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Research Article

An Enhanced Amperometric Triglyceride Biosensor by Graphene Oxide Nanoparticles

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Abstract

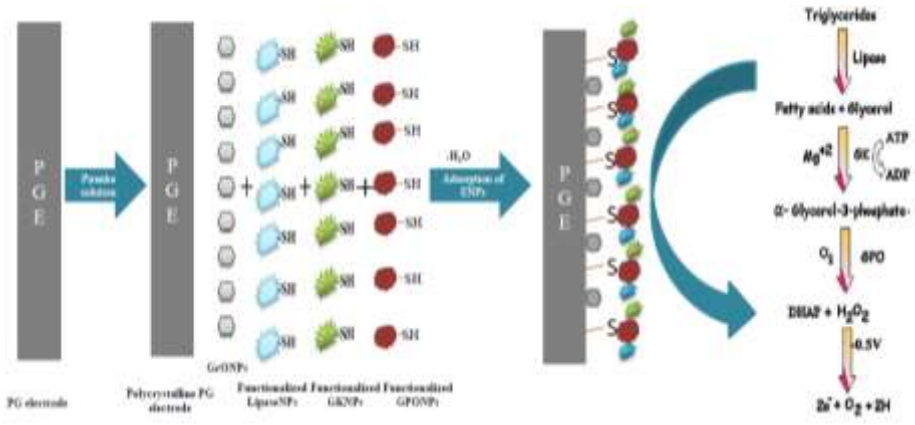
An enhanced amperometric triglyceride (TG) biosensor was created by electrodepositing graphene oxide nanoparticles (GrONPs) onto pencil graphite electrode (PGE) and then co-immobilizing enzyme nanoparticles (ENPs) of lipase, glycerol kinase (GKNPs), and glycerol 3-oxidase (GPONPs), onto this modified electrode. Electrochemical impedance spectroscopy (EIS), cyclic voltammetry, and scanning electron microscopy (SEM) were employed to examine the working electrode (ENPs/GrONPs/PGE) at various phases of its creation. At an applied potential of 0.3V, the biosensor showed its best current response within 2s. The electrode's ideal pH and temperature were 7.5 and 40˚C respectively, and its linearity was seen for triolein in the concentration range of 0.01 to 60 mM, with a detection limit (LOD) of 0.2 nM. In human serum, the biosensor showed an analytical recovery of 98% for added triolein. The coefficients of variability both within and between batches were 0.048% and 0.05%, respectively. The TG levels in sera as determined by the current biosensor and the conventional enzymic colorimetric technique showed a significant correlation (R^2 = 0.99). This biosensor measured the levels of TG in the sera of patients with cardiogenic shock and hyperlipidemia as well as those who appeared to be in apparently healthy condition. The storage stability of electrode revealed only 25% decrease in its initial activity, after its regular uses for 240 days of storage in dry condition at 4˚C.

Keywords: Triglycerides (TG); TG Biosensor; Graphene oxide nanoparticles; Pencil graphite electrode; Hyperlipidemia; Serum.

Graphical Abstract

Construction of triglyceride biosensor based on LipaseNP/GKNPs/GPONPs/GONS/PGE.

LipaseNP = Lipase nanoparticles;
GKNPs = Glycerol kinase nanoparticles;
GPONPs = Glycerol-3-phospate kinase nanoparticles;
GrONPs = Graphene oxide nanoparticles;
PGE = Pencil graphite electrode.



1. Introduction

The determination of triglycerides (triacylglycerol, TG) in blood plays a crucial role in the diagnosis and medical management of atherosclerosis and heart strokes. A normal blood triglyceride level is 150 mg/dL or less. Hyperlipoproteinemia is anticipated, when the TG level ranges between 150 and 199 mg/dL. A TG level exceeding 500 mg/dL indicates hyperlipidemia, while levels surpassing 1000 mg/dL or 5000 mg/dL are associated with eruptive xanthoma, enlarged liver, and spleen, respectively (Haim *et al.*, 1999) Immobilised enzyme-based biosensors have benefits over existing TG measurement techniques in terms of use, sensitivity, specificity, and storage stability (Pundir and Narwal, 2018). Lipase and NAD^+ dep

endent glycerol dehydrogenase biosensors and lipase, glycerol kinase (GK), and glycerol-3-P oxidase (GPO) TG biosensors are the two types of enzyme-based biosensors for TG measurement (Pundir and Narang, 2013). The latter is considered to be better than the former, since it shows irreversible electron flow and doesn't require an electron mediator.

Enzyme molecules in water combine by dehydration to generate enzyme nanoparticles (ENPs). These ENPs have unique optical, electrical, electronic, thermal, chemical, and catalytic properties that improve electron transfer and a greater surface area. ENPs have therefore been chosen for biosensor building over simple or natural enzymes. High activity retention and biosensor reusability are further benefits of these ENPs (Pundir, 2015). We have constructed improved amperometric biosensors for cholesterol using ENPs of cholesterol esterase (CEase) & cholesterol oxidase kinase (COD) (Chawla *et al.*, 2013; Aggarwal *et al.*, 2016), glycerol using ENPs of glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO) (Narwal and Pundir, 2018), pyruvate using ENPs of pyruvate oxidase (Malik *et al.*, 2019) and lactose using ENPs of Beta-galactosidase and glucose oxidase (Ahlawat *et al.*, 2022). In order to construct improved amperometric TG biosensors, we have prepared ENPs of lipase, GK and GPO nanoparticles (NPs) and immobilised onto Au electrodes) (Pundir and Aggarwal, 2017) and pencil graphite electrodes (PGE) (Narwal and Pundir, 2016).

