Fabrication of bienzymatic cholesterol biosensor based on gold nanoparticles decorated graphene-nanostructured polyaniline nanocomposite

Deepshikha*
Amity Institute of Biotechnology,
Amity University Uttarpradesh,
Noida, 201303, India
E-mail: deepshikhasaini80@gmail.com
E-mail: deep.shikha80@rediffmail.com
*Corresponding author

Ruchika Chauhan and Pratima R. Solanki
Amity Institute of Nanotechnology,
Amity University Uttarpradesh,
Noida, 201303, India
E-mail: ruchikachauhan@hotmail.com
E-mail: prsolanki@amity.edu

Tinku Basu
Amity Institute of Technology,
Amity University Uttarpradesh,
Noida, 201303, India
E-mail: tbasu@amity.edu
E-mail: basu002@yahoo.com

Abstract: A novel amperometric cholesterol biosensor based on bienzyme system such as cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) was developed based on the gold nanoparticles decorated graphene-nanostructured polyaniline nanocomposite (NSPANI/AuNP/GR) film, electrochemically deposited onto indium-tin-oxide (ITO) electrode. The cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) have been coimmobilised onto the NSPANI/AuNP/GR nanocomposite electrode using gluteraldehyde as a crosslinking agent. The nanobioelectrodes ChOx-HRP/NSPANI/AuNP/GR/ITO and ChOx-/NSPANI/AuNP/GR/ITO have been characterised by differential pulse voltammetry (DPV), cyclic voltammetry (CV) and scanning electron microscopy (SEM). The bienzymatic nanobioelectrodes ChOx-HRP/NSPANI/AuNP/GR/ITO have exhibited higher sensitivity than mono enzymatic bioelectrode (ChOx-/NSPANI/AuNP/GR/ITO). Minimum interferences have been observed from ascorbic acid, uric acid, sodium pyruvate, glucose and urea for both single and bienzyme systems. It is inferred that bienzyme based nanobioelectrodes offer wider linearity (35 to 500 mg/dl), higher sensitivity (4.22 μAmM⁻¹), high shelf life (8 weeks), low response time (19s) and high accuracy for testing of blood serum samples than mono enzyme system. Mechanism of the overall biochemical reaction has been proposed to illustrate the enhanced bio-sensing performance of the bienzyme system.

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Keywords: polyaniline nanocomposite; cholesterol oxidase; ChOx; horseradish peroxidase; HRP; electrodeposition; repeatability; reusability and shelf life.


Biographical notes: Deepshikha received her MSc (Chemistry) from Guru Nanak Dev University, Amritsar, India in 2005. She is a gold medalist in MSc Chemistry. She has completed her PhD degree from Amity University, Noida, India in 2012. She has got five years of research and seven years of teaching experience. At present, she is pursuing her postdoc in Peking University, Beijing, China. Her specialisation is on nanostructured conducting polymers and graphene chemistry.

Ruchika Chauhan is a research scholar in Amity Institute of Nanotechnology, Amity University Uttarpradesh. She has completed her MSc in Chemistry and MPhil degree from Kumayun University, Uttarakhand, India. She has research experience of more than five years. She has four publications in international journals. She has attended several national and international conferences. She has two patents.

Pratima R. Solanki is an Assistant Professor in Amity Institute of Nanotechnology, Amity University Uttarpradesh, India. She has completed her MSc in Zoology from MD University, India. She has research experience of more than 11 years. She has 65 publications in international journals. She has attended several national and international conferences. She has a couple of patents. Her area of research interest is biosensors and nanobio composite.

Tinku Basu is the Deputy Director in Amity Institute of Nanotechnology, Amity University Uttarpradesh. She received her PhD degree from IIT, Kharagpur, India. She has 48 publications in international journals. She has attended several national and international conferences. She has five patents. She has guided four PhD students and four MTech students. Her area of research interest is conducting polymers biosensors and nano composite for industrial application.

1 Introduction

Graphene, one of the most exciting nanostructures of carbon, is a two-dimensional honeycomb crystalline single layer of carbon lattice (Geim, 2009; Zhang et al., 2005; Rao et al., 2009). Recently, it has received enormous interest in various areas of research, such as biosensors, bioelectronics, energy storage and conversion, drug delivery (Stoller et al., 2008; Bunch et al., 2007; Liu et al., 2008; Ang et al., 2008), ultrafast electronic devices (Novoselov et al., 2004), molecular resolution sensors (Schedin et al., 2007; Novoselov et al., 2006; Zhang et al., 2005), and electromechanical resonators (Bunch et al., 2007) etc. owing to its large specific surface area, extraordinary electrical and thermal conductivities (Novoselov et al., 2004; Kim et al., 2008), high mechanical
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stiffness (Lee et al. 2008), good biocompatibility (Chen et al., 2008), and low manufacturing cost (Segal, 2009). The high electrical and thermal conductivities of graphene originate from the extended long-range $\pi$-conjugation.

