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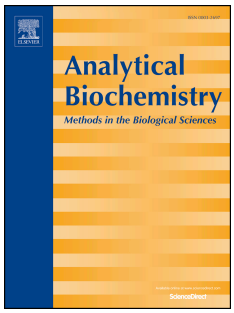

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Fabrication of glycerol biosensor based on co-immobilization of enzyme nanoparticles onto pencil graphite electrode

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Running Title: Enzyme nanoparticles based glycerol biosensor

Keywords: Glycerol; Glycerol kinase nanoparticles (GKNPs); Glycerol 3-phosphate oxidase nanoparticles (GPONPs); Glycerol biosensor; Pencil graphite electrode, Serum

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Fabrication of glycerol biosensor based on immobilization of enzyme nanoparticles nanocomposites onto pencil graphite electrode

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Abstract

Glycerol kinase (GK) and glycerol-3- phosphate oxidase (GPO) nanoparticles (NPs) were prepared, characterized and immobilized onto pencil graphite (PG) electrode to fabricate an improved amperometric glycerol biosensor (GKNPs/GPONPs/PGE). GKNPs/GPONPs/PGE worked in optimum conditions of pH 7.0, temperature 30^0C, at an applied potential of -0.3V. The biosensor exhibited wide linear response in a concentration range of glycerol (0.01-45mM) with detection limit 0.0001μM. The biosensor revealed high sensitivity (7.24μAm$M^{-1}cm^{-2}$), low response time (2.5s) and a good agreement with the standard enzymic colorimetric method with a correlation coefficient (R^2 = 0.99). The evaluation study of biosensor offered a good analytical recovery of 98.73% when glycerol concentration was added to the sera sample. In addition, within and between batches study of working electrode showed coefficients of variation as 0.105% and 0.14%, respectively. The application of biosensor is performed in the serum of apparently healthy subject and patients affected by cardiogenic shock. There was a 20% loss in initial activity of biosensor after its regular use over a time period of 180 days, while being stored at 4ºC.

Keywords: Glycerol; Glycerol kinase nanoparticles (GKNPs); Glycerol 3-phosphate oxidase nanoparticles (GPONPs); Glycerol biosensor; Pencil graphite electrode, serum

Introduction

Glycerol, also known as glycerin or 1,2,3-propanetriol embracing hydroxyl group on each of its carbon atom. The esterification of each hydroxyl group of glycerol in presence of fatty acids forms triacyl-glycerol or triglyceride(TG) [1]. It acts as a pioneer precursor during the synthesis of phospholipids (PL) and TG in the liver and adipose tissue, which constitutes to the storage fats of the body and is used as source of energy. [2]. Normal level of glycerol in human serum is 0.05-0.1mmol/L. The elevated glycerol level over 0.327 ± 0.190mmol/L in serum is considered as a biomarker for cardiac problems [3]. The determination of glycerol is essential in therapeutics, as its prognostic value is an important biomarker for cardiovascular diseases [4]. Various analytical methods are available for determination of glycerol, which includes gas chromatography, gas liquid chromatography, beside, spectrophotometric and enzymatic methods [5-10]. On the contrary, these methods have some drawbacks as requirement of skilled personnel, expensive instrument set up, tedious and prolonged protocols. Moreover, kits used in enzymatic analysis of glycerol are very costly and involve only one time use of enzymes, hence become costly for few sample analysis [11]. To overcome these drawbacks, biosensors and specifically amperometric biosensors have been designed for glycerol detection [12]. In earlier reported glycerol biosensors, the native enzymes were immobilized onto different nanocomposites electrodeposited on electrodes [13-15]. However, direct immobilization of native enzymes onto nanocomposites can cause their denaturation, prompting loss of their activity and stability [16-18]. The problem was overcome by aggregating and glutataraldehyde crosslinking the enzyme molecules into their nanoparticles (NPs) and then direct immobilization of their functionalized form onto the electrode [19]. The enzyme naopartcles (ENPs) showed their unique electronic, optical, mechanical, electrical, thermal and catalytic properties, beside increased surface area [20]. The use of enzyme nanoparticles (ENPs) in place of native enzymes in fabrication of enzyme electrodes has led the improved analytic performance of biosensors [21, 22]. Pencil graphite (PG) electrode possesses various fascinating characteristics for instance high conductivity, cost effectiveness, low signal to noise ratio and easy accessibility, which renders it a better option over electrodes such as Au, glassy-carbon and Pt electrodes [23]. Hence, we describe herein the fabrication of an ultrasensitive, rapid and cost effective glycerol biosensor based on immobilization of nanoparticles (NPs) of glycerol kinase (GK) and glycerol-3- phosphate oxidase (GPO) for detection of glycerol in cardiogenic

