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Construction of triglyceride biosensor based on nickel oxide–chitosan/zinc oxide/zinc hexacyanoferrate film

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ABSTRACT

A method is described for construction of an amperometric triglyceride (TG) biosensor based on co-immobilization of lipase, glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO) onto nickel oxide nanoparticles (NiONPs)–chitosan (CHIT) nanocomposite adsorbed onto zinc oxide/zinc hexacyanoferrate (ZnO–ZnHCF) hybrid film electrodeposited on the surface of an Au electrode. The NiONPs–CHIT/ZnO–ZnHCF hybrid film was characterized by cyclic voltammetry (CV), atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS). The biosensor showed optimum response within 4 s at pH 6.0 and 35 °C, when polarized at +0.4 V against Ag/AgCl. There was a linear relationship between sensor response and triolein concentration in the range 50–700 mg/dl with sensitivity of 0.05 μA/mg/dl. The sensor was employed for determination of TG in serum. The detection limit of the biosensor was 10 mg/dl. The biosensor was evaluated with 95–96% recovery of added triolein in sera and 2% and 3% within and between batch coefficients of variation (CVs) respectively. There was a good correlation ($r = 0.99$) between serum TG values by standard enzymic colorimetric method and the present method. The biosensor lost 50% of its initial activity after its 100 uses over a period of 180 days, when stored at 4 °C.

1. Introduction

Triglycerides (TG) are generated by esterification of three hydroxyl (—OH) groups of glycerol with three molecules of fatty acids. The normal level of TG in serum is in the range, 40–190 mg/dl [1]. The elevated level of triglyceride in serum is an important diagnostic marker for atherosclerosis associated with human vascular disorders such as coronary artery disease (CHD) [2]. Among several methods available for determination of TG such as titrimetric [3], enzymic colorimetric [4], spectrophotometric [5], fluorometric [6], HPLC [7] and mass fragmantography [8], electrochemical sensing methods have the advantages over these methods, because of their simplicity, sensitivity, rapidity and no requirement of sample preparation. There are two types of enzyme based TG biosensors, (i) potentiometric biosensors using porous silicon [9] and (ii) amperometric biosensors using enzymes immobilized onto artificial membranes such as collagen [10], cellulose acetate (CA) [11], polyvinylchloride (PVC) [12], polyvinyl alcohol (PVA) [13] and eggshell membranes mounted on metal electrodes [14] Prussian blue modified screen printed electrode [15] iridium nano-particle [16], polyaniline (PANI)/single-walled carbon nanotubes (SWCNT) [17], cerium oxide film [18] and zinc oxide nanoparticles [19]. However these immobilization methods allow leakage of enzyme, resulting in a low stability of the enzyme electrode. Covalent immobilization of enzyme not only overcomes this problem but also leads to better biomolecule activity and greater stability. There is a growing considerable interest in the application of chemically modified enzyme electrodes prepared by deposition of a thin film of mixed-valance compounds [20–22]. Metal hexacyanoferrates have fascinated significant attention as exceptional surface modifier, ever since its first report by Neff [23] on deposition of a thin film of Prussian blue (PB) on an electrode surface. Since the formation of zinc hexacyanoferrate (ZnHCF) film on a carbon electrode [24] and polynuclear mixed-valent hybrid film of ZnO/ZnHCF and ruthenium oxide hexacyanoferrate (RuO-HCF) deposited on GC electrode [25], the films have been used as an effective protecting layer to prevent the anodic dissolution of zinc substrate [26]. Metal oxide nanoparticles show evidence of higher ratios of surface area to volume than their bulk counterparts, so metal oxide nanoparticle modified electrochemical interfaces are expected to provide larger electrochemically active areas and consequently lead to higher detection sensitivity for target molecules. Nickel oxide nanoparticles (NiONPs) are magnetic and have very large surface areas; hence these nanoparticles will also have very high surface energy [27]. The high surface active area of NiONPs is

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available for enzymes and provides responsive microenvironment to maintain its bioactivity and improve its stability [28]. Chitosan (CHIT) along with nanoparticles has been utilized as a stabilizing agent due to its excellent film-forming ability, mechanical strength, biocompatibility, non-toxicity, high permeability toward water, susceptibility to chemical modifications, cost-effectiveness, for enzyme immobilization [29]. Moreover, amino groups of CHIT provide a hydrophilic environment compatible with the biomolecules and in acidic condition, CHIT showed a cationic nature that may provide an electrostatic core environment to zwitterions molecules such as enzyme. We describe herein the construction of an amperometric TG biosensor based on covalent co-immobilization of lipase, glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO) onto thin film of NiONPs–CHIT/ZnO–ZnHCF hybrid and its application for determination of serum TG.

