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An improved amperometric triglyceride biosensor based on co-immobilization of nanoparticles of lipase, glycerol kinase and glycerol 3-phosphate oxidase onto pencil graphite electrode

Vinay Narwal, C.S. Pundir*

Department of Biochemistry, M.D. University, Rohtak, Haryana, India

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ABSTRACT

Nanoparticles (NPs) of commercial lipase from *Candida rugosa*, of glycerol kinase (GK) from *Cellulomonas* species, of glycerol-3- phosphate oxidase (GPO) from *Aerococcus viridans* were prepared, characterized and co-immobilized onto a pencil graphite (PG) electrode. The morphological and electrochemical characterization of PG electrode was performed by scanning electron microscopy (SEM), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) before and after co-immobilization of enzyme nanoparticles (ENPs). An improved amperometric triglyceride (TG) biosensor was fabricated using Lipase NPs/GKNPs/GPONPs/PG electrode as the working electrode, Ag/AgCl as the standard electrode and Pt wire as auxiliary electrode. The biosensor showed optimum response within 2.5 s at a pH 7.0 and temperature of 35 °C. The biosensor measured current due to electrons generated at 0.1 V against Ag/AgCl, from H_2O_2, which is produced from triolein by co-immobilized ENPs. A linear relationship was obtained over between a wide triolein concentration range (0.1 mM–45 mM) and current (mA) under optimal conditions. The Lipase NPs/GKNPs/GPONPs/PG electrode showed high sensitivity (1241 ± 20 mA cm^{-2} mM^{-1}); a lower detection limit (0.1 nM) and good correlation coeficient ($R^2 = 0.99$) with a standard enzymic colorimetric method. Analytical recovery of added triolein in serum was 98.01%, within and between batch coefficients of variation (CV) were 0.05% and 0.06% respectively. The biosensor was evaluated and employed for determination of TG in the serum of apparently healthy subject and persons suffering from hypertriglyceridemia. The biosensor lost 20% of its initial activity after its continued uses over a period of 240 days, while being stored at 4 °C.

1. Introduction

Triacylglycerols or triglycerides (TGs), known as natural fats, are composed of one molecule of glycerol that is attached to three molecules of fatty acids (Saturated/unsaturated or both) through ester bonds. TGs are the major transporters of dietary fats throughout the bloodstream. The very low density lipoproteins (VLDL) and chylomicrons are made up of TGs mainly [1]. Besides transporting fat, TGs can be used as fuel, whenever the body's energy demand is not fulfilled by carbohydrates [2]. Normal level of TGs in the blood is less than 150 mg/dl (1.7 mM), while 150–199 mg/dl (1.7–2.2 mM) is considered border line high and 200–499 mg/dl (2.3–5.6) as high. The elevated TGs level over 500 mg/dL (5.6 mM) in the blood is used as a biomarker for cardiovascular disease [3], Alzheimer disease [4], pancreatitis [5] and diabetes. TGs are accumulated in the blood due to abnormal lipoprotein metabolism and also by its high rate of biosynthesis and low rate of catabolism [6,7]. A decreased activity of triacyl glycerol lipase catalyzing the hydrolysis of TG into free fatty acids (FFA) and glycerol may result into an accumulation of TGs [8,9]. In addition, excess the TGs level causes decrease in nitric oxide level but increase in concentration of many inflammatory compounds contributing to vascular injury and endothelial dysfunction [10]. Therefore, the measurement of TG level in the blood is very important. Various methods are available for determination of TG such as titrimetric method [11], colorimetric method [12], spectrophotometric method [13], chromatographic methods [14], fluorometric method [15], nuclear magnetic resonance method [16], micro method [17], mass fragmentographic method [18] and enzymatic centrifugal method [19–21]. However, most of these methods are complicated, require time-consuming sample pre-treatment, expensive instrumental set-up and skilled persons

* Corresponding author.
E-mail addresses: chandraspundir@gmail.com, pundircs@rediffmail.com (C.S. Pundir).

