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Flower like Bi structures on Pt surface facilitating effective cholesterol biosensing

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ABSTRACT

This work demonstrates effective biosensing of cholesterol with the help of an efficient inorganic H_2O_2 transducer based on Pt-Bi combined with the organic enzyme platform. It could be shown that the Bi (bismuth) adatoms modified Pt (platinum) surface displays enhanced catalytic oxidation of H_2O_2 at neutral pH and the catalytic oxidation of H_2O_2 occurs at a lower potential of 0.25 V vs NCE (normal calomel electrode). The sensing platform is highly sensitive and shows linear response towards [H_2O_2] in the absence of any redox mediator or enzyme. The H_2O_2 sensing platform, further modified with cholesterol oxidase led to cholesterol biosensing with a sensitivity of 3.41 μA mM^{-1} cm^{-2}. The apparent Michaelis-Menten constant (K_m^{app}) was calculated to be 0.43 mM which indicates high binding affinity with the substrate. The cholesterol biosensor does not suffer from the interferences due to other common electroactive species and is highly stable.

1. Introduction

Coronary heart disease is the main cause of death worldwide which is due to the accumulation of excess cholesterol in the vasculature. As a result cholesterol has become one of the main parameters which are measured in routine clinical laboratory testing. So quantification of blood cholesterol is important for the diagnosis of various life threatening diseases. The normal concentration of cholesterol in a healthy human serum is about 5.17 mM, in which 30% is present as sterol and 70% is esterified with fatty acids [1], along with interfering species such as ascorbic acid, uric acid and glucose. Usually cholesterol is determined by employing nonenzymatic spectrophotometric techniques using colour reagents. These methods involve difficult procedures for the precipitation of lipoproteins which are expensive and lack specificity and stability. Hence the development of a simple, specific and highly sensitive cholesterol biosensor is essential for the regular examination of cholesterol levels in blood. For ensuring selectivity of detection, usually enzymes are being used in most of the techniques. In the fabrication of a cholesterol biosensor, cholesterol oxidase (ChOx) will be used as the biosensing element. Cholesterol oxidase catalyzes the oxidation of cholesterol to cholest-4-en-3-one and H_2O_2 in the presence of oxygen. The concentration of cholesterol can be quantified by following the concentration of either H_2O_2 generated or oxygen consumed during the enzymatic reaction. Since the monitoring of the concentration of oxygen is difficult the concentration of H_2O_2 is preferred as the precise method. Enzymatically generated H_2O_2 can be electrochemically quantified either by its reduction or oxidation. The monitoring of H_2O_2 oxidation would be convenient if the modified electrode surface can enhance the oxidation at favourable potential without any interference from other coexisting analytes. Also it would be appropriate to find a material that can work at neutral pH as we want to propose an enzyme based cholesterol sensor. Platinum is a well-known catalyst for the electrooxidation of H_2O_2. According to Hall et al. the bare macro Pt electrode shows H_2O_2 oxidation at a potential of 0.7 V vs Ag/AgCl [2]. This is a great drawback, since at this high overpotential many electroactive substances, e.g., ascorbic acid (AA), and uric acid (UA), which are usually present in real samples, may be oxidized to give interfering signals. Hence the objective of this work is to find a bimetallic, catalytic electrode that can exhibit high electrocatalytic activity towards the oxidation of H_2O_2 at lower overpotentials. It is known that bimetallic electrodes obtained either by underpotential deposition (upd) of foreign metal ad-atoms or by metal alloying exhibit enhanced catalytic activity for the oxidation of small molecules and the active sites of these surfaces are not fouled by reactants, intermediates or products. Foreign metal adatoms that can irreversibly adsorb onto noble metal electrode substrates have been employed both to catalyze the electrooxidation processes and/or prevent the poison intermediate pathway. The enhancement of electrocatalytic efficiency of noble metals by foreign metal adatoms such as As, Sb, Te, Pb, Cd, Tl, and Bi, depends upon the specific crystallographic orientation of the platinum substrate, adatoms employed, and type of functional groups involved in the electrocatalytic process [3–12]. Among them, bismuth adatoms significantly enhance the catalytic activity of single crystalline [4,10], or polycrystalline platinum electrodes [13,11] towards the electrooxidation of formic acid, glucose, and other small aliphatic alcohols [14] in alkaline

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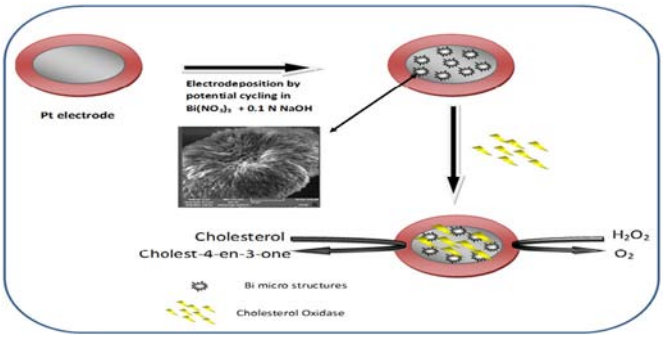