shocks patients. The use of ENPs without incorporating nanomaterials facilitates a new simple approach towards the biosensor designing for clinical applications.

Materials and Methods

Experimental Methods

Materials

Glycerol kinase from E.coli and glycerol-3-phosphate oxidase from Pediococcus sp. from Sigma- Aldrich,USA, glycerol, ascorbic acid, uric acid, glycine, glucose, silica gel from SRL, Mumbai, H_2SO_4 and HCl from Qualigens Fine Chemicals, Mumbai were used. 6B graphite pencil (Make: Camlin, India) with a graphite rod of 2mm diameter was bought from the local stationary market. The double distilled water (DW) (ohmic resistance: 1.8×10^{-5} for every ohm) was used throughout the study. The sera samples from apparently healthy people and persons suffering from cardiogenic shock were collected from Pandit Bhaghwat Dayal Sharma Post Graduate Institute of Medical Science, Rohtak hospital.

Instruments used

Potentiostat/Galvanostat (Make: Autolab, model: AUT83785, made by Eco Chemie, The Netherland) with NOVA 1.4 software was used. UV Spectrophotometer (Make: Shimadzu, Japan, Model 1700), Scanning electron microscopy (SEM) (Zeiss EV040, USA), Fourier Infra-red spectrometer (FTIR) (Thermo Scientific, USA), were utilized. The electrochemical impedance spectra (EIS) was recorded by FRA software in 5mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 25ml solution at -0.2 V between frequency range, 0.01 Hz–10 kHz.

Preparation of GKNPs and GPONPs

The GKNPs and GPONPs were synthesized separately using desolvation method [17]. To 2.0 ml dissolved GK/ GPO, 6.0 ml absolute ethanol was added drop wise at a rate of 0.1-0.2ml/min under constant stirring at a speed of 500rpm.It was followed by the addition of 1ml, 2.5% glutaraldehyde solution in the mixture under the identical stirring conditions at 4°C for 24h to ensure complete crosslinking of respective ENPs. The glutaraldehyde provided intermolecular crosslinking of enzyme molecules via Schiff base. The synthesized ENPs were thiol

functionalized by adding 0.2ml of cysteamine solution (0.02g/ml) to each suspension under constant stirring for 5-6h. ENPs were precipitated from mixture by their centrifugation at 1200×g for 10 min at 4°C. The process was followed by dispersion of ENPs in 2ml 0.1M phosphate buffer, pH 7.3 and sonication for 5 min. It is assumed that alpha-NH_2 group of cysteamine reacts with excess unreacted –CHO groups of glutaraldehyde cross-linked ENPs to form Schiff base. Thus, the glutaraldehyde cross-linked ENPs get functioned with –NH_2 groups. These functionalized ENPs were stored at 4°C, until use.

Characterization of GKNPs and GPONPs

The GKNPs and GPONPs were characterized by recording their images using transmission electron microscope (TEM), FTIR spectra (4000-500 cm^{-1}) and UV –visible spectra (200-600nm).

Preparation of GKNPs/GPONPs /PG electrode

The GKNPs and GPONPs modified PG electrode was prepared by physical adsorption of enzyme nanoparticles onto the surface of pencil graphite electrode. The bare PG electrode was pretreated with a potential range of -1.1 to 0.1V in solution of freshly prepared 0.2M H_2SO_4, until the cyclic voltammogram characteristics of the bare PG electrode was developed. The PG electrode was dipped into the GKNPs and GPONPs suspension at 4 °C for 24h to get physical adsorption of ENPs onto PG electrode. This immobilization could be possible by adsorption due to an electrostatic attraction between negatively charged -COOH groups of PG electrode and positively charged –NH_2 groups on the surface of ENPs, introduced by cysteamine di-hydrochloride. The GKNPs/GPONPs/PG electrode was washed with 0.1M of sodium phosphate buffer (SPB, pH 7.0) to remove extra molecules of ENPs. The working electrode was stored in the PB (pH 7.0) buffer at 4 °C, when not in use. The GKNPs/GPONPs/PG electrode was characterized using techniques SEM, FTIR and EIS before and after its physical adsorption with PNPs.