2. Materials and methods

2.1. Reagents and materials

Lipase (EC 3.1.1.3) (40–70 U/mg) from *Candida rugosa*, glycerol kinase (GK) from *Cellulomonas* species (25–75 U/mg), glycerol-3-phosphate oxidase (GPO) from *Aerococcus viridans* (113 U/mg), Triton X-100 from Sigma–Aldrich (St. Louis, MO, USA), 3,5-dichloro-2-hydroxy benzene sulfonic acid (DHBS) from E-Merk, Germany, triolein, ATP, chitosan ($M_W \sim 1 \times 10^6$; 75–85% deacetylation), zinc nitrate ($Zn(NO_3)_2{\cdot}4H_2O$) and nickel chloride ($NiCl_2$) from SISCO Research Laboratory Pvt. Ltd., Mumbai and kit for enzymic colorimetric method (Enzo kit) for TG from Erba Transasia, Daman, India were used. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) (ohmic resistance: 1.8×10^5 per ohm) was used throughout the study.

2.2. Apparatus and methods

Cyclic voltammetry (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat/Galvanostat (Autolab, Eco Chemie, The Netherland; model no: AUT83785) with a three electrode system consisting of a Pt wire as auxiliary electrode, Ag/AgCl electrode as reference electrode and modified Au wire 1.5 cm height × 0.05 cm diameter (23 carat) as working electrode. All the electrochemical experiments were performed at an ambient temperature (25 °C). Atomic force microscopes (AFM) studies of bare Au electrode and NiONPs–CHIT/ZnO–ZnHCF film modified Au electrode were carried out at Physics Department, IIT, New Delhi using NanoScope III, Veeco Metrology Group (model no. NS3, Inc., Santa Barbara, CA, USA). An aqueous solution of NiONP was subjected to transmission electron microscopy (TEM) at Punjab University, Chandigarh to determine the size of NiONP. X-ray diffraction (XRD) study of NiONP was carried out at Physics Department G. J. University, Hisar, using X-ray diffractometer (make: Rigaku Mini Flex II, Americas Corporation).

2.3. Preparation of triolein solution

Triolein was used as a substrate for lipase. Triolein emulsion was prepared as described [30]. Solutions of different concentrations of triolein ranging from 10 to 800 mg/dl were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and stored at 4 °C until use.

2.4. Preparation of ZnO–ZnHCF film

Prior to film deposition, the Au electrode (0.05 mm) was polished with alumina slurry and then ultrasonically cleaned (amplitude 3.5 μm; rotation 100 rpm) for about a min in DW. Finally, the electrode was washed thoroughly with DW and used. The electrochemical deposition of zinc oxide/zinc hexacyanoferrate films on the surface of Au electrode was accomplished by potentiodynamic cycling of the working electrode range between potential +0.4 V to +1.6 V at a scan rate of 20 mV/s in a suitable aqueous H_2SO_4 solution (0.1 M pH 2.0) containing $Zn(NO_3)_2$ and $K_3[Fe(CN)_6]$. After 30 cycles, the electrode was taken out and rinsed thoroughly with DW. Cyclic voltammetric study of ZnO–ZnHCF hybrid film-modified electrode was carried out during electrodeposition of this hybrid film.