Scheme 1. Schematic representation of cholesterol biosensor fabrication based on Bi micro structures on Pt electrode.

conditions. In addition, bismuth adsorbs irreversibly onto the platinum electrode surface and the resulting adlayer is sufficiently stable during potentiodynamic experiments [5]. In this work we explore the catalytic activity of Pt enhanced by monolayer of Bi in neutral pH for the catalytic oxidation of H_2O_2 which is subsequently combined with the enzymatic oxidation of cholesterol for the development of a stable cholesterol biosensor. Various modifying approaches based on conducting polymers, metal oxides and ferrocene like mediators have been employed for H_2O_2 transduction in combination with cholesterol oxidase for the detection of cholesterol [15–21]. However these approaches have resulted in higher overpotential with most of them offering only low sensitivity of detection. Also it is seen from the literature that the enhanced catalytic activity of Bi adlayers are well investigated only in alkaline medium for the catalytic oxidation of carbohydrates, alcohols etc. which is not suitable for enzyme based biological applications. For the first time the high catalytic activity of Bi adlayers on Pt in neutral pH, and the feasibility of developing a highly sensitive and selective cholesterol biosensor is described in this work (Scheme 1). It has been demonstrated that the Bi adlayers lowers the overpotential for the oxidation of H_2O_2 by blocking some of the active sites of Pt and enhancing the catalytic activity. Also the Bi adlayers exhibit an interesting flower like morphology along with its good catalytic activity.

2. Experimental

2.1. Materials and reagents

Solutions were prepared from analytical-reagent grade chemicals without further purification by using Millipore water (Milli-Q system). Sodium hydroxide pellets (99%), bismuth (III) nitrate pentahydrate, hydrogen peroxide (30%), cholesterol, cholesterol oxidase (from *Streptomyces* sp., 25 units/mg), Nafion (5 wt% solution in lower aliphatic alcohols), were obtained from Sigma Aldrich. Phosphate buffer solution (PBS, 0.1 M) of pH 7.2 was used as supporting electrolyte for all voltammetric and amperometric measurements. A stock standard of 200 μM $Bi(NO_3)_3 \cdot 5H_2O$ was prepared by dissolving the relevant amount of the salt in 50 mM $HClO_4$ solution. The electrolyte solutions containing Bi(III) species were prepared in 0.1 M NaOH. The stock solution of cholesterol was prepared in deionized water having 10% Triton X-100. All experiments were carried out at room temperature.

2.2. Instrumentation

The voltammetric and chronoamperometric experiments were performed with an Autolab PGSTAT 30 Potentiostat/Galvanostat (Eco Chemie, Utrecht, The Netherlands) and the data were acquired using Autolab GPES software package version 4.8. Cyclic voltammetry (CV) was carried out in a three-electrode cell using a polycrystalline platinum electrode modified with an adlayer of bismuth as a working electrode, a normal calomel (NCE) reference electrode and a platinum foil counter-electrode. The polycrystalline platinum working electrode was purchased from BAS (USA). All impedance measurements were carried out using PG-STAT 12/30/302 Autolab potentiostat/galvanostat using FRA software. Impedance measurements were carried out using an ac signal of 10 mV amplitude at the formal potential of the redox probe using a wide frequency range from 100 kHz to 0.1 Hz. The electrolyte solution containing equal concentrations of both the oxidized and reduced forms was employed for study.

SEM images of the electrode surface were taken using Hitachi S3000-H, Japan and images were recorded at 25 kV using secondary electron detector. A platinum foil was used as the deposition platform.

X ray photoelectron spectroscopy (XPS) of the modified electrode was recorded using Multilab-2000 (Thermo Scientific). The modified electrode was affixed to the sample holder. All spectra were recorded using an X-ray source (Al Kα radiation) with a scan range of 0–1200 eV binding energy. The collected high-resolution XPS spectra were analysed using the XPS peak fitting software program. The energy scale has been adjusted on the carbon peak (C 1s) spectra at 284.5 eV.

