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Design and characterization of a biosensor with lipase immobilized nanoparticles in polymer film for the detection of triglycerides

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ABSTRACT

High levels of triglycerides in blood can harden and block the arteries increasing the risk of heart disease and strokes. Triglycerides are important constituents of oils and fats used in various foods. The triglyceride content in commercial preparations of oils is estimated using conventional methods. In the present study, an electrochemical biosensor with lipase immobilized novel conductive polymer film has been developed for estimating triglyceride content in a variety of products. The portable biosensor can bring down the detection costs dramatically and can be used for varied purposes. It is based on cyclic voltammetry and has a three-electrode configuration system. Glassy carbon electrode is functionalized with nanoparticles embedded in polyethyleneimine and lipase is immobilized using glutaraldehyde. The strategy increases the electrochemical conductance manifold and overcomes the hindrance to lipase posed by membranes as it is oriented on the outside of the membrane. Thus, it increases the sensitivity and selectivity of detection. Results of scanning electron microscopy and FT-IR spectroscopy were used for characterizing the electrode surface. Linear range of the electrode for triglycerides is 100–500 mg/dL. The sensor was used successfully to determine triglyceride content in several real samples and the average recovery values lie from 95.47 % to 101.05 %.

1. Introduction

Triglycerides (TGAs) are natural esters of fat, formed when a molecule of glycerol binds three molecules of fatty acids. TGAs play a major role in metabolism as energy sources and in the transport of dietary fat in the human body [1]. High concentration of triglycerides (TGA) is related to fatty liver disease or steatosis, liver dysfunction, diabetes, nephrosis, elevated risk of atherosclerosis, heart disease and various endocrine disorders. Thus, estimating triglyceride levels is considered important in the routine medical check-ups. Due to improved awareness of health and implementation of strict regulatory laws for public health, triglyceride estimation in food has become an important practice of modern life [2,3]. Standard methods for the determination of TGAs developed so far are methods such as enzyme-based colorimetry, nuclear magnetic resonance, chromatography, titrimetry, spectrophotometry and fluorometry [1]. The tests used for the determination of TGAs are laborious, slow, complex, non-portable and expensive. Moreover, most of these methods require sample pre-treatments to be carried out [2]. Thus, simpler, cheaper, portable, rapid and reliable methodologies should be developed to ease the testing procedures. There are several nanomaterials-based biosensors developed in the recent past for the detection of pathogens like SARS-CoV-2 [4], toxic substances in food such as mycotoxins [5], pesticides in foods [6,7], endocrine disruptor Bisphenol A [8], ractopine in pork [9], antioxidants like rutin [10], metabolites like glucose [11] and heavy metals in drinking water [12]. Fabrication of electrochemical biosensor for the estimation of TGA levels aids in overcoming all these limitations and make TGAs quantification cheaper, rapid and reliable.

Electrochemical biosensors use lipase or multienzymes in combination with nanoparticles and polymer to detect TGAs [1–3,13]. Lipases belong to the family of hydrolases and cause hydrolysis of esters producing fatty acids and glycerol. The multienzyme reaction follows the steps given below [1–3,13]:

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + 3\ \text{Fatty acids} + 3H^+ \quad \text{(i)}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol 3} - \text{Phosphate} + \text{ADP} \quad \text{(ii)}$$

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$$\text{Glycerol 3–Phosphate} + O_2 \xrightarrow{\text{Glycerol 3–Phosphate oxidase}} H_2O_2 + \text{Dihydroxyacetone phosphate} \quad \text{(iii)}$$

In multi-enzyme TGA biosensors, lipase in combination with glycerol kinase and glycerol-3-phosphate oxidase is used. Here, glycerol generated by lipase-catalyzed hydrolysis is converted by glycerol kinase to glycerol-3-phosphate. Then, Glycerol-3-phosphate oxidase converts it to dihydroxyacetone phosphate and hydrogen peroxide. This hydrogen peroxide is oxidized to release oxygen and electrons which are measured by current change (Eqs. (i)-(iii)) [1–3,13].

The performance of an enzyme-based sensor and its stability is largely influenced by the chemistry, method used and efficiency of coating of the enzyme over the electrode surface. This can be achieved by either adsorption, covalent attachment, or entrapment. Covalent attachment provides less chance of enzyme leaching and offers properly oriented catalytic sites for the reaction to take place [14]. The use of nanotechnology in biosensor fabrication plays a crucial role.

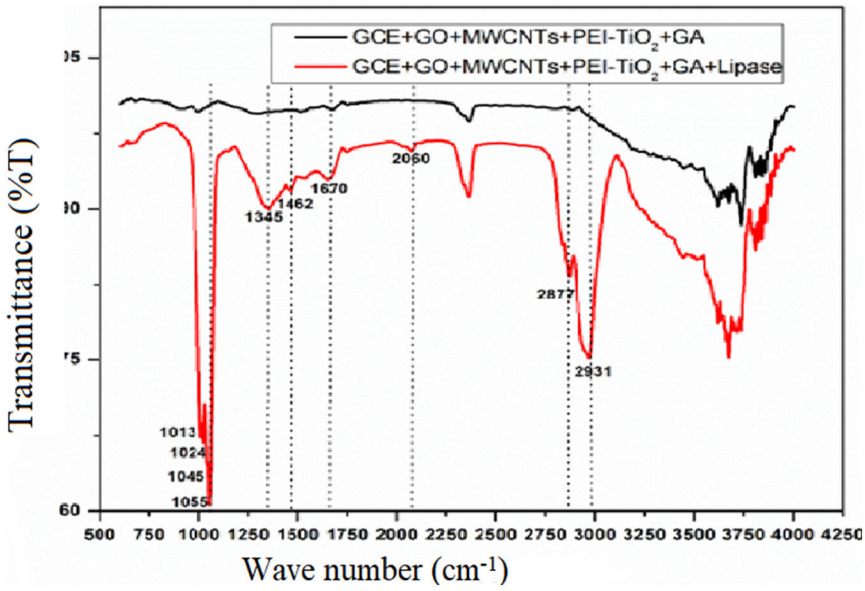

Fig. 1. FT-IR Spectra of nano-polymer film with and without the enzyme lipase.