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to operate. Biosensing methods overcome these drawbacks, as these are simple, fast, economic, specific and highly sensitive. In TG biosensors, the enzyme has been immobilized onto different nanocomposites, for detecting TG in biological fluids [22]. However, direct immobilization of enzymes onto nanocomposites might bring about their denaturation, prompting to the loss of their activity and stability. This problem was overcome by aggregating the enzyme molecules and followed by glutaraldehyde crosslinking to form their nanaoparticles (NPs), before immobilization. Due to their unique electronic, optical, mechanical, electrical, thermal and catalytic (ability to facilitate electron transfer) properties, beside the increasing surface area, enzyme nanoparticles (ENPs) have shown great promises in improving enzyme electrodes [23,24]. Accordingly, use of ENPs rather than native enzyme could enhance the analytic performance of biosensors. Pencil graphite (PG) electrode offers numerous attractive features such as good conductivity, ultra low cost, low background current and easy availability, which renders it a better option over electrodes such as Au, glassy-carbon and Pt electrodes [25]. We describe herein the preparation of NPs of lipase, glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO), their characterization and co-immobilization onto a PG electrode to construct an improved amperometric biosensor for measurement of the TG level in serum.

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 corrosive (DHBS) from E-Merk, Germany, triolein, ATP, and kit for enzymic colorimetric method for TG from Erba Transasia, Daman, India was used. 6 B graphite pencil (Make: Camlin, India) with a graphite rod of 2 mm diameter was purchased from the local stationary market. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) (ohmic resistance: 1.8×10^{-5} for every ohm) was used through the study.

2.2. Instruments used

Potentiostat/Galvanostat (Make: Autolab, model: AUT83785, made by Eco Chemie,The Netherland) with a three electrode system comprising of Lipase NPs/GKNPs/GPONPs/PG electrode as a working anode, an Ag/AgCl as reference electrode and a Pt wire as an auxiliary electrode. The electrochemical experiments were performed by general purpose electrochemical software (GPES). Scanning electron microscopy (SEM) (Zeiss EV040, USA). UV Spectrophotometer (Make: Shimadzu, Japan, Model 1700), X-Ray diffractometer (XRD), (Make: 122 Rigaku, D/Max2550, Tokyo, Japan), Fourier Infra-red spectrometer (FTIR) and spectronic-20 (Thermo Scientific, USA), were utilized. The electrochemical impedance spectra (EIS) was recorded by GPES (FRA) software in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 25 ml solution at −0.5 V between frequency range, 0.01 Hz–10 kHz.

2.3. Preparation of triolein solution

Triolein was utilized as a substrate for lipase. Triolein emulsion was prepared as described [26]. Solutions of various concentrations of triolein ranging from 0.1 mM to 60 mM were set up in 0.1 M sodium phosphate buffer (pH 7.0) and kept at 4 °C until use [27].

2.4. Preparation of lipase NPs, GKNPs and GPONPs

The Lipase NPs, GKNPs and GPONPs were prepared separately by desolvation method using ethanol [28]. To GK, GPO and Lipase solution (1 ml each separately), 5 ml of absolute ethanol was added drop wise at a rate of 0.1–0.2 ml/min under continuous stirring at a speed of 500 rpm. This was followed by the addition of 1 ml, 2.5% glutaraldehyde solution in the above solutions under the same stirring condition at 4 °C for 24 h to ensure complete crosslinking of respective ENPs. Such a high concentration of glutaraldehyde is liable to give intermolecular crosslinking of enzyme molecules through Schiff base. The ENPs, thus formed were thiol functionalized by adding 0.2 ml of cysteamine solution (0.02 g/ml) to each suspension under constant stirring for 5–6 h. ENPs were isolated from free enzyme solution by centrifuging NPs suspension at 1200 × g for 10 min at 4 °C, followed by redispersion of ENPs in 2 ml 0.1 M phosphate buffer, pH 7.3 and sonication for 5 min. It is assumed that alpha-NH_2 group of cysteamine reacts with excess unreacted —CHO groups of glutaraldehyde cross-linked ENPs to form Schiff base. Thus, the glutaraldehyde cross-linked ENPs get functioned with —NH_2 groups. These functionalized ENPs were stored at 4 °C, until use.

2.5. Characterization of lipase NPs, GKNPs and GPONPs

Lipase NPs, GKNPs and GPONPs were characterized by taking their images in a transmission electron microscope (TEM) and recording their UV–vis (200–600 nm) spectra.

