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Development of a stable cholesterol biosensor based on multi-walled carbon nanotubes–gold nanoparticles composite covered with a layer of chitosan–room-temperature ionic liquid network

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ABSTRACT

A novel amperometric biosensor was fabricated based on the immobilization of cholesterol oxidase (ChOx) into a cross-linked matrix of chitosan (Chi)–room-temperature ionic liquid (IL) (1-butyl-3-methylimidazolium tetrafluoroborate). Initially, the surface of bare electrode (indium tin oxide coated glass) was modified with the electrodeposition of Au particles onto thiol (–SH) functionalized multi-walled carbon nanotubes (MWNTs). The biosensor electrode is designated as MWNT(SH)–Au/Chi–IL/ChOx. Scanning electron microscopy image of MWNT(SH)–Au/Chi–IL/ChOx reveals that Chi–IL exists as the interconnected wires covering the Au particles on the surface of MWNT(SH)–Au. Cyclic voltammetry and chronoamperometry were used for the electrochemical determination of cholesterol at the biosensor electrode, MWNT(SH)–Au/Chi–IL/ChOx. The presence of Au particles in the matrix of CNTs provides an environment for the enhanced electrocatalytic activities. The MWNT(SH)–Au/Chi–IL/ChOx biosensor exhibited a linear response to cholesterol in the concentration range of 0.5–5 mM with a correlation coefficient of 0.998, good sensitivity (200 $\mu A\,M^{-1}$), a low response time (~7 s), repeatability (R.S.D value of 1.9%) and long term stability (20 days with a decrease of 5% response). The synergistic influence of MWNT(SH), Au particles, Chi and IL contributes to the excellent performance for the biosensor.

1. Introduction

Cholesterol is a structural component of biological membranes. It is present in nerve tissues, brain, skin, adrenal glands and liver and is the main ingredient in the fatty sheet that insulates the nerve. Determination of cholesterol level in human blood receives significant attention in clinical diagnosis of heart disease, brain thrombosis etc. (Arya et al., 2007; Li et al., 2003). The traditional method of determination of cholesterol by spectrometry suffered from poor specificity, instability of color forming agent and standardization difficulties. Enzymatic procedures for the determination of cholesterol have practically replaced the chemical methods due to the simplicity, rapidness and cost.

Immobilization of an enzyme onto the substrate (matrix) is a crucial step for the construction of enzyme based biosensors. Physisorption (Su et al., 2005) and entrapment into polymer matrices (Dhand et al., 2007; Singh et al., 2006a) have been tried to construct cholesterol biosensors. Physical adsorption is simple and fast. However, it suffers from desorption of proteins during operation or by changes in pH, temperature and ionic strength. Development of a suitable matrix that can hold the enzyme without leaching is highly warranted. Different matrices have been utilized to immobilize enzymes towards fabrication of cholesterol sensors. Support matrices consisting of either a single component or multi components have been tried. A recent review presents the details on varieties of matrices used for the immobilization of cholesterol oxidase (ChOx) (Arya et al., 2008) and characteristics of the fabricated cholesterol biosensors.

From a critical literature survey, it is understood that each of the matrices has its advantages and disadvantages. Hence, intensive research activities are continuously pursued to develop new matrices. For imparting comprehensive performances such as sensitivity, selectivity and stability to the biosensors, multi-components are required to be incorporated into the matrix of enzyme immobilization. Au nanoparticles, multi-walled carbon nanotubes (MWNT), chitosan (Chi) and ionic liquids (IL) have individually been known to improve the electrocatalytic activity (Liu et al., 2008), electron conduction path (Muguruma et al., 2008; Zhu

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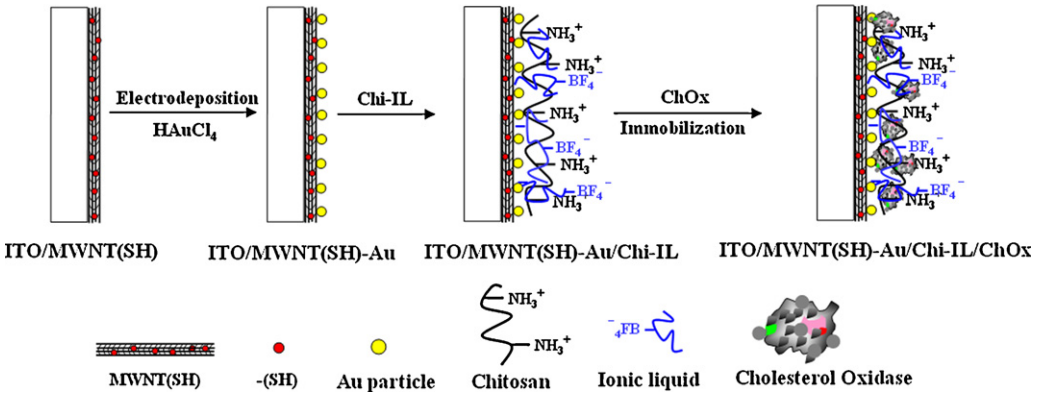

Scheme 1. Fabrication of MWNT(SH)–Au/Chi–IL/ChOx biosensor electrode.

et al., 2007), stability (Wang and Tang, 2008) and biocompatibility of biosensors (Shangguan et al., 2008), respectively.