2.3. Electrode modification

Surface modification of the Pt electrode with adsorbed bismuth was carried out using a slightly modified reported procedure [10]. Electrochemical cycling and irreversible adsorption were attempted for electrode modification with Bi. During irreversible adsorption, the bare Pt electrode is simply dipped in Bi ions solution for different time intervals. In case of Bi modification by electrochemical cycling, the electrode was subjected to potential cycling (50 mV/s) between −1 and 1 V vs NCE in non-deaerated 0.1 M NaOH solution containing 10–50 μM Bi(III). The resulting modified electrodes were then rinsed with deionized water and then transferred to an electrochemical cell containing the test solution free of Bi(III) ions. Thus formed Bi adlayer was stable in neutral medium when the modification was carried out by electrochemical cycling and hence this method was chosen for further studies. The Bi modified

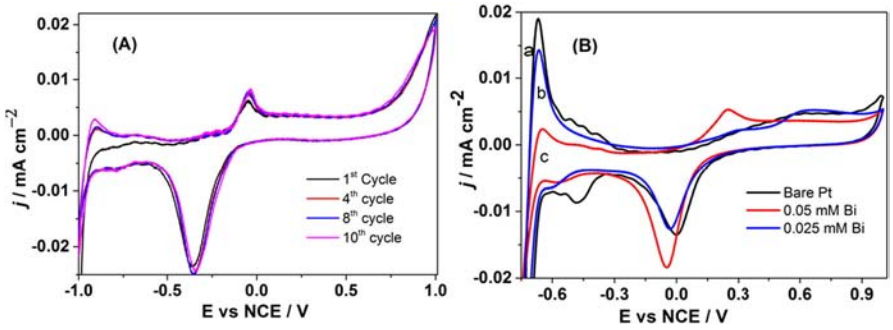

Fig. 1. (A) Cyclic voltammograms showing the formation of Bi adlayers during potential cycling in 0.1 M NaOH at 50 mV/s. (B) Cyclic voltammograms showing the voltammetric response of a) unmodified Pt, b) Bi-Pt modified electrode prepared with [Bi] = 0.025 mM, and c) Bi-Pt modified electrode prepared with [Bi] = 0.05 mM in neural pH (phosphate buffer pH = 7.0).

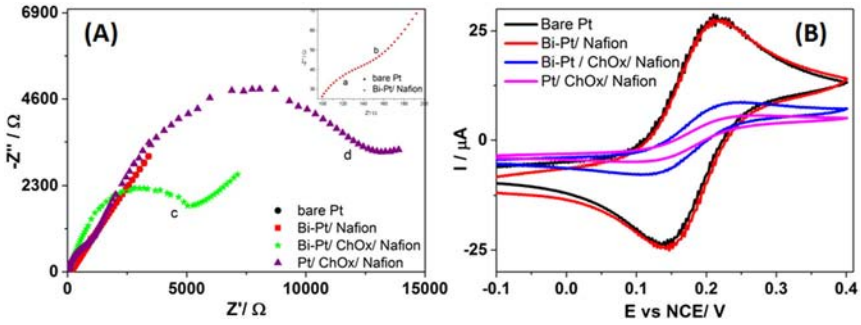

Fig. 2. (A) EIS of (a) bare Pt, (b) Bi-Pt/Nafion, (c) Bi-Pt/ChOx/Nafion and (d) Pt/ChOx/Nafion in 0.1 M KCl containing 5 mM $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ solution and (B) CVs of (a) bare Pt, (b) Bi-Pt/Nafion, (c) Bi-Pt/ChOx/Nafion and (d) Pt/ChOx/Nafion in 0.1 M KCl containing 5 mM $K_3[Fe(CN)_6]$ solution.

Pt electrode was covered with an overlayer of Nafion by drop casting 5 µl of 0.05% ethanolic solution to remove the effect of interferences during H_2O_2 sensing. For the development of cholesterol biosensor, cholesterol oxidase (ChOx) enzyme was immobilized by dropcasting 5 µl of ChOx (1 mg/ml in PBS) on the Bi–Pt electrode. To prevent the leaching out of ChOx, Nafion was coated over the enzyme layer. The resulting biosensor was denoted as Bi- Pt/ChOx/Nafion.

3. Results & discussion

3.1. Electrochemical characterization of Bi adlayers

Cyclic voltammetry was used to show the presence of adlayers of Bi and to compare the catalytic activity of unmodified and modified polycrystalline Pt with bismuth adlayers. Subsequent to the transfer of Pt electrode into the 0.1 M NaOH solution containing Bi(III) ions, signals develop for the oxidation of the Bi layer during potential cycling (Fig. 1(A)). The reduction signal corresponding to Bi overlaps with the platinum oxide reduction. Since the signals for the Bi adlayer increases during cycling until saturation is reached, it can be concluded that Bi species remain confined to the Pt electrode even after oxidation of the Bi adlayer.