Out of all conducting polymers, polyaniline (PANI) is one of the promising matrices for biosensor applications due to its simple and reversible acid/base doping/dedoping chemistry [pH paper] enabling control over properties such as free volume (Janata and Josowicz, 2002), solubility (Huang et al., 2004), electrical conductivity (Liu et al., 2003a) and optical activity (Dhand et al., 2008). In recent years, nanostructured polyaniline (NSPANI) has aroused much scientific interest since it combines the properties of low-dimensional organic conductors and high surface area materials and offers the possibility of enhanced performance wherever a large interfacial area between NSPANI and its environment is required. Various synthesis methods are used for the synthesis of nanostructured polyaniline as discussed in the previous chapters (Mahasweta et al., 2008; Deepshikha and Basu, 2011a, 2011b).

Noble metal nanoparticles are known to be excellent catalysts, due to their high ratio of surface atoms with free valences to the cluster of total atoms. As well known, gold nanoparticles (AuNP) have many unique properties such as high surface free energy, strong adsorption ability, well suitability and good conductivity (Boisselier and Astruc, 2009; Perez-Juste et al., 2005). Besides, they can provide more binding sites and more congenial microenvironment for biomolecules immobilisation to retain the bioactivity of the proteins, which can prolong the life time of biosensors (Penn et al., 2003; Lu et al., 2009). Nanocomposites based on metal nanoparticles and exfoliated graphene nano sheet with synergistic effect have exhibited particular promise in biosensing characteristics as they can play very interesting role such as

1. a biocompatible enzyme friendly platform
2. fast electro catalytic oxidation or reduction of the product generated during biochemical recognition process at the electrode surface to reduce overvoltage and avoid interference from other coexisting electroactive species
3. an enhanced signal because of its fast electron transfer and large working surface area.

Lu et al. (2008) have reported highly sensitive and selective amperometric glucose biosensor using exfoliated graphite nanoplatelets decorated with Pt and Pd nanoparticles. Besides that, graphene-based nanocomposites have been used to fabricate alcohol dehydrogenase biosensors for glucose, alcohol, etc. (Zhang et al., 2010; Zhou et al., 2009; Liu et al., 2010; Du et al., 2010; Wang et al., 2010; Shao et al., 2010). Biosensors, based on graphene-encapsulated nanoparticle arrays, for highly sensitive and selective detection of breast cancer biomarkers are successfully demonstrated. The increased surface-to-volume ratio significantly has helped in lowering the detection limits (1pM) for the target biomarkers (Myung et al., 2011). A glucose electrochemical biosensor has been reported based on zinc oxide nanoparticles (ZnO NPs) doped in graphene (NGs) nano sheets. The results show that the linear response range of the biosensor lies between 0.1 to 20 $\mu$M and the detection limit has calculated 0.02 $\mu$M at a signal-to-noise ratio of 3 (Norouzi et al., 2011).
Cholesterol and its ester are essential constituents of all animal cells, and it is present in brain and nerve tissues. The level of cholesterol in serum is an important parameter in the diagnosis and prevention of heart diseases. The development of electrochemical biosensor received significant interest for precise and smart determination of cholesterol in serum and food sample (Dey and Retna, 2010). However, the so far developed cholesterol biosensors suffer from low reliability, poor shelf life and low sensitivity. There is a scope to pay attention to the above properties in order to fabricate a reliable cholesterol biosensor for clinical diagnosis. There are two key factors in the fabrication of a biosensor, firstly enzyme system and secondly transducer matrix to monitor biosensor performance. In amperometric biosensor, cholesterol quantification is usually performed by measurement of the current associated with the oxidation of hydrogen peroxide. One of the major drawbacks of electrochemical biosensor is the overpotential necessary for the oxidation of H₂O₂ which can cause interferences from other oxidisable species such as ascorbic acid (AA), uric acid (UA), and acetaminophen (AAP). To avoid interferences, some improved biosensors based on the coupled enzyme reactions have been reported to detect hydrogen peroxide at low potential (Yang et al., 2006; Sun et al., 2006). In such cases, the primary product, i.e., produced by the reaction of the analyte with the first enzyme is further converted by a second enzyme to produce products detectable by a transducer (Antiocchia and Gorton, 2007). Coupled enzyme reactions are also employed to filter out chemical signals by eliminating the interference on the enzyme (Liu et al., 2003b; Chen et al., 2006).

In the previous communication, the authors have reported a novel nanocomposite NSPANI-AuNP-GR based on NSPANI (S1I2), graphene nano sheet and gold nanoparticles (NSPANI-AuNP-GR) with high catalytic activity (Deepshikha and Basu, 2011c). It has been found out that the NSPANI-AuNP-GR nanocomposite can be successfully electrodeposited on the ITO surface. In the manuscript, attempt been made to develop a reliable and reusable amperometric bienzymatic cholesterol biosensor based on nanocomposite film on ITO electrode for estimation of free cholesterol. In order to achieve the commercial viability, the developed electrochemical biosensor performance has been compared with photometric technique and tested on blood serum sample of various pathological labs. The novelty of the sensor lies on the method of fabrication of transducer matrix, enzyme system, the performance with special reference to reusability, reliability, shelf life and sensitivity and successful application to blood serum testing.