Carbon nanotubes (CNTs) based modified electrode exhibited excellent electron transfer capabilities for the oxidation of biomolecules (Vasilis et al., 2004; Manesh et al., 2008; Shobha and Sriman Narayanan, 2008; Deng et al., 2008; Zou et al., 2008). CNTs could be combined with Au nanoparticles and the nanohybrids exhibited improved electro catalysis (Miguel and Luis, 2006; Choi et al., 2002; Santhosh et al., 2006; Gopalan et al., 2007; Muguruma et al., 2008; Wu et al., 2007; Chen et al., 2007; Xiang et al., 2008). Chi is an attractive biocompatible, biodegradable and non toxic natural biopolymer with excellent film forming ability, high water permeability, good adhesion and susceptibility to chemical modifications. Chi forms a biocompatibile matrix in conjunction with CNTs (Kang et al., 2007) or other redox mediators or metal nanoparticles (Yin et al., 2008) for the fabrication of electrochemical biosensors. ILs possess unique properties like wide potential windows, high thermal stability, viscosity, good conductivity and solubility. IL can be easily incorporated into carbon materials (Zhang and Zheng, 2008), chitosan (Xi et al., 2008), CNT (Liu et al., 2007). To the best of our knowledge, the combination of CNT–metal particle composite along with Chi–IL composite has not been explored as the matrix for the construction of a biosensor.

In the present study, we developed a new kind of matrix for the immobilization of ChOx toward fabrication of a cholesterol biosensor. The new matrix consists of multi-components such as (i) gold (Au) particles distributed MWNT and (ii) Chi (a biopolymer) (iii) and an (IL). We report on the fabrication of a cholesterol biosensor by combining the advantages features of MWNT, Au nanoparticles, Chi and IL. Further, we have employed a new methodology for the preparation of MWNT-Au/Chi–IL matrix. A biosensor electrode was fabricated (Scheme 1) by entrapment of ChOx onto MWNT(SH)–Au/Chi–IL matrix and the biosensor is designated as MWNT(SH)–Au/Chi–IL/ChOx. The existence of Chi–IL adds stability and reproducibility to the biosensor. The MWNT(SH)–Au/Chi–IL/ChOx biosensor exhibited an excellent linear response to cholesterol (Ch) for a wide concentration range with good selectivity and sensitivity.

2. Experimental

2.1. Materials

Cholesterol oxidase (ChOx) (sptreptomyces species: 100 U mg^{-1}; U: enzyme units), chitosan (85%) (Chi) and Triton-X 100 (polyoxyethylene isooctylphenyl ether) were obtained from Sigma (Korea). Cholesterol (95%) and glutaraldehyde were purchased from Aldrich. Uric acid, ascorbic acid, acetaminophen and 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM-BF_4, ionic liquid, IL) were of analytical grades. Serum samples were obtained from the Kyungpook National University Hospital, Daegu, Korea. The serum was diluted with a buffer solution (pH 6.8, 0.05 M) containing Triton-X (1% (m/v)) and 2-propanol (2% (v/v)). MWNTs (10–50 nm in diameter, CNT Co. Ltd. Incheon, Korea) were functionalized and used. Indium-doped tin oxide (ITO)-coated glass plate (specific surface resistance of about 10 Ω) was purchased from Daekyong Ltd. (Korea).

2.2. Preparation of solutions

2.2.1. Chitosan

0.5 g of Chi was dissolved in 100 mL of 1.0% (v/v) acetic acid and ultrasonicated for 30 min.

2.2.2. Cholesterol

Cholesterol (5 mM) was prepared in phosphate buffer (PB) (0.05 M; pH 6.8) by dissolving 0.0967 g of cholesterol in a 50 mL volumetric flask containing 1 mL isopropanol and 5 mL Triton X-100 and the solution was kept at 60 °C for 1 h.

2.3. Instrumentation

Electrochemical experiments were performed using an IVIUMSTAT electrochemical interface (Netherlands). UV–visible spectra were recorded using Varian Cary UV–visible spectrophotometer. Surface investigation was done by using scanning electron microscopy (SEM) on a TEOL JSM-5600LV instrument with the operation of 25 kV.

2.4. Fabrication of MWNT(SH)–Au/Chi–IL/ChOx biosensor

The fabrication of MWNT(SH)–Au/Chi–IL/ChOx biosensor electrode involves three sequential stages: formation of (i) ITO/MWNT(SH)–Au, (ii) ITO/MWNT(SH)–Au/Chi–IL and (iii) ITO/MWNT(SH)–Au/Chi–IL/ChOx.