2.6. Preparation of lipase NPs/GKNPs/GPONPs/PG electrode

Lipase NPs/GKNPs/GPONPs were immobilized onto the surface of PG electrode (0.2 × 2.5 cm, diameter × height; area = 3.14 cm^2) to prepare an enzyme NPs-based enzyme electrode. Before the experiment, the potential of the cleaned bare PG electrode was scanned over the −1.1–0 V range in freshly prepared 0.2 M H_2SO_4 until the voltammogram characteristic of the clean PG electrode was established.The PG electrode was placed in the Lipase NPs/GKNPs/GPONPs suspension (1:1:1 ratio) under mild stirring at 4 °C for 12 h for co-immobilization of cysteamine functioned Lipase NPs, GKNPs, and GPONPs onto PG electrode. This immobilization could be possible by adsorption due to an electrostatic attraction between negatively charged —COOH groups of PG electrode and positively charged —NH_2 groups on the surface of ENPs, introduced by cysteamine di-hydrochloride. The Lipase NPs/GKNPs/GPONPs/PG electrode was rinsed with 0.1 M of phosphate buffer (PB, pH 7.0) carefully and stored in a PB at 4 °C, when not in use. The characterization of Lipase NPs/GKNPs/GPONPs/PG electrode was carried out at different stages of its construction by SEM, CV and EIS.

2.7. Construction of TG biosensor and its response measurements

An amperometric TG biosensor was constructed using Lipase NPs/GKNPs/GPONPs/PG electrode as working electrode, Ag/AgCl as standard electrode and Pt wire as an auxiliary electrode connected through potentiostat. Fig. 1 provides the schematic representation of fabrication of LipaseNPs/GKNPs/GPONPs/PG electrode and electro-chemical reactions involved in its response measurement. The electrochemical characterizations and measurements on the modified Lipase NPs/GKNPs/GPONPs/PG electrode were carried out by cyclic voltammetry. Electrochemical experiments were carried out in 0.1 M phosphate buffer saline containing 1 mM TG (0.1 ml), 6.6 mM ATP, 10 mM $MgCl_2$ and 1 mM Triton X100 at a scan rate of 20 mV s^{-1}. The current response was measured between 0 s and 10 s at an interval of 2.5 s.

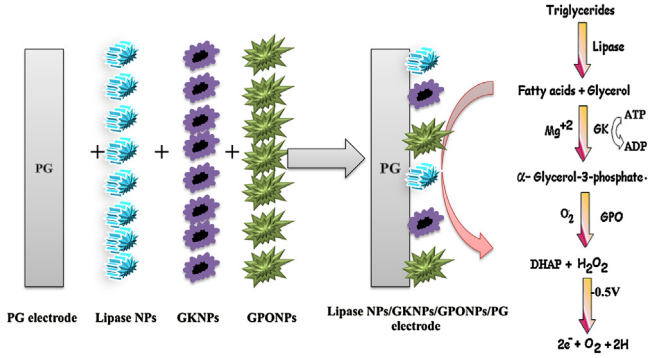

Fig. 1. Schematic representation of fabrication of LipaseNPs/GKNPs/GPONPs/PG electrode and electro-chemical reactions involved in its response measurement. (NPs = Nanoparticles, GK = Glycerol kinase, GPO = Glycerol-3-phosphate oxidase, PG = Pencil graphite, DHAP = Dihydroxyacetone phosphate).

2.8. Optimization of TG biosensor

Various kinetic properties of ENPs modified PG 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. To determine the optimum pH, the pH was varied between pH 4.0–9.0 at an interval of pH 0.5 using the following buffer, each at a final concentration of 0.1 M: pH 4.0–5.0 sodium acetate buffers, pH 5.5–7.5 sodium phosphate buffer and pH 8.5–9.0 Tris HCl buffer. Similarly to determine optimum temperature and incubation time the reaction mixture was incubated at different temperature (5–70 °C) and time duration (0–10 s). The effect of TG concentration on biosensor response was determined by varying the concentration of TG in the range 0.1 mM–65 mM. The limit of detection (LOD) was calculated using the equation: LOD = 3 × sigma/slope.

2.9. Amperometric determination of TG in serum

Blood samples (1 ml each) from the apparently healthy adults (male and female,n = 20) and persons suffering from hypertriglyceridemia in various age groups (n = 20) 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 × *g* for 5 min and their supernatants (sera) were collected and stored at 4 °C until use. TG content in sera 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 a standard curve between triolein concentrations and current (in mA) prepared under optimal working conditions (Fig. 2a).