2 Experimental

2.1 Materials

Few layered graphene (Quantum Materials Corporation, Bangalore), Aniline (Sigma-Aldrich), sodium dodecyl sulphate (SDS) (Qualigen), ammonium persulfate (NH₄)₂S₂O₈ (E-Merck), hydrochloric acid (Qualigen), chloroauric acid HClO₄ (Sigma-Aldrich) were used in the present experiment. Cholesterol oxidase (ChOx; EC 1.1.3.6, from Pseudomonas fluorescens) with specific activity of 24 U/mg, horseradish peroxidase (HRP, E.C1.11.1.7, 250 U/mg, from horseradish) were purchased from Sigma, potassium ferricyanide (K₃[Fe(CN)₆]₃), potassium ferrocyanide (K₄[Fe(CN)₆]₄), sodium dihydrogen orthophosphate(NaH₂PO₄), disodium hydrogen orthophosphate
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(Na₂HPO₄) were purchased from Qualigens (India). Deionised water from a Millipore-MilliQ was used in all cases to prepare aqueous solutions. Monomer was double distilled before polymerisation.

2.2 Instrumentation

Fourier transform infrared spectroscopic (FTIR) measurements were performed with a Perkin-Elmer FTIR spectrophotometer. Morphological imaging of the fabricated electrodes were obtained by Scanning electron microscope (LEO 440 Model) and Atomic force microscopy (AFM) was performed by Park Systems XE-70 Atomic Force Microscope in non-contact mode. Cyclic voltammetry (CV) and Differential Pulse Voltammetry (DPV) measurements were conducted in phosphate buffer (50mM, 0.9% NaCl) containing 5mM[Fe(CN)₆]³⁻/⁴⁻ in a three-electrodes cell consisting of Ag/AgCl as reference, platinum (Pt) as counter electrode and ITO as a working electrode (0.25cm²) using Autolab Potentiostat/Glavanostat Model AUT83945 (PGSTAT302N).

3 Synthesis of gold nanoparticles decorated graphene-nanostructured polyaniline nanocomposite (NSPANI/AuNP/GR)

3.1 Synthesis of NSPANI/AuNP/GR nano dispersion

In a typical synthesis, graphene was first dissolved into a dilute aqueous solution of SDS (0.02 M). The aniline solution in the dopant (0.02M) was added to an aqueous solution of SDA under stirring condition. The mixture was then placed in the low temperature bath, so that the temperature was maintained at 0° to 5°C. 70 µl of aqueous 0.05 M HAuCl₄ was added into aqueous dispersion. An aqueous solution of the oxidising agent, (NH₄)₂S₂O₈, in ice-cold water was added to the above mixture. The polymerisation was allowed to proceed for 3 to 4 h with stirring. After that the stirring was stopped and the mixture was kept under static condition for 1–3 days at 277–278°K for polymerisation to complete.

3.1.1 Preparation of NSPANI-SDS/ITO electrode

The electrodeposition of NSPANI/AuNP/GR dispersion on the indium tin oxide plates (ITO) was carried out electrochemically using cyclic voltammetric technique. The potential was swept from -0.4 to + 1.0 V at scan rate of 80 mV/s for the required number of cycle to fabricate NSPANI/AuNP/GR/ITO electrode.

3.1.2 Preparation of solutions

Stock solution of cholesterol was prepared in deionised water having 10% Triton X-100 and was stored at 4°C. This stock solution was further diluted to make different concentrations of cholesterol solution. o-dianisidine (1%) solution was prepared freshly in deionised water. Buffers of various pH values were prepared by dissolving different ratios of sodium dihydrogen orthophosphate (NaH₂PO₄) and disodium hydrogen orthophosphate (Na₂HPO₄) in millipore water.
4 Fabrication of NSPANI/AuNP/GR/ITO electrodes

The NSPANI/AuNP/GR film was electrochemically deposited onto ITO coated glass plates by sweeping a potential from −200 mV to +1,000 mV (vs. Ag/AgCl) at a scan rate of 80 mV/s, in a three-electrodes cell consisting of Ag/AgCl as reference, platinum (Pt) as counter electrode and ITO as a working electrode (0.25cm²). So, electrodeposited NSPANI/AuNP/GR film on ITO has been investigated for biosensor application. The electrodeposition curves of NSPANI/AuNP/GR/ITO exhibit characteristics electrochemistry for NSPANI (Shao et al., 2010) with the main peaks a and b corresponding to the transformation of leucoemeraldine base (LB) to ES and ES to pernigraniline salt (PS), respectively. On the reverse scan, peaks b’ and a’ correspond to the conversion of PS to ES and ES to LB, respectively. The presence of a small redox peak around +350 mV (C and C’) is associated with the formation of p-benzoquinone and hydroquinone as a side product upon cycling the potential to +1,000 mV. The increase in current density with successive scans suggests that the polymer film build up on the electrode surface. Figure 1 Inset shows the plot of maximum anodic peak current vs. number of cycles. Maximum peak current was observed at 28 cycles indicating a continuous film deposition. It can also be observed that the shifts in peak potentials began to occur after a number of cycles. This may be the result of increased resistance of the
electrode, as the film deposited becomes thicker. Currents reached for the electrodeposition (28 cycles) of the nanoparticles up to 0.261 mA cm\(^{-2}\). On further increase in number of cycles, anodic peak current decreases. This decrease in peak current is ascribed to the degradation of polymer film. In the present study, 28 cycles was used for film deposition for biosensor application.