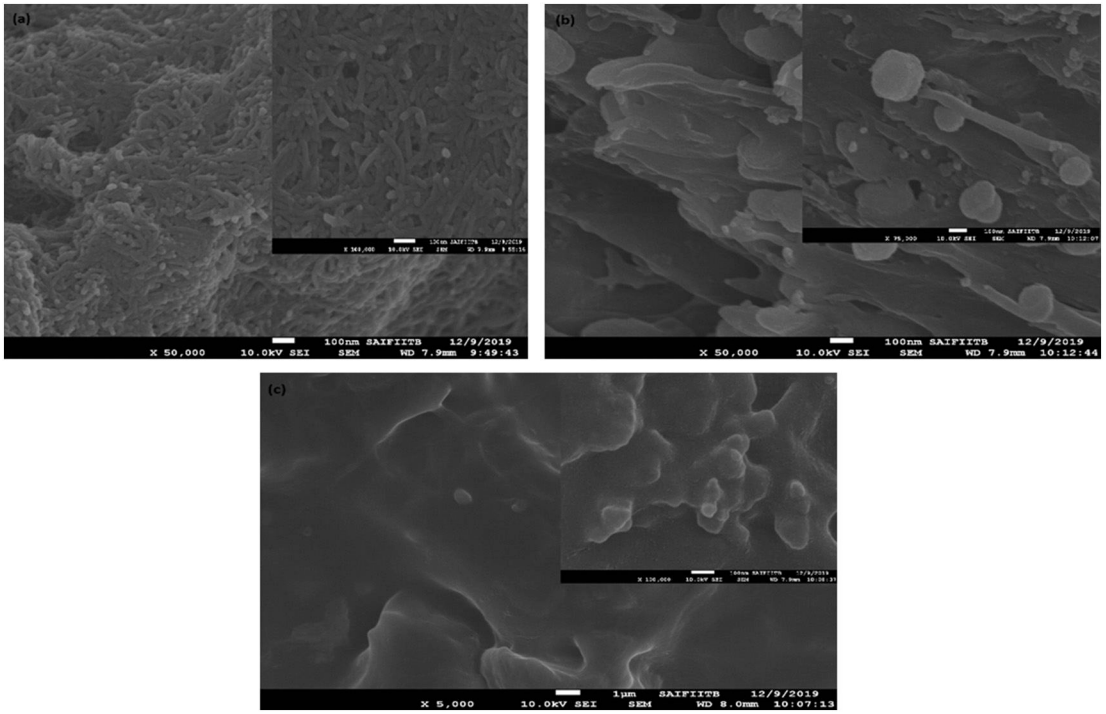

Fig. 2. FE-SEM photographs of (a) GCE/GO/MWCNTs, (b) GCE/GO/MWCNTs/PEI-TiO_2, (c) GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase.

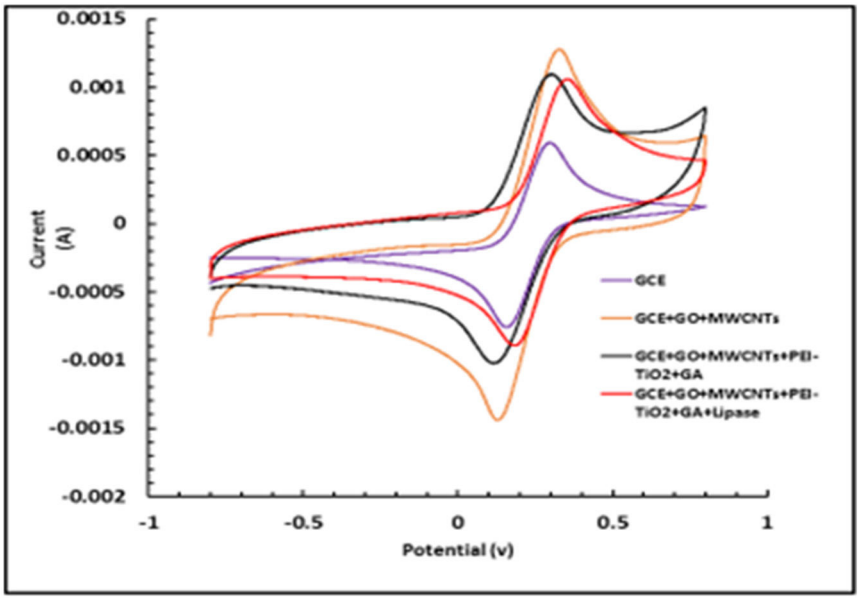

Fig. 3. Cyclic voltammetry of bare GCE, GCE/GO/MWCNTs, GCE/GO/MWCNTs/PEI-TiO_2/GA and GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase in 10 mL system of the electrolyte prepared in 50 mM PB, pH 6.5, containing 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl at 150 mV/s scan rate.

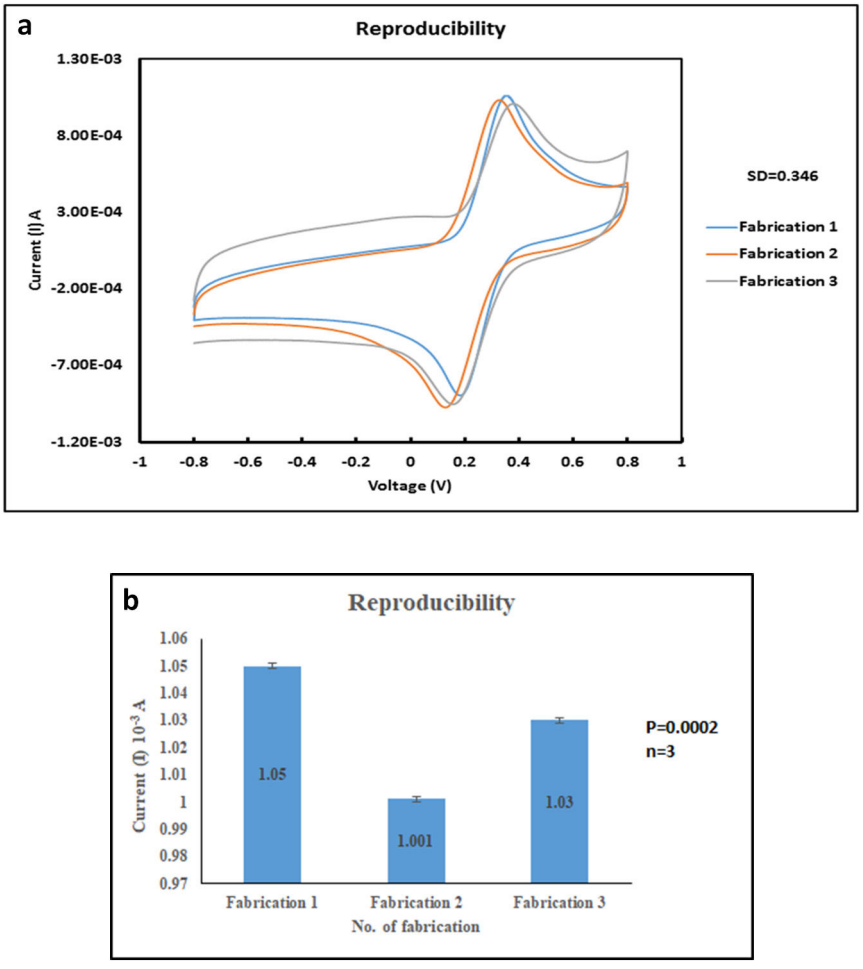

Fig. 4. (a) Cyclic voltammetric measurements of GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase electrode fabricated in triplicates in 50 mM PB, pH 6.5, containing 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl at 150 mV/s scan rate. (b) Anodic peak currents of GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase electrode in triplicates in 50 mM PB, pH 6.5, containing 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl at 150 mV/s scan rate.