The electrochemical response for Bi layer remains stable in neutral medium as shown in Fig. 1(B) and it is also observed that the Bi modification depends on the initial concentration of Bi used for deposition. At low concentrations (0.025 mM) the peaks corresponding to Bi and Pt oxide formation are seen separately at 0.27 V and 0.6 V respectively. As the concentration of Bi increases to 0.05 mM, Bi oxidation becomes predominant and the oxidation peak occurs at 0.25 V. Also the current due to Pt oxide decreases indicating the replacement of oxide sites by Bi. The comparison of catalytic activities of Bi modified Pt electrodes prepared at these two concentrations of Bi (0.025 and 0.05 mM Bi) showed that the electrode modified with 0.025 mM Bi exhibited higher catalytic activity. Hence further experiments have been conducted with this composition. Compared to bare Pt electrode hydrogen adsorption region of the Bi modified electrode shows a decrease indicating probably the hydrogen adsorption sites are covered by Bi ad atoms. Surface coverage of the Bi modified electrode was calculated using the following equation [22].

$$\Gamma = \frac{Q}{nFA} \quad (1)$$

where Γ is the surface coverage on the electrode, Q is the charge measured by integrating the area under the oxidation peak, n is the number of electrons, F is the Faraday's constant and A is the geometric area of electrode used for the modification. The surface coverage was found to be 4.840×10^{-12} mol cm^{-2} showing the presence of monolayer of Bi over Pt.

The characterization of the modified electrode in $K_4[Fe(CN)_6]$/$K_3[Fe(CN)_6]$ solution using electrochemical impedance spectroscopy (EIS) and CV was carried out (Fig. 2A & B). Electrochemical impedance spectroscopy (EIS) is an effective tool for studying the interfacial properties of surface-modified electrodes as a function of frequency. EIS spectra provide the value of charge transfer resistance (R_{CT}) that indicates the electron-transfer kinetics of redox probe at the electrode interface. The modification of electrode surface results in change in the value of R_{CT}. Fig. 2(A) shows the impedance spectra of bare Pt, Bi-Pt/Nafion, Bi-Pt/ChOx/Nafion, and Pt/ChOx/Nafion. The Nyquist diagrams of bare Pt and Bi-Pt/Nafion exhibit almost similar R_{CT} of 163 Ω. This indicates that the Bi adlayer does not influence the charge transfer process. The curves c and d represent the ChOx enzyme modified Bi-Pt and bare Pt respectively. The Pt/ChOx/Nafion shows two overlapping semicircles with R_{CT} values of 1525 Ω and 10,000 Ω. The lower R_{CT} may be due to the enzyme free active sites of Pt and higher R_{CT} represents the insulating characteristics of ChOx enzyme. Bi-Pt/ChOx/Nafion exhibit R_{CT} of 5182 Ω and it is lower compared to Pt/ChOx/Nafion. This reveals that Bi-Pt provides better environment for ChOx loading resulting in an enhanced electron transfer between the electrode and medium, compared to bare Pt.

Fig. 2(B) presents the results of cyclic voltammetric (CV) studies of Pt, Bi-Pt/Nafion, Bi-Pt/ChOx/Nafion, and Pt/ChOx/Nafion in 0.1 M KCl

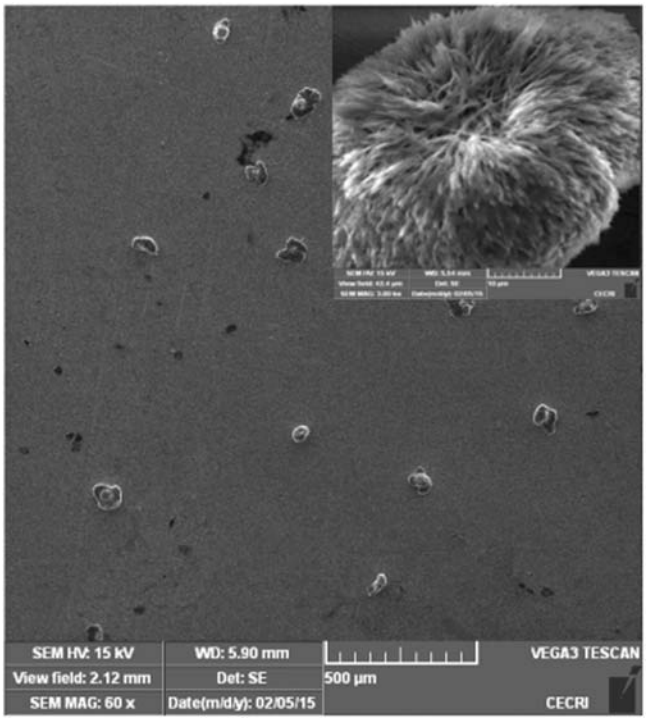

Fig. 3. SEM image of Bi-Pt electrode showing the uniform distribution of Bi microflowers on the Pt surface. Inset flower like structure of Bi.