5 Fabrication of ChOx/NSPANI/AuNP/GR/ITO and ChOx-HRP/NSPANI/AuNP/GR nano bioelectrodes

The NSPANI/AuNP/GR/ITO electrode was treated with 10 μl of aqueous glutaraldehyde (0.1%) as a cross-linker. 10 μl of freshly prepared ChOx (1 mg/mL) was uniformly spread onto glutaraldehyde treated NSPANI/AuNP/GR /ITO electrode and is kept in a humid chamber for 12 h at 4°C to fabricate ChOx/NSPANI/AuNP/GR/ITO nanobioelectrode. 10 μL freshly prepared solution of HRP (1 mg/mL) and ChOx (1 mg/mL) (1:1) was uniformly spread onto glutaraldehyde treated NSPANI/AuNP/GR/ITO electrode and was kept in a humid chamber for 12 h at 4°C to prepare ChOx/NSPANI/AuNP/GR/ITO and ChOx-HRP/NSPANI/AuNP/GR/ITO nanobioelectrodes. The nanobioelectrodes were immersed in 5 mM phosphate buffer solution (pH 7.0) in order to wash out unbound enzyme from the electrode surface. When not in use, the electrode was stored at 4°C in a refrigerator.

6 Photometric studies

Photometric measurements were conducted using a UV-visible spectrophotometer. Photometric experiments were carried out cholesterol concentration using PBS buffer (50 mM, 0.9% NaCl, pH 7.4). These measurements were also used to estimate the enzyme activity. To carry out photometric enzymatic assay of the immobilised enzyme, ChOx/NSPANI/AuNP/GR/ITO and ChOx-HRP/NSPANI/AuNP/GR/ITO nanobioelectrodes were dipped in 3 ml of PBS solution containing 20 μl of HRP (1 mg dl/1), 20 μl of o-dianisidine dye, and 100 μl of cholesterol. The difference between the initial and final absorbance values at 500 nm after 3 min. incubation of cholesterol were recorded and plotted.

7 Results and discussion

7.1 Characterisation of NSPANI/AuNP/GR/ITO, ChOx/NSPANI/AuNP/GR/ITO and ChOx-HRP/NSPANI/AuNP/GR electrodes

7.1.1 FT-IR study

Figure 2 represents the FT-IR absorption spectra of the NSPANI-AuNP-GR/ITO (curve a), ChOx/NSPANI-AuNP-GR/ITO (curve b) and ChOx-HRP/NSPANI-AuNP-GR (curve c) electrodes. The FT–IR spectrum of electrochemically deposited NSPANI-AuNP-GR/ITO nanocomposite (curve a) shows benzenoid and quinoid ring stretching bands (C = C) present at 1,447.6 and 1,560 cm\(^{-1}\). The presence of peak at
3,123 cm$^{-1}$ is attributed to –N–H stretching vibrations of NSPANI in the composite (Wu et al., 2010). A peak at 1,534 cm$^{-1}$ due to the skeletal vibration of graphene nanosheet is observed (Aravind et al., 2011) in the FT-IR spectra of NSPANI-AuNP-GR/ITO (curve a). In the FTIR spectra, apart from the above mentioned functional groups, a peak appears at 655 cm$^{-1}$ which may correspond to stretching vibration of Au-O-Au (Aravind et al., 2011). The presence of these peaks reveals the existence of NSPANI, graphene nanosheet and AuNPs on the ITO electrode. In the FTIR spectrum of ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR nanobioelectrode [Figure 2(b) and Figure 2(c)], enzyme binding is indicated by the appearance of additional absorption bands at 1,524 and 1,630 cm$^{-1}$ assigned to the carbonyl stretch (amide I band) and N–H bending (amide II band), respectively (Wisitsoraat et al., 2010). Also, a broadband seen around 3,560 cm$^{-1}$ is attributed to amide bond present in ChOx (Singh et al., 2011b).

Figure 2 FTIR spectra of (a) NSPANI-AuNP-GR/ITO (b) ChOx/NSPANI-AuNP-GR/ITO (c) ChOx-HRP/NSPANI-AuNP-GR electrodes (see online version for colours)

7.1.2 SEM study

SEM images of NSPANI-AuNP-GR/ITO [Figure 3(a)], ChOx/NSPANI-AuNP-GR/ITO [Figure 3(b)] and ChOx-HRP/NSPANI-AuNP-GR [Figure 3(c)] are shown in Figure 3. The electrodeposition of NSPANI-AuNP-GR matrix on ITO electrode has been confirmed by the homogeneous rough surface [Figure 3(a)]. SEM image shows NSPANI deposited on a few layered graphene nanosheet which provide large surface area for the incorporation of metal nanoparticles. SEM image reveals the uniform loading of AuNP over NSPANI-GR matrix [Figure 3(c)] (Aravind et al., 2011). The nano scale surface roughness of the NSPANI-AuNP-GR nanocomposite film is suitable for the immobilisation of biomolecules. From the Figure 3(b) and Figure 3(c), it is found that the enzymes are uniformly distributed on the electrode surfaces. The surface morphology of
ChOx/NSPANI-AuNP-GR/ITO [Figure 3(b)] and ChOx-HRP/NSPANI-AuNP-GR [Figure 3(c)] shows full coverage of the surface by the single and bienzyme bioconjugates. The presence of globular structure can be attributed to the covalently bound enzyme molecule since most of the proteins and enzymes possess globular structure (Singh et al., 2009; Solanki et al., 2007).

