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Benzymatic triglyceride biosensor based on electrochemically reduced graphene oxide

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Abstract : Triglycerides (triacylglycerols) estimation is extremely important since its high concentration [normal range in men (40–160 mg/dL) and in women (35–135 mg/dL)] can cause hyperlipidemia[1]. Apart from coronary diseases, hyperlipidemia is useful for diagnosis and treatment of diabetes mellitus, nephrosis, liver obstruction and other diseases involving lipid metabolism of various endocrine disorder[2]. Reduced graphene oxide has recently triggered research interest for electrochemical biosensing applications due to its high electron mobility, excellent electrochemical properties and high surface area. Graphene oxide has been synthesized via modified Hammer's method. A uniform thin film of reduced graphene oxide (RGO) has been electrodeposited using chronoamperometric technique on indium tin oxide(ITO) electrode. The structural and morphological characterization of ERGO/ITO electrode electrodeposited on ITO electrode have been carried out by Field effect scanning electron microscopy,Raman spectroscope, FTIR and cyclic voltammetry to fabricate ERGO/ITO electrode with optimum electronic, chemical and structural properties. Glycerol dehydrogenase (GDH) and Lipase (LIP) have been co-immobilized on ERGO/ITO electrode via carbodiimide chemistry to prepare bio electrode (LIP-GDH/TB/ERGO/ITO) for sensing triglyceride. In this investigation, a novel biochemical path has been developed through potassium ferro/ferri ($[Fe(CN)_6]^{3-/4-}$) and toluidine blue in place of NADH which deteriorates biosensor shelf life and causes fouling effect. Response studies have been done using cyclic voltammetry revealing that this fabricated electrode can detect tributyrin in the range of 50-300mg/dL with high sensitivity and a low value of apparent Michaelis–Menten constant 0.152mM moles indicating high enzyme affinity of bioelectrodes (LIP-GDH/TB/ERGO/ ITO) towards tributyrin. The (LIP-GDH/TB/ERGO/ITO) bioelectrode has been also utilized to estimate triglyceride in serum samples.

Introduction

Triglyceride (TG), an ester derived from glycerol and three fatty acids molecules is a component of very-low-density lipoproteins (VLDLs) and chylomicrons, which play an important role in metabolism as energy source and also help in transporters of dietary fat. High levels of triglycerides in the bloodstream leads to atherosclerosis, which indicates the risk of heart disease, coronary heart disease (CAD) and hypolipoprotenimia.

Hyperlipidemia is associated with several disorders including diabetes mellitus, nephrosis, liver obstruction and endocrine. So the estimation of triglycerides (triacylglycerols) is extremely important since its high concentration [normal range in men (40–160 mg/dL) and in women (35–135 mg/dL)] may cause various disorders. Analytical methods which are already existing for determination of triglycerides are tedious, time consuming sample preparation, require expensive reagents, equipments and skilled person to operate. But enzyme based biosensors are comparatively simpler, sensitive, rapid and does not require sample preparations. Recently chemically modified electrodes have received extensive attention due to high sensitivity, selectivity, stability, low over potential for smooth electron transfer and less prone to fouling.

Most triglyceride biosensors till reported are based on multi-enzymes detection platform wherein a biochemical reaction depends upon enzyme kinetics of the other enzymatic reaction. The single enzyme based detection format such as Lipase (LIP) based is pH sensitive. It is required to develop a comparatively simple and reliable biorecognition process for the detection of triglyceride. In the present investigation, a bi-enzyme system i.e. Lipase(LIP) and Glycerol dehydrogenase (GDH), co-immmobilized on electrochemically deposited reduced graphene oxide nano sheet on Indium tin oxide(ERGO/ITO) has been employed for the electrochemical determination of triglycerides in the absence of NADH mediator. NADH is a biological mediator. The direct oxidation of NADH at a solid electrode surface is irreversible and frequently results in electrode fouling. In the present investigation, first time an attempt is taken to replace NADH by Toluidine blue (TB) as a chemical mediator and ferro-ferri as a redox electrolyte.