2.5. Preparation of nanocomposite of NiONPs and chitosan

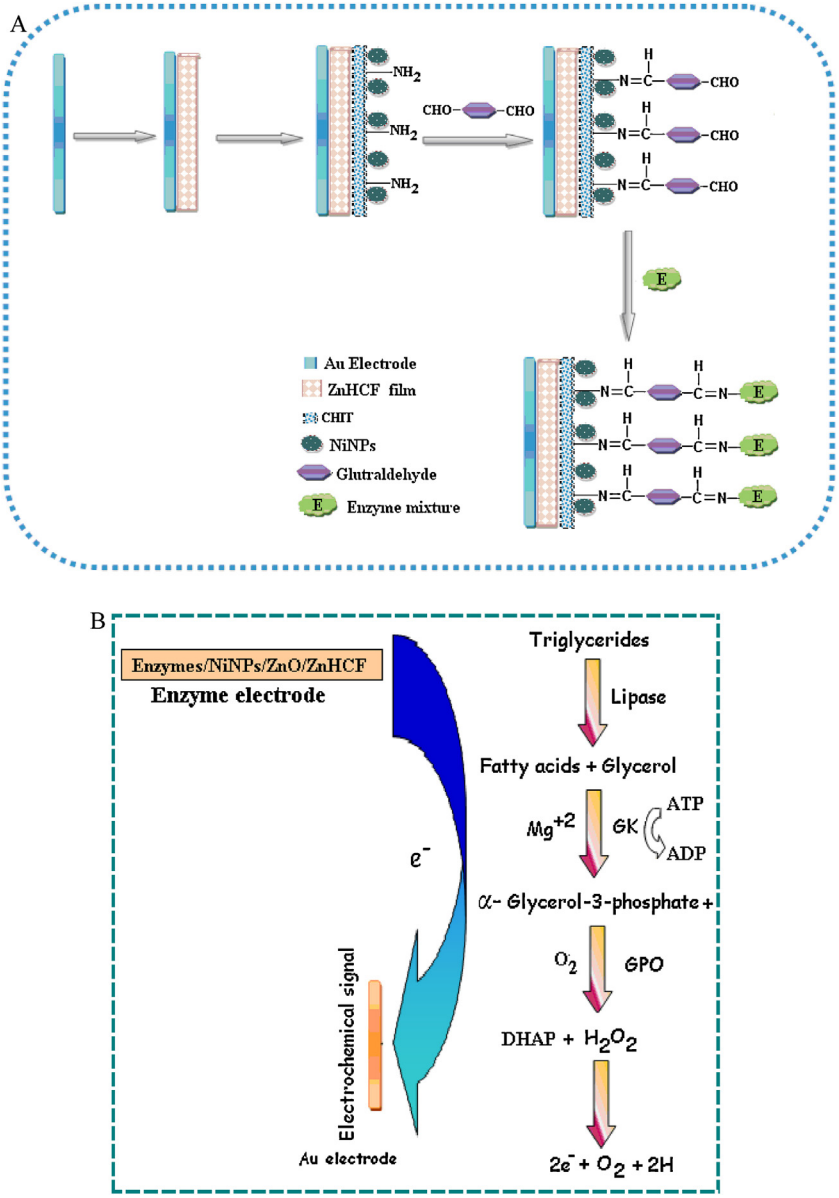
NiONPs were synthesized by two-step method [24]. Firstly 2.3 g $NiCl_2$ was dissolved in 10 ml DW and 1.5 g $NaHCO_3$ was dissolved in 10 ml DW in separate glass beakers. The $NiCl_2$ solution was stirred on a magnetic stirrer for 15 min and then $NaHCO_3$ solution was added to $NiCl_2$ solution drop wise under constant stirring. After 15 min, the products were collected by centrifugation at 5000 × *g* for 10 min and washed thoroughly with DW and dried in air. The structural property of NiONPs was studied. The NiONPs (5 mg) were dispersed into transparent chitosan (CHIT) solution (0.5 g of CHIT dissolved in 10 ml of 0.05 M acetate buffer solution pH 5.0) and kept overnight at room temperature followed by magnetic stirring for about 30 min and then sonication for about 1 h at room temperature. A hybrid nanocomposite of NiONPs and CHIT was formed.

2.6. Adsorption of NiONPs–CHIT nanocomposite onto ZnO–ZnHCF hybrid film modified Au electrode

The NiONP–CHIT nanocomposite was adsorbed on the surface of ZnO–ZnHCF hybrid film modified Au electrode. Different durations of physisorption of nanocomposite resulted into in different amounts of nanocomposite being deposited onto ZnO–ZnHCF hybrid film modified Au electrode, which would eventually generate different catalytic activities toward TG. The optimal time for physisorption was determined and found to be 24 h. ZnO–ZnHCF film could not be covered thoroughly by hybrid nanocomposite with a shorter physisorption time, causing insufficient active sites for TG catalysis. However, a longer physisorption time caused the hybrid nanocomposite to aggregate into much larger particles or bundles, which negates the relative advantage of larger reactive surface area of smaller NiONPs.

2.7. Immobilization of enzymes on NiONPs–CHIT decorated ZnO–ZnHCF film modified Au electrode

The hybrid of NiONPs and CHIT nanocomposite bound to ZnO–ZnHCF film modified Au electrode was dipped into 2.5% glutaraldehyde in 0.02 M phosphate buffer pH 7.0 and kept it for 2 h at room temperature. The Au electrode was taken off from glutaraldehyde solution and washed thoroughly with DW and finally with sodium phosphate buffer (50 mM, pH 7.0). This activated hybrid nanocomposite film on to Au electrode was dipped into 5 ml enzyme solution (10 mg protein) containing lipase (40 U), GK (25 U) and GPO (110 U) in 4:2.5:11 unit ratio and kept overnight at 4 °C for covalent immobilization of enzyme. The hybrid nanocomposite modified film electrode was taken off from enzyme solution and washed with DW followed by sodium phosphate buffer (50 mM, pH 7.0) and stored dry at 4 °C until use. There was 60.2% retention of the protein (total 1.8 mg protein) after immobilization. Glutaraldehyde provided covalent immobilization of enzyme. One —CHO group of glutaraldehyde was linked to $-NH_2$ group of surface of enzymes (lipase + GK + GPO), while it's other —CHO group was bound to $-NH_2$ group of CHIT (Scheme 1A).