Fabrication of glycerol biosensor and its response measurements

An amperometric glycerol biosensor was fabricated using GKNPs/GPONPs/PG electrode as working electrode, Ag/AgCl as standard electrode and Pt wire as an auxiliary electrode

connected through galvanostat potentiostat. Figure 1 represents the schematic presentation of construction of working electrode and electro-chemical reactions involved in its response measurement. The working electrode was optimized and characterized using various cyclic voltammetry methods. All electrochemical procedures were performed in 0.1M phosphate buffer containing 0.1ml of 0.1mM glycerol and optimized concentration of ATP and $MgCl_2$ at an optimized scan rate. The current response was measured between 0s and 10s at an interval of 2.5s.

Optimization of glycerol biosensor

The kinetic properties such as ATP concentration, $MgCl_2$ concentration, applied potential, scan rate, pH, temperature and effect of glycerol concentration of GKNPs/GPONPs/PG electrode were studied electrochemically to optimize the operational conditions of the biosensor. The concentrations of ATP and $MgCl_2$ were varied from 0.1 to 4.0mM to get the meaningful response at optimum concentrations. The effect of applied potential was studied between -0.1 to 0.6V. Similarly, effect of scan rate was studied between 5mV/s to 20mV/s. To get the optimum pH, the pH was varied in the range pH 4.0 to 9.0 at an interval of pH 0.5 using the suitable buffers (strength- 0.1M) such as: pH 4.0 to 5.0 sodium acetate buffers, pH 5.5 to 7.5 sodium phosphate buffer for pH 8.0. Likewise to get optimum temperature and incubation time the master mix (buffers, substrate, ATP and $MgCl_2$) was incubated at different temperature (5–70ºC) and time duration (0-10s). The effect of glycerol concentration on biosensor response was determined by varying the concentration of glycerol in the range 0.1mM-45mM. The limit of detection (LOD) was calculated using the equation: LOD = 3xσ/slope, where σ=standard deviation

Evaluation of glycerol biosensor

The evaluation of the working electrode was done by analyzing its analytical performance in terms of analytical recovery, reproducibility, precision and correlation coefficient. The analytical recovery and coefficient of variation were calculated by the equations:

$$\%\ Recovery = \frac{Sample\ absorbance \times 100}{Standard\ absorbance}$$

$$Coefficient\ of\ variation = \frac{Standard\ deviation\ \times 100}{Mean\ of\ samples}$$

The effect of various interferants found in the blood such as glucose, glutamic acid, ascorbic acid and urea were measured at their physiological concentrations into the blood.

Application of glycerol biosensor

Blood samples (1 ml each) of apparently healthy people's and patients suffering from cardiogenic shocks in different age groups (n=20 each) were collected from local Pt. BDS Post Graduate Institute of Medical Sciences (PGIMS) Rohtak Hospital. The hospital has ethical clearance from competent ethical committee/board to collect samples from outdoor patients. The samples were centrifuged at 5000×g for 5 min to get their supernatants (sera) and kept at 4 ◦C until use. The quantity of glycerol in sera was measured by the present biosensor in the same fashion as elucidated above for its response measurement, under optimized operational conditions except that serum was used instead of glycerol. The current (mA) was recorded and the quantity of glycerol in serum was interposed from the standard curve between glycerol concentrations and current (in mA) under optimal operational conditions (Fig.2a).

Storage stability of enzyme electrode

The storage stability and long term stability of the biosensor were analyzed over a period of 180 days at its regular use, while being stored dry at 4°C.

Results

Characterization of GKNPs and GPONPs

The size of GKNPs and GPONPs as measured by TEM was in the range, 5 to 100nm (Fig. 3a), with an average size of 20nm. UV and visible spectra of GKNPs and GPONPs exhibited strong absorbance peak at 280nm (Fig. 3b).