Prior to modification, ITO plate was rinsed with acetone and washed with distilled water. MWNTs were thiol (–SH) functionalized (MWNT(SH)) using 4-aminothiophenol as the linker (Santhosh et al., 2006). 5 mg of MWNT-SH was suspended in dimethylformamide (5 mL) containing 50 μL of Nafion (5%) and sonicated for 5 min. 50 μL of the suspension was dropped onto the surface of ITO and casted as film by drying at 60 °C for 12 h. Au particles were electrochemically deposited onto the ITO/MWNT(SH) from a solution of $HAuCl_4$ (5.0×10^{-4} M) by cycling the potentials between 1.10 and 0.0 V (vs. Ag/AgCl as reference electrode) with a scan rate of 100 mV/s.

0.2 mL of Chi solution was mixed with 50 μL of IL and 50 μL of glutaraldehyde (0.1%). Chi–IL mixture was drop coated onto ITO/MWNT(SH)–Au electrode. After being washed with water and dried, 8 μl of ChOx was dropped onto the surface of MWNT(SH)–Au/Chi–IL to fabricate MWNT(SH)–Au/Chi–IL/ChOx electrode. The modified electrodes were washed thoroughly with PB solution (pH 6.8) containing 1.0% NaCl and 1% Triton-X 100 and stored at 4 °C.

2.5. Electrochemical measurements and determination of cholesterol

Electrochemical experiments were carried out using ITO, Pt wire and Ag/AgCl as working, counter and reference electrode, respectively. EIS measurements were performed in 1 mM $K_3Fe(CN)_6$ (PB, pH 6.8). Hydrodynamic voltammograms were obtained by allowing the background current to reach a stable value (by 5–10 min) at each applied potential and recording the steady state current after the injection of 100 μL of cholesterol (5 mM) into 0.05 M PB (pH 6.8) solution (5 mL) containing Triton-X (1% (m/v)) and 2-propanol (2% (v/v)). A small magnetic stirrer and bar provide the convective transport.

Cholesterol was amperometrically determined at −0.05 V by monitoring the reduction current response of hydrogen peroxide. Amperometric experiments were carried out for successive injection of 100 μL of cholesterol to a 5 mL of 0.05 M PB (pH 6.8) solution containing Triton-X (1% (m/v)) and 2-propanol (2% (v/v)). The solution was gently stirred with a magnetic bar at a constant speed. The steady state current was recorded.

3. Results and discussion

3.1. Fabrication of MWNT(SH)–Au/Chi–IL/ChOx biosensor electrode

Fabrication of biosensor electrode consists mainly of three stages: (i) deposition of Au particles onto the surface of MWNT(SH) to obtain MWNT(SH)–Au electrode, (ii) coating a layer of Chi–IL over MWNT(SH)–Au and (iii) immobilization of ChOx into the matrix of MWNT(SH)–Au/Chi–IL (Scheme 1).

Achieving uniform distribution of Au particles onto CNTs remains a challenge. Recently, we have adopted a γ-ray induced reduction method to disperse Au nanoparticles onto the surface of MWNTs (Showkat et al., 2007). In an another approach, polyaniline has been used as a linker to bind Au nanoparticles onto MWNT (Lee et al., 2006). In the present work, Au particles were electrodeposited onto the surface of MWNT(SH) by cyclic voltammetry (Supporting Information: S1). The broad band around 0.90 V represents the reduction of adsorbed $AuCl_4^-$ ions to Au^o (Chen et al., 2002). The sharp peak at+0.58 V corresponds to the reduction of Au(III) to Au^o (Chen et al., 2007). The increase in peak current at +0.58 V with increasing number of potential cycles, informed that Au^o particles were increasingly loaded onto MWNT(SH). The amount of Au^o particles deposited onto MWNT(SH) was calculated from the following equation

$$m = \frac{Q_{dep}M}{FZ}$$

where Q_{dep} is the charge used for the adsorption of Au^o, M is molar mass of Au, F is the Faradic constant and Z is the number of electrons transferred for the formation of Au^o (Au(III) to Au^o, Z = 3). At the end of first potential cycle, 9 μg/cm^2 of Au^o particles were deposited into MWNT(SH). After 10 potential cycles, 19 μg/cm^2 of Au^o particles were electrodeposited. The surface functionalization of MWNT with –SH groups resulted an enhanced deposition of Au^o particles due to interaction between –SH groups in MWNT(SH) and Au^o (Showkat et al., 2007). In the subsequent step of fabrication of the biosensor electrode, the composite consisting of IL, Chi and glutaraldehyde was coated as a layer over MWNT(SH)–Au. The amine groups in Chi form covalent cross-links with free aldehyde groups in glutaraldehyde to form a 3D network. In the final stage of biosensor fabrication, ChOx was immobilized into MWNT(SH)–Au/Chi–IL matrix to obtain MWNT(SH)–Au/Chi–IL/ChOx electrode.