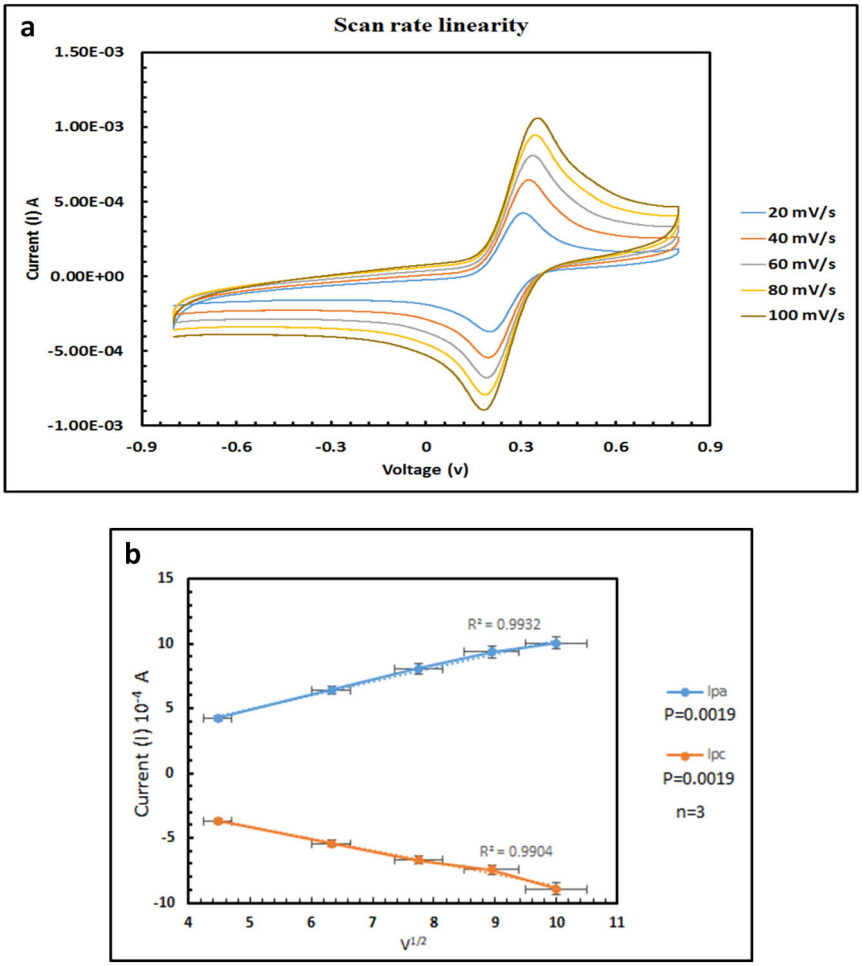

Fig. 5. (a) Cyclic voltammetry of GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase electrode at different scan rates from 20 mV/s to 100 mV/s in 50 mM PB (50 mM $K_3[Fe(CN)_6]$, 9 % NaCl, pH 5). (b) Square root of scan rate (20 mV/s to 100 mV/s) Vs current magnitude of GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase electrode in 50 mM PB, pH 6.5, containing 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl.

Nanomaterials like carbon nanotubes, graphene oxides and metal nanoparticles in combination with polymers provide biocompatibility, higher sensitivity, more surface area, increased conductivity, mechanical strength and amenability for chemical modification and functionalization [14]. The main objective of the work presented here is to develop a single enzyme biosensor for TGA using novel fabrication chemistry. The glassy carbon electrode was coated with reduced graphene oxide (rGO), multi-walled carbon nanotubes (MWCNTs) and the nano-polymer mixture of polyethylene imine (PEI) and titanium dioxide (TiO_2). Glutaraldehyde was used to cross link the polymer and lipase. Enzyme lipase hydrolyses the substrate tributyrin into glycerol and fatty acids and releases protons. The proton release causes changes in the pH of the electrolyte solution which then results in the increase of current. The magnitude of increase in the current was found to be proportional to triglyceride concentration. The sensor was exposed to various concentrations of triglyceride to measure its performance by cyclic voltammetry.

2. Experimental

2.1. Materials and methods

Amano lipase PS, from *Burkholderia cepacia* (534641-10G) was purchased from Sigma Aldrich, India. Glycerol tributyrate was purchased from LOBA Chemie PVT. LTD., India. MWCNTs (size <100 nm and 5–8 μm) were purchased from Sigma Aldrich, India. Reduced graphene oxide or rGO (38818) and nano-TiO_2 suspension (SRL 94632-15 nm) were purchased from Sisco Research Laboratory, India. Polyethylenimine or PEI (P3143) and Glutaraldehyde (03965) were obtained from Sigma Aldrich and LOBA Chemie PVT. LTD., India respectively.

Glycerol tributyrate stock was prepared by mixing it in absolute ethanol to form 1 g/mL stock solution and was stored at 4 °C. Triton X-100 was added along with glycerol tributyrate to a final concentration of 0.2 % (*w/v*) in the phosphate buffer system just before use. Lipase stock solution (20 mg/mL) was prepared in 0.1 M phosphate buffer or PB (pH 7.2) and was stored at 4 °C. Autoclaved double distilled water was used throughout. Potential used for cycling the voltage was −0.8 to +0.8 with the sample interval of 0.001 V at the scan rate of 80 mV/s. The electrolyte composition used during the experiments was 50 mM Phosphate buffer (pH 8.0) (9 % NaCl + 50 mM $K_3[Fe(CN)_6]$). Groundnut oil was purchased from the local market of Vadodara, India.

2.2. Apparatus

FT-IR spectra were recorded using FT/IR-4700 type A (C019161788) (ATR PRO ONE-A062861809) spectrophotometer [7,12]. All the electrochemical measurements were recorded by potentiostat CHI 660C (CH Instruments, Austin, TX, USA), following the method described earlier [7,12]. The three electrodes configuration system containing a glassy carbon electrode (CHI 104), an Ag/AgCl reference electrode (CHI 111) and a platinum counter electrode was used. Ultrasonicator (Digital sonifier 450, Branson 50/60 HZ, USA) was used to disperse

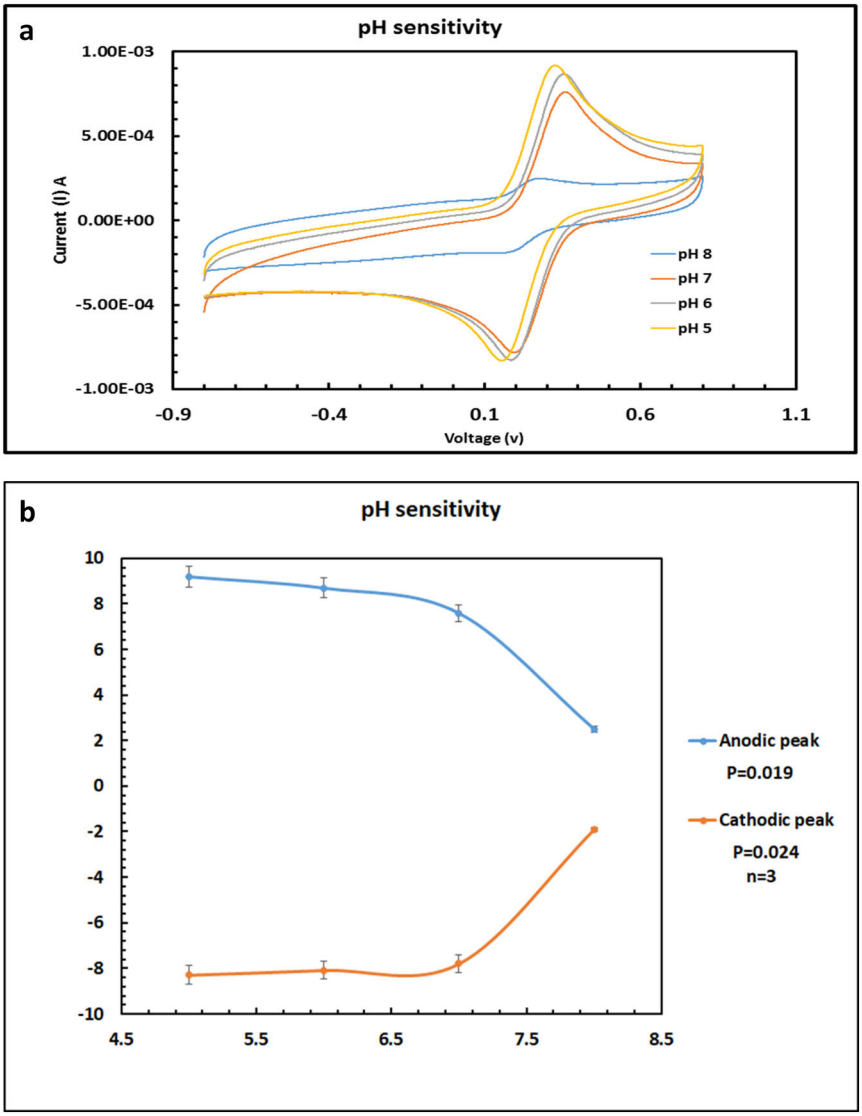

Fig. 6. (a) Cyclic voltammetry of GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase electrode as a function of pH in 50 mM $K_3Fe(CN)_6$ and 9 % NaCl in 50 mM PB (pH 5 to 8) at the scan rate of 80 mV/s. (b) Effect of pH on the current density of both the peaks of GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase electrode.

nanoparticles [7,12]. Field emission gun scanning electron microscope (FE-SEM) JSM-7600F; Jeol, USA was used to study the surface of the coated electrode [7,12].