7.1.3 AFM study

AFM is employed to establish the thickness, surface morphology and surface roughness of the NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO electrodes. The two-dimensional (2D) and three-dimensional (3D) atomic force microscopy [AFM, Figure 4(a)] studies reveal that film NSPANI-AuNP-GR/ITO (2 × 2 μm) shows nanoporous morphology with roughness [root mean square (RMS)] of about 29.3 nm, though their spherical shape appears to be partially distorted. The size of the spherical
nanoparticles varies from 25 to 50 nm with average particle size of 35 nm. However, after the immobilisation of ChOx-HRP, the surface morphology of NSPANI-AuNP-GR/ITO film changes into well-arranged regular morphology wherein the average particle size increases to 100 nm and roughness decreases to 6.1 nm revealing that ChOx-HRP is adsorbed onto NSPANI-AuNP-GR/ITO [Figure 4(b)] via electrostatic interactions. AFM image of ChOx-HRP/NSPANI-AuNP-GR/ITO bioelectrode (2 × 2 μm) exhibits well-arranged uniform surface indicating that NSPANI-AuNP-GR/ITO film provides a desired microenvironment for strong adsorption of ChOx-HRP in a particular orientation and wherein it retains its better configuration with more active sites.

**Figure 4** 2D and 3D AFM images of (a) NSPANI-AuNP-GR/ITO (b) ChOx-HRP/NSPANI-AuNP-GR electrodes (see online version for colours)

7.1.4 DPV study

DPV experiments have been conducted in phosphate buffer (50 mM, pH 7.0) containing 5 mM Fe[Fe(CN)$_6$]$_{3/4}^-$ in the range −0.4 to 1.2V (Figure 5). The high value of maximum
anodic peak current obtained as $1.63 \times 10^{-4}$ A for NSPANI-AuNP-GR/ITO electrode (curve a) suggests high conducting nature of NSPANI-AuNP-GR/ITO electrode and enhanced electron transfer towards the electrode. The magnitude of peak current decrease to $1.32 \times 10^{-4}$ A (curve b) and $1.01 \times 10^{-4}$ A (curve c) for ChOx-HRP/NSPANI-AuNP-GR/ITO and ChOx/NSPANI-AuNP-GR/ITO nanobioelectrodes respectively indicating slow redox process at the nanobioelectrodes due to insulating characteristics of ChOx-HRP and ChOx revealing immobilisation of ChOx and ChOx-HRP on NSPANI-AuNP-GR/ITO electrode. The magnitude of peak current ($1.32 \times 10^{-4}$ A) of ChOx-HRP/NSPANI-AuNP-GR/ITO (curve b) is found to be higher as compared to ChOx/NSPANI-AuNP-GR/ITO (1.01 $\times 10^{-4}$ A) (Curve c). The enhanced peak current indicates the increased surface activeness of the nanobioelectrode and increased number of electron transfer.

**Figure 5** Differential pulse voltammetry of (a) NSPANI-AuNP-GR/ITO (b) ChOx-HRP/NSPANI-AuNP-GR/ITO (c) ChOx/NSPANI-AuNP-GR/ITO electrodes (see online version for colours)

### 7.2 Electrochemical response studies

The DPV curves of ChOx/NSPANI-AuNP-GR/[Figure 6(a)] and ChOx-HRP/NSPANI-AuNP-GR/ITO [Figure 6(b)] nanobioelectrodes recorded in the range of –0.4 to 1.2V using phosphate buffer of pH 7.0 containing 5 mM [Fe(CN)$_6$]$^{3-}$/$^{4-}$ are shown in Figure 6. Change in current ($\Delta$I) is plotted against concentration values. A linear relationship between the cholesterol concentration and the increase in response current ($\Delta$I) for both the mono as well as bienzyme-based nanobioelectrodes is observed. The linear regression curve [Figure 6(a)] of the ChOx/NSPANI-AuNP-GR/ITO nano bioelectrode which is used to detect cholesterol in the range of 35–350 mg/dl, follows the equation; $\Delta$I [current (mA) = 0.12(mA) + 0.0031 (mA mgdl$^{-1}$) × cholesterol concentration (mgdl$^{-1}$)] with 99.4 µA and 0.981 as standard deviation and correlation coefficient respectively. The
sensitivity of the bioelectrode has been found to be 3.10 μA mgdl⁻¹. The linear equation of ChOx-HRP/NSPANI-AuNP-GR/ITO in Figure 6(b) is represented by the equation:

\[ \Delta I \text{(current)} (mA) = 0.36 (mA) + 0.0042 (mA \text{ mgdl}^{-1}) \times \text{cholesterol concentration (mgdl}^{-1}) \]

with 65.2 μA and 0.995 as standard deviation and correlation coefficient respectively. Furthermore, the ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes exhibit a higher sensitivity of 4.22 μA mgdl⁻¹, than the single enzyme-based electrodes (ChOx/NSPANI-AuNP-GR/ITO) and linear range of 35–500 mg/dl. The response current and sensitivity are higher for bienzyme sensor than the mono enzymatic nano biosensor suggesting effective reduction of H₂O₂ catalysed by HRP. All of the experiments have been carried out in triplicate sets, and the results reveal reproducibility of the system. The values of response time of the (ChOx/NSPANI-AuNP-GR/ITO) and (ChOx-HRP/NSPANI-AuNP-GR/ITO) are found to be as 28 and 19 s, respectively, which are measured by measuring the time taken to reach the steady state current after applying a steady voltage of 250 mV for 100 mg/dl of cholesterol solution in 7.0 pH PBS buffer containing 5 mM [Fe(CN)₆]³⁻/⁴⁻.