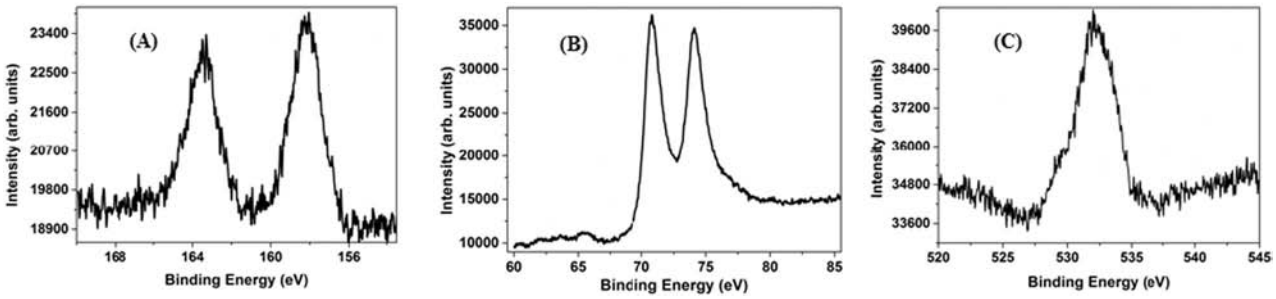

Fig. 4. XPS data of (A) Bi4f for bismuth, (B) Pt 4f for platinum, and (C) O 1s for oxygen.

containing 5 mM $[Fe(CN)_6]^{3-}$ at 50 mV/s. The oxidation peak current obtained for ferricyanide electron transfer is 27.7 μA for the bare Pt electrode and is almost the same (27.2 μA) for Bi-Pt/Nafion electrode. The Bi-Pt/ChOx/Nafion and Pt/ChOx/Nafion electrode show an oxidation peak current of 8.2 μA and 5.1 μA respectively which indicates that after immobilization of ChOx, Bi-Pt modified electrode displays a higher current response resulting in enhanced electron transport between enzyme and electrode due to the synergetic effect of Bi and Pt. The electrochemical active area (A_e), of the bare Pt and Bi-Pt/Nafion electrodes calculated from the anodic peak current, i_{pa}, as per the Randles-Sevcik equation is given by (Eq. (2))

$$i_{pa} = \left(2.65 \times 10^5\right) A_e n^{3/2} D^{1/2} C^* \nu^{1/2} \quad (2)$$

where n the number of electrons participating in the redox process, is 1 for $[Fe(CN)_6]^{3-/4-}$ redox system, C* is the concentration of ferricyanide (5×10^{-3} M), D is the diffusion coefficient of potassium ferricyanide (7.6×10^{-6} cm^2 s^{-1}) and ν is the scan rate (50 mV/s). The calculated surface areas of the bare Pt electrode and Bi-Pt/Nafion electrode are 0.034 cm^2, 0.033 cm^2 respectively.

3.2. Structural and morphological characterization

The structural and morphological characteristics of the Bi-modified Pt electrode surface was analysed by SEM and XPS. The SEM images indicate that bismuth adlayers are uniformly distributed over the polycrystalline Pt surface in the shape of flowers (see Fig. 3 and the inset). Each flower like structure of Bi is nearly 40 to 50 μm in dimensions.

XPS data confirm the presence of bismuth (Bi4f), oxygen (O1s), carbon (C1s), and platinum (Pt4f). The Bi4f spectrum shows a doublet consisting of a low energy band ($Bi4f_{7/2}$) and a high energy band ($Bi4f_{5/2}$) (Fig. 4(A)).Typically the binding energies of $4f_{7/2}$ level for the metallic Bi range from 156.6 to 157.1 eV. The one observed by us appears at 158.3 eV. The higher BE of Bi monolayer on a Pt substrate compared to bulk Bi can be understood as a consequence of an electronic charge transfer to the Pt metal [10].

Subsequently, the Pt4f spectra display a doublet of a high energy band ($Pt4f_{5/2}$) and low energy band ($Pt4f_{7/2}$) (Fig. 4(B)). The presence of the two peaks indicates that Pt is present in two different oxidation states, Pt(0) and Pt(II). However, the lower BE peak clearly dominates and can be attributed to metallic Pt, while the higher BE peak can be related to PtO [23].

The XPS peak appearing between 525 and 540 eV recorded with the Bi-Pt electrode is shown in Fig. 4(C). We observe a peak at 531.8 eV. This peak can be assigned to binding energy of O1s of oxygen. The shift from 529.2 eV to 531.8 eV may be associated with the oxidation of Bi adlayer on the modified electrode surface. The above discussions demonstrate that the adatoms of bismuth on Pt electrode surface may exist in various oxidized states.