2.10. Evaluation of TG biosensor

The biosensor was evaluated by studying analytic recovery, precision and correlation using a standard kit for enzymic colorimetric method for TG. The effect of various interferrants found in the blood such as citric acid, glutamic acid, uric acid, ascorbic acid and urea was studied at their physiological concentrations.

2.11. Reusability and storage stability of enzyme electrode

The reusability and long-term storage and stability of the biosensor were investigated over a period of 240 days at an interval of 30 days, during its storage in dry condition at 4 °C.

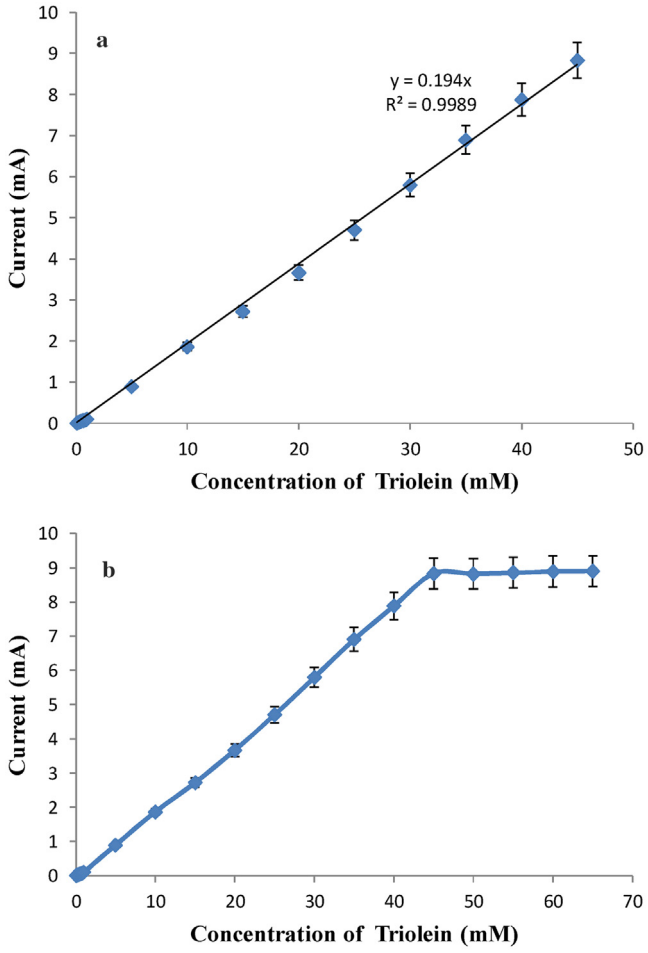

Fig. 2. a) Standard curve of triolein by TG biosensor based on Lipase NPs/GKNPs/GPONPs/PG electrode. (NPs = Nanoparticles, GK = Glycerol kinase, GPO = Glycerol-3-phosphate oxidase, PG = Pencil graphite) All readings are mean of five observations (n = 5). b) Effect of triolein concentration on current response of Lipase NPs/GKNPs/GPONPs/PG electrode. (NPs = Nanoparticles, GK = Glycerol kinase, GPO = Glycerol-3-phosphate oxidase, PG = Pencil graphite) All readings are mean of five observations (n = 5).

3. Results and discussion

3.1. Characterization of lipase NPs, GKNPs and GPONPs

The size of Lipase NPs, GKNPs and GPONPs as measured by TEM was between 5 nm to 100 nm (Fig. 3a), and showed an average size of 20 nm, which enhanced the surface area of the electrode as compared to the native enzyme. UV and visible spectra of Lipase NPs, GKNPs and, GPONPs exhibited strong absorbance peak at 280 nm (Figure not given), revealing a high degree of absorption of aromatic amino acids of peptide chain/free enzyme following their NPs formation.