Since its discovery in 2004, Graphene is the most promising material. Graphene has many applications in various fields, such as electronic devices, nanocomposite materials, sustainable energy storage, conversion (ultracapacitors, batteries, fuel cells, solar cells), and bioscience/biotechnologies due to its superior thermal, electrical conductivity, good mechanical properties and high specific surface area. Graphene, distinctive carbon-based nanomaterial, is a honeycomb structure sheet of sp^2 bonded carbon atoms, showing tremendous attention from both the experimental and theoretical scientific communities for the designing of high sensitive and selective biosensors. Reduced graphene oxide (RGO) can be prepared by the reduction of GO by Hydrazine hydrate, $NaBH_4$ solution, Thermal annealing, Vitamin C, glucose, Pyrogallol, KOH, 55% approach for ERGO synthesis. In this paper, we report the development of electrochemically reduced graphene oxide (ERGO) film on ITO electrode from GO dispersion.

Experimental

Chemicals and Instruments

$NaNO_3$, H_2SO_4(98%), $KMnO_4$, H_2O_2(30%), HCL were purchased from Merck and Graphite powder was from Alfa Aser. LIP(from Pseudomonas) with specific activity of 48 U mg^{-1}, Glycerol dehydrogenase (from Cellulomonas) with specific activity of 77 U mg^{-1}, Toluidine Blue O, N-hydroxysuccinimide (NHS), N-ethyl-N-(3-dimethylaminopropyl carbodiimide (EDC) and Tributyrin have been purchased from Sigma–Aldrich. Electrochemical analysis was conducted on an Autolab Potentiostat/Galvanostat using a three-electrode system with ITO as working electrode, platinum (Pt) wire as the auxiliary electrode and Ag/AgCl as reference electrode in phosphate buffer saline (PBS, 50mM,pH6.5,0.9%NaCl)containing 5mM $[Fe(CN)_6]^{-3/-4}$ as redox probe. FTIR spectroscopy was done using Varian-7000 UMA-600 IR microscope with excitation wavelength of 1064 nm. Raman studies by Renishaw Invia Raman microscope, FESEM by MIRA3 TESCAN and Cyclic Voltammetry by Autolab Potentiostat/Galvanostat Model AUT83945 (PGSTAT302N) were conducted.

Fabrication of electrochemically reduced graphene oxide (ERGO) film on ITO electrode

Water dispersed GO solution was synthesized by Hummer's modified process. Briefly, small amount of graphite powder was vigorously stirred overnight in the solution of $NaNO_3$ and $KMnO_4$ in concentrated H_2SO_4. 30% of H_2O_2 was added to complete the oxidation. Impurities and other inorganic ions were removed through washing with 5% HCL and deionized water using centrifugation. Washed GO was ultrasonicated with deionized water. Colloidal solution of GO in water (1mg/mL) was obtained.The ITO electrodes was cleaned with acetone. GO suspension (1mg/mL) was reduced electrochemically and deposited on ITO through crono ampherometric technique using a three-electrode system with ITO as working electrode, platinum (Pt) wire as the auxiliary electrode and Ag/AgCl as reference electrode -1.5 V was applied for 60 s.

Preparation of solutions

The solutions of LIP (48 Umg^{-1}) and GDH (77 U mg^{-1}) were freshly prepared in PBS (50mM, pH 7.0) prior to being used. Stock solution of tributyrin was prepared in ethanol and stored at 4 °C. This stock solution was further diluted to make different concentrations of the analyte (tributyrin). Solution of TB (0.1%), EDC (0.4 M) and NHS (0.1 M) were prepared freshly in deionized water.