Figure 6 Calibration curve of (a) ChOx/NSPANI-AuNP-GR/ITO
(b) ChOx-HRP/NSPANI-AuNP-GR/ITO bioelectrodes (see online version for colours)

The value of the enzyme-substrate kinetics parameter (Michaelis-Menten constant, Km) estimated using the Lineweaver-Burke plot reveals affinity of enzyme for desired analyte. It may be noted that Km is dependent both on matrix and the method of immobilisation of enzymes that often results in their conformational changes resulting in different values of Km. The Km value was determined by the analysis of the slope and intercept for the plot of the reciprocals of change in current vs cholesterol concentrations, i.e., Lineweaver–Burke plot of 1/ΔI vs. 1/C. The values of apparent Michaelis-Menten constant (Km) have been estimated using Lineweaver-Burke plot for ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO as 0.02 mM and 0.01 mM, respectively. The observed lower value of Km for bienzyme system indicates high affinity for cholesterol attributed to the immobilisation of ChOx-HRP onto NSPANI-AuNP-GR/ITO for faster
biochemical reaction. This result can be assigned to the uniform distribution of enzyme molecules on to the NSPANI-AuNP-GR/ITO nanocomposite film surface. The overall biochemical reaction for ChOx-HRP/NSPANI-AuNP-GR is shown by the equations (1 to 5) and Figure 7.

**Figure 7** Proposed biochemical reaction on the ChOX-HRP/NSPANI-AuNP-GR (see online version for colours)

\[
\text{Cholesterol} + O_2 \rightarrow \text{Cholest-4en-3-one} + H_2O_2 \quad (1)
\]

\[
H_2O_2 + HRP(F^3_ε) \rightarrow HRP - I + H_2O \quad (2)
\]

\[
HRP - I + NSPANI - AuNP - GR / ITO \rightarrow \quad (3)
\]

\[
HRP - II + NSPANI - AuNP - GR / ITO(+) \rightarrow \quad (4)
\]

\[
NSPANI - AuNP - GR / ITO(+) + e \rightarrow NSPANI - AuNP - GR / ITO (at the electrode) \quad (5)
\]

7.3 Photometric response studies

The response characteristics of ChOx-HRP/NSPANI-AuNP-GR/ITO and ChOx/NSPANI-AuNP-GR/ITO bioelectrodes were studied as a function of cholesterol concentration (Figure 8) and the value of absorbance resulting from the oxidised form of dye has been found to be increasing linearly with increase in cholesterol concentration for both the bioelectrodes. It has been found that the ChOx-HRP/NSPANI-AuNP-GR/ITO bioelectrode in the range of 35-400 mg/dl for cholesterol concentration follows the equation [Change in absorbance = 0.022 + 0.00016 × cholesterol concentration (mg/dl) with 0.003 as standard deviation whereas ChOx/NSPANI-AuNP-GR/ITO bioelectrode in the range of 35-350 mg/dl for cholesterol concentration follows the equation]. Change in absorbance = 0.002 + 0.000088 × cholesterol concentration (mg/dl) with 0.0025 as standard deviation. The value of apparent Michaelis-Menten constant (Km) has been estimated using the Lineweaver-Burke plot, graph between inverse of absorption and
inverse of cholesterol concentration. The lower value of $K_m$ (0.012 mM) for ChOx-HRP/NSPANI-AuNP-GR/ITO biosensor as compared to ChOx/NSPANI-AuNP-GR/ITO biosensor (0.023 mM) suggest that the NSPANI-AuNP-GR matrix is facilitating the enzymatic reaction.

Figure 8  Photometric response of (a) ChOx/NSPANI-AuNP-GR/ITO  
(b) ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes as a function of cholesterol concentration (see online version for colours)

Figure 9  Effect of pH on (a) ChOx/NSPANI-AuNP-GR/ITO  
(b) ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes (see online version for colours)
7.4 Studies of pH, interference, reusability and shelf life of biosensors

7.4.1 pH studies

The response current of the ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO nano bioelectrodes studied in the pH range 6.0–7.8 (Figure 9), suggests that both the bioelectrodes exhibit maximum activity at around pH 7.0. At this pH, the biomolecules retain their natural structures and do not get denatured. Thus all experiments have been conducted out at the optimum pH value of 7.0 for cholesterol estimation.