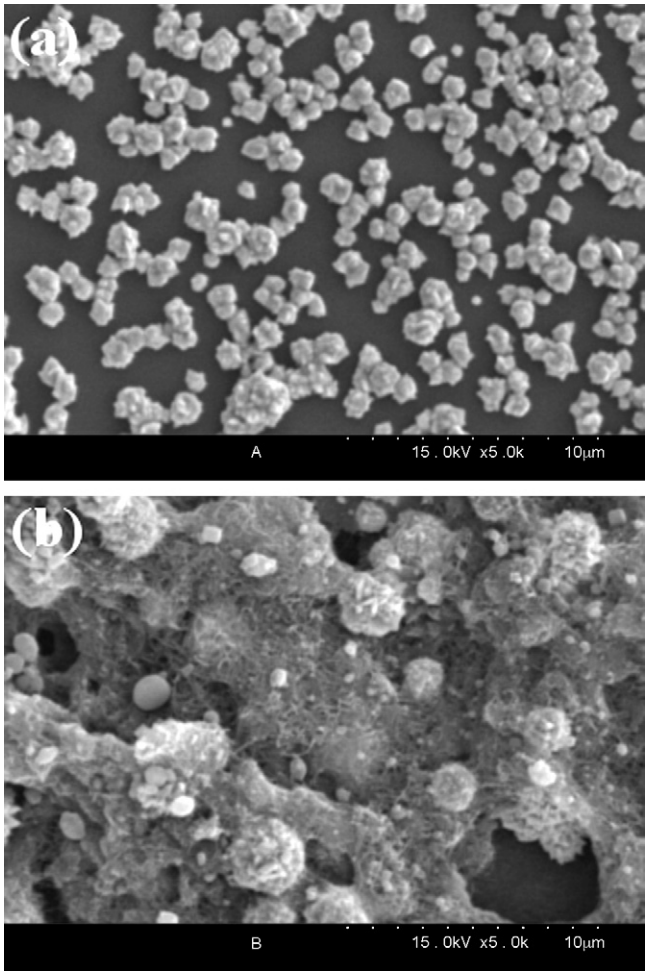

Fig. 1. SEM images of MWNT(SH)–Au (a) and (b) MWNT(SH)–Au/Chi–IL/ChOx.

We have adopted a simple three stage method for the fabrication of MWNT(SH)–Au/Chi–IL/ChOx. The first stage of making MWNT(SH)–Au electrode involved a shorter period (2 min) for the electrodeposition of Au particles. The next two stages, coating a layer of Chi–IL and immobilization of ChOx, are simpler physical processes and performed under mild conditions. Also, this three stage method for the fabrication of electrode is beneficial as compared to fabrication of MWNT(SH)–Au/Chi–IL/ChOx through mixing of all components (MWNT, Au nanoparticles, Chi and IL) in a single step. ChOx molecules are expected to undergo unpreferred conformational changes when they are entrapped into the complex matrix consisting of MWNT, Au particle, Chi and IL. This could reduce the enzyme activity of ChOx. However, minimum conformational changes in ChOx are possible at the surface of MWNT(SH)–Au/Chi–IL.

3.2. Morphology

SEM image of MWNT(SH)–Au (Fig. 1a) shows the presence of uniformly distributed spherical clusters of Au particles with cluster diameters in the range 250 nm to 1 μm. SEM image of

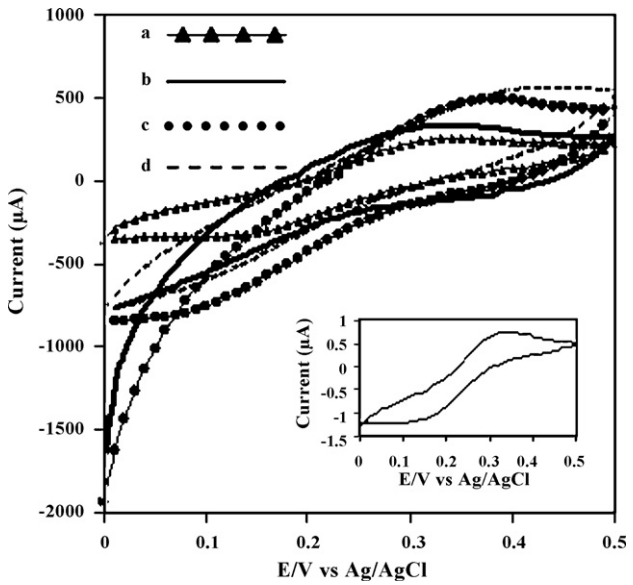

Fig. 2. Cyclic voltammograms of different modified electrodes: (a) MWNT(SH), (b) MWNT(SH)–Au and (c) MWNT(SH)–Au/Chi–IL (d) MWNT(SH)/Au/Chi–IL/ChOx) and bare electrode (inset); Supporting electrolyte = 0.1 M KCl containing 1 mM of $Fe(CN)_6^{3-/4-}$; Scan rate = 50 mV/s.

MWNT(SH)–Au/Chi–IL (Fig. 1b) reveals the presence of interconnected wires of Chi–IL composite on the surface of MWNT(SH)–Au.