Fabrication of bi-enzymatic triglyceride bioelectrode on ITO based ERGO film

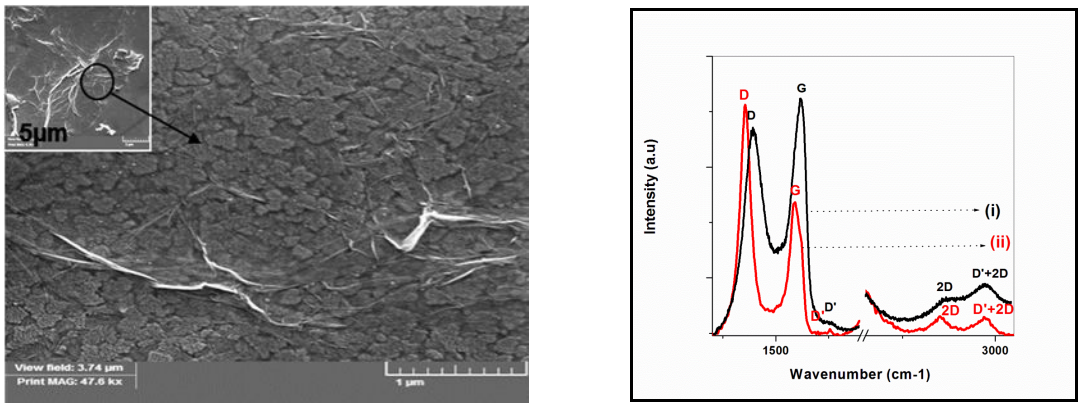
TB was immobilized onto ERGO/ITO electrode by immersing the electrode into 0.1% aqueous solution of TB for about 12 h followed by rinsing with water. After this immobilization of mediator on the electrode surface, LIP and GD was covalently attached to ERGO/ITO electrode via carbodamide chemistry. Bond formation between the –NH– and $–NH_2$ groups of enzyme and –COOH, –COH group of ERGO has occurred using EDC as the coupling agent and NHS as the activator. For the enzyme immobilization, the optimal binding is achieved when 30 µL solution of LIP(1mg/ml) and 10 µL solution of GDH (1mg/mL) mixed with 0.4 M EDC and 0.1 M NHS was dropped on 0.25 cm^2 of ERGO /ITO electrode and kept in a humid chamber for 4 h. Thus fabricated bio electrode (LIP-GDH/TB/ERGO/ITO) is then washed with phosphate buffer saline (PBS) solution of (50 mM, 0.9% NaCl, pH 7.0) containing 0.05% Tween 20 to remove any unbound enzymes.

Result and Discussion

Characterization

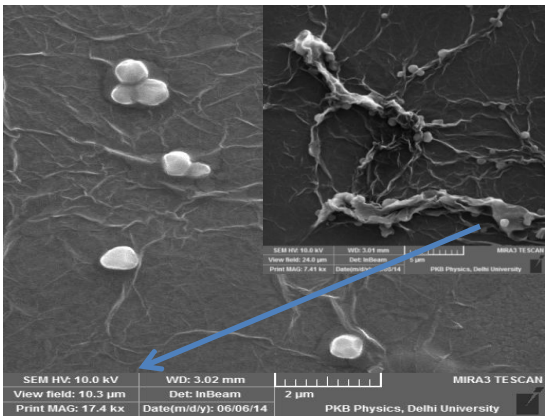
FESEM and RAMAN studies

Structural morphology of ERGO film on ITO, deposited by electrochemical reduction and the attachment of enzyme with matrix has been studied by FESEM. Fig 1(a) shows the FESEM images of the ERGO film on ITO and Fig. 1 (b) demonstrates the Raman spectra of ERGO as well as of GO film on ITO. Fig 1 (c) revels the attachment of enzymes with ERGO film. Film with 25µm×25µm area is scanned for imaging in FESEM. The inset of Fig 1(a) shows an uniform deposition of transparent reduced graphene oxide sheets on ITO substrate. An oriented, continuous, uniform and transparent film on ITO, named ERGO/ITO is observed. Its magnified visualization supports partially overlapped morphology with a low contrast, an indication of few layer thicknesses. In Fig. 1(b)(i) Raman Peaks of cast GO film on ITO are observed at 1380 cm^{-1}, 1587 cm^{-1}, 1660 cm^{-1}, 2715 cm^{-1}, 2943 cm^{-1} and in Fig. 1(b)(ii) at 1355 cm^{-1}, 1592 cm^{-1}, 1664 cm^{-1},2695 cm^{-1}, 2943 cm^{-1} for ERGO film. In Fig 1(b)(i) the prominent D peak at 1380 cm^{-1} clearly indicates the presence of structural imperfections induced by the attachment of hydroxyl, epoxy group, carboxyl groups on carbon basal plane. This band is a second order process originated by an A_1g–phonon at the K-point and is activated by the presence of defects. The intensity of this mode is therefore related to the number of sp^2C rings with the presence of defects. The peak around at 1587 cm^{-1} represents G-band which involves an E2g phonon and is associated with the stretching of sp2-C bonds in both rings and chains[3]. The other band, around at 2715 cm^{-1}, usually is denoted as 2D, is a second order Raman process whose shape, width and position are related to the order and the stacking of the graphene domains. Other less intense bands appear at 1660 cm^{-1} (D') and 2943 cm^{-1} (D+D') which are activated by defects. The I_D/I_G is 1.12 for GO cast film on ITO. The Raman spectrum confirms the exfoliated graphene oxide structure (4). In Fig 1(b)(ii) the intensity of D peak is increased and that of G is decreased resulting in a higher degree of structural disorder. The I_D/I_G ratio is increased upto 1.73 indicating the high degree of defects due to permanent loss of sp2 network during electro-chemical reduction. The values of D, G, D', 2D peaks are also shifted. 2D peak is sharp in case of ERGO/ITO as revealed in Fig 1(b)(ii). The increased ratio of I_D/I_G favours the accretion of structural disorder. The Raman study confirms the conversion of GO to ERGO. Fig 1(c) clearly revels the attachment of enzymes (i.e. LIP & GDH) with the ERGO film surface. Inset of Fig 1 (c) shows that the enzymes are attached to the edges of the film only. The Fig 1(a) shows that the ERGO film has a typical crumpled and wrinkled sheet structure which provides a large rough surface area for immobilization of enzyme.