With their distinct structural and functional characteristics such as their electrical, magnetic, optical, and catalytic capacities, nanoparticles are perfect for a range of biological sensing applications. Graphene nanoparticles (GrONPs) have exceptional mechanical, electrical, optical, and thermal characteristics, making them extremely attractive for a range of applications (Batra *et al.*, 2016; Alshammary, 2022). Therefore, it is anticipated that the addition of GrONPs to pencil graphite electrodes will improve the TG Biosensor's overall analytical performance. We have reported an improved amperometric glycerol biosensor by co-immobilizing GKNPs and GPONPs onto a pencil graphite electrode (PGE) decorated with GrONPs (Narwal and Pundir, 2019).

We present herein the construction of amperometric TG biosensor using GrONPs modified PGE with lipaseNPs, GKNPs, and GPONPs immobilized in combination. The constructed biosensor demonstrated enhanced analytical performance in terms of response time, sensitivity, limit of detection (LOD) and storage stability.

2. Materials and Methods

2.1. Sources of Chemicals and Bio-Chemicals

The lipase enzyme derived from porcine pancreas, glycerol kinase enzyme obtained from Cellulomonas sp., and glycerol-3 phosphate oxidase (GPO) enzyme from Aerococus viridians were used in this study. The following chemicals were purchased from Sigma Aldrich Co. USA: 3,5-dichloro-2-hydroxybenzene sulfonate (DHBS) and 4-amino-phenazone. Glycerol, ascorbic acid, uric acid, glycine, acrylamide, glucose, and silica gel were obtained from SRL, Mumbai India. H_2SO_4, $KMNO_4$, $NaNO_3$, H_2O_2, and HCl were acquired from Qualigens Fine Chemicals, Mumbai, India. A 6B graphite pencil (Make: Camlin, India) with a graphite rod of 2 mm diameter and a 2HB pencil were purchased from the local stationary market for the preparation of graphene oxide nanoparticles (GrONPs). Distilled water (DW) with an ohmic resistance of 1.8×10^{-5} ohm was used throughout the study. Triolein, glycerol, and ATP-sodium salt were obtained from SISCO Research Laboratory Pvt. Ltd. Mumbai. All other chemicals used were of analytical reagent (AR) grade. The left-over sera samples from both healthy individuals and individuals with hyperlipidemia were collected from the local hospital Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Science (Pt. BDS PGIMS) Rohtak, Haryana, India.

2.2. Instruments and Equipments

The potentiostat/galvanostat (Make: Autolab, model: AUT83785, made by Eco Chemical, The Netherlands) was employed in the current investigation along with NOVA 1.4 software. The following instruments were used: digital water bath shaker (NSW New Delhi), digital pH metre (335D, Systronics, Ahmadabad), Spectronic-20 (Thermo USA), Transmission electron microscope (Pananalytical Phillip CM12), Fourier transform infrared spectroscopy spectrometer (model Is10, Thermoelectron, USA), and scanning electron microscope (SEM) (Zeiss EV040, USA). In a 25 ml solution of 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at -0.2 V between the frequency ranges of 0.01 Hz and 10 kHz, the electrochemical impedance spectra (EIS) were recorded using FRA software.

2.3. Preparation of Graphene Oxide Nanoparticles (GrONPs)

GrONPs were synthesized by Hummer's method with modifications as described earlier (Narwal and Pundir, 2019)

2.4. Characterization of GrONPs

GrONPs were subjected to comprehensive characterization utilizing the techniques of Transmission Electron Microscopy (TEM) and UV spectroscopy, following the methodology outlined in the reference (Narwal and Pundir, 2019).

2.5. Electrodeposition of GrONPs onto PG Electrode

The preparation and cleaning of the PGE followed the methodology outlined in reference (Narwal and Pundir, 2019).

2.6. Preparation of Lipase NPs, GKNPs and GPONPs

The nanoparticles of lipase, GK and GPO were synthesized through the desolvation method employing ethanol as the dehydrating agent, following the procedure detailed in a previous published study (Pundir and Aggarwal, 2017).

2.7. Immobilization of Lipase NPs, GKNPs and GPONPs onto GrONPs Modified PG Electrode

The co-immobilization of lipaseNPs, GKNPs, and GPONPs (with same proportions) was performed by attaching their free -NH_2 to the unbound carboxyl (-COOH) groups of GrONPs electrodeposited on the surface of PGE. This covalent coupling process was achieved using EDC-NHS chemistry, as described earlier (Narwal and Pundir, 2019).

2.8. Characterization of EnzymeNPs/GrONPs/PG Electrode

The characterization of the enzymeNPs/GrONPs/PG electrode was conducted using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and electrochemical impedance spectroscopy (EIS) at Jawaharlal Nehru University (JNU), New Delhi, India.

2.9. Construction and Response Measurements of Amperometric TG Biosensor

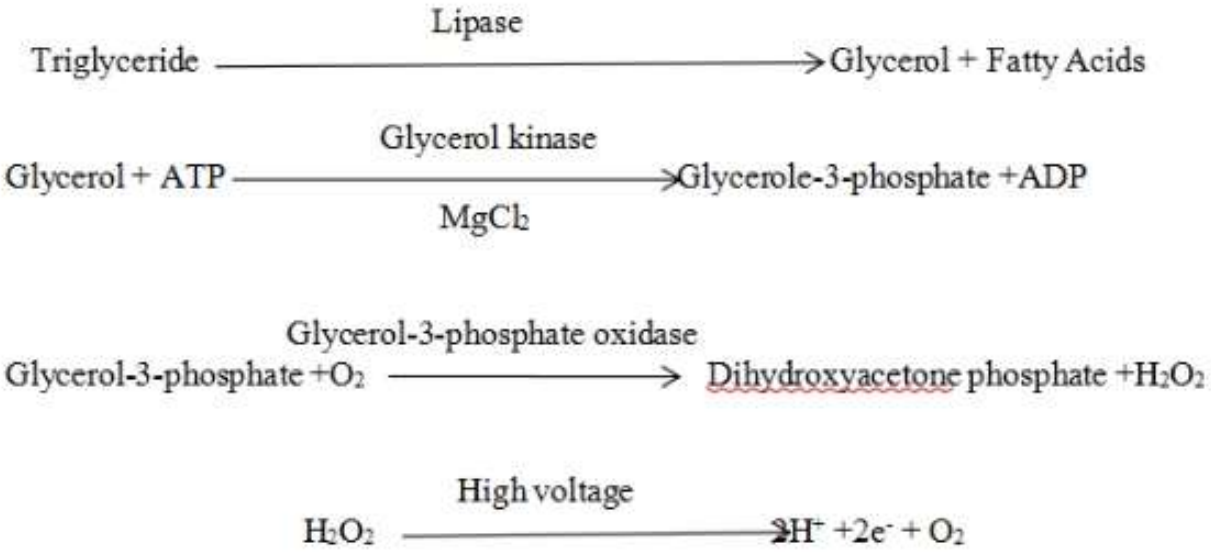
An amperometric TG Biosensor was fabricated using ENPs (lipase NPs, GKNPs and GPONPs) immobilized onto GrONPs decorated PGE, as the working electrode. The auxiliary electrode consisted of a platinum (Pt) wire, and the reference electrode was Ag/AgCl. The connection between the electrodes was established using a potentiostat (Eco Chemie, The Netherlands). To measure the generated current (in mA), the electrodes were polarized at various potentials (in volts) relative to the Ag/AgCl reference electrode. The current measurements were based on specific electrochemical reactions that occurred during the experimental procedure (Pundir and Aggarwal, 2017).