7.4.2 Interference studies

Different interferents which are mostly present in blood such as AA (0.05 mM), glucose (5 mM), UA (0.1 mM), sodium ascorbate (0.05 mM) and urea (1 mM) were tested for both the nanobioelectrodes such as ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO using cholesterol solution (100mg/dl) in a 1:1 ratio. Figure 8 shows the effect of interferents on the observed response of ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO bioelectrodes. In the Figure 10, the first bar (Cholesterol) shows the current obtained with 100mg/dl cholesterol. The remaining bars show the current corresponding to the mixture of cholesterol and interferents in a 1:1 ratio. The percentage interference (% interference) was calculated using equation (6) for various interferents.

\[
\text{\% Interference} = \left( \frac{\Delta I_{\text{cholesterol}} - \Delta I_{\text{interferent}}}{\Delta I_{\text{cholesterol}}} \right) \times 100
\]

where \( \Delta I_{\text{cholesterol}} \) is the change in current obtained with 100 mg/dl cholesterol and \( \Delta I_{\text{interferent}} \) is the change in current corresponding to the mixture of cholesterol and interferents in a 1:1 ratio. A maximum of 6% interference for ChOx-HRP/NSPANI-AuNP-GR/ITO bioelectrode and 9% interference for ChOx/NSPANI-AuNP-GR/ITO bioelectrode are observed.

7.4.3 Reusability studies

The unique features of both the type of nanobioelectrodes are their reusability (Figure 10) which is attributed due to composition of the transducer matrix. It has been found that ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes can be reused in number of times with 100 % efficiency. Figure 10 reflects the response of nanobioelectrodes for 15 times testing using the same nanobioelectrode with 100 mg/dl cholesterol concentration in PBS solution (50mM, 0.9% NaCl, 5mM \([\text{Fe(CN)}_6^{3-4-}]\)) at room temperature (25°C). The reusability of the nanobioelectrodes can be attributed to robust properties of the transducer matrix. The reusability indicates that NSPANI-AuNP-GR matrix offers a favourable microenvironment for enzymes which does not cause denaturing of enzymes. The reusability can be explained by the enhanced stability of the enzymes, indicating unique electrochemical properties and biocompatibility of NSPANI-AuNP-GR/ITO electrode.
Figure 10  Interferent study of (a) ChOx/NSPANI-AuNP-GR/ITO (b) ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes (see online version for colours)

Figure 11  DPV curves for reusability testing (current vs. potential plot with 100 mg/dl analyte for 8 times) (a) ChOx/NSPANI-AuNP-GR/ITO (b) ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes (see online version for colours)
7.4.4 Shelf life studies

The shelf lives of ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes have been determined by measuring the response current at regular interval of one week for about two months. Figure 12 demonstrates the shelf life of the ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes. The ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes are stored at 4°C when not in use. The nanobioelectrodes have been found to be stable up to 12 weeks without any loss in activity.

Table 1  A comparative evaluation of single and bienzymatic biosensor performance

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Characteristics</th>
<th>ChOx/NSPANI-AuNP/GR/ITO</th>
<th>ChOx-HRP/NSPANI-AuNP/GR/ITO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Linearity</td>
<td>35–400 mg/dl</td>
<td>35–500 mg/dl</td>
</tr>
<tr>
<td>2</td>
<td>Detection limit</td>
<td>35 mg/dl</td>
<td>25 mg/dl</td>
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<tr>
<td>3</td>
<td>Response time</td>
<td>28 secs.</td>
<td>19 secs.</td>
</tr>
<tr>
<td>4</td>
<td>Sensitivity</td>
<td>3.10µA mgdl⁻¹</td>
<td>4.22µA mgdl⁻¹</td>
</tr>
<tr>
<td>5</td>
<td>Km</td>
<td>0.02mM</td>
<td>0.01mM</td>
</tr>
<tr>
<td>6</td>
<td>Shelf life</td>
<td>8 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>S. no.</td>
<td>Components of biosensor</td>
<td>Characteristics</td>
<td>References</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------</td>
<td>----------------</td>
<td>------------</td>
</tr>
<tr>
<td>1</td>
<td>(Mat) ChOx-HRP/NSPANI-AuNP-GR/ITO (E) ChOx-HRP (M) Ampero. vs. Ag/AgCl</td>
<td>(L) upto 500 mg/dl (S) 4.22 μA mg/dl⁻¹ (Km) 0.01 mM (DL) 25 mg/dl (RT) 19 secs (SL) 2 months</td>
<td>Present investigation</td>
</tr>
<tr>
<td>2</td>
<td>(Mat) ChOx/f-G/GC ChOx/Au/f-G/GC (E) ChOx (M) Ampero. vs. Ag/AgCl</td>
<td>(L) upto 135 μM (S) 314 nA/μM cm² (SL) 1 month</td>
<td>Aravind et al. (2011)</td>
</tr>
<tr>
<td>3</td>
<td>(Mat) ChOx/NSPANI-SDS (E) ChOx (M) Photometric</td>
<td>(L) 05–10.5 mM (S) 9 mM (Km) 1.32 mM (RT) 59 secs (SL) 5 weeks</td>
<td>Deepshikha and Basu (2011d)</td>
</tr>
<tr>
<td>4</td>
<td>(Mat) GR-Pt nanoparticle hybrid material (E) ChOx, ChEt (M) Ampero. vs. Ag/AgCl</td>
<td>(L) upto 12 mM (S) 2.07 ± 0.1 μA/μM cm² (Km) 5 mM (DL) 0.2 μM</td>
<td>Aravind et al. (2011)</td>
</tr>
<tr>
<td>5</td>
<td>(Mat) GOx-HRP/MWCNT/PPY/ITO (E) GOx, HRP (M) Ampero. vs. Ag/AgCl</td>
<td>(L) 1–10 mM (S) 13.8 mA/μM (Km) 0.52 mM (DL) 0.1 mM (RT) 10 secs (SL) 5 weeks</td>
<td>Singh et al. (2011a)</td>
</tr>
<tr>
<td>S. no.</td>
<td>Components of biosensor</td>
<td>Characteristics</td>
<td>References</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------</td>
<td>----------------</td>
<td>------------</td>
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<tr>
<td>6</td>
<td>(Mat) ChOx/NanoFe$_3$O$_4$/ITO (E) ChOx (M) Ampero. vs. Ag/AgCl</td>
<td>(L) 2.5–400 mg/dl (S) 86Ω/mg/dl/cm$^2$ (Km) 0.8 mg/dl (DL) 0.25 mg/dl (RT) 25 secs (SL) 55 days</td>
<td>Kaushik et al. (2010)</td>
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<td>7</td>
<td>(Mat) ChEt–ChOx/MWCNT/SiO$_2$–CHIT/ITO (E) ChEt–ChOx (M) Ampero. vs. Ag/AgCl</td>
<td>(L) 10–500 mg/dl (S) 2.12 μA/mM (Km) 0.052 mM (DL) 0.1 mM (RT) 10 secs (SL) 10 weeks</td>
<td>Solanki et al. (2009)</td>
</tr>
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<td>8</td>
<td>(Mat) ChOx/PANI-NS/ITO (E) ChOx (M) Ampero. vs. Ag/AgCl</td>
<td>(L) 25–500 mg/dl (S) $1.3 \times 10^{-3}$ mA/mg$^{-1}$dl (Km) 2.5 mM (RT) 10 secs. (SL) 12 weeks</td>
<td>Dhand et al. (2010)</td>
</tr>
<tr>
<td>Sample no.</td>
<td>ChOx/NSPANI/Au-GR/ITO (amperometric)</td>
<td>Error (%)</td>
<td>ChOx/NSPANI/Au-GR/ITO (photometric)</td>
</tr>
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<td>------------------------------------</td>
<td>-----------</td>
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</tr>
<tr>
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<td>5</td>
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</table>
8 Comparative evaluation of mono and bienzymatic biosensor