3.3. Activity of ChOx and stability of MWNT(SH)–Au/Chi–IL/ChOx electrode

The existence of Chi–IL layer did not influence the enzyme activity of ChOx. UV–visible spectroscopy as well by cyclic voltammetry were used to test enzyme activity. UV–visible spectroscopy was used to monitor activity of ChOx at ITO/MWNT(SH)–Au/Chi–IL/ChOx electrode for 30 min (details are presented in Supporting Information: S2). The absorbance at 240 nm was used to determine the enzyme activity, as this corresponds to the cholestenone formed due to interaction between enzymes and cholesterol (Ram et al., 2001; Solanki et al., 2008). A decrease of 0.03% activity was witnessed after 30 min as inferred from the decrease in absorbance value at 240 nm. Cyclic voltammetry was also used to study the stability of ITO/MWNT(SH)–Au/Chi–IL/ChOx electrode (details are presented in S2). CV pattern remain nearly identical for 30 min and ensured the stability of ChOx.

3.4. Electrochemical characteristics of MWNT(SH)–Au/Chi–IL/ChOx electrode

3.4.1. Cyclic voltammetry

Electrochemical performances of the electrodes, ITO (bare), MWNT(SH), MWNT(SH)–Au, MWNT(SH)–Au/Chi–IL and MWNT(SH)–Au/Chi–IL/ChOx, were investigated using $Fe(CN)_6^{3-/4-}$ system as a redox marker (Fig. 2). Well defined redox characteristics of $Fe(CN)_6^{3-/4-}$ were observed for all the modified electrodes but with variations in the values of difference between the anodic and cathodic peaks (ΔE_p). CV of $Fe(CN)_6^{3-/4-}$ redox probe at ITO (bare) exhibits peaks with ΔE_p as ~200 mV (Fig. 2, inset).

A marked increase in ΔE_p (~230 mV) was noticed at MWNT(SH) electrode (Fig. 2, curve a) as compared to ITO (~200 mV) (Fig. 2, inset). The differences in uncompensated solution resistance and electron transfer kinetics between the bare ITO and ITO/MWNT(SH) electrodes might be the reasons for the large ΔE_p at ITO/MWNT(SH). The anodic peak current of $Fe(CN)_6^{3-/4-}$ is 1.5 time higher at MWNT(SH)–Au than at MWNT(SH) electrode. MWNT(SH)–Au exhibited a decreased ΔE_p (190 mV) (Fig. 2, curve b) as compared to MWNT(SH) electrode (230 mV). Thus, presence of Au^0 on the surface of MWNT caused an increased electrocatalytic activity. When a layer of Chi–IL was present on MWNT(SH), the electrode did not show much variations in ΔE_p and current values as compared to MWNT(SH)–Au (Fig. 2, curve c). Earlier, a decrease in electroactivity has been noticed by the existence of Chi due to its blocking influence on electrode surface. However, in the present study, cyclicvoltametric results informed that the layer of Chi–IL layer could retain electron transfer path (Fig. 2, curve c).

MWNT(SH)–Au does not have the functional capability to hold the enzyme. However, MWNT(SH)–Au/Chi–IL could effectively immobilize ChOx into its 3D network through electrostatic interactions (Scheme 1). The peak current at MWNT(SH)–Au/Chi–IL/ChOx (Fig. 2, curve d) is nearly the same as noticed at MWNT(SH)–Au/Chi–IL (Fig. 2, curve c). Thus, the electron transfer path is not blocked by the presence of ChOx onto MWNT(SH)–Au/Chi–IL. We presume that ChOx molecules are suitably configured onto the surface of MWNT(SH)–Au/Chi–IL to provide electron transfer path to the electrode. Also, peak current noticed at MWNT(SH)–Au/Chi–IL/ChOx is much higher than the bare (ITO) electrode.

CVs of MWNT(SH)–Au/Chi–IL/ChOx and MWNT(SH)–Au/ChOx electrodes were recorded at different scan rates in 1 mM $[Fe(CN)_6]^{3-/4-}$ (pH 6.8) (Supporting Information: S3). The anodic and cathodic peak current values increased linearly with scan rates in the range of 10–100 mV/s. This infers that the electron transfer at these electrodes is a surface confined process. ΔE_p is lower for MWNT(SH)–Au/Chi–IL/ChOx electrode as compared to MWNT(SH)–Au/ChOx (Supporting Information: S3). The existence of Chi–IL over MWNT(SH)–Au provides a favorable microenvironment for the ChOx to transfer electron to the electrode.