2.3. Fabrication of lipase/glutaraldehyde/PEI-TiO2/MWCNTs/rGO/GCE electrode

In the present study, novel fabrication chemistry to coat the electrode combined the use of rGO, carboxylic acid modified MWCNTs and nano-TiO_2 dispersed into 0.03 % (*w/v*) PEI. Finally, the lipase enzyme was covalently attached to modified MWCNTs for triglyceride detection. 0.03 % (w/v) of polyethyleneimine (PEI) stock solution was prepared to disperse titanium dioxide nanoparticles (TiO_2). 500 μL of readily purchased TiO_2 suspension was mixed with 500 μL of 0.03 % (*w/v*) of PEI and 500 μL of autoclaved double distilled water. The mixture was sonicated for 90 min. in an ultrasonicator bath before use. 1 mg/mL stock of rGO was prepared by ultrasonicating it in autoclaved double distilled water for 1 h. 6 mg/mL stock of carboxylic acid modified MWCNTs was prepared by ultrasonicating in DMF (dimethyl formamide) for 2 h. The amplitude used for ultrasonication of all the nanoparticles was kept the same as 30 % at 1 s intervals.

Before coating, the glassy carbon electrode (GCE) was polished with 0.3–0.05 μm alumina slurry followed by rinsing it in autoclaved double distilled water and ethanol. The polished electrode was then dried at room temperature before coating. 5 μL of rGO dispersion was drop casted onto GCE and was allowed to dry at room temperature for 15 min. It was then followed by 5 μL MWCNTs-COOH cast-off to the surface and dried at room temperature for 15 min. Then, 5 μL TiO_2 nano-suspension prepared in polyethyleneimine polymer was layered onto GO/MWCNT-COOH modified electrode surface. It was then allowed to dry at room temperature for 15 mins. Then, 5 μL of lipase solution (0.1 M PBS, pH 7.2) (20 mg/mL) was layered onto it. The lipase was allowed to air dry at room temperature. Following it, 5 μL of 0.1 % Glutaraldehyde as a crosslinker was layered onto the surface. Change in the current at each step was also measured in the 10 mL system of the electrolyte prepared in 50 mM PB (pH 6.5) having 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl at 150 mV/s scan rate. Glutaraldehyde crosslinks both polyethyleneimine polymer and lipase enzyme. The resulting electrode was washed with PBS (pH 6.5) and stored at 4 °C in air. Fabrication of the electrode was confirmed initially by cyclic voltammetry (CV). The fabricated electrode with and without an enzyme were compared using CV. Other techniques such as FT-IR and FE-SEM were also used for confirming the fabrication chemistry.

All experiments were done at least three times in independent sets.

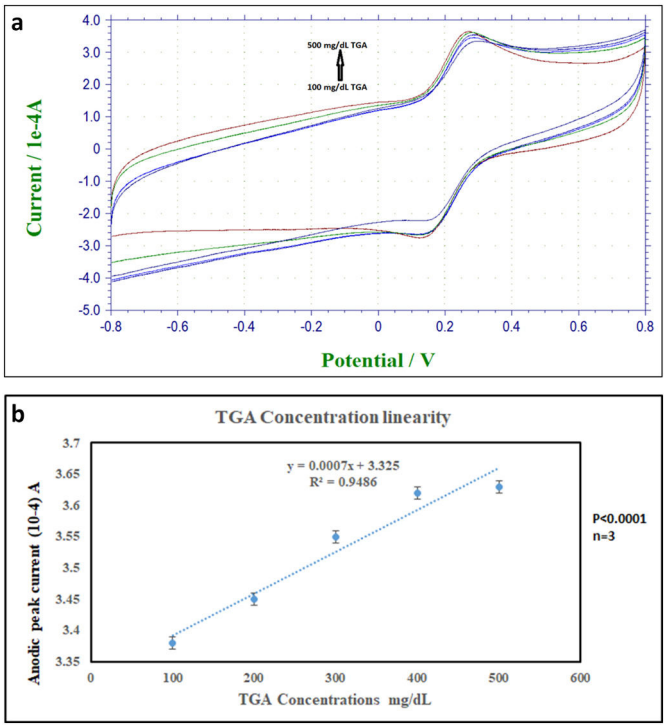

Fig. 7. (a) Anodic sweep segments of cyclic voltammetry of the electrode GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase as a function of TGA concentration at the scan rate 80 mV/s in 50 mM PB ($K_3[Fe(CN)_6]$, 9 % NaCl, pH 8) (100, 200, 300, 400, 500 mg/dL). (b) Linear range of the biosensor GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase for TGA detection (100–500 mg/dL).

Statistical analysis of the results was carried out using the software available in the site. https://www.graphpad.com/quicalcs/oneSampleT1/?Format=50. The significance values are given in the figures.

3. Results

3.1. FT-IR studies

FT-IR spectra of the nanopolymer films were recorded with and without doping the lipase enzyme (Fig. 1). The strong vibrational peaks observed from 1000 to 1100 cm^{-1} correspond to C—C and C—N composite vibrations of the protein chains. The absorbance peak of the amide I region can be observed from 1600 to 1700 cm^{-1} due to the vibrational bending of C═O. The peak observed at 1670 cm^{-1} corresponds to the β-turn of the enzyme. The less intense peak at 2060 cm^{-1} exhibits stretching of N═C between GA and lipase suggesting covalent binding between them. Strong peak observed at 1345 cm^{-1} corresponds to the –OH bending of the lipase. C—H vibrations and imidazole ring stretching were observed at 2931, 2877 and 1462 cm^{-1} respectively. Thus, the FT-IR results confirm the covalent attachment of the lipase onto the porous electrode surface [15,16]. Higher intensity of the peaks indicates a high amount of enzyme loading onto the membrane surface.

3.2. Surface morphology studies

Field emission gun scanning electron microscope was used to study the morphological features of the electrodes GCE/GO/MWCNTs, GCE/GO/MWCNTs/PEI-TiO_2/GA and GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase (Fig. 2(a), (b) & (c)). As evident from Fig. 2, at every step of electrode fabrication, the morphology of the surface showed changes. In the first step of electrode modification, the electrode was first layered with GO, followed by layering with MWCNTs dispersion. The filamentous layer of MWCNTs can be seen in Fig. 2(a). Subsequently, the porous structure of the electrode surface was observed (Fig. 2(b)) when the electrode was layered with PEI-TiO_2 suspension. Then, Fig. 2(c) shows the electrode with nano-polymer-enzyme film. It is evident from Fig. 2 (c) that the enzyme was attached to the surface of the electrode altering its previous surface morphology.