Table 1 represents a comparative evaluation of mono and bienzymatic biosensor performance. It has been found that bienzymatic electrodes ChOx-HRP/NSPANI-AuNP-GR/ITO exhibit better performance in terms of linearity, shelf life, response time and sensitivity as compared to mono enzyme-based ChOx/NSPANI-AuNP-GR/ITO electrode. The immobilisation of the ChOx together with HRP is thought to either help the protein to assume a favourable orientation or to make possible conducting channels between the prosthetic groups and the electrode surface. Both can reduce the effective electron transfer distance and thereby facilitates the charge transfer between the electrode and the enzyme (Deepshikha and Basu, 2011).

Table 2 shows the characteristics of ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrode including reported in the literature for ChOx-HRP system.

9 Blood serum testing

The response of the ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrodes to the cholesterol in human blood serum has been investigated by amperometric and photometric studies and results were compared. Five serum samples obtained from pathological lab were analysed. Table 3 shows the results from the blood serum samples using ChOx/NSPANI-AuNP-GR/ITO and ChOx-HRP/NSPANI-AuNP-GR/ITO biosensors. Both the nanobioelectrodes provide excellent performance in evaluation of cholesterol in blood serum samples which may be due to the high electrocatalytic effect of NSPANI-AuNP-GR/ITO nanocomposite electrode. The results obtained from amperometric determination of free cholesterol in blood serum are compared with the results obtained from the photometric response studies considering as standard values of free blood cholesterol. The results obtained for ChOx-HRP/NSPANI-AuNP-GR/ITO by using amperometric and photometric studies are very close to each other with minimum error while ChOx/NSPANI-AuNP-GR/ITO shows comparatively higher deviation.

10 Conclusions

Gold nanoparticles decorated Graphene-Nanostructured Polyaniline Nanocomposite electrodes have been fabricated for the development of reusable cholesterol biosensor. Both single ChOx and ChOx-HRP-based nano biosensors are developed using covalent bonding through gluteraldehyde. The bienzyme-based nanobioelectrodes (ChOx-HRP/NSPANI-AuNP-GR/ITO) offer better performance in terms of detection limit, sensitivity, and response time than single enzyme system. This is attributed to the
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presence of HRP along with ChOx to enhance the overall biochemical reaction. It has been shown that this nanobiocomposite electrode can be used to estimate cholesterol in blood serum samples. The unique features of the ChOx-HRP/NSPANI-AuNP-GR/ITO nanobioelectrode lie with the novelty of fabrication, minimum interference, very low $K_m$ value, low response time, excellent reusability and the its usefulness for blood serum samples. The large specific surface area, excellent conductivity, stable and reliable redox properties of NSPANI-AuNP-GR nanocomposite electrode allow the rapid transit of electron and enhance current response for the immobilised enzymes. It should be interesting to utilise these nanocomposite electrodes for development of other biosensors.

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References