(a) **(b)**

Fig.1(a) FESEM image of ERGO/ITO: inset : 5µm scale enlargement as marked. (b) Raman spectra of GO/ITO (i) and ERGO/ITO (ii)



1(c)

Fig. 1(c) FESEM image of LIP and GDH immobilized on ERGO/ITO inset: 5µm scale

Fourier transform infrared spectroscopy (FTIR) Analysis

Fig. 2 shows representative FTIR spectra of ERGO/ITO and LIP-GDH/TB/ERGO/ITO electrodes. Fig 2(a) shows the characteristic peaks of ERGO around 1,141 cm^{-1} for C-O ($\nu_{(alkoxy\ and\ epoxy)}$), 1,403 cm^{-1} for bending vibration of $\nu_{(O\text{-}H\ of\ carboxyl)}$, vibration modes of epoxide (ν_{C-O-C}) peak at 1,246 cm^{-1}, *sp*2-hybridized $\nu_{C=C}$ at 1,549 cm^{-1}, ketonic species ($\nu_{C=O}$) at 1,634 cm^{-1}[5,6]. Additional peaks in Fig. 2(b) at 1,641 and 1,539/cm can be assigned to the carbonyl stretching (amide I band) and –N–H bending (amide II band), the broader peak at 3400 cm^{-1} in spectra of LIP-GDH/TB/ERGO/ITO is attributed to N-H stretching vibrations indicating immobilized enzyme molecules onto ERGO/ITO surface[7].

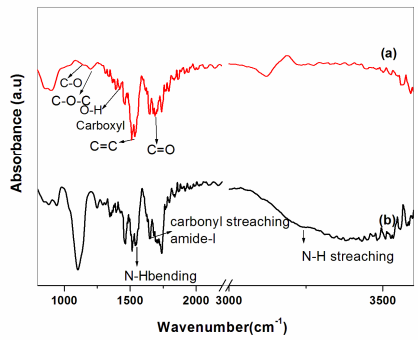


Fig. 2(a) FTIR spectra of ERGO/ITO and (b)FTIR spectra of LIP- GDH/TB/ERGO/ITO electrodes

Cyclic voltammetry studies

Fig 3 shows the cyclic voltammogram of ITO (a), ERGO/ITO (b), ERGO/TB/ITO (c) and LIP-GDH/TB/ERGO/ITO (d) electrodes. CV of all the electrodes have been investigated in PBS (50mM, pH 7.8, 0.9% NaCl) containing 5mM [Fe(CN)6]3−/4− at scan rate of 50 mV/s from potential -0.4V to 0.8V. Fig. 3(a) shows the anodic peak current value of ITO electrode at $1.05x10^{-4}$A. The 3 times raise in peak current value up to $2.9x10^{-4}$A of ERGO/ITO as shown in Fig 3(b) confirms electrodeposition of highly conductive ERGO film onto ITO. The large increase in anodic peak current is due to enhanced electroactive surface area and facile heterogeneous electron transfer properties of ERGO flakes leading to the large scale redox conversion[8]. The introduction of redox mediator TB molecules in ERGO/ITO matrix results in further enhancement in the anodic peak current i.e. $3.32x10^{-4}$A as revealed in Fig 3(c). TB, being a mediator facilitates the electron transfer process during the electrochemical reaction. The CV of LIP-GDH/ TB /ERGO/ ITO bio electrode shows reduction in the anodic peak value i.e. $2.6x10^{-4}$ (redox) ensuring successful immobilization of enzymes. This decreased electrochemical response is attributed to the hindrance caused by macromolecular structure of enzymes[9].