3.2. Characterization of lipase NPs/GKNPs/GPONPs/PG electrode

3.2.1. By scanning electron microscopy (SEM)

The SEM images of the surface of the bare PG electrode and Lipase NPs/GKNPs/GPONPs/PG electrode are shown in respectively. The stepwise modification of electrode was seen clearly from these SEM images (Fig. 3b). The SEM image of the bare PG electrode showed a smooth and featureless morphology. The Lipase NPs/GKNPs/GPONPs/PG electrode exhibited globular structural morphology, which appeared due to adsorption of LipaseNPs/GKNPs/GPONPs onto PG electrode. The porous elec-

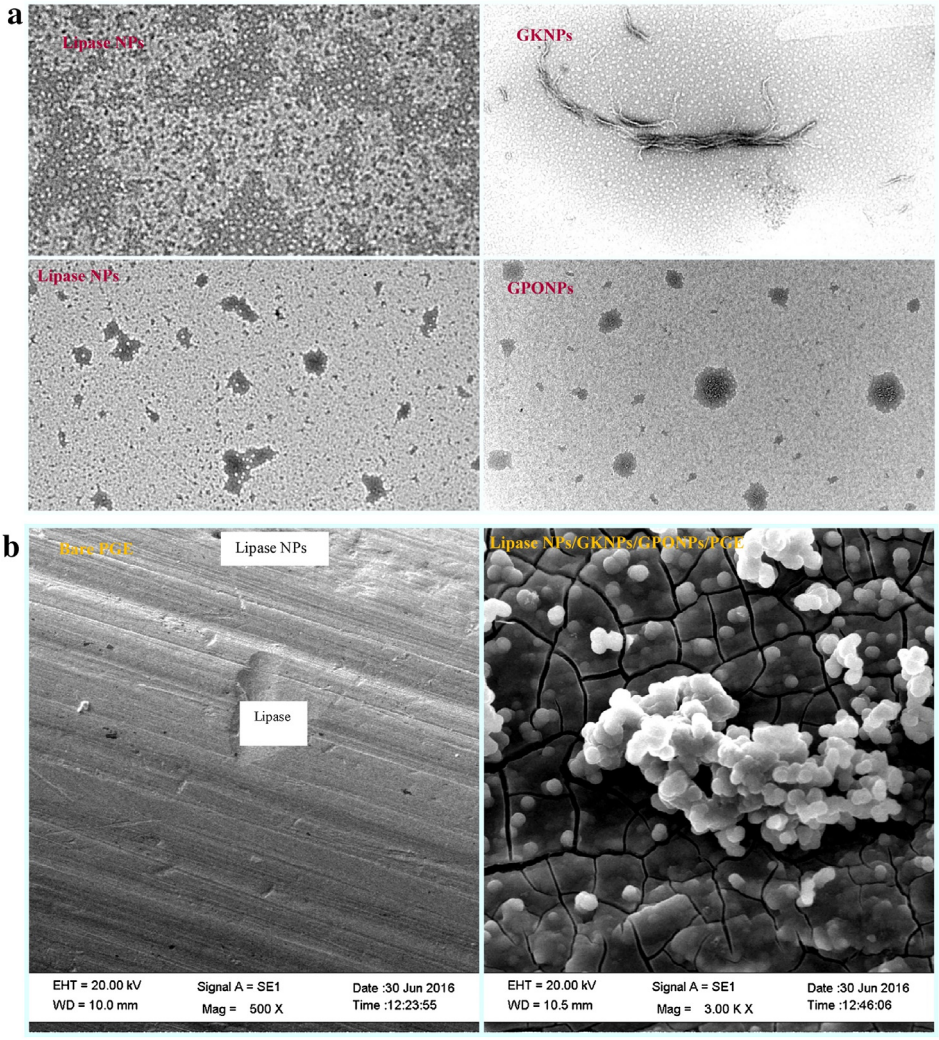

Fig. 3. a Transmission electron microscopic (TEM) images of Lipase NPs, GKNPs, and GPONPs. (NPs = Nanoparticles, GK = Glycerol kinase, GPO = Glycerol-3-phosphate oxidase). b Scanning electron microscopic (SEM) images of bare PG electrode and Lipase NPs/GKNPs/GPONPs/PG electrode. (PG = Pencil graphite, NPs = Nanoparticles, GK = Glycerol kinase, GPO = Glycerol-3-phosphate oxidase).

trode revealed larger and effective surface area after absorption of GKNPs/GPONPs/Lipase NPs