3.3. Electrochemical detection of H_2O_2

Usually oxidase-based enzymes catalyze the oxidation of corresponding substrates in the presence of molecular oxygen resulting in the generation of H_2O_2 that can undergo electrochemical oxidation and the current produced due to oxidation is directly proportional to the analyte concentration. Hence any electrochemical system which is highly sensitive towards H_2O_2 oxidation can be used as an efficient platform in the development of amperometric oxidase based biosensors. To examine the response of the modified Bi-Pt electrode against H_2O_2, we have initially conducted the voltammetric and amperometric experiments. The Bi-Pt electrode shows voltammetric response for the oxidation of H_2O_2 at the potential of 0.25 V as shown in Fig. 5(A), which is significantly less positive than that of the bare Pt electrode. While increasing the concentration of H_2O_2, gradual increase in the voltammetric peak current was noticed. The oxidation of H_2O_2 occurs at a higher potential on bare Pt electrode and the voltammetric response increased only for few additions of H_2O_2. The voltammetric response is very feeble and occurs at higher potential possibly due to the

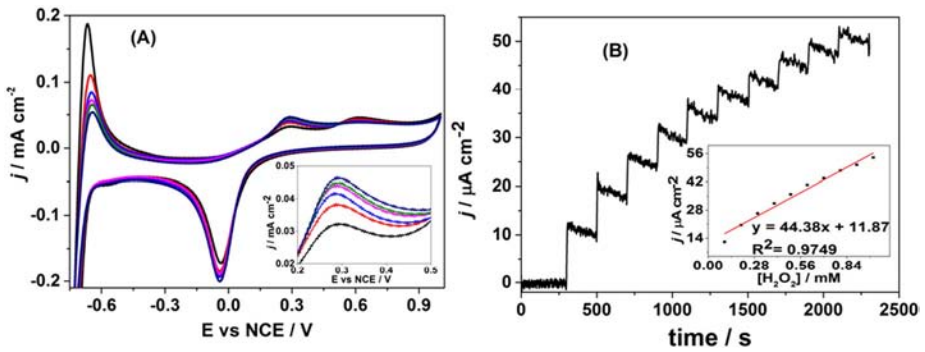

Fig. 5. (A) Cyclic voltammetric response for the oxidation of H_2O_2 on the Bi-Pt electrode in PBS. Inset shows the increasing oxidation current signals with increasing H_2O_2concentrations. (B) Amperometric response of H_2O_2 with the Bi-Pt electrode. Aliquots of 0.1 mM H_2O_2 were added into the stirred supporting electrolyte (PBS, pH 7). The potential of the electrode was held at 250 mV. The inset is the corresponding calibration plot.

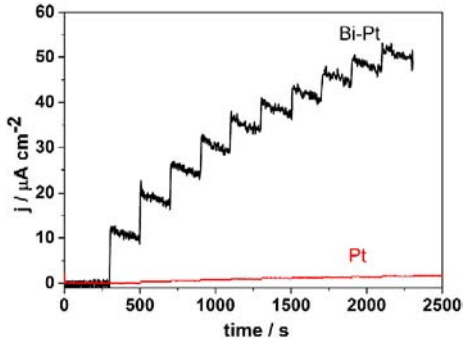

Fig. 6. Comparison of amperometric response of H_2O_2 with the Bi-Pt electrode in PBS, pH 7 at 250 mV and Pt electrode in PBS, pH 7 at 300 mV.

passivating layer (figure not shown). The influence of Bi ad atoms on the oxidation of H_2O_2 was reflected in the enhanced oxidation current and shift in the peak potential by 50 mV in the negative direction.

The performance of the Bi-Pt electrode was further analysed by measuring the amperometric response against H_2O_2. Fig. 5(B) is the amperometric response obtained for the oxidation of H_2O_2 on the Bi-Pt electrode. The electrode potential was held at 0.25 V, and aliquots of H_2O_2 were added at regular intervals to the stirring supporting electrolyte solution. Stable amperometric response was obtained upon sequential additions of H_2O_2. The sensitivity of detection obtained was 44.38 μA mM^{-1} cm^{-2}. The linear range of detection obtained in this case is from 0.1 mM to 1 mM. In the case of bare Pt electrode, the amperometric experiment can be carried out only at a higher potential of 0.3 V and it shows a sensitivity of only 1.4 μA mM^{-1} cm^{-2} (see Supplementary Fig. 1).

The amperometric response on the Bi-Pt electrode is nearly 30 times higher than that of the bare Pt electrode highlighting the electrocatalytic activity of the Bi ad atoms on platinum (Fig. 6). The high sensitivity and low detection limit can be ascribed to the enhanced catalytic activity exhibited by flower like Bi microstructures on Pt. It should be highlighted here that the electrode shows a very fast response time of 3 s which indicates the good catalytic activity of the ad-atom modified Pt electrode assisting the electron-transfer kinetics for the oxidation of H_2O_2. Bi-Pt electrode can also be used to sense H_2O_2 over different concentration ranges varying from micromolar to tens of millimolar levels in the ranges, 12.5 μM–200 μM and 0.1–16 mM (see Supplementary Figs. 2 and 3). It shows a sensitivity of 7.9 μA mM^{-1} cm^{-2} at micromolar levels and 9.5 μA mM^{-1} cm^{-2} at millimolar levels of concentration H_2O_2.

