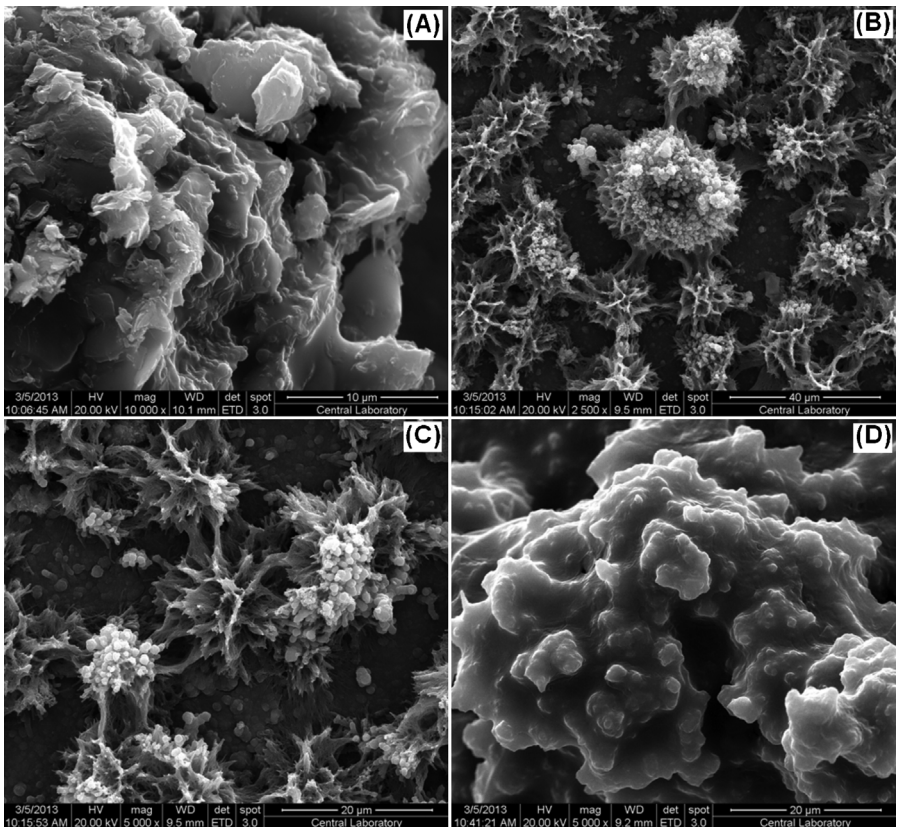


Fig. 4. Surface characteristics of (A) sepiolite (B) conducting polymer (PPHED) (with 2500× magnification) (C) PPHED (with 5000× magnification) (D) ChOx immobilized conducting polymer coated sepiolite modified via SEM images.

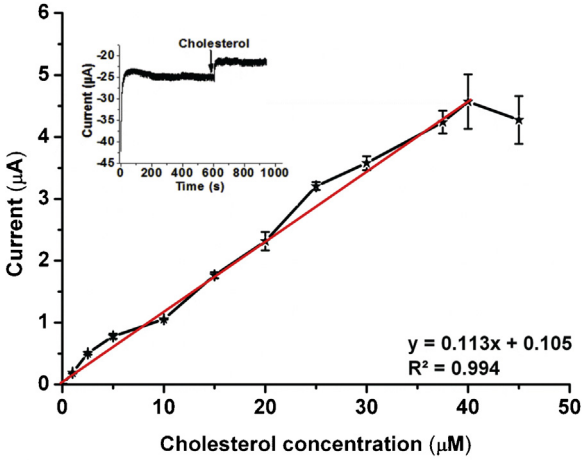


Fig. 5. Calibration curve for cholesterol (in 50 mM phosphate buffer, pH 7.0, 25 °C, −0.7 V). Error bars show standard deviation (SD) of three measurements. (A typical amperometric signal to 50 μM cholesterol in phosphate buffer, 50 mM, pH 7.0 given as inset).

Table 3
Determination of cholesterol in food samples by constructed biosensor and reference procedure.

Sample	Reference method (mM)	PPHED-sepiolite biosensor (mM)	Relative error (%)
Liquid oil	0.75 ± 0.120	0.67 ± 0.078	10.60
Margarine	3.66 ± 0.013	3.72 ± 0.085	1.64
Butter 1	2.21 ± 0.041	2.09 ± 0.113	5.42
Butter 2	2.68 ± 0.117	2.33 ± 0.007	13.06

3.4. Detection of cholesterol in food samples

The constructed biosensor was tested for food samples including butter, margarine and liquid oil. Determination of the cholesterol was done according to a previously described method [37]. 7.5 g of the samples were added to 1 M KOH solution in methanol. After the mixture was boiled under reflux for 30 min, centrifugation was done at 4000 rpm for 5 min and then the extracts were diluted to 50 mL with ethyl acetate. These food samples were injected (as the substrate) into 50 mM PBS (pH 7.0) for the amperometric measurement and current change was monitored. Also, these food samples were analyzed for cholesterol by using cholesterol kit as the reference method. It was clearly shown that amperometric results and reference method were very close to each other.

4. Conclusions

A novel sepiolite modified conducting polymer based highly sensitive and fast response amperometric cholesterol biosensor was developed successfully. After combination of conducting polymer and sepiolite on the electrode surface, the quality of the immobilization of the enzymes and stability of the biosensor were enhanced. Surface characteristic of this constructed biosensor was confirmed by SEM. The fabricated biosensor exhibits excellent kinetic parameters such as K_M^{app}, I_{max}, low LOD and high stability. Hence, the biosensor was successfully applied for the detection of cholesterol in food samples.

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