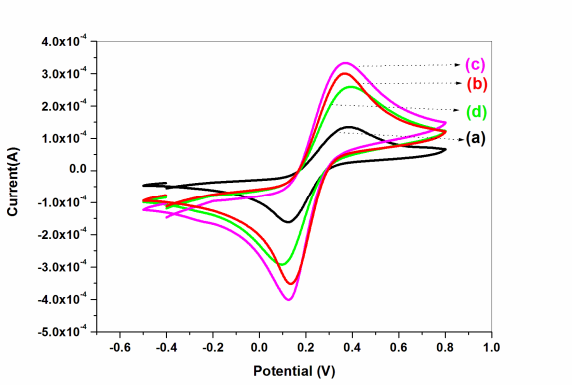
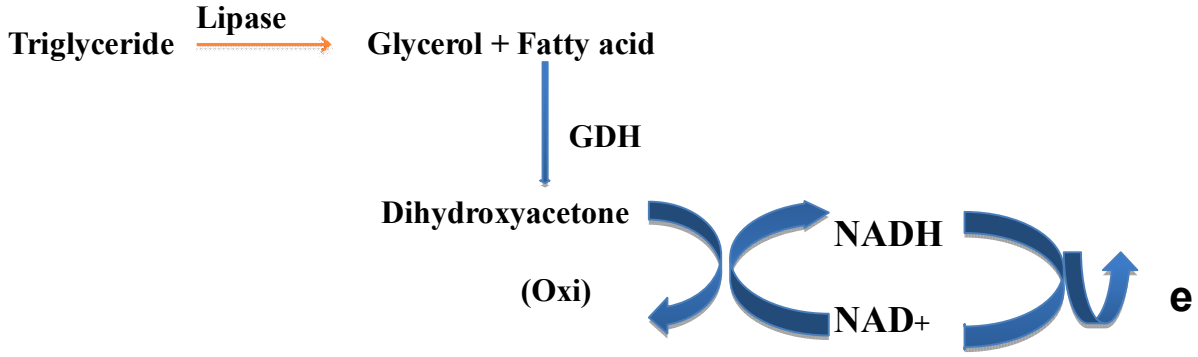


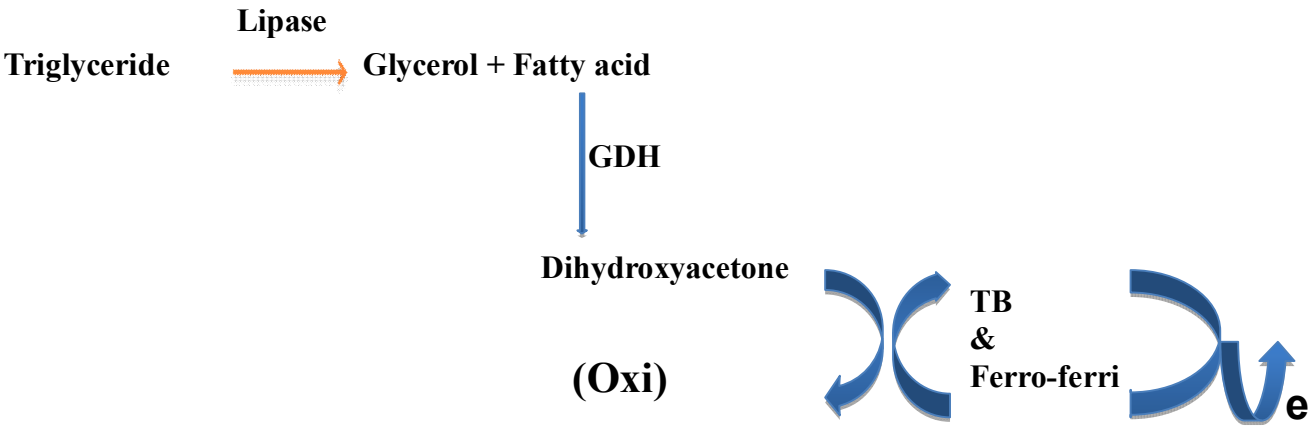
Fig 3 CV of ITO (a), ERGO/ITO (b), TB/ERGO/ITO (c) and LIP-GDH/TB/ERGO/ITO (d) electrodes.
ESPONSE STUDIES

Development of alternative bio-chemical pathway of triglyceride bi-enzymatic system Scheme I shows the reaction mechanism of triglyceride breakdown. LIP enzyme helps in the hydrolysis of tributyrine (triglyceride) which results in the production of fatty acids along with glycerol molecules. Glycerol dehydrogenase (GDH) enzyme further catalyses the oxidation of glycerol into dihydroxyacetone with the participation of NAD+ as the electron acceptor.