2.10. Optimization of Biosensor

The optimization of present Biosensor (ENPs/GrONPs/PGE) was done as described in previous study reference (Pundir and Aggarwal, 2017).

2.11. Determination of Triglyceride Level in Sera

The serum supernatant was obtained through centrifugation of 1 ml intravenous blood samples from male and female individuals with hyperlipidemia, representing a range of ages and appearances, at the local hospital of Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Science (Pt. BDS PGIMS) Rohtak, Haryana, India. The centrifugation was performed at 5000 rpm for duration of 5 minutes. To assess the concentration of TG in these serum samples, a state-of-the-art Biosensor was employed. The methodology employed for determining the triolein concentration in serum samples was identical to the procedure outlined for measuring triolein levels in TG Biosensor (Pundir and Aggarwal, 2017) under optimal operational conditions, by serum samples instead of triolein. Prior to analysis, all serum samples were stored at temperature of 4˚C.



2.12. Evaluation of the TG Biosensor

The assessment of the working electrode was conducted through the examination of its analytical performance, encompassing attributes such as linearity, limit of detection, analytical recovery, reproducibility, precision, and correlation coefficient. The impact of diverse potential interfering substances presents in blood samples, namely glucose, glutamic acid, ascorbic acid, and urea, was evaluated at concentrations corresponding to their physiological levels.

2.13. Reusability and Storage Stability of the TG Biosensor

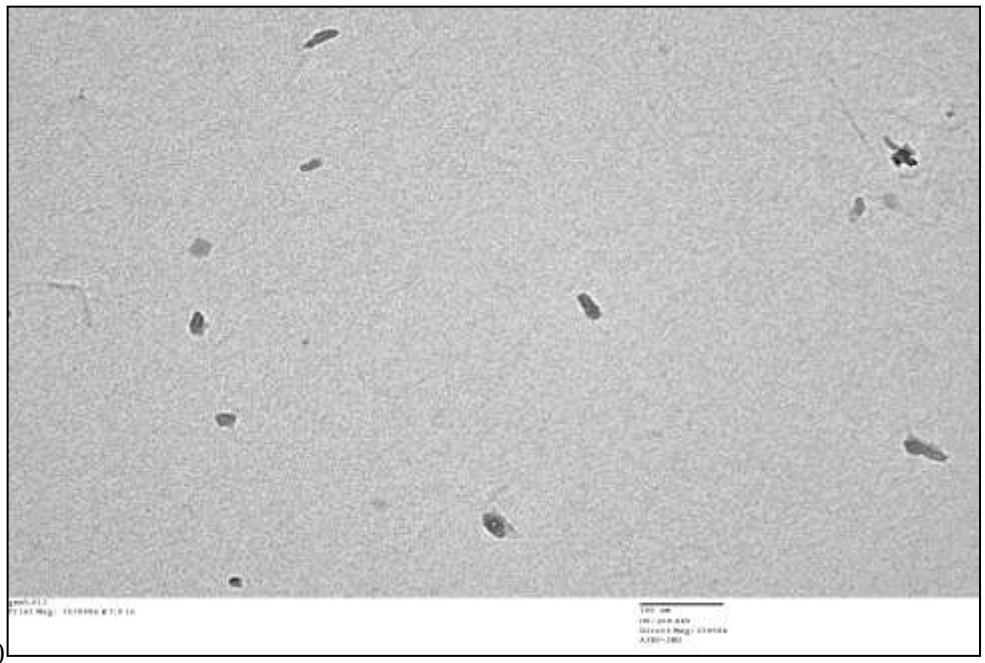
The investigation focused on assessing the reusability and storage stability of the biosensor over a duration of 240 days under regular usage conditions and while stored in a dry environment at a temperature of 4°C.

3. Results and Discussion

3.1. Preparation and Characterization of GrONPs

The synthesis of graphene oxide nanoparticles (GrONPs) was conducted through the utilization of Hammer's method. Transmission Electron Microscopy (TEM) analysis of GrONPs revealed that the size of GrONPs ranged from 5 to 100 nanometers (Fig. 1a), with an average size of 20 nm. Furthermore, examination of the UV and visible spectra of GrONPs exhibited an absorbance peak at 230 nm (Fig. 1b), similar to earlier report (Narwal and Pundir, 2016).