3.3. Electrochemical studies of GCE/GO/MWCNTs/PEI-TiO_2/GA/lipase

Electrochemical properties of the bare GCE, GCE/GO/MWCNTs, GCE/GO/MWCNTs/PEI-TiO_2/GA and GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase were studied by running cyclic voltammetry in 50 mM PBS of pH 6.5 containing 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl at 150 mV/s scan rate (Fig. 3). The magnitude of current increased after layering graphene oxide and MWCNTs. The higher electroactive surface offered by the nanoparticles onto the electrode resulted in enhanced electron transportation between the electrode surface and electrolyte. Following it, PEI-TiO_2 was layered onto the electrode and cross-linked with glutaraldehyde. The current was found to decrease after coating with PEI-TiO_2/GA layer compared to GCE/GO/MWCNTs. The decrease in the magnitude of current shows PEI and GA causing an insulating effect over the electrode surface. After that, when the electrode had lipase immobilized onto the surface through glutaraldehyde, it again showed a further decrease in current. Thus, PEI, GA and lipase here hinder the electron transfer involved in the redox process. The electrode GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase was fabricated in triplicates to check the replicability of the sensor. The current response of each electrode was observed in 50 mM PBS (50 mM $K_3[Fe(CN)_6]$, 9 % NaCl, pH 6.5) at a 150 mV/s scan rate. As shown in Fig. 4(a) and (b) the sensor can be replicated with the SD of 0.346 at its anodic peak.

The coated electrode GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase was then tested for different scan rates from 20 to 100 mV/s in 50 mM PBS (50 mM $K_3[Fe(CN)_6]$, 9 % NaCl, pH 5). It is evident from Fig. 5(a) and (b) that redox peak current magnitude depends on the scan rate. From Fig. 5(b) showing the plot for the square root of scan rate versus magnitude of redox peaks, the R^2 values for both the anodic and cathodic peaks were calculated and found to be 0.9932 and 0.9904 respectively. Thus, an anodic peak showing more correlation will be considered for further experiments to estimate TGA concentration. Moreover, the ratio of cathodic and anodic peak current (Ipc/Ipa) approximates 1. These results suggest that the electron transfer follows surface- controlled process [17,18].

3.4. Effect of pH on GCE/GO/MWCNTs/PEI-TiO_2/GA/lipase

The effect of pH on GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase was investigated by running cyclic voltammetry at a scan rate and sample interval of 80 mV/s and 0.001 V respectively. The electrolyte used was 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl in 50 mM PB (pH 5 to 8). It is clear from Fig. 6(a) and (b) that the current peak of both the anode and cathode decrease with increasing pH. This may be due to a decrease of positive charge present on the electrode surface, leading to increased hindrance in electron transportation between the electrolytic medium and electrode. Thus, decreased value of the electrochemical signal was observed at elevated pH [2]. This result confirmed that the electrode is sensitive to pH changes. During the biochemical reaction, lipase hydrolyses triglyceride into fatty acid, glycerol and protons (Eq. (i)). These released protons would lower the pH of the medium and as a result, the electrochemical signals of the biosensor would also change. Fig. 6(a) and (b) show that the current was lowest at pH 8 for both the peaks. Thus, to have a positive correlation between the TGA concentration and current peak density, pH 8 was chosen as the optimized pH for the bioelectrode. All further measurements were taken at pH 8 and at the anodic sweep segment of cyclic voltammetry [2,19].

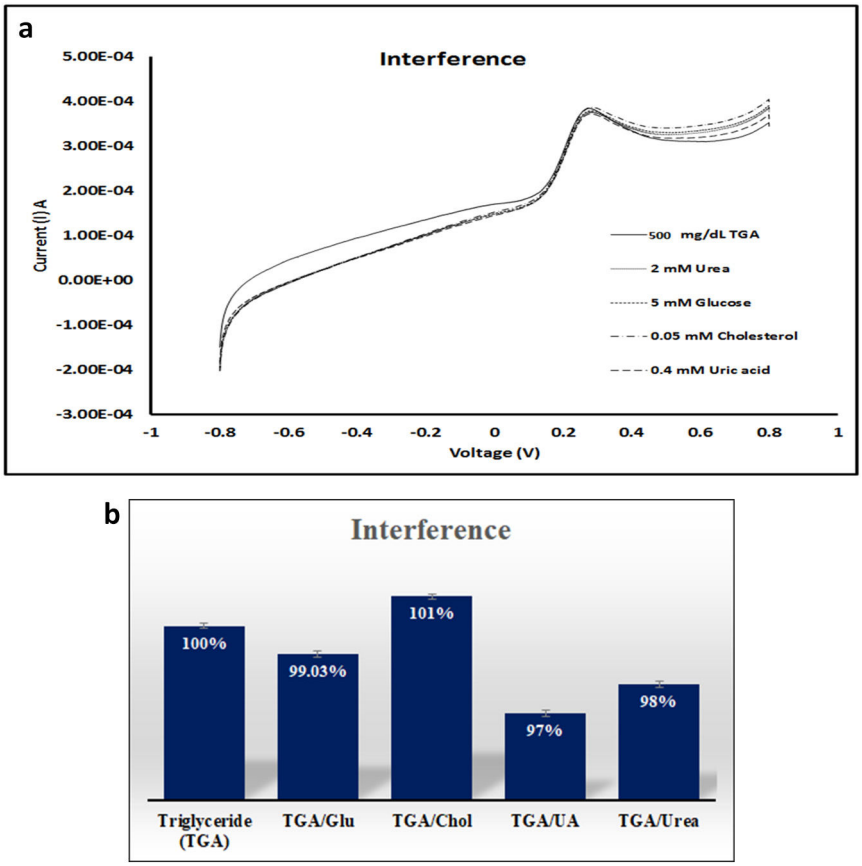

Fig. 8. (a) Anodic current changes at anodic sweep segments after adding the interfering species namely, urea, glucose, cholesterol and uric acid. The interfering species were added in 500 mg/dL TGA at GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase bioelectrode, (b) Effect on anodic current due to interfering species on GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase bioelectrode was presented as bar graph.

Table 1
TGA content of real samples was determined at three different concentrations and recovery was calculated.

Sample	Triglycerides added (mg/dL)	Triglycerides found (mg/dL)	Recovery
Coconut oil	220	222.857 ± 1.29	101.29 %
	260	265.71 ± 2.2	102.2 %
	410	408.57 ± 0.35	99.65 %
Groundnut oil	250	251.42 ± 0.56	100.56 %
	350	351.4 ± 0.4	100.4 %
	500	465.71 ± 6.86	93.14 %
Sunflower oil	200	180.0 ± 10.0	90 %
	230	222.86 ± 3.98	96.02 %
	350	351.428 ± 0.4	100.4 %

3.5. Cyclic voltammetric detection of triglyceride

Cyclic voltammetric response at anodic sweep segment of the GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase bioelectrode as a function of triglyceride (TGA) concentration was recorded in 50 mM $K_3[Fe(CN)_6]$ and 9 % NaCl in 50 mM PB (pH 8) at the scan rate of 80 mV/s. It is evident from Fig. 7(a) that as the concertation of the TGA increases, the anodic peak current increases. This increase in magnitude of the current can be explained by the pH sensitive behavior of the bioelectrode. Protons are released during the enzymatic reaction by lipase which can alter the pH of the reaction system. This change in pH ultimately leads to a change in the magnitude of the current [2]. According to Fig. 7(b), change in the anodic peak current magnitude with respect to the TGA concentrations follows a linear regression model with the R^2 of 0.9485. The lower and higher detection limits found for TGA are 100 and 500 mg/dL respectively.