Scheme I General reaction of triglyceride breakdown

The redox mechanism of NADH/NAD^+ at a solid electrode surface is irreversible and frequently results in electrode fouling. It is, therefore, necessary to replace the NADH by some other mediator. In this paper, TB and $[Fe(CN)6]^{3-/4-}$ are used as mediators in place of NADH in the path of this biological cycle. TB as mediator on electrode surface and $[Fe(CN)_6]^{3-/4-}$ as a mediator in electrolyte may replace NADH. So the modified biological process is interpreted in Scheme II.



Scheme II Replacement of NADH/NAD$^+$ by TB and [Fe(CN)6]$^{3-/4-}$

In order to prove the concept following analysis *have* been conducted. Firstly, ERGO is exposed to glycerol to perform the control test of ERGO/ITO electrode and to see effect of matrix(ERGO/ITO electrode) on glycerol. Secondly, the bio-electrode based on only GDH/TB/ERGO/ITO is exposed to various concentrations of glycerol. Fig. 4 shows the control testing of the electrode and Fig 5 shows the CV studies of alternate bio-chemical scheme. CV studies of ERGO/ITO and GDH/TB/ERGO/ITO electrodes have been investigated in PBS (50mM, pH 7.8, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ at scan rate of 50 mV/s with various concentration of glycerol. The Fig 4(a) represents the peak current values of ERGO/ITO electrode and 4(b) exhibits the cyclic voltammogram of ERGO/ITO in presence of glycerol. No significant change in peak current is detected in the presence of glycerol. Increased value of peak potential is due to the presence of insulating glycerol in the medium. This clarifies that ERGO/ITO electrode is not showing response to glycerol.

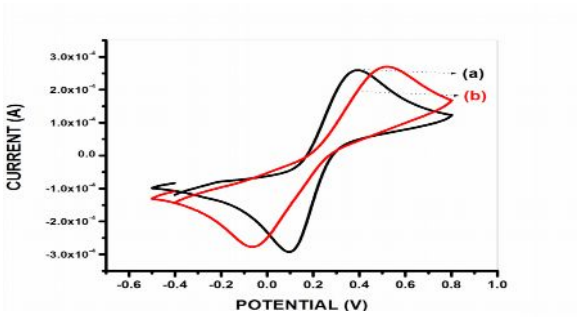


Fig 4. CV of ERGO/ITO (a) and ERGO/ITO in glycerol (b)

Fig 5 shows the CV studies of alternate bio-chemical scheme(scheme II). Fig 5(a) shows the cyclic voltammogram of GDH/TB/ERGO/ITO bioelectrode. The anodic peak current magnitude of GDH/TB/ERGO/ ITO bioelectrode is decreased from 3.14 x 10^{-4} A[Fig 5(a), ERGO/ITO] to 2.6x 10^{-4} A[Fig 5(b)] and potential is shifted to higher value after the GDH immobilization due to the hindrance caused by macromolecular structure of GDH indicating successful immobilization of GDH onto TB/ERGO/ITO surface. When the GDH/TB/ERGO/ITO bioelectrode is exposed to various concentrations of glycerol (150mg/dL- 300mg/dL), the peak current gradually increases with increase in concentration of glycerol which proves that GDH/TB/ERGO /ITO bioelectrode oxidises glycerol to dihydroxy acetone in presence of TB and [Fe(CN)6]3−/4−. The Fig 5(c) represents the response of GDH/TB/ERGO/ITO to glycerol of only one concentration(other datas are not shown) and the raise of peak current from 2.6 x 10^{-4} A to 2.9 x 10^{-4} is observed in presence of glycerol (200mg/dl) in $[Fe(CN)6]^{3-/4-}$ medium. It can be inferred that the GDH/TB/ERGO/ITO bioelectrode catalyses oxidation of glycerol in presence of chemical mediators i.e. toluidine blue on the surface and ferro-ferri in the electrolyte which enhances the heterogeneous electron transfer process in presence of glycerol.