3.3. By electrochemical impedance spectroscopy (EIS)

The EIS provides the useful information on impedance changes of the electrode surface during the fabrication of an enzyme/ENPs electrode and was carried out to investigate absorption of GKNPs/GPONPs/Lipase NPs onto PG electrode. The diameter of the semicircle at higher frequencies corresponds to the electron transfer resistance (R_{CT}), which controls the electron transfer kinetics of the redox probe at the electrode interface, while the linear part at lower frequencies correspond to Warberg diffusion process. The Nyquist plot (Supplementary Fig. S1a and b) displays EIS of bare PG electrode and Lipase NPs/GKNPs/GPONPs/PG electrode in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)6$ respectively. The R_{CT} value for the Lipase NPs/GKNPs/GPONPs/PG electrode was obtained as 70 Ω, which was higher compared to bare electrode (R_{CT} = 50 Ω). These results showed that the increased R_{CT} value of Lipase NPs/GKNPs/GPONPs/PG electrode was due to the immobilization of enzyme nanoparticles onto PG surface. This increase in R_{CT} is attributed to the fact that most biological molecules, including enzyme nanoparticles, are poor electrical conductors at low frequencies (at least <10 kHz; applied voltage: −0.5 V) and cause hindrance to the electron transfer.

3.4. Voltammetric response of the lipase NPs/GKNPs/GPONPs/PG electrode

No peak was observed in case of the bare/unmodified electrode. However, a well defined oxidation and reduction peak with a peak potential of −0.5 V was observed in case of Lipase NPs/GKNPs/GPONPs modified PG electrode (Fig. 4). The Lipase NPs/GKNPs/GPONPs might provide a large available surface and enhance the electrocatalytic activity for electro-oxidation of TG. The results of the cyclic voltammetry (CV) also proved that Lipase NPs/GKNPs/GPONPs could provide a conductive path through the PG electrode. So, the PG electrode acts as an 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 Lipase NPs/GKNPs/GPONPs/PG electrode confirmed that the enzyme nanoparticles got immobilized on the surface of the modified PG electrode.

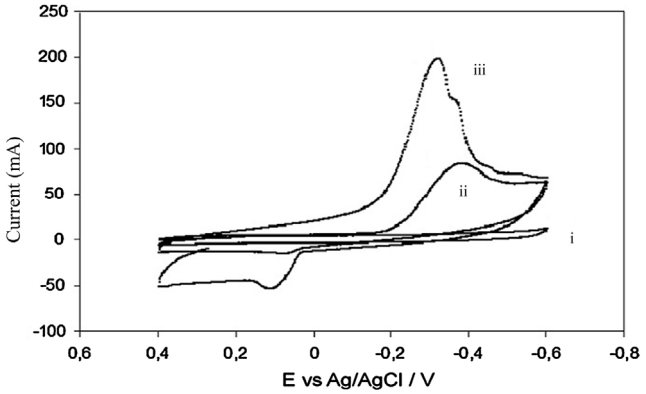

Fig. 4. Cyclic voltammogram of (i) bare electrode (ii) Lipase NPs/GKNPs/GPONPs/PG electrode without substrate in 25 ml 0.1 M sodium phosphate buffer (pH = 7.0) containing, 6.6 mM ATP, 10 mM $MgCl_2$ and 1 mM Triton X100 (iii) Lipase NPs/GKNPs/GPONPs/PG electrode containing 10 mM triolein (0.1 ml)at a scan rate of 20 m Vs^{-1}. (NPs = Nanoparticles, GK = Glycerol kinase, GPO = Glycerol-3-phosphate oxidase, PG = Pencil graphite).

3.5. Current response measurement of lipase NPs/GKNPs/GPONPs/PG electrode

The electrochemical response of the Lipase NPs/GKNPs/GPONPs/PG electrode was investigated as a function of triolein concentration (0.1 mM–65 mM) by recording CV at 20 mV/s scan rate in PBS (0.1 M, pH 7.0) containing 5 mM [Fe(CN)6]3−/4− (the electrical conductivity of PBS = 25Ω). TG biosensor is based on measurement of electrons i.e. the current generated from H_2O_2 under high potential. At the surface of ENPs electrode, co-immobilized lipase NPs hydrolyses TG into free fatty acids and glycerol, which get phosphorylated into glycerol-3-phosphate by co-immobilized GKNPs in presence of ATP. This glycerol-3-phosphate is then, oxidized into dihydroxyacetone phosphate and H_2O_2 by GPONPs. The electron i.e. current is generated from electro-oxidation of H_2O_2 under high potential, which is measured. The current response curves of PG electrodes without and with a mixture of ENPs (with substrate and without substrate) are shown in Fig. 4(i, ii, iii). When TG concentration was 10 mM, the current response of the PG electrode with native enzymes (Lipase/GK/GPO) was 0.54 mA, while the same electrode with mixture of ENPs (LipaseNPs/GKNPs/GPONPs) was 2.85 mA. The results indicated that the hybrid ENPs had an intense effect on the electrode response.