Scheme 1. (A) Schematic representation of enzymes/NiONP–CHIT/ZnO–ZnHCF hybrid film modified Au electrode. (B) Electrochemical reactions at enzymes/NiONP–CHIT/ZnO–ZnHCF hybrid film modified Au electrode.

2.8. Electrochemical measurements

The electrochemical characterizations and measurements on the modified electrode were carried out by cyclic voltammetry using Potentiostat/Galvanostat. All experiments were carried out using a conventional three-electrode system consisting enzymes/NiONPs–CHIT/ZnO–ZnHCF hybrid film modified Au electrode as the working electrode, a Pt wire as the auxiliary electrode and an Ag/AgCl (saturated KCl) electrode as the reference electrode. Electrochemical experiments were carried out in phosphate buffer saline (PBS, 50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ as redox probe. The current response was measured between 2 s and 12 s at an interval of 2 s.

2.9. Optimization of working conditions of TG biosensor

Various kinetic properties of hybrid nanocomposite film modified Au electrode such as optimum pH, incubation temperature, response time and effect of substrate (triolein) concentration were studied amperometrically in order to optimize the working conditions of the biosensor.

2.10. Amperometric determination of TG in serum

Blood samples (1 ml each) from apparently healthy male and apparently healthy female and persons suffering from hypertriglyceridemia, peripheral vascular disease and pancreatitis in various age groups ($n = 8$) were collected from local Pt. BDS Post Graduate Institute of Medical Sciences (PGIMS) Rohtak Hospital. After keeping at room temperature for 1 h, these blood samples were centrifuged at $5000 \times g$ for 5 min and their supernatants (sera) were collected and stored at 4 °C until use. TG content in serum was determined by the present biosensor in the similar manner as described above for its response measurement, under its optimal working conditions except that triolein was replaced by serum. The current (mA) was measured and the amount of TG in serum was interpolated from standard curve between triolein concentrations and current (in mA) prepared under optimal working conditions.

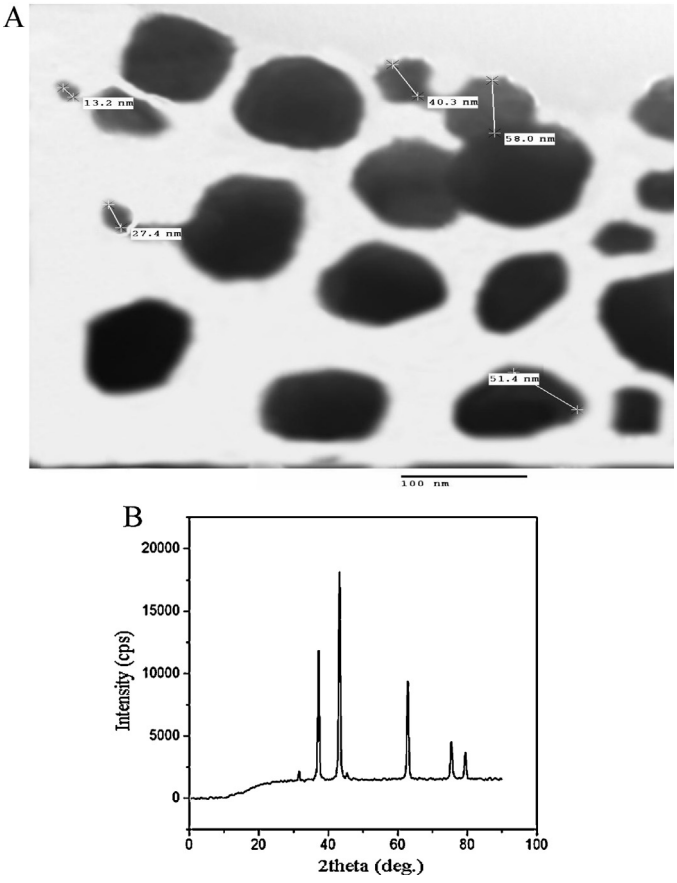


Fig. 1. (A) Transmission electron microscopy of NiONPs (B) XRD pattern of NiONPs.

2.11. Evaluation of TG biosensor

The biosensor was evaluated by studying analytic recovery, precision and correlation. The effect of various interferrants found in blood such as urea, uric acid, glucose, fructose, ethanol, cholesterol, citric acid, ascorbic acid, lactic acid, malic acid, tartaric acid, alanine, leucine, bilirubin, creatinine and pyruvic acid was studied at their physiological concentrations.

2.12. Reusability and storage stability of enzymes/NiONPs–CHIT/ZnO–ZnHCF hybrid film modified Au electrode

The reusability and long-term storage and stability of the biosensor were investigated over a period of 180 days at an interval of 30 days, during its storage in dry condition at 4 °C.