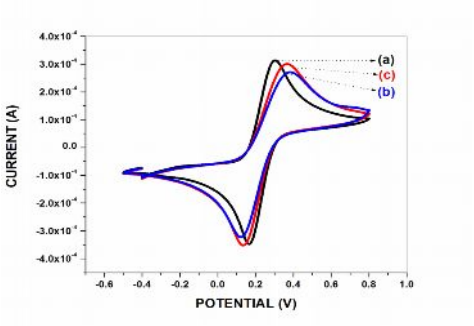


Fig5. CV of TB/ERGO/ITO (a) GDH/TB/ERGO/ITO (b) GDH/TB/ERGO/ITO in glycerol

Response Studies of LIP-GDH/TB/ERGO/ITO bio electrode using CV technique

Fig 6 shows the cyclic voltammogram of LIP-GDH/ERGO/ITO bioelectrode in the presence of various concentrations of tributyrine. The inset of Fig 6 shows the calibration curve between the magnitude of response peak current as a function of tributyrine concentration. The amperometric response of LIP-GDH/TB/ERGO/ITO has been investigated in PBS (50mM, pH 7.8, 0.9% NaCl) containing 5mM $[Fe(CN)6]^{3-/4-}$ at scan rate of 50 mV/s. Scheme II reveals biochemical reaction at LIP-GDH/ERGO/ITO for tributyrine detection where in LIP enzyme catalyses the hydrolysis of tributyrine (triglyceride) which results in the production of fatty acids along with glycerol molecules. Glycerol dehydrogenase (GDH) enzyme co-immobilized with LIP on the TB/ERGO/ITO further oxidises the glycerol into dihydroxyacetone in presence of mediators i.e. TB and ferro-ferri. The overall biochemical reaction is accelerated by the mediators TB and $[Fe(CN)6]^{3-/4-}$. The Figure 6:inset presents the calibration plot of anodic peak current at 0.37 V for LIP-GDH/ERGO/TB/ITO bio electrode as a function of tributyrine concentration. With the increasing concentration of tributyrine from 50 to 300 mg dL^{-1} the anodic peak current is increased gradually and beyond 300 mgdL^{-1} the anodic peak current value is found to decrease as shown in calibration curve (Fig 6 :inset). This bioelectrode can detect tributyrine in the range of with low response time of 12 s. Response time as been calculated on the bases of the time taken to reach the optimum current when LIP-GDH/ERGO/TB/ITO bio electrode is exposed to 150mg/dL in the PBS (50mM, pH 7.8, 0.9% NaCl) containing 5mM $[Fe(CN)6]^{3-/4-}$ at the potential 0.3V. Each experiment is repeated for three times.

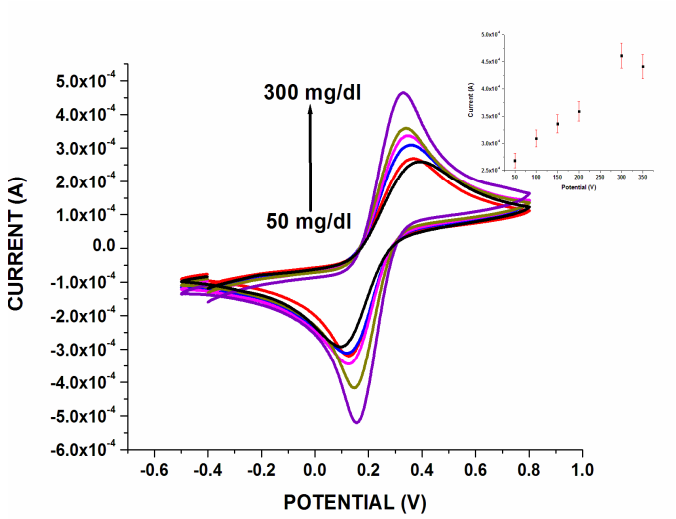


Fig 6. Electrochemical response studies of LIP-GDH/ERGO/ITO bioelectrode as function of tributyrin concentration using CV, Inset: calibration curve between magnitude of current and tributyrin concentration (50–300mg/dL).