3.4.2. Electrochemical impedance spectroscopy

EIS has been utilized to investigate the interfacial properties of MWNT(SH)–Au/ChOx and MWNT(SH)–Au/Chi–IL/ChOx electrodes. Fig. 3 displays the typical Nyquist plots (z" vs. z') of the electrodes recorded in 1 mM $[Fe(CN)_6]^{3-/4-}$. In all the cases, Nyquist plots of the electrodes have a semi-circle and linear portion. The semi-circle at higher frequencies and linear part at lower

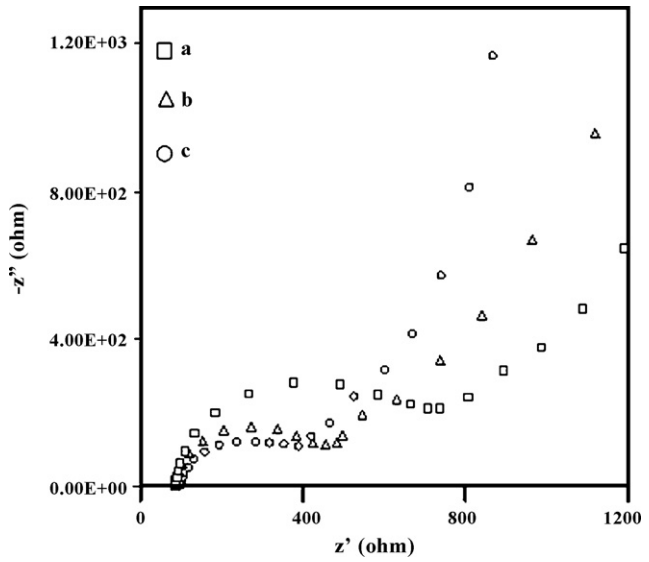

Fig. 3. The Nyquist plots (z" vs. z') of modified electrodes in 1 mM $K_3Fe(CN)_6$ with 0.1 M KCl: (a) bare ITO, (b) MWNT(SH)–Au/ChOx and (c) MWNT(SH)–Au/Chi–IL/ChOx).

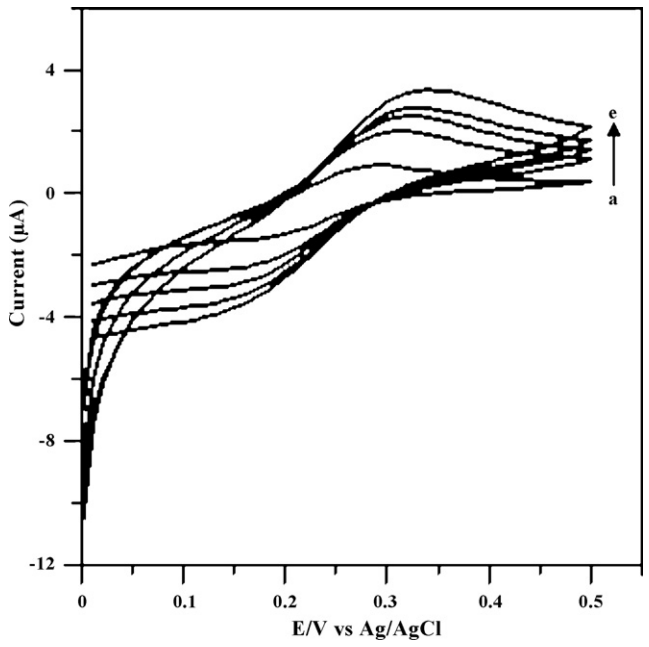

Fig. 4. Cyclic voltammograms of MWNT(SH)–Au/Chi–IL/ChOx electrode in phosphate buffer (pH 6.8) for different cholesterol concentrations. a: no cholesterol, (b–d). Each addition increased the concentration of cholesterol by 0.1 mM (inner to outer).

frequencies correspond to electron transfer and diffusion processes, respectively. The diameter of semi-circle part infers the electron transfer resistance (R_{ct}). The reproducibility of impedance data on electrode modification was tested. Each electrode modification was repeated three times and impedance measurements were recorded. R_{ct} values were evaluated and the relative standard deviation (RSD) was estimated (Supporting Information: S4). The average value of R_{ct} was considered for comparison between the electrodes. R_{ct} of bare (ITO) electrode (~735 Ω) (Fig. 3, curve a) was much larger than for other modified electrodes. After the modification to ITO/MWNT(SH)–Au/ChOx, R_{ct} decreased to 449 Ω (Fig. 3, curve b) indicating the influence of MWNT(SH)–Au to improve the conductivity of the surface. The resistance of ITO/MWNT(SH)–Au/Chi–IL/ChOx was lower (390 Ω) (Fig. 3, curve c) as compared to ITO/MWNT(SH)–Au (449 Ω). These observations informed that the existence of MWNT(SH)–Au could cause an increase in conductivity of ITO.

3.5. Performance of MWNT(SH)–Au/Chi–IL/ChOx as cholesterol biosensor

3.5.1. Cyclic voltammetry

Fig. 4 shows the representative CVs of MWNT(SH)–Au/Chi–IL/ChOx in PB solution (pH 6.8) recorded for different concentrations of cholesterol. After the addition of cholesterol (0.1 mM), the anodic peak current increased two times (Fig. 4, curve b) as compared to the oxidation current in the absence of cholesterol (Fig. 4, curve a). Fig. 4, curves b–e illustrate that the successive addition of cholesterol (0.1 mM) resulted in a linear increase in peak current.