3. Results and discussion

The typical TEM images of NiONPs of (Fig. 1 A) showed the spherical shape of NiONPs. The TEM image, shows a large number of NiONPs, consider single one of them, the calculated average size was between 20–40 nm. The XRD patterns of the prepared samples clearly showed the characteristics peaks of NiONPs appeared at 32.66°, 37.96°, 43.28°, 64.34°, 76.76°, and 80.56° (Fig. 1B). No characteristic peaks of impurities were observed, suggesting that high purity of the as-prepared NiONPs samples. All peaks were consistent with the peaks of NiONPs with high crystallinity [31]. An amperometric triglyceride biosensor was constructed by glutaraldehyde coupling of enzymes onto NiONPs–CHIT composite decorated ZnO–ZnHCF hybrid film electrodeposited on Au

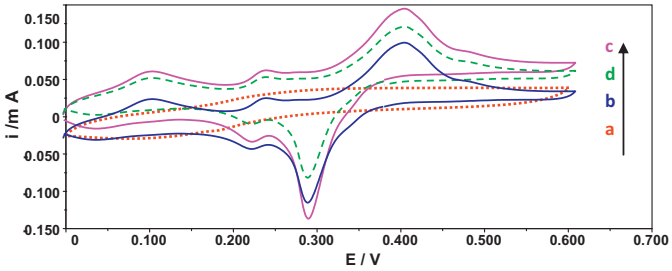


Fig. 2. Cyclic voltammograms of (a) bare Au electrode, (b) ZnO–ZnHCF, (c) NiONP–CHIT/ZnO–ZnHCF and (d) enzymes/NiONP–CHIT/ZnO–ZnHCF modified Au electrode in PBS (50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4}$ at a scan rate of 20 mV/s.

electrode. The following electrochemical studies were carried out during the process.

3.1. Electrochemical characterization of ZnO–ZnHCF hybrid film modified Au electrode

The cyclic voltammetric technique was used for studying the electrodeposition process of ZnO–ZnHCF hybrid film. Typical cyclic voltammograms obtained with the ZnO–ZnHCF modified Au electrode at different scan rates after immersing in a H_2SO_4 solution (0.1 M, pH 2) containing 10^{-4} M Zn $(NO_3)_2$ and 10^{-4} M K_3 $[Fe(CN)_6]$ solution. The repeatedly increasing currents for both anodic and cathodic peak demonstrated the deposition of ZnO–ZnHCF hybrid film continuously on the electrode surface. Experiments using the ZnO–ZnHCF/Au electrode produced two redox couples at potentials of +1.2 V and +0.91 V vs. Ag/AgCl. By the reduction of nitrate, ZnO was deposited through the potentiodynamic cycling, due to base generation [32]. The following electrochemical reaction could be related to growth of ZnO film.

$$Zn^{2+} + NO_3^- + 2e^- \rightarrow ZnO + NO_2^- \quad (1)$$

With the increase in scan rates, both the cathodic and anodic current was increased. Voltammogram results were recorded after several preliminary scans. No voltammetric response was observed for the unmodified electrode.

3.2. Voltammetric response of the enzymes/NiONPs–CHIT/ZnO–ZnHCF hybrid film modified Au electrode

No peak was observed in case of unmodified electrode (Fig. 2, curve a). However, a well defined oxidation and reduction peak with a peak potential of +0.1 V, 0.25 V and +0.4 V, respectively was observed in case of ZnO–ZnHCF film modified Au electrode (Fig. 2, curve b). Both the oxidation and reduction current for the NiONPs–CHIT/ZnO–ZnHCF film were increased after physical absorption of hybrid nanocomposite on the surface of ZnO–ZnHCF film (Fig. 2, curve c). The NiONPs might provide a large available surface and enhance the electrocatalytic activity for TG electro oxidation. The results of the CV also proved that NiONPs could provide a conductive path through the composite membrane matrix. So the NiONPs acting as electron-transfer accelerator help in enhancing the current response of enzyme electrode and thus increasing the sensitivity of the biosensor. The low reduction and oxidation current of enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode confirmed that the enzyme got immobilized on the surface of modified Au electrode (Fig. 2, curve d). These results indicated that a slow redox process occurred at the electrode surface due to slow electron transfer from the surface showing the immobilization of enzyme.