(a)



(b)

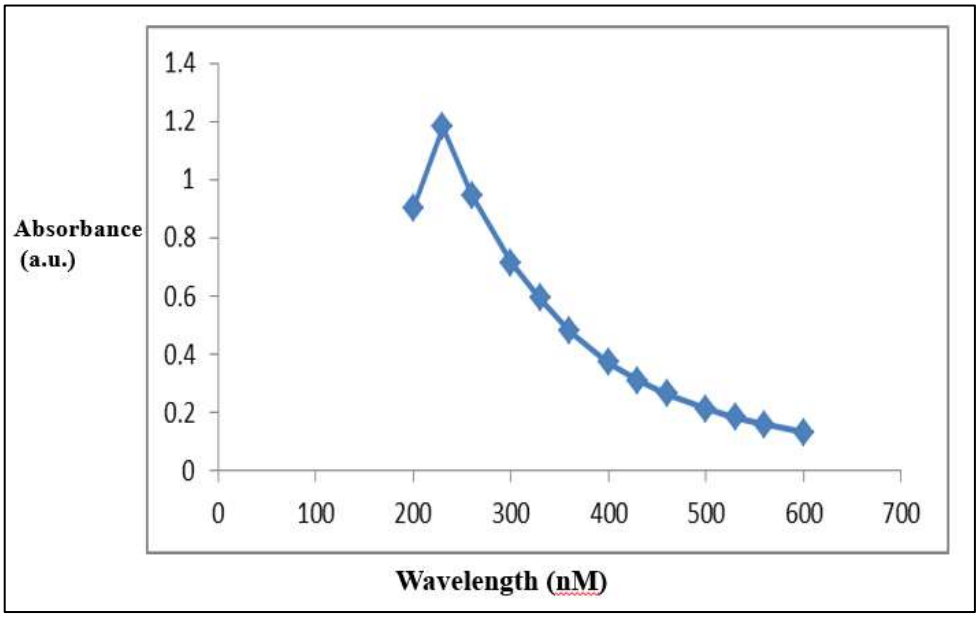


Fig. 1: (a) TEM image of graphene oxide nanoparticles (GrONPs) (**b)** UV-Visible spectra of GrONPs showing absorbance peak at 230 nM wavelength.

3.2. Electro Deposition of GrONPs on PGE and Covalent Co-Immobilization of Lipase NPs, GKNPs, GPONPs onto GrONPs Modified PG Electrode

The covalent coupling (CO-NH-) method was employed to co-immobilize lipase NPs, GKNPs, and GPONPs onto the surface of a GrONPs-modified PG electrode. This immobilization was achieved through the utilization of EDC-NHS chemistry.

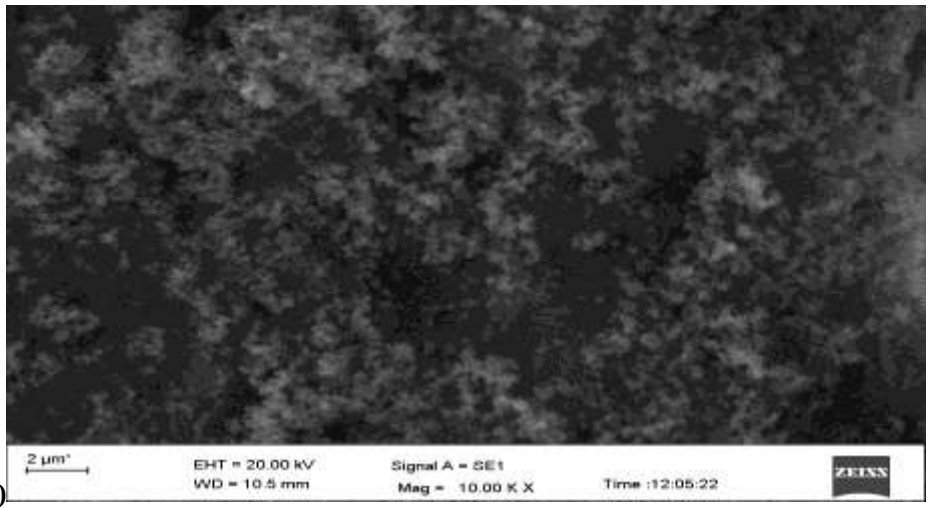
3.3. Characterization of Lipase NPs/GKNPs/GPONPs/GrONPs/PG Electrode

3.3.1. By scanning electron microscopy (SEM)

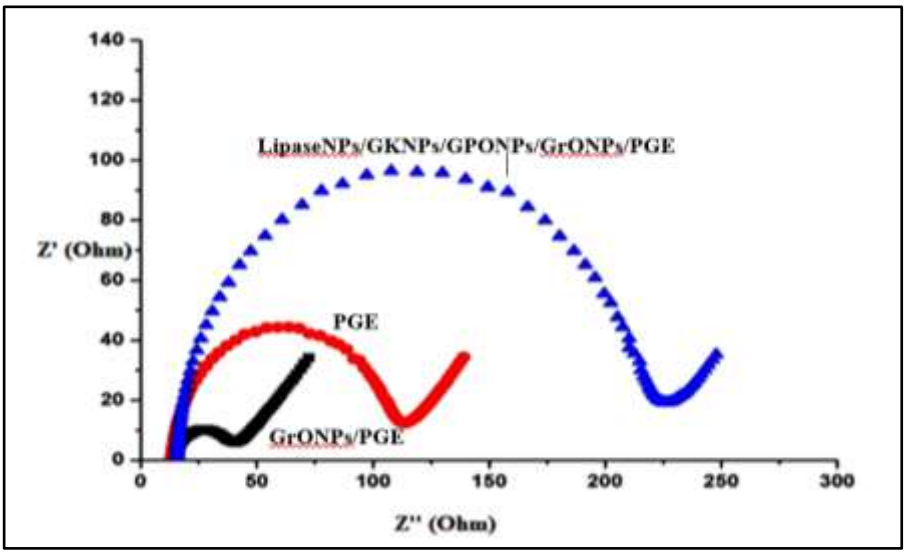
SEM images were acquired to examine the morphological characteristics of the uncoated PG electrode and the PGE modified with various ENPs (lipaseNPs, GKNPs, GPONPs) and GrONPs. Fig. 2a depicts the obtained images, which reveal a distinctive globular structural morphology. This particular morphology can be attributed to the presence of ENPs (lipase NPs, GKNPs, and GPONPs) that were successfully immobilized onto the GrONPs-coated PGE.