3.6. Interference study

The specificity of the biosensor is extremely important when it comes to practical use. The property of the enzyme decides the performance of the sensor. Though lipase is known to be highly specific for its substrate, it has to perform in the presence of various interfering species in real samples. Therefore, its specificity in the presence of various interfering species was studied. 2 mM urea, 5 mM glucose, 0.05 mM cholesterol and 0.4 mM uric acid were added separately to 500 mg/dL TGA in 50 mM PB (50 mM $K_3[Fe(CN)_6]$, 9 % NaCl, pH 8). Interference in the anodic peak current due to each interfering species was checked by running an anodic sweep segment of cyclic voltammetry. It is evident from Fig. 8(a) and (b) that the magnitude of the anodic peak current varies from 100 % to 99.03 %, 101 %, 97 % and 98 % in the presence of glucose, cholesterol, uric acid and urea respectively. Thus, the performance of the biosensor seems to be affected negligibly in the presence of interfering species. This result confirms that the bioelectrode GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase has strong anti-interference characteristics.

3.7. Real sample detection

To check the performance of the bioelectrode GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase for its practical use, the electrode was tested against commercially available double-filtered ground nut oil, coconut oil and sunflower oil. The oils were separately mixed in 50 mM PB ($K_3[Fe(CN)_6]$, 9 % NaCl, pH 8). The samples were scanned at an 80 mV/s scan rate. Stock solutions of the ground nut oil, coconut oil and sunflower oil were made in ethanol and were dissolved at three different

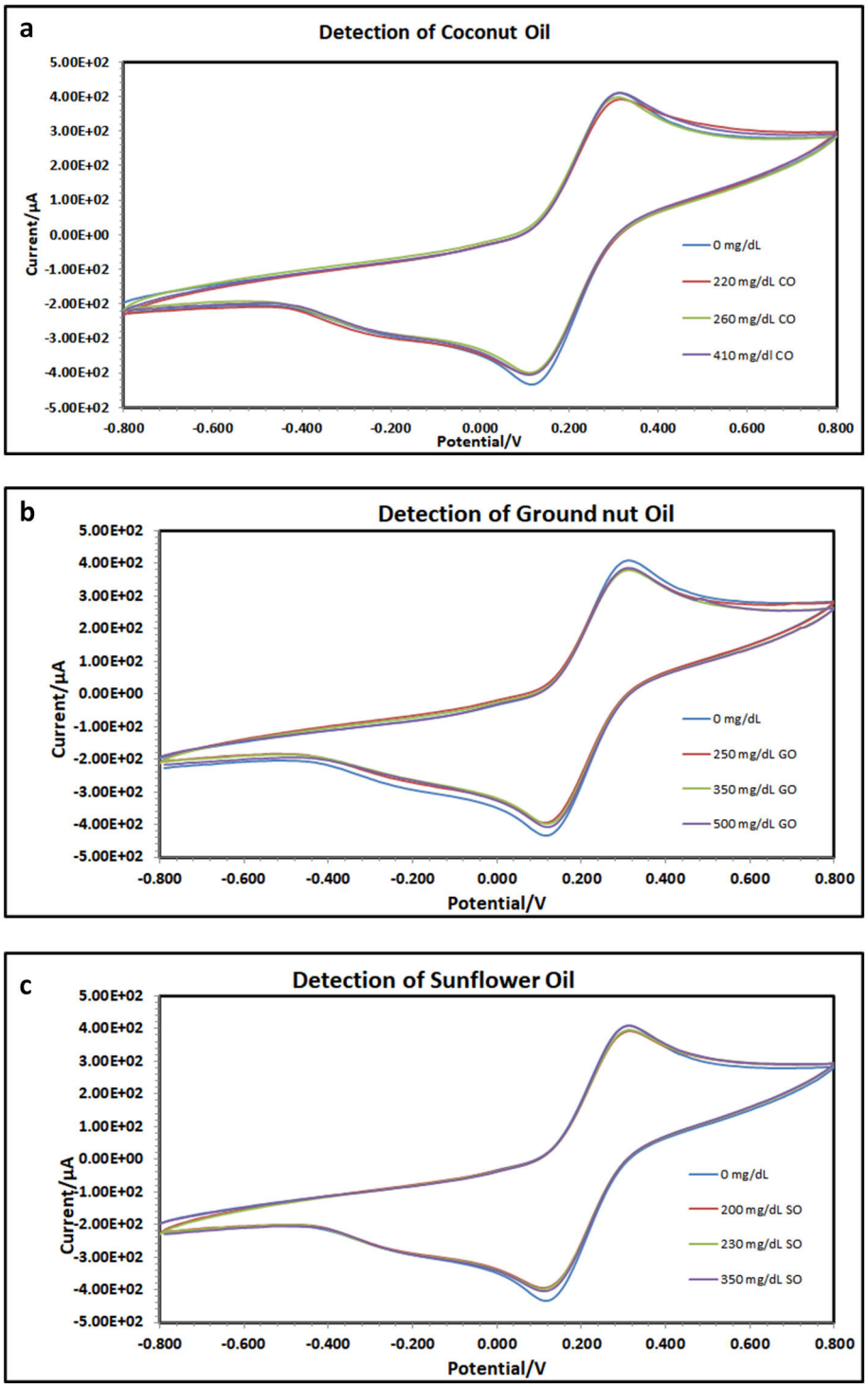

Fig. 9. Real sample detection. Real samples coconut oil (a), groundnut oil (b) and sunflower oil (c) were used in the study at three different concentrations.

concentrations as shown in Table 1, into the reaction system just before use with the help of Triton X-100 (0.2 % *W/V*). The results showed relatable current change at the anodic sweep segment of cyclic voltammetry. The average recovery of the real sample was found to be 101.05 %, 98.03 % and 95.47 % with coconut oil, groundnut oil and sunflower oil respectively (Table 1 and Fig. 9(a), (b) and (c)).

4. Discussion

The field of nanobiotechnology is rapidly growing with a variety of nanomaterials being successfully used for several biological applications [20]. Nanomaterials are used in such novel medical applications as biosensors to detect levels of drugs in blood [21] and in the design of a flow sensor to monitor intravenous infusions to avoid drug overdose [22]. Further, nanobiotechnology is expanded into areas of preservation of food [23], monitoring biofouling of ultrafiltration membranes [24], removal of organic pollutants [25], photocatalytic degradation of dye pollutants present in water [26], sonocatalytic degradation of pesticides [27] and conversion of biowastes into useful bio-nano-catalytic materials [28]. The application of nanomaterial-based biosensors for the

detection of various analytes was presented in the Introduction. Biosensors offer simpler, cheaper, portable, rapid and reliable methodologies when compared to standard biochemical assays.