3.6. Optimization of TG biosensor

A novel amperometric TG biosensor was developed based on co-immobilization of LipaseNPs, GKNPs and/GPONPs onto PG electrode. The maximum current was observed within 2.5 s at 0.1 V against Ag/AgCl. Hence, in subsequent electrochemical studies the current was recorded within 2.5 s at this voltage. The biosensor showed the optimum current at pH 7.0 in 0.1 M sodium phosphate buffer, which is close to the physiological pH. The optimum incubation temperature and time for the biosensor was observed at 35 °C and 2.5 s. Hence, the subsequent electrochemical experiments were carried out in 0.1 M sodium phosphate buffer, pH 7.0 at 35 °C. There was a linear relationship between biosensor response and TG concentration in the range 0.1 mM–45 mM, after which the response was constant (Fig. 2b). The Lipase NPs/GKNPs/GPONPs/PG electrode generated a jmax of 7.76 ± 0.12 mA with app. Km of 0.1 ± 0.1 mM and showed high sensitivity of 1241 ± 20 mA cm^{-2} mM^{-1} at a potential of 0.1 V.

3.7. Evaluation of TG biosensor

A linear relationship was obtained over between a wide triolein concentration range, 0.1 mM–45 mM, under optimum conditions (Fig. 2a),which is better than earlier biosensors [29–37]. The limit of detection limit(LOD) of the present biosensor was 0.1 nM, which is also better than earlier biosensors [29–37].The average recoveries of TG added in sera at levels of 0.5 mM and 1.0 mM were 98.01 and 98.77% respectively demonstrating the reliability of the present biosensor (Supplementary Table S1). Within and between batch coefficients of variation for the determination of TG in sera on the same day and after one week of storage at −20 °C were 0.05% and 0.06%, respectively (Supplementary Table S2). These values of precision study show the good reproducibility and consistency of the present method, which could be attributed to the electrostatic attraction of Lipase NPs/GKNPs/GPONPs onto PG electrode.

3.8. Correlation of TG biosensor

The TG level in 20 sera of apparently healthy subjects and persons suffering from hypertryglycerdemia as measured by present biosensor were compared with those obtained by standard enzymic colorimetric kit method. A good correlation coefficient (R^2 = 0.99) was obtained with the standard enzymic colorimetric method (Figure not given), revealing the high accuracy of the present biosensor.

3.9. Applications of TG biosensor

The sera TG content in apparently healthy adults as measured by the present biosensor was in the range 0.5 ± 0.03–1.7 ± 0.05 (n = 20) mM for sera of apparently healthy persons, which is in normal established range. The sera TG level in persons suffering from hypertriglyceridemia was in the range 3.3 ± 0.03–19 ± 0.04 mM (n = 20), which is significantly higher than those in apparently healthy adults (p > 0.001) (Supplementary Table S3).

3.10. Interference study of TG biosensor

Citric acid, glutamic acid, uric acid, ascorbic acid and urea had practically no effect on TG biosensor response at their physiological concentrations, under the standard assay conditions. (Supplementary Fig. S2).

3.11. Storage stability of LipaseNPs/GKNPs/GPONPs/PG electrode

The Lipase NPs/GKNPs/GPONPs/PG electrode lost 20% of its initial activity after its continued uses, over a period of 240 days, while being stored at 4 °C. These observations indicate the better stability of the present ENPs electrode over earlier electrodes [29–37].

4. Conclusion

The use of Lipase NPs/GKNPs/GPONPs modified PG electrode in fabrication of TG biosensor has resulted into its better analytical performance in terms of faster response time (2.5 s),the lower detection limit (0.1 nM), wider linear range (0.1 mM–45 mM) and higher storage stability (240 days). Thus enzyme nanoparticles modified PG electrode could be used for improvement of other biosensors also.

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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.enzmictec.2017.01.009.

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