3.3.2. By electrochemical impedance spectroscopy (EIS)

The electrochemical impedance spectroscopy (EIS) was employed to investigate the changes in impedance of PGE before and after modification through the immobilization of ENPs and GPONPs. The EIS measurements revealed that the semicircle diameter at higher frequencies corresponded to the electron transfer resistance (RCT), which influenced the electron transfer kinetics of the redox probe at the electrode surface. Conversely, the linear component observed at lower frequencies indicated the Warburg diffusion process. The Nyquist plot in Fig. 2b illustrates the EIS results obtained for the bare PGE, GrONPs/PGE, and lipaseNPs/GrONPs/GKNPs/GPONPs/PGE immersed in a 5 mM solution of ($K_3Fe(CN)_6$ and $_/K_4Fe(CN)_6$). The RCT value for the bare PG electrode was measured as 120 ohms, which decreased to 40 ohms upon electrodeposition of GrONPs onto PGE due to their high electrical conductivity. However, upon co-immobilization of lipaseNPs, GKNPs, and GPONPs onto the GrONPs/PG electrode, the RCT value increased to 225 ohms owing to the high insulation capability due to presence of ENPs**.**



(a)



(b)

Fig. 2: (a) SEM images of bare PG electrode and ENPs/GrONPs/PGE (**b)** Electrochemical Impedance Spectra of bare PG electrode, GrONPs modified PG electrode and ENPs/GrONPs/PGE

3.4. Current Response of ENPs/GrONPs/PG Electrode

The unmodified PGE exhibited a considerably low current, in contrast to the ENPs (lipase NPs,GKNPs and GPONPS) and GrONPs coated PGE, which demonstrated well-defined oxidation and reduction currents at a potential of 0.3 V. Notably, the current exhibited a linear increase with the elevation of the scan rate from 20 mV/s to 40 mV/s, as shown in Fig. 3a. Therefore, all electrochemical investigations were conducted using an optimized scan rate of 40 mV/s.

Fig. 3b illustrates the cyclic voltammetry (CV) response of the unmodified PGE, the PGE modified with lipase NPs/GKNPs/GPONPs/GrONPs in the absence of substrate(triolein), and the PGE modified with lipase NPs/GKNPs/GPONPs/GrONPs in the presence of substrate (triolein). The results clearly indicate that the current response of ENPs and GrONPs coated PGE with substrate was significantly higher compared to the same modified electrode without substrate.