3.5.2. Hydrodynamic voltammetry

In order to fix a most suited working potential for the detection of cholesterol, hydrodynamic voltammetric measurements were performed at MWNT(SH)–Au/Chi–IL/ChOx (a) and MWNT(SH)–Au/ChOx (b) biosensor electrodes. Generally, amperometric biosensors based on immobilized ChOx have been used to follow the current response of hydrogen peroxide generated by the enzyme catalyzed reaction. The amperometric detection of H_2O_2 is normally carried out by following the oxidation current responses at positive potentials (>0.5 V). However, current responses at these positive potentials are strongly influenced by oxidizable interferrents present in real samples (Wang and Mu, 1999; Vidal et al., 1999). Hence, sensitive and selective detection of H_2O_2 has been reported based on monitoring the reduction current of H_2O_2 (Karyakin, 2001; Vidal et al., 2002). In this study, we intended to follow the reduction current response of H_2O_2 at MWNT(SH)–Au/Chi–IL/ChOx biosensor, because this would avoid interferences from the other electroactive components (Vidal et al., 2002). Hence, hydrodynamic voltammetric studies were performed for an injection of 100 μL of cholesterol into PB solution (5 mL) with a start potential of 0.0 V and changing the potentials to more negative values (Supporting Information: S5). The electrochemical current response of the reduction of H_2O_2 increased from 0.0 V, reached a maximum at −0.05 V and remained nearly the same thereafter. Therefore, a potential of −0.05 V was fixed to follow the amperometric current response of reduction of H_2O_2.

3.5.3. Amperometric response

Fig. 5 displays the amperometric response of MWNT(SH)–Au/Chi–IL/ChOx biosensor for the successive step changes of cholesterol concentrations in PB (pH 6.8) containing 1% Triton X (m/v) and 2% (v/v) 2-propanol at an operating potential of −0.05 V. For a comparative purpose, the current–time curve at the MWNT(SH)–Au/ChOx electrode was recorded (Fig. 5, curve b). At the MWNT(SH)–Au/ChOx electrode, a small current response was noticed as compared to MWNT(SH)–Au/Chi–IL/ChOx. This clearly demonstrates the importance of the presence of Chi–IL over the surface of MWNT(SH)–Au on augmenting the current response to cholesterol. MWNT(SH)–Au/Chi–IL/ChOx biosensor exhibited a linear response to cholesterol in the concentration range of 0.5–5 mM with a correlation coefficient of 0.9981 (Fig. 5, inset). The sensitivity of MWNT(SH)–Au/Chi–IL/ChOx biosensor was 200 $\mu A\,M^{-1}$ as compared to 40 $\mu A\,M^{-1}$ for MWNT(SH)–Au/ChOx. The higher sensitivity for MWNT(SH)–Au/Chi–IL/ChOx electrode documents the importance of the layer of Chi–IL over MWNT(SH)–Au.

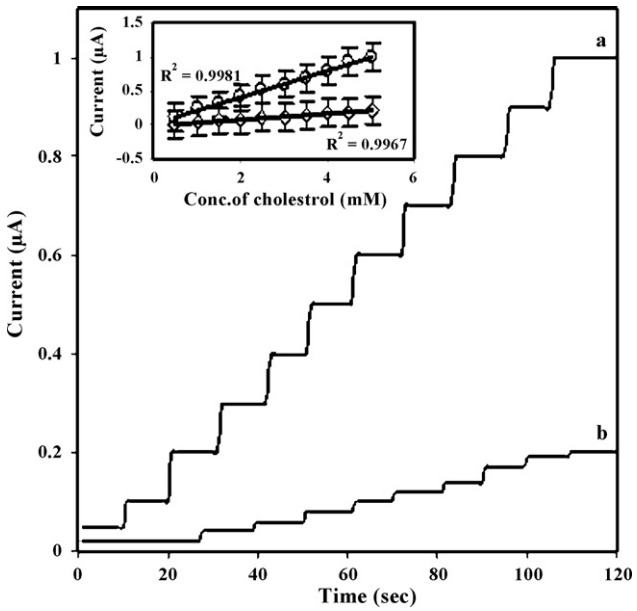

Fig. 5. Chronoamperometric current responses of electrodes, (a) MWNT(SH)–Au/Chi–IL/ChOx and (b) MWNT(SH)–Au/ChOx biosensor for successive addition of 0.5 mM cholesterol. Inset shows calibration plot, concentration of cholesterol (0.5–5 mM) vs. current for (a) MWNT(SH)–Au/Chi–IL/ChOx and (b) MWNT(SH)–Au/ChOx.

The current at MWNT(SH)–Au/Chi–IL/ChOx modified electrode increased immediately after the addition of cholesterol and reached a steady value. The average time required to reach 90% of the saturation current value was determined as ~7 s (response time, RT). On analysis of the reports on cholesterol sensor characteristics (Arya et al., 2008), it is evident that sensors with polypyrrole based matrices exhibited linear current responses to only a limited concentration range (upto 0.35 mM) (Vidal et al., 2002). Few other sensors showed linear responses to current for a wider cholesterol concentration range (beyond 0.35 mM) (Arya et al., 2007; Guo et al., 2004) but with a high RT (>13 s). In the present investigation, MWNT(SH)–Au/Chi–IL/ChOx biosensor exhibited linear current responses to a wide cholesterol concentration (0.5–5.0 mM) together with a much shorter RT (~7 s). The RT of cholesterol at MWNT(SH)–Au/Chi–IL/ChOx is much lower than reported for the cholesterol biosensors based on sol–gel/chitosan hybrid composite film (13 s) (Tana et al., 2005), polypyrrole-hydrogen (30 s) (Brahim et al., 2001) and ChOx in sol–gel film (51 s) (Yao and Takashima, 1998). The fast response is attributed to the synergetic influence of MWNT(SH), Au-particles, Chi and IL.