In earlier studies, a few biosensors for the estimation of triglycerides were designed by different groups. A lipase-based biosensor was developed using a nanostructured cerium oxide film. The researchers coated cerium oxide nano-film onto an indium tin oxide glass plate. The nano-film was derived by the sol-gel method. Lipase was immobilized onto the film for a triglyceride detection assay. The fabricated electrode ITO/nano-CeO_2/lipase was characterized by scanning electron microscopy and cyclic voltammetry. The biosensor showed detection limit, linearity and shelf life as 32.8 mg/dL, 50–500 mg/dL and 84 days respectively [29]. One more biosensor was constructed using electrophoretically coated polyaniline nanotube film on to indium tin oxide glass tube. Lipase was immobilized on the film using glutaraldehyde by covalent bonding. Impedance was used to detect triglyceride in the samples. The sensors exhibited response time, sensitivity and linearity as 20 s, 2.59×10^{-3} $K\Omega^{-1}$ mg/dL and 25–300 mg/dL respectively [2]. In another study, lipase was co-immobilized with Glycerol 3-Phosphate oxidase and Glycerol kinase onto zinc oxide-chitosan nano-film. This whole film was coated on to platinum electrode and was characterized by cyclic voltammetry and electrochemical impedance spectroscopy against Ag/AgCl as a reference electrode. The biosensors showed detection range and detection limit as 50–650 mg/dL and 20 mg/dL respectively at pH 7.5 [30]. Wu and his co-workers [19] modified a glassy carbon electrode (GCE) with conductive, biocompatible and nonporous gold composite (NPG) for the determination of tributyrin. For this purpose, the electrode was immobilized with lipase (GCE/NPG/lipase). The biosensor showed shelf life with the detection limit and linear range as 2.68 mg/dL and 50–250 mg/dL respectively [19]. While, Narwal and Pundir coated commercially available nanoparticles of the enzyme lipase, glycerol kinase and glycerol 3-phosphate kinase onto a pencil electrode for the estimation of triolein. As in the earlier study, the biosensor was characterized by cyclic voltammetry and impedance spectroscopy against Ag/AgCl as a reference electrode at pH 7. The biosensor exhibited the lowest detection limit and linear range at 0.1 nM and 0.1–45 nM respectively. The sensor showed stability over 240 days having 80 % of its original current [31]. The release of fatty acid generated by lipase reaction causes changes in pH which can be detected by the three-electrode system (Eq. (i)). This kind of sensor uses only a lipase enzyme rather than multiple enzymes [1].

An alternative promising way of detecting TGAs is to measure glycerol produced by lipase activity. Glycerol has high oxidation potential and due to that its direct oxidation at bare working electrodes is not suitable for analytical applications. The development of metal nanoparticles such as copper, gold, rhodium and titanium oxide modified electrodes offer oxidation of alcohols and thus glycerol as well [1]. Di Tocco and his team immobilized lipase onto chitosan coated magnetic nanoparticles present in combination with multi-walled carbon nanotubes (MWCNTs), pectin and copper oxide nanoparticles on a glassy carbon electrode. The sensor thus developed hydrolyzed triglycerides from serum samples into fatty acids and glycerol. Glycerol oxidized in the reaction was measured by cyclic voltammetry and amperometry. The electrode showed the detection limit from 3.2×10^{-3} g L^{-1} to 3.6×10^{-3} g L^{-1} [1]. Ono and his co-workers employed Nortropine-N-oxyl (NNO) to oxidize glycerol produced by lipase reaction with tributyrin on a gold working electrode against Ag/AgCl reference electrode. The proposed method could sense the concentration of tributyrin from 0.1 to 10 nM [13].

The problem with multi-enzyme systems is the difference in their stabilities, K_M values and optimal experimental conditions. Our laboratory has been working with nanomaterial-based biosensors [7,12,20], hence we decided to fabricate an electrochemical biosensor that can overcome all these limitations making TGAs quantification cheaper, rapid and reliable. The design and chemistry take into account the shortcomings associated with other electrodes like the environmental toxicity and the cost. This is a single enzyme biosensor, unlike many other electrodes described here. Further, most TGA biosensors still face problems like enzyme leaching, degradation, active site orientation as well as a hindrance or less facilitation of electron transfer between the electrode surface and enzyme. These problems end up affecting the performance of the sensor in terms of sensitivity, detection limits, shelf life, specificity and others. The triglyceride biosensor presented here can successfully be used for the estimation of triglyceride content in commercially available cooking oils and fats. Further, the chemistry and design of this biosensor offer a successful prototype of a biosensor, that can be adopted for the estimation of other molecules.

5. Conclusion

In summary, nanoporous GCE/GO/MWCNTs/PEI-TiO_2/GA/Lipase bioelectrode was fabricated successfully using a novel chemistry of fabrication. This structure formed a good interface between the active site of the enzyme and the electrode surface. Enzyme lipase was immobilized on the PEI with glutaraldehyde covalent crosslinking. The electrode showed the linearity for triglyceride (TGA) from 100 mg/dL to 500 mg/dL with a higher detection limit of 500 mg/dL. The electrode was checked for its specificity in the presence of various interfering species. The bioelectrode showed relatively good specificity for the triglycerides. The bioelectrode was checked against the real sample of ground nut oil, coconut oil and sunflower oil for its practical application. The bioelectrode can be replicated with an SD of 0.346. This biosensor is expected to serve as an effective tool for the detection of triglycerides. Further, the fabrication chemistry is promising for developing biosensors of different metabolites and compounds using other enzymes.

Declaration of competing interest

The authors declare no conflict of interest.

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References

[1] A. Di Tocco, S.N. Robledo, Y. Osuna, J. Sandoval-Cortez, A.M. Granero, N. R. Vettorazzi, H. Fernández, Development of an electrochemical biosensor for the determination of triglycerides in serum samples based on a lipase/magnetite-chitosan/copper oxide nanoparticles/multiwalled carbon nanotubes/pectin composite, Talanta 190 (2018) 30–37.
[2] C. Dhand, P.R. Solanki, K.N. Sood, M. Datta, B.D. Malhotra, Polyaniline nanotubes for impedimetric triglyceride detection, Electrochem. Commun. 11 (2009) 1482–1486.
[3] F. Kartal, A. Kilinç, S. Timur, Lipase biosensor for tributyrin and pesticide detection, Int. J. Environ. Anal. 87 (2007) 715–722.
[4] Y. Orooji, H. Sohrabi, N. Hemmat, F. Oroojalian, B. Baradaran, A. Mokhtarzadeh, M. Mohaghegh, H. Karimi-Maleh, An overview on SARS-CoV-2 (COVID-19) and other human coronaviruses and their detection capability via amplification assay, chemical sensing, biosensing, immunosensing, and clinical assays, Nanomicro Lett. 13 (1) (2021) 18, https://doi.org/10.1007/s40820-020-00533-y.
[5] H. Sohrabi, O. Arbabzadeh, P. Khaaki, A. Khataee, M.R. Majidi, Y. Orooji, Patulin and trichothecene: characteristics, occurrence, toxic effects and detection capabilities via clinical, analytical and nanostructured electrochemical sensing/biosensing assays in foodstuffs, Crit. Rev. Food Sci. Nutr. 62 (20) (2022) 5540–5568, https://doi.org/10.1080/10408398.2021.1887077.
[6] H. Sohrabi, P. Salahshour Sani, Y. Orooji, M.R. Majidi, Y. Yoon, A. Khataee, MOF-based sensor platforms for rapid detection of pesticides to maintain food quality and safety, Food Chem. Toxicol. 165 (2022), 113176, https://doi.org/10.1016/j.fct.2022.113176.
[7] J.B. Thakkar, S. Gupta, C.R. Prabha, Acetylcholine esterase enzyme doped multiwalled carbon nanotubes for the detection of organophosphorus pesticide using cyclic voltammetry, Int. J. Biol. Macromol. 13 (2019) 895–903.
[8] V. Sanko, A. Şenocak, S.O. Tümay, Y. Orooji, E. Demirbas, A. Khataee, An electrochemical sensor for detection of trace-level endocrine disruptor bisphenol a using Mo2Ti2AlC3 MAX phase/MWCNT composite modified electrode, Environ. Res. 212 (Pt A) (2022), 113071, https://doi.org/10.1016/j.envres.2022.113071.