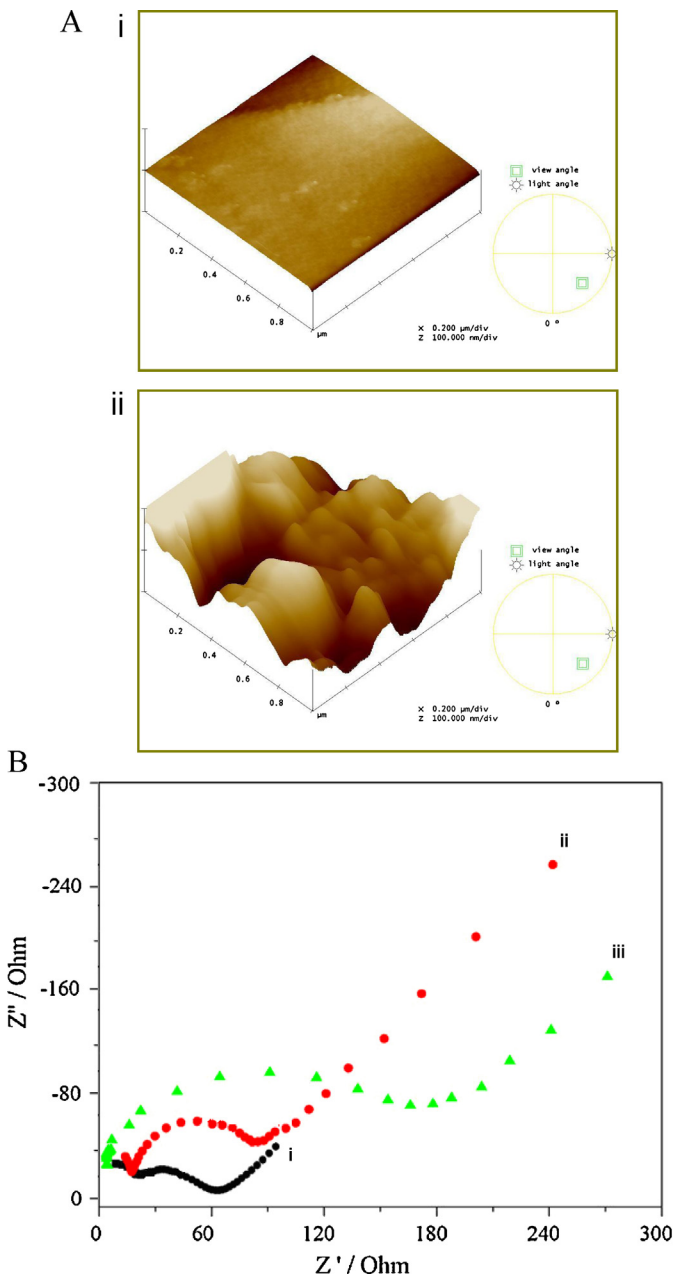


Fig. 3. (A) Three-dimensional amplification of the AFM image of the bare Au electrode (i) and NiONP–CHIT/ZnO–ZnHCF hybrid film modified Au electrode (ii). (B) Electrochemical impedance spectra of bare Au electrode (i), electro-deposited ZnO–ZnHCF (ii) and NiONP–CHIT/ZnO–ZnHCF (iii) electrodes in PBS (50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4}$. Frequency range: 0.01 Hz–10 kHz.

3.3. AFM study of CHIT–NiONPs/ZnO–ZnHCF hybrid film

A qualitative indication of the differences in roughness of two samples was obtained from the gray-scale index that depicts the height profile of the surface features. The surface of NiONPs–CHIT/ZnO–ZnHCF hybrid film modified Au electrode was found to be rougher compared with bare Au electrode (Fig. 3A, i). The surface of NiONPs–CHIT/ZnO–ZnHCF hybrid film modified Au electrode (Fig. 3A, ii) exhibited humps, undulations and features with greater heights (max. height of 28.75 nm) in comparison with bare Au electrode. The highly undulated surface of NiONPs–CHIT/ZnO–ZnHCF hybrid film modified Au electrode represents increase in surface area of modified Au electrode.

3.4. Electrochemical impedance studies of modified Au electrode

The charge transfer process in enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode has been studied by monitoring charge transfer resistance (Rct) at the electrode and electrolyte interface. The value of the electron transfer resistance (semicircle diameter) (Rct) depends on the dielectric and insulating features at the electrode/electrolyte interface. The Rct values for the ZnO–ZnHCF/Au electrode (Fig. 3B, i), NiONPs–CHIT/ZnO–ZnHCF/Au electrode (Fig. 3B, ii) and enzymes/NiONPs–CHIT//ZnO–ZnHCF/Au electrode (Fig. 3B, iii) were as 60, 90 and 170 Ω, respectively. These results showed that the electron transfer via redox couple is hindered by the presence of enzymes on electrode surface. The increased Rct value of enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode was due to the immobilization of enzymes onto NiONPs–CHIT/ZnO–ZnHCF/Au surface. This increase in Rct is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz; applied voltage: 0.4 V) and cause hindrance to the electron transfer.