The influence of pH, buffer concentration and concentration of Triton X on the performance of ChOx immobilized enzyme electrodes have earlier been extensively investigated (Wang and Mu, 1999; Singh et al., 2006b; Li et al., 2003; Arya et al. 2007; Vidal et al., 1999). In the present study, optimum conditions for the cholesterol detection were chosen based on the information available from earlier literature (Arya et al., 2007). In the amperometric studies, 100 μL of 5 mM cholesterol was injected into PB buffer (0.05 M, pH 6.8) containing 1% (m/v) Triton X 2% (v/v) 2-propanol and the current response was recorded. Thus, pH and concentration of buffer were maintained as constant. However, upon successive addition of cholesterol, the biosensor response was expected to be the consequence of two opposite processes; an increase in current corresponding to increase in cholesterol concentration and a decrease in current due to increase in Triton X concentration. As a result, a constant biosensor response has been reported (Vidal et al., 1999). Further, there could be changes in the concentration of 2-propanol by the successive addition of cholesterol. After completion of successive addition of cholesterol, the concentration of 2-propanol decreased by ~0.01% (v/v) through dilution effect. Earlier studies (Vidal et al., 1999) revealed that a stable response could be noticed upto 35% (v/v) concentration of 2-propanol. The marginal changes in the concentration of 2-propanol (~0.01%) might probably not influence the current response in the present study.

3.6. Cholesterol in serum

The MWNT(SH)–Au/Chi–IL/ChOx biosensor was used to determine cholesterol in serum sample. The serum sample was diluted in a PB solution (0.05 M, pH 6.8) containing Triton X 1% (m/v) and 2-propanol (2% (v/v). The determinations were carried out using standard addition method, with three times addition of a standard cholesterol solution. The results are presented in (Supporting Information: S6). The results showed that RSD for three measurements were in the range of 1.0–4.7% and recoveries in the range of 97.2–104.4.

3.7. Interference, stability and reproducibility

Amperometric responses for cholesterol (0.5 mM), uric acid (UA) (0.5 mM), ascorbic acid (AA) (0.5 mM), and acetaminophen (AP) (0.5 mM) at the MWNT(SH)Au/Chi–IL/ChOx modified electrode in phosphate buffer (pH 6.8) (Supporting Information: S7). A well-defined current response was observed for cholesterol (0.5 mM) at the potential of −0.05 V. The successive injection of relevant physiological level (0.5 mM) concentration of UA, AA, and AP did not produce any additional signal or change in the current response. The synergetic influence of MWNT, Au Chi–IL and ChOx permits the detection of cholesterol at a much lower potential (−0.05 V). The detection of cholesterol at a far negative potential limits the effects from potential intereferents (Fig. 5). Thus, MWNT(SH)Au/Chi–IL/ChOx biosensor exhibited high selectivity towards the determination of cholesterol.

The repeatability and stability of the biosensor were evaluated. A set of 12 different amperometric measurements for 1 mM cholesterol on PB (pH 6.8) with a single biosensor resulted a R.S.D. value of 1.9% indicating the good repeatability of the MWNT(SH)–Au/Chi–IL/ChOx biosensor. The stability of the MWNT(SH)–Au/Chi–IL/ChOx biosensor electrode was investigated by the storing the electrode at 4 °C for 20 days. MWNT(SH)–Au/Chi–IL/ChOx sensor shows a decrease in the current response (for 0.5 mM of cholesterol) up to 5% after 20 days. Thus, MWNT(SH)–Au/Chi–IL/ChOx biosensor electrode reveals the excellent stability and repeatability. The presence of 3D network of Chi–IL imparts limits the leaching of ChOx and enhance the stability and repeatability.

4. Conclusions

We developed a new amperometric cholesterol biosensor utilizing the synergetic influence of the high surface area MWNT, catalytic Au nanoparticles, biocompatible chitosan (Chi) and a room temperature ionic liquid (IL). The biosensor electrode, MWNT(SH)Au/Chi–IL/ChOx, exhibited high selectivity, sensitivity, fast response and repeatability. The excellent performance of the cholesterol biosensor originates from the new strategy used for the fabrication of biosensor. An effective immobilization of ChOx and an enhanced electron transfer process between enzyme and electrode were achieved by the presence of 3D interconnected network in Chi–IL layer. We anticipated that the methodology adopted here for the fabrication of biosensor would be extended for the development of other biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.11.034.

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