[9] Y. Orooji, P.N. Asrami, H. Beitollahi, S. Tajik, M. Alizadeh, S. Salmanpour, M. Baghayeri, J. Rouhi, A. L. Sanati, F. Karimi, An electrochemical strategy for toxic ractopamine sensing in pork samples; twofold amplified nano-based structure analytical tool. J. Food Meas. Charact. 15, 4098-4104. doi: 10.1007/s11694-021-00982-y.

[10] A. Şenocak, V. Sanko, S.O. Tümay, Y. Orooji, E. Demirbas, Y. Yoon, A. Khataee, Ultrasensitive electrochemical sensor for detection of rutin antioxidant by layered Ti3Al0.5Cu0.5C2 MAX phase, Food Chem Toxicol. 164 (2022) 113016, https://doi.org/10.1016/j.fct.2022.113016.

[11] H. Sohrabi, A. Khataee, S. Ghasemzadeh, M.R. Majidi, Y. Orooji, Layer double hydroxides (LDHs)- based electrochemical and optical sensing assessments for quantification and identification of heavy metals in water and environment samples: a review of status and prospects, TrendsEnviron. Anal. Chem. 31 (2021), e00139, https://doi.org/10.1016/j.teac.2021.e00139.

[12] S. Gupta, C.R. Prabha, C.N. Murthy, Functionalized multi-walled carbon nanotubes/polyvinyl alcohol membrane coated glassy carbon electrode for efficient enzyme immobilization and glucose sensing, J. Env. Chem. Engg. 4 (2016) 3734–3740.

[13] T. Ono, K. Sato, Y. Sasano, K. Yoshida, T. Dairaku, Y. Iwabuchi, Y. Kashiwagi, Electrochemical detection of triglycerides based on an enzymatic reaction and electrocatalytic oxidation with nortropine-N-oxyl, Electroanalysis 31 (2019) 603–606.

[14] B. Ma, L.Z. Cheong, X. Weng, C.P. Tan, C. Shen, Lipase@ZIF-8 nanoparticles-based biosensor for direct and sensitive detection of methyl parathion, Electrochim. Acta 283 (2018) 509–516.

[15] Y.P. Cao, Y.P. Xia, X.F. Gu, L. Han, Q. Chen, G.Y. Zhi, D.H. Zhang, PEI-crosslinked lipase on the surface of magnetic microspheres and its characteristics, Colloids Surf. B: Biointerfaces 189 (2020), 110874.

[16] E. Ondul, N. Dizge, N. Albayrak, Immobiliztion of Candida antarctica a and thermomyces lanuginosus lipases on cotton terry cloth fibrils using polyethyleneimine, Colloids Surf. B: Biointerfaces 95 (2012) 109–114.

[17] M. Li, S. Xu, M. Tang, L. Liu, F. Gao, Y. Wang, Direct electrochemistry of horseradish peroxidase on graphene modified electrode for electrocatalytic reduction towards H2O2, Electrochim. Acta 56 (2011) 1144–1149.

[18] Q. Liu, X. Lu, J. Li, X. Yao, J. Li, Direct electrochemistry of glucose oxidase and electrochemical biosensing of glucose on quantum dots/carbon nanotubes electrodes, Biosens. Bioelectron. 22 (2007) 3203–3209.

[19] C. Wu, X. Liu, Y. Li, X. Du, X. Wang, P. Xu, Lipase-nanoporous gold biocomposite modified electrode for reliable detection of triglycerides, Biosens. Bioelectron. 53 (2014) 26–30.

[20] S. Gupta, C.N. Murthy, C.R. Prabha, Recent advances in carbon nanotube based electrochemical biosensors, Int. J. Biol. Macromol. 108 (2018) 687–703.

[21] Y. Orooji, M.H. Irani-Nezhad, R. Hassandoost, A. Khataee, S.R. Pouran, S.W. Joo, Cerium doped magnetite nanoparticles for highly sensitive detection of metronidazole via chemiluminescence assay, Spectrochim. Acta A Mol. Biomol. Spectrosc. 15 (234) (2020), 118272, https://doi.org/10.1016/j.saa.2020.118272.

[22] R. Hagihghi, A. Razmjou, Y. Orooji, M.E. Warkiani, M. Asadnia, A miniaturized piezoresistive flow sensor for real-time monitoring of intravenous infusion, J. Biomed. Mater. Res. B Appl. Biomater. 108 (2020) 568–576, https://doi.org/10.1002/jbm.b.34412.

[23] M. Khalili, A. Razmjo, R. Shafiei, M.H. Shahavi, M.C. Li, Y. Orooji, High durability of food due to the flow cytometry proved antibacterial and antifouling properties of TiO2 decorated nanocomposite films, Food Chem. Toxicol. 168 (2022), 113291, https://doi.org/10.1016/j.fct.2022.113291.

[24] Y. Orooji, A. Movahedi, Z. Liu, M. Asadnia, E. Ghasali, Y. Ganjkhanlou, A. Razmjou, H. Karimi-Maleh, N.T.H. Kiadeh, Luminescent film: biofouling investigation of tetraphenylethylene blended polyethersulfone ultrafiltration membrane, Chemosphere 267 (2021), 128871, https://doi.org/10.1016/j.chemosphere.2020.128871.

[25] Z. Ansarian, A. Khataee, S. Arefi-Oskoui, Y. Ooroji, H. Lin, Ultrasound-assisted catalytic activation of peroxydisulfate on Ti3GeC2 MAX phase for efficient removal of hazardous pollutants, Mater. Today Chem. 24 (2022), 100818, https://doi.org/10.1016/j.mtchem.2022.100818.

[26] Y. Orooji, R. Mohassel, O. Amiri, A. Sobhani, M. Salavat-Niasari, Gd2ZnMnO6/ZnO nanocomposites: green sol-gel auto-combustion synthesis, characterization and photocatalytic degradation of different dye pollutants in water, J. Alloys Compd. 835 (2020), 155240, https://doi.org/10.1016/j.jallcom.2020.155240.

[27] R. Keyikoglu, A. Khataee, H. Lin, Y. Orooji, Vanadium (V)-doped ZnFe layered double hydroxide for enhanced sonocatalytic degradation of pymetrozine, Chem. Eng. J. 434 (2022), 134730, https://doi.org/10.1016/j.cej.2022.134730.

[28] Y. Orooji, N. Han, Z. Nezafat, N. Shafei, Z. Shen, M. Nasrollahzadeh, H. Karimi-Maleh, R. Luque, A. Bokhari, J.J. Klemes, Valorisation of nuts biowaste: prospects in sustainable bio(nano)catalysts and environmental applications, J. Clean. Prod. 347 (2022), 131220, https://doi.org/10.1016/j.jclepro.2022.131220.

[29] P.R. Solanki, C. Dhand, A. Kaushik, A.A. Ansari, K.N. Sood, B.D. Malhotra, Nanostructured cerium oxide film for triglyceride sensor, Sens. Actuators B: Chem 141 (2009) 551–556.

[30] J. Narang, C.S. Pundir, Construction of a triglyceride amperometric biosensor based on chitosan-ZnO nanocomposite film, Int. J. Biol. Macromol. 49 (2011) 707–715.

[31] V. Narwal, C.S. Pundir, An improved amperometric triglyceride biosensor based on co-immobilization of nanoparticles of lipase, glycerol kinase and glycerol 3-phosphate oxidase on to pencil graphite electrode, Enzym. Microb. Technol. 100 (2017) 11–16.