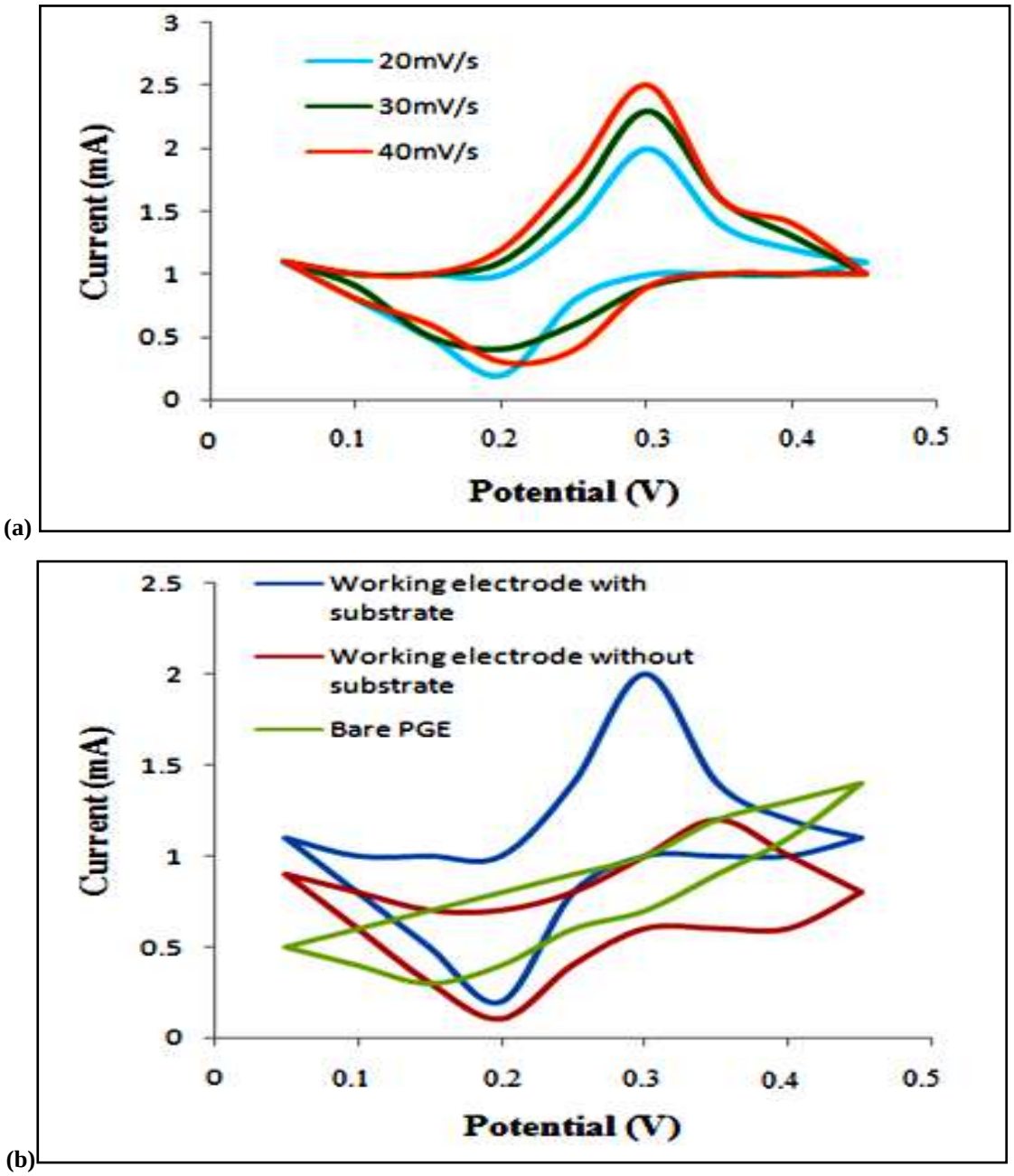


Fig. 3: (a) CVs of lipase NPS/GKNPs/GPONPs/GroNPS modified PG electrode at various scan rate ranging from 20-40 mV/s. (**b)** CVs response of bare PG, lipase NPs/GKNPs/GPONPs/GrONPs modified electrode with substrate.

3.5. Construction of TG Biosensor Using ENPs/GrONPs/PGE as Working Electrode

In the present study, we the constructed of enhaced/improved amperometric bio-sensor for TG detection employing GrONPs. The method involves the co-immobilization of ENPs (lipase NPs, GKNPs and GPONPs onto a GrONP decorated PGE. This technique is depicted in Scheme 1.

3.6. Optimization of TG Biosensor

The kinetic properties of co-immobilized engineered ENPs onto graphene oxide nanoparticles (GrONPs) modified PG electrode was investigated in this study. The optimal pH for the biosensor was found to be 7.5, as shown in Fig. 4a. This is quite close to physiological pH (7.3) and also falls within range of pH 6.5-9.0 of earlier amperometric TG biosensors (Batra *et al.*, 2016). Similarly, the optimal incubation temperature was determined to be 40 °C (Fig. 4b), which is within the range of 25-40°C commonly observed for earlier amperometric biosensors (Batra *et al.*, 2016). Therefore, subsequent experiments were conducted at pH 7.5 and 40 °C.

Furthermore, the biosensor exhibited a rapid response, reaching maximum current within 2 seconds (Fig. 4c). This response time is shorter compared to previously reported amperometric triglyceride (TG) biosensors, which typically ranged from 2.5 to 45 seconds (Pundir and Aggarwal, 2017; Narwal and Pundir, 2016; Solanki *et al.*, 2016; Narang *et al.*, 2013; Narang and Pundir, 2011; Phongphut *et al.*, 2013a and 300s by Shambhavi, *et al.*, 2024). Moreover, a linear correlation between the current response and triolein concentration was observed within the range of 0.01 mM to 60 mM (Fig. 4d).

3.7. Evaluation of Proposed TG Biosensor

The present study aimed to evaluate various performance parameters of the TG Biosensor, including linearity, limit of detection (LOD), analytical recovery, precision, and correlation. The LOD was determined using the following formula: LOD = 3.3 × SD/S, where SD represents the standard deviation in the current response and S corresponds to the slope of the calibration curve.

3.7.1. Linearity

A direct correlation was observed between the magnitude of current (mA) and the concentration of triolein within the range of 0.01 to 60 mM. This relationship exhibits superior performance compared to previously documented biosensors, with concentration ranges reported as 0.11-11.3 mM (Narang *et al.*, 2014), 0.225-1.10 mM (Narang, 2012), 0.56-2.83 mM, (Manoj *et al.*, 2020), 0.56-7.91 mM (Jalarand *et al.*, 2023), and 0.11-5.65 mM (Pundir and Aggarwal, 2017).