3.5. Current response measurement of enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode

The electrochemical response of the enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode has been investigated as a function of triolein concentration (50–750 mg/dl) using CV technique at 20 mV/s scan rate in PBS (50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ (the electrical conductivity of PBS = 25 Ω). Scheme 1B shows proposed electrochemical reactions at the enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode during triolein detection. TG biosensor is based on measurement of electrons i.e., current generated from H_2O_2 under high potential. At the surface of enzyme electrode, co-immobilized lipase hydrolyses TG into free fatty acids and glycerol, which is phosphorelated into glycerol-3-phosphate by co-immobilized GK. This glycerol-3-phosphate is oxidized into dihydroxyacetone phosphate and H_2O_2 by GPO. The electrons thus generated from H_2O_2, are facilitated from solution to Au electrode through transducer (NiONPs–CHIT/ZnO–ZnHCF hybrid film).

The current response curves of enzyme electrodes without and with hybrid nanocomposite and only with NiONPs are shown in Fig. 4A (i, ii and iii). When TG concentration was 100 mM, the current response of the electrode without nanoparticles was 0.75 mA, while the same with hybrid nanocomposite was 1.55 mA. The results indicated that the amount of hybrid nanocomposite had an intense effect on the electrode response.

3.6. Optimization of the biosensor

Effect of working potential, response time, pH and temperature on the biosensor was observed. The influence of applied potential on TG detection at hybrid nanocomposite ZnO–ZnHCF modified electrode was studied and an optimal potential of +0.4 V vs. Ag/AgCl was found. The working potential was stepped from 0.0 to +0.5 V. This optimum applied potential of +0.4 V ensures a minimizing of interference effects. This may be due to decrease in the concentration of positively charged moieties present onto NiONPs–CHIT/ZnO–ZnHCF matrix as pH of PBS approaches toward its isoelectric point (9.2) resulting into decreased interaction between redox ions $[Fe(CN)_6]^{3-/4-}$ onto ZnO–ZnHCF surface. The effect of time on the enzymes was also determined by recording the current response from 2 s to 12 s at an interval of 2 s. The modified electrode showed maximum response at 4 s. This low response time could be due to co-immobilization of enzymes on the surface of modified Au electrode, where the product of the first enzyme

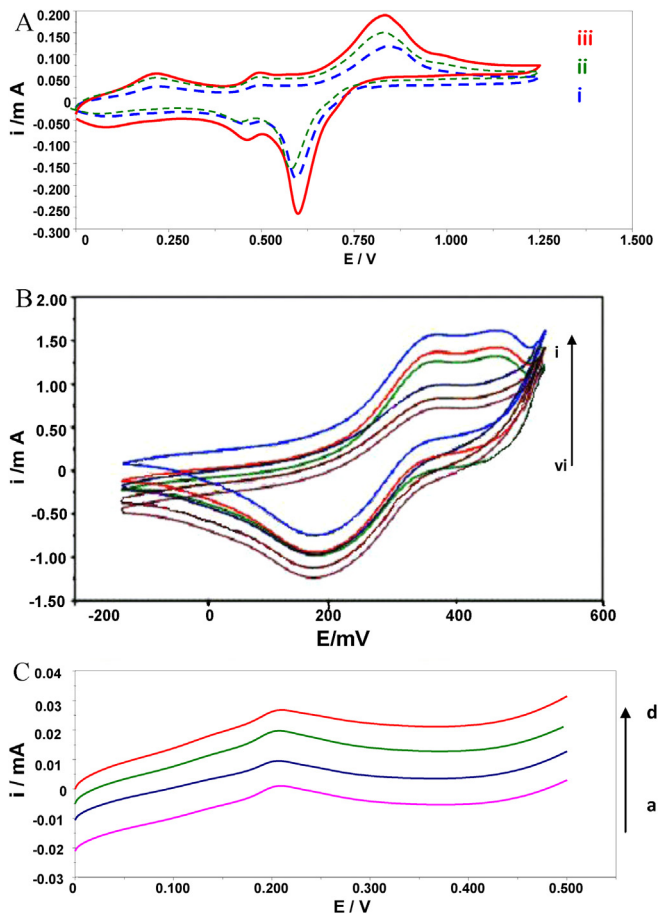


Fig. 4. (A) Cyclic voltammograms of (i) ZnO/ZnHCF film/AuE, (ii) NiONPs/AuE and (iii) NiONPs–CHIT–ZnO/ZnHCF film/AuE in PBS (50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 μl of triolein (0.5 mM). Scan rate: 20 mV/s. (B) Cyclic voltammetry studies of NiONP–CHIT/ZnO–ZnHCF modified electrode as a function of pH as 6.5 (i), 5.5 (ii), 6.0 (iii), 7.5 (iv), 8.5 (v) and 9.0 (vi) of PBS (50 mM) at scan rate 20 mV/s between −0.2 and +0.6 V. (C) Square wave voltammetry of modified electrode using different concentrations of substrate (a) 50 (b) 350 (c) 650 (d) 750 mg/dl in PBS (50 mM, pH 6.5, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ at a potential range 0 to +0.6 V, scan rate 20 mV/s vs. Ag/AgCl.