In Section 3.1 it has been shown clearly that the electrochemical surface area for the Pt and Bi-Pt electrode are almost the same by carrying out voltammetric experiments with the redox probe potassium ferricyanide. Hence it can be concluded that the higher catalytic activity of Bi-Pt electrode for the oxidation of H_2O_2 is only due to the synergetic effect of free Pt sites and Bi ad atoms. During the preparation of Bi-Pt electrode it has been observed that a lower concentration of Bi (Section 3.1) equal to 0.025 mM led to partial replacement of Pt active sites and exhibited higher catalytic activity. Further Bi adlayer exhibited an interesting flower like morphology. The synergistic effect of the bimetallic Bi-Pt electrode helps in decreasing the overpotential of the H_2O_2 oxidation besides leading to a highly sensitive detection. Further Bi adlayer modified Pt electrode is found to be stable and catalytically active at neutral pH.

To study the selectivity of the modified electrode, the amperometric responses were measured for the common analytes such as ascorbic acid, uric acid and glucose along with H_2O_2. The oxidation of ascorbic acid and H_2O_2 on the unmodified electrode occurs at similar potentials, and the oxidation of uric acid occurs at a higher value. The coexistence of these analytes is expected to interfere in the measurement of H_2O_2. Obviously, Bi-Pt modified electrode doesn't exhibit any interference due to uric acid and in the case of ascorbic acid there was no interference up to 0.2 mM level. Further elimination of ascorbic acid could be achieved by an overlayer of Nafion on Bi-Pt modified electrode (see Supplementary Fig. 4). The Nafion used as a binder in the modification of the Bi-Pt electrode, would effectively hamper the negatively charged ascorbate and urate by electrostatic repulsion. The Bi-Pt electrode is highly stable, and the same electrode can be used for repeated measurements.

3.4. Amperometric detection of cholesterol

Since the Bi-Pt electrode shows high sensitivity and low detection limit towards H_2O_2, a cholesterol biosensor was developed with an immobilized layer of the enzyme cholesterol oxidase. Fig. 7(A) is the amperometric response obtained for the sensing of cholesterol. Stable amperometric response was obtained for cholesterol which exhibits a fast response time (4.2 s). The facile access of the sensing platform for the reactant favours a good amperometric response. The sensitivity and linear range of cholesterol detection obtained in this case were 3.41 μA mM^{-1} cm^{-2} and 0.05 mM to 22 mM respectively.

The apparent Michaelis-Menten constant (K_m^{app}) for cholesterol oxidase was calculated using a Lineweaver-Burk plot (see Supplementary Fig. S5) according to the following equation (Eq. (3)):

$$\frac{1}{I} = \frac{I}{I_{max}} + \frac{K_m^{app}}{I_{max[S]}} \tag{3}$$

where I is the steady-state current after addition of cholesterol, I_{max} is the maximum current obtained at saturated level of substrate, and [S] is the concentration of substrate. The graphical analysis of the Lineweaver-Burk plot yields the value of K_m^{app} as 0.4 mM for cholesterol. It may be noted that the K_m^{app} value strongly depends on the nature of the immobilization matrix and the immobilization method. The value

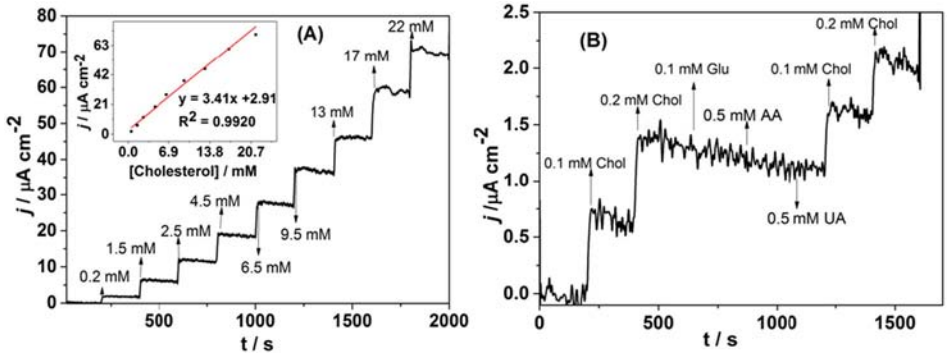

Fig. 7. (A) Amperometric response of cholesterol with the Bi-Pt/ChOx/Nafion electrode for successive additions of cholesterol into the stirred supporting electrolyte (PBS, pH 7) at 250 mV. The inset is the corresponding calibration plot. (B) Amperometric response showing the interference free sensing of cholesterol. Different concentrations of cholesterol (Chol), ascorbic acid (AA), uric acid (UA) and glucose (Glu) were injected during the amperometric experiment.

