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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).