becomes the substrate for the second enzyme and so on leading to fast response. Fig. 4B shows the effect of pH of the buffer solution on the performance of the biosensor. The effect of pH (ranging from 5.5 to 9) onto NiONPs–CHIT/ZnO–ZnHCF/Au electrode and enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au bioelectrode has been investigated using CV technique in 5 mM $[Fe(CN)_6]^{3-/4-}$ at scan rate of 20 mV/s. It can be seen that magnitude of the peak current decreases and the peak potential shifts toward higher potential on increasing pH. This may be due to decrease in the concentration of positively charged moieties present onto NiONPs–CHIT/ZnO–ZnHCF matrix as pH of PBS approaches toward its isoelectric point (9.2) resulting into decreased interaction between redox ions $[Fe(CN)_6]^{3-/4-}$ onto ZnO–ZnHCF surface. This results in increased hindrance in electron transport between the medium and electrode leading to decreased value of electrochemical signal at elevated pH. The pH dependence of the sensor response was evaluated over the pH range from 5.5 to 9.0. The sensor response was found optimum at pH 6.5. The effect of incubation temperature on the biosensor was examined between 25 and 45 °C. The current response of the biosensor increased with increasing temperature and reached a maximum at approximately 35 °C, and then went down as the temperature turned higher. In addition, the current response kept correspondingly steady between 25 and 45 °C, indicating that enzyme bioconjugate with NiONPs had good thermodynamic stability and life span.

3.7. Effect of substrate concentration

Fig. 4C shows SWV response of enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode for different concentration of triolein (50–750 mg/dl) with a sensitivity of 0.05 μA/mg/dl at scan rate of 20 mV/s. Detection limit of the biosensor was 10 mg/dl with a signal to noise ratio of 3.0, which is better than earlier reported biosensors [17–19].

3.8. Analytic recovery and precision study

The analytic recovery of added triolein in serum (100 mg/dl) was 95–96%. The precision of the present method was determined over seven days. Each day seven replicate quality control serum samples were analyzed for TG content. The within and between batch precision were less than 2% and 3% respectively. These results demonstrated that the described method was precise and reproducible.

3.9. Accuracy

To study the accuracy of the present method, TG level in 10 sera samples were determined by both the commercial enzymic colorimetric method (x) and the present method (y). The values obtained by both the methods matched with each other with a good correlation ($r = 0.9807$) (Supplementary Fig. 1).

3.10. Interference study

Among the various serum substances tested such as urea, uric acid, glucose, fructose, ethanol, cholesterol, citric acid, ascorbic acid, lactic acid, malic acid, tartaric acid, alanine, leucine, bilirubin, creatinine and pyruvic acid, at their physiological concentration, none had practically any significant interference.

3.11. Determination of serum TG

TG serum level in apparently healthy adults as measured by the present biosensor ranged from 53 to 190 mg/dl, which is in established normal range (40–190 mg/dl). The TG level in the serum of persons suffering from hypertriglyceridemia was in the range 200–1050 mg/dl (Supplementary Table 1).

Three similarly constructed enzyme electrodes (enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode) were employed for determination of TG level in serum in order to establish the reproducibility and variability of the present electrode. The results presented in Supplementary Table 2 show no significant variation (CV less than 6% in all cases), confirming the reproducibility of present electrode.

3.12. Stability of the enzyme electrode

The shelf life of the enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode was investigated by measuring its electrochemical current response for 6 months at a regular interval of 30 days. The enzymes/NiONPs–CHIT/ZnO–ZnHCF/Au electrode retained about 50% of its initial activity even after 6 months, when stored in dry conditions at 4 °C. The enzyme electrode was constructed thrice and effect of storage at 4 °C was studied on the response of these three similarly constructed electrodes. The results given in Supplementary Fig. 2 show practically no variation in storage stability of the three electrodes.

A comparison of various analytical properties of present biosensor with those of recent amperometric TG biosensors is summarized in Supplementary Table 3.

4. Conclusion

The use of NiONPs–CHIT/ZnO–ZnHCF hybrid film in construction of an amperometric TG biosensor has led to its improved analytical performances in terms of response time (2 s), sensitivity (0.05 μA/mg/dl) and stability (6 months). The biosensor was unaffected by a number of serum substances. Based on these observations, this film could also be employed for the improvement of other biosensors.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2013.05.007.

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