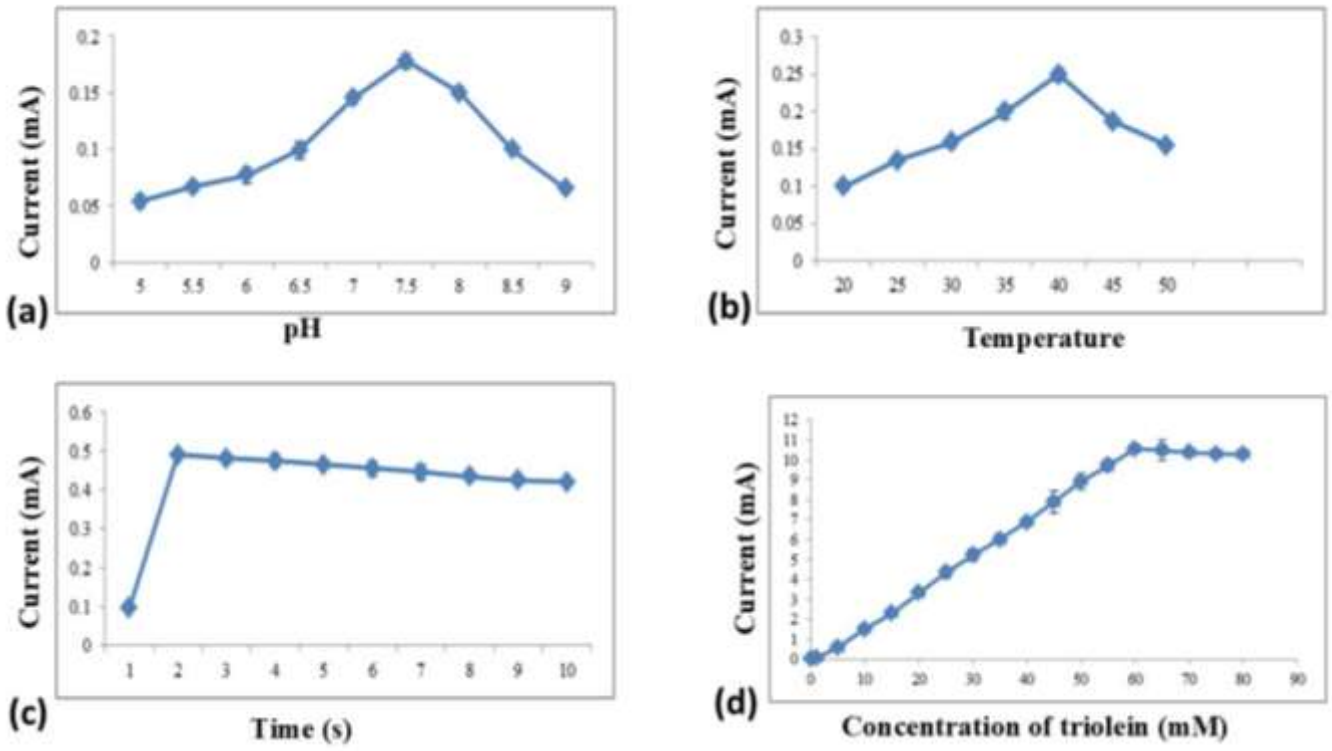


Fig. 4: (a) effect of pH on current response of lipaseNPs/GKNPs/GPONPs/GrONPs/PG electrode **(b)** Effect of incubation temperature on current response of lipaseNPs/GKNPs/GPONPs/GrONPs/PG electrode **(c)** Effect of incubation time on current response of lipaseNPs/GKNPs/GPONPs/GrONPs/PG electrode (**d)** Effect of triolein concentration on current response of triglycerides bionanosensor based on lipaseNPs/GKNPs/GPONPs/GrONPs/PG electrode

3.7.2. Limit of detection

The Biosensor currently employed in this study exhibited a detection limit [signal-to-noise ratio (S/N)] of 3, which was measured to be 0.2 nM. Notably, this detection limit surpasses the previously reported detection limits of various TG biosensors, such as 110 nM (Narang et.al.2014), 90 nM (Chauhan *et al*., 2013), 30 nM (Wu *et al*., 2014), 45.5 nM (Smart *et al*., 2023),280 nM(Chauhan *et al*., 2013), 1 nM (Pundir and Aggarwal, 2017), and 0.1-1.500 mM (Manoj *et al.*, 2020) and 0.53mM (Pliego-Sandoval *et al*., 2023).

3.7.3. Analytical recovery

The analytical recovery of triolein, when added to serum samples at concentrations of 40 and 80 mg/dl, was determined to be 98.02% and 98.04%, respectively. These results indicate the excellent reliability and accuracy of the biosensor employed in the study.

3.7.4. Precision

The triglyceride content of the sample was assessed to evaluate the repeatability and reliability of the methodology. The testing was performed on multiple occasions, including five times on a single day (within-batch) and five times after the storage of samples at -20°C for one week (between-batch). The results revealed a coefficient of variation (CV) of 0.048% for within-batch testing and 0.05% for between-batch testing. These findings highlight the exceptional reproducibility and repeatability of the Biosensor utilized in the study.

3.7.5. Correlation

Triglyceride levels in 15 serum samples obtained from hyperlipidemic patients were assessed utilizing state-of-the-art Biosensor technology (y) and contrasted with measurements obtained through the conventional enzymic colorimetric method (x) to investigate the precision of the latter. A robust relationship was observed, as indicated by the regression equation: y = 0.993x + 0.967, which exhibited a high level of association (R^2 = 0.999) (Fig. 5). The results obtained from this investigation provide compelling evidence regarding the exceptional accuracy of the currently available biosensor in quantifying triglyceride levels.

3.8. Determination of Serum Triglyceride

The serum triglyceride (TG) levels in a cohort of asymptomatic adults were evaluated using present biosensor. The results revealed that the TG levels ranged from 50 to 160 mg/dl in males and 40 to 140 mg/dl in females, which fell within the established normal range of 40-160 mg/dl. In individuals diagnosed with hyperlipidemia, the TG levels measured by the present biosensor, were found in the range from 210 to 550 mg/dl in males and 220 to 490 mg/dl in females.

3.9. Interference Study

The amperometric response was evaluated in the presence of various potential interferents, namely glucose, fructose, uric acid, urea, ascorbic acid, ethanol, cholesterol, citric acid, lactic acid, alanine, leucine, creatinine, pyruvate, and bilirubin, all existing at concentrations representative of physiological levels. The obtained results demonstrated that these metabolites exhibited negligible influence on the measured response.