Optimization of biosensor with pH

Fig 7 shows the effect of pH on the LIP-GDH/TB/ERGO/ITO bio electrode. The pH effect was evaluated on the sensor response in the pH range from 6.5 to 8. The optimum response of LIP-GDH/TB/ERGO/ITO bio electrode is achieved at 7.8 pH as shown in Fig 7. So the entire experiments were carried out at pH 7.8.

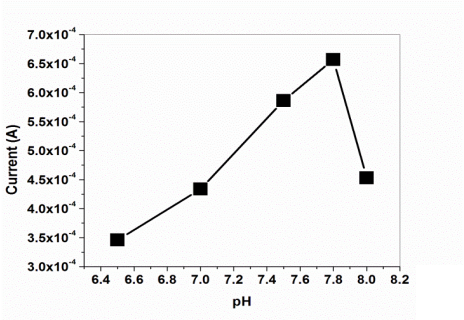


Fig. 7 LIP-GDH studies from pH 6.5 to 8.0

Interference study

Fig 8 shows the effect of interferents on the response of LIP-GDH/ERGO/TB/ITO bio electrode. All the readings are carried in PBS (50mM, pH 7.8, 0.9% NaCl) containing 5mM [Fe(CN)6]3−/4− with various serum substances of urea, glucose, ascorbic acid, sodium pyruvate, sodium ascorbate, cholesterol at their physiological concentrations. The change in the anodic peak current is measured in PBS containing same amount of triglyceride (100mg/dL) and interferents i.e. 1:1 ratio. In Fig 8 the first bar shows the change of current obtained with 150 mg dL^{-1} tributyrin (TG). The remaining bars show the change in current corresponding to the mixture of tributyrine (150 mg dL^{-1}) and interferents in 1:1 ratio. The percentage interference (% Int) has been calculated using equation (1) for various interferents where, ΔI_{TG} is the change in current obtained with 150 mg dL^{-1} triglyceride concentration and Δ_{Int} is the current obtained for 1:1 mixture of tributyrin and interferent. The variation in observed current in the presence of desired interferents, reveals a maximum of 5.4% interference due to sodium pyruvate.

$$\% I_{Int} = \frac{I_{Int+TG} - I_{TG}}{I_{TG}} \times 100$$

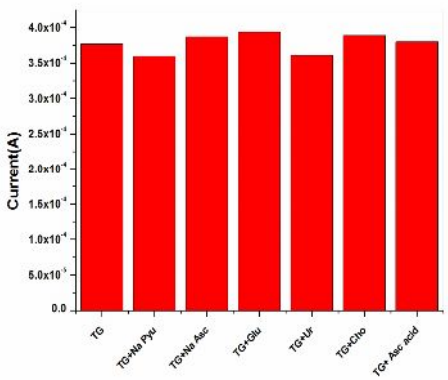


Fig 8. Effect of interferents onto LIP-GDH/TB/ERGO/ITO bio-electrode

Blood sample testing

To estimate the tributyrin using LIP-GDH/TB/ERGO/ITO bio electrode human blood serum obtained from a clinic located in Delhi. The LIP-GDH/TB/ERGO/ITO bio electrode is dipped in the solution of PBS (50mM, pH 7.8, 0.9% NaCl) containing 5mM $[Fe(CN)6]^{3-/4-}$ and serum at scan rate of 50 mV/s. It can be seen that the magnitude of current obtained with and without serum samples are in a equitable agreement. The values of triglyceride obtained by LIP-GDH/ERGO/TB/ITO bio electrode are harmonizing with clinical values.

Conclusions

Electrochemically Reduced graphene oxide (ERGO) based electrochemical biosensor for triglyceride detection has been demonstrated here. An uniform, transparent and few layered reduced graphene oxide nano sheet with high conductivity have been deposited on ITO electrode from well dispersed GO solution (1mg/dL) in water by electrochemical reduction process. The FESEM, RAMAN, FTIR and CV studies reveal the successful formation of ERGO. The ERGO/ITO electrode was utilized for co-immobiliozation of LIP and GDH through covalent bonding to fabricate LIP-GDH/TB/ERGO/ITO bio electrode. A novel biochemical pathway based on TB and $[Fe(CN)6]^{3-/4-}$ mediators, is developed for triglyceride detection without using the NADH. The linear range of biosensor is from 50 to 300 mg dL^{-1}. The stability of ERGO/ITO electrode is up to 2 months. The result is not shown.

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