Table 1
Comparison of the analytical performance of different cholesterol biosensors based on cholesterol oxidase enzyme.

Immobilization matrix	Applied potential (vs Ag/AgCl)	Sensitivity ($\mu A\ mM^{-1}\ cm^{-2}$)	K_m (mM)	Response time (s)	Detection range (mM)	Detection limit (μM)	Ref
Overoxidized py/Pt	0.7 V	0.574	0.53[a]	–	0.025–0.3	5.7	[14]
PPy-p(HEMA)-TEGDA/Pt	0.7 V	0.019	–	30	0.5–15	120	[15]
Pol pyrrole/poly(1,5-diamino-polynaphthalene)	0.7 V	0.449	0.49[a]	10.6	Up to 2.25	9.7	[16]
Pt/poly(3-TPAA)	0.7 V	449.0	14.53[a]	70–90	0–8	420	[17]
NS-CeO_2/ITO	0.5 V	79.0	2.08[b]	15	0.55–22	–	[18]
Si/Ag/ZNT/	0.5 V	79.4	0.25[b]	2	0.001–13	–	[19]
CoOx/GC	0.8 V	0.043	0.49	15	Up to 50	4.2	[20]
MWCNTs/Pt–Au@ZnONRs	0.4 V	0.213	1.84[a]	15	0.27–16	0.27	[21]
Bi-Pt/Nafion	0.3 V	3.41	0.43[b]	4.2	0.05–22	50	This work

[a] *Pseudomonas fluorescens* (source of cholesterol oxidase).
[b] *Streptomyces species* (source of cholesterol oxidase).

obtained with the Bi-Pt biosensor for cholesterol oxidase is lower than those reported in the literature (Table 1).The lower value implies the strong affinity of ChOx towards cholesterol, indicating a facilitated enzymatic reaction. Effect of interferences arising from analytes such as glucose, ascorbic acid, uric acid were analysed by amperometry and the results presented in Fig. 7(B) shows the anti-interference capability of Bi-Pt/ChOx/Nafion electrode.

The performance of this biosensor is excellent with respect to the existing Pt and Pt nanoparticle-based biosensors for cholesterol. Table 1 shows a comparison of the performance of the Bi-Pt electrode reported in this work with that of the available literature reports. Several modifications using polymeric materials, metal oxides, carbon nanotubes have been used in the available literature. This work reports for the first time the enhanced catalytic activity of H_2O_2 which has led to a highly sensitive detection of cholesterol in neutral medium using Bi-Pt electrode. Cholesterol oxidase from two different sources viz., *Pseudomonas fluorescens* and *Streptomyces* species are usually used in the literature reports. In the present work cholesterol oxidase isolated from *Streptomyces* species has been used for all the experiments. Comparison of the K_m values in Table 1 reveals that low values of K_m ranging from 0.25 to 0.53 has been reported with enzymes obtained from both the sources. However K_m value has been found to depend upon the immobilization matrix and the electrode modification. In this respect overoxidized poly pyrrole modified electrodes, zinc nanotubes on silver sputtered silicon substrates and Bi-Pt electrodes have provided better environment for enzyme immobilization with favourable conformational changes leading to increased interaction between the substrate and the active sites of the enzymes leading to lower K_m values. However the comparison of the overpotentials reveals that higher overpotentials for H_2O_2 oxidation has been observed in cases with lower K_m values [14,16,19,20]. Silver sputtered Silicon substrates with zinc nanotubes score high in terms of K_m value and score low in terms of overpotential. Bi-Pt electrode scores better in terms of K_m value and overpotential. Comparison of the response times reveals that Bi-Pt electrodes exhibit a low response time. Shortest response time has been observed in the case of silver sputtered silicon substrates with zinc nanotubes. The sensitivity of Bi-Pt electrode is lower compared to references 17–19. However it exhibits a wide linear range and relatively a lower limit of detection. The enzyme modified electrode is stable and can be used for repeated measurements. The sensitivity of detection is decreased by 8% after four days. Comparison of overall performance reveals that Bi-Pt electrodes are more promising for biosensing of cholesterol.

4. Conclusions

The enhanced catalytic activity of Pt modified with Bi ad layers and the use of this electrode for a sensitive detection of cholesterol are demonstrated in this paper. The success of a biosensor is seen by its satisfactory performance at biological pH where the enzyme would remain stable. The Bi adatoms present on Pt electrode blocks few active site of Pt and enhances the catalytic activity of Pt and lowers the over potential. Also the Bi adlayers on Pt present an interesting flower like morphology along with the high catalytic activity at neutral pH. The H_2O_2 sensing platform along with the immobilized cholesterol oxidase presents an effective matrix for cholesterol biosensing at the physiological levels and is found to be free from interferences. The overall performance characteristics of the biosensor are found to be superior when compared with the literature reports.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2016.03.075.

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