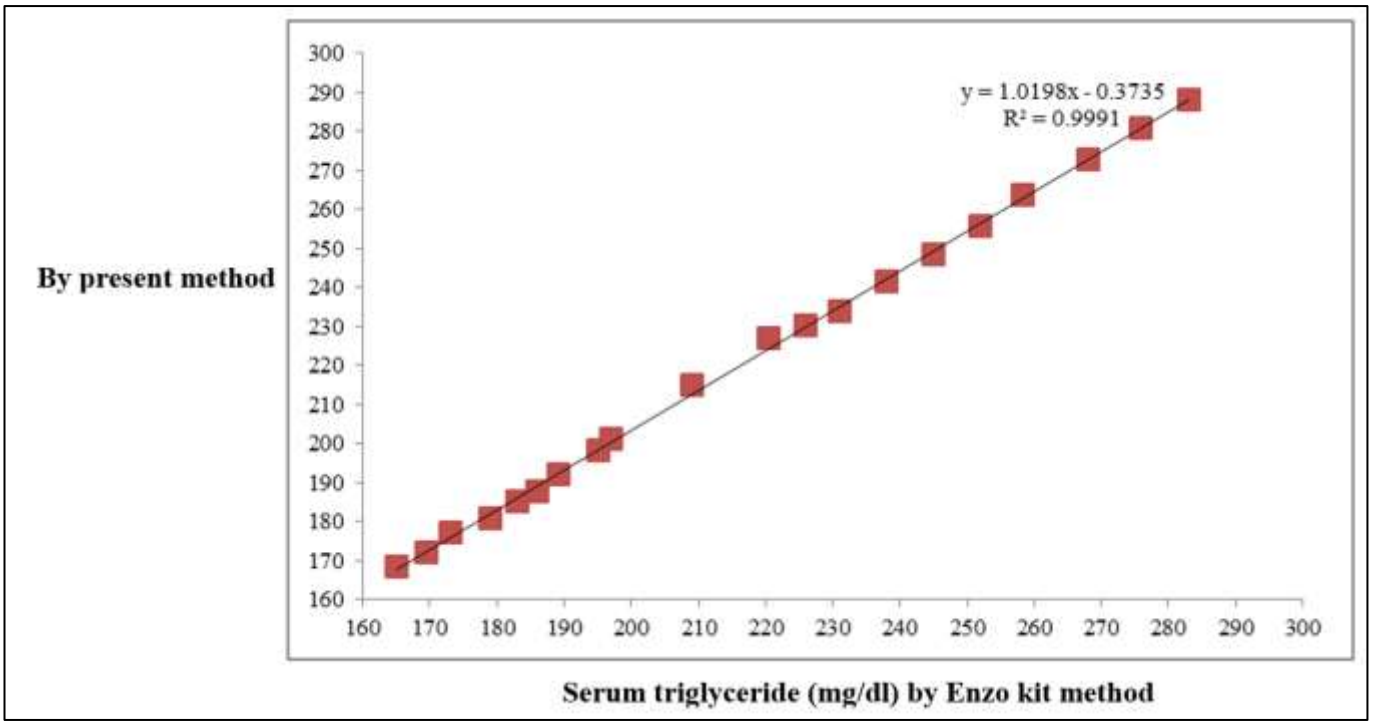


Fig. 5: Correlation between serum triglyceride values as measured by standard enzymic colorimetric method (x axis) and those measured by present bionanosensor based on Lipase NPs/GKNPs/GPONPs/GrONPs/ PG electrode

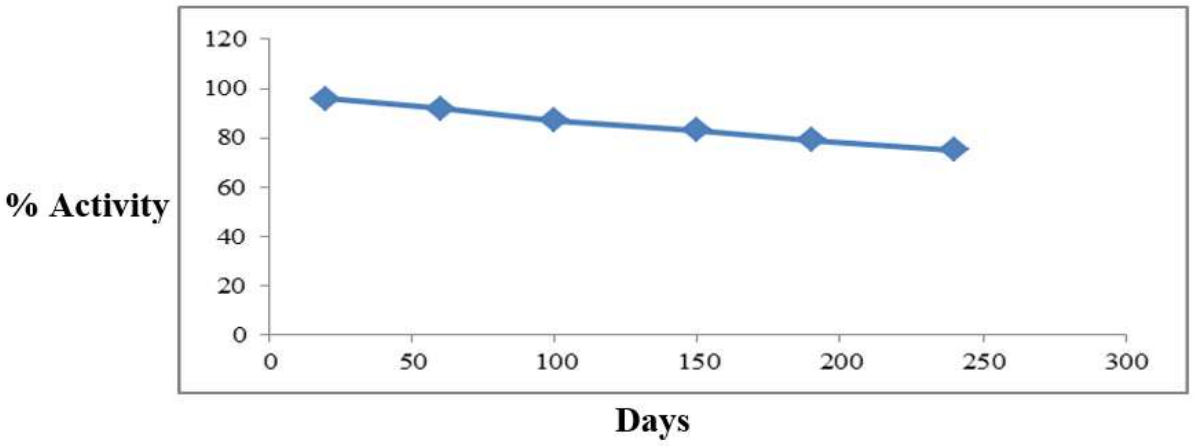


Fig. 6: Effect of storage stability on triglyceride bionanosensor based on lipaseNPs/GKNPs/GPONPs/GrONPs/PG electrode

3.10. Reusability and Storage Stability of ENPs/GrONPs/ PGE

To facilitate the reuse of the working electrode, it was subjected to a cleaning procedure inv olving immersion in a 0.1 M sodium phosphate buffer solution with a pH of 7.0. This cleaning process was repeated three to four times at room temperature. Following regular use for duration of 240 days, involving 180 instances of utilization, the working electrode exhibited a reduction in activity by only 25% (as shown in Fig. 6). The enzyme electrode, stored in a refrigerator at a temperature of 4 °C in a 0.1 M sodium phosphate buffer with a pH of 7.0, demonstrated superior storage stability and reusability compared to previously reported TG biosensors. The earlier biosensors exhibited storage durations of 77 days (Solanki *et al.*, 2016), 180 days (Narang *et al.*, 2013), 30 days (Phongphut *et al.*, 2013a), and 90 days (Pundir and Aggarwal, 2017).

4. Conclusion

An enhanced amperometric TG biosensor was developed through the co-immobilization of nanoparticles of lipase, GK, and GPO onto a graphene oxide nanoparticle (GrONPs) modified pencil graphite electrode (PGE). The constructed biosensor exhibited enhanced analytical characteristics, including improved sensitivity, lower limit of detection (LOD), a wider working range, excellent reproducibility, and superior storage stability. Based on these findings, it can be concluded that the utilization of GrONPs/PG electrode significantly enhances the analytical performance of the biosensor.

Acknowledgement

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Authors' Contributions

CSP: Conception, design, critical revision of MS for important intellectual content. **VN**: Conception and revision of MS. **PK**: Assistance in experimental work and drafting of MS and **Deepa:** Execution of experimental work, acquisition, analysis and interpretation of data.

Conflict of Interest

Authors have no conflict of interest for this MS.

Supplementary information

Not Applicable

Ethical Approval

In this study, residual human sera samples were obtained from the outpatient department (OPD) Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Science (Pt. BDS PGIMS) Rohtak, Haryana, India through a Memorandum of Understanding (MoU) between MDU Rohtak and PGIMS, Rohtak. Pt. BDS PGIMS Rohtak possesses its own ethical clearance from a competent ethical committee for the collection of biological samples, specifically for the diagnosis of various diseases and their treatment.

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