Characterization of GKNPs/GPONPs/PG Electrode

By scanning electron microscopy (SEM)

Figure 3c showed the SEM images of bare PG electrode and GKNPs/GPONPs modified PG electrode. Bare electrode depicted the smooth and flat morphology, whereas GKNPs/GPONPs/PG electrode revealed globular structural morphology. The globular morphology was due to enzyme nanoparticles of GK and GPO, which were clearly adsorbed in pencil graphite electrode.

By electrochemical impedance spectroscopy (EIS)

The EIS provides the change in impedance of electrode before and after its modification with immobilization of GKNPs/GPONPs. The semicircle diameter at higher frequencies contributed to the electron transfer resistance (R_{CT}), which directed the electron transfer kinetics of the redox probe at the surface of electrode, while at lower frequencies linear part corresponded to Warburg diffusion process [21]. In Fig.3d, the Nyquist plot presented EIS of bare PG electrode and GKNPs/GPONPs/PG electrode in 5mM ($K_3Fe(CN)_6/K_4Fe(CN)_6$) respectively. The R_{CT} value for the GKNPs/GPONPs/PG electrode was obtained as 70Ω, which was higher compared to bare electrode (R_{CT}=50 Ω).

Voltammetric response of the GKNPs/GPONPs/PG electrode

The unmodified/ bare PG electrode had showed very low current, while on the contrary, a well defined oxidation and reduction current appeared for GKNPs/GPONPs decorated PG electrode at a potential of -0.3V (Fig. 4a). The current increases linearly with increase in scan rate from 5mV/s to 20 mV/s (Fig. 4b). At lower scan rate only oxidation current appeared, while at higher scan rate of 20mV/s both oxidation and reduction current appeared. Hence, all electrochemical studies were performed at an optimized scan rate of 20mV/s.

Current response measurement of GKNPs/GPONPs /PG electrode

The amperometric response of the GKNPs/GPONPs/PG electrode was analyzed as a function of glycerol concentration (0.1mM) by measuring CV at 20 mV/s scan rate in 0.1M PBS of pH 7.0 containing 5 mM [Fe(CN)6]3−/4− (the electrical conductivity of PBS = 25Ω). Glycerol biosensor was based on the principle of measurement of electrons, which were generated from H_2O_2 at a fixed potential. The GKNPs in the presence of ATP and $MgCl_2$ phosphorylated glycerol into glycerol- 3-phosphate, which was oxidized by GPONPs into dihydroxyacetone phosphate and H_2O_2. The electron i.e. current is generated from electro-oxidation of H_2O_2 under low potential, which is measured. The amperometric response of PG electrodes without and with a mixture of ENPs (with substrate and without substrate) was recorded. At a glycerol concentration of 10mM, the amperometric response of the PG electrode with native enzymes (GK/GPO) was 0.14 mA, while the same electrode with mixture of ENPs (GKNPs/GPONPs) was 1.75mA.

Optimization of glycerol biosensor

An amperometric biosensor was constructed by decorating GKNPs and/GPONPs onto PG electrode. The optimum concentration of ATP was determined by investigating the response of biosensor at different ATP concentrations when applied for 0.1mM glycerol. The maximum current was predicted at 1.0mM concentration of ATP (Fig. 4c). Thus, 1.0mM ATP concentration was kept for further work. Similarly, optimized $MgCl_2$ concentration was found 2.0mM $MgCl_2$ for ensuing work (Fig. 4d), which was also lower than GK/GPOx/Horse Radish Peroxidase (3.00 mM) biosensor [13] . The maximum current was observed within 2.5s (Fig.5c) at -0.3V against Ag/AgClFig. The biosensor expressed the optimum current at pH 7.0 in 0.1M sodium phosphate buffer (Fig.5a) and incubation temperature of 30ºC(Fig.5b). While at other lower and higher pH and temperature, there was gradual shift in the oxidation and reduction peaks with decline in the current range. Hence, the succeeding electrochemical studies for glycerol detection were performed in 0.1M sodium phosphate buffer of pH 7.0 and 30°C at optimal potential of −0.3V. The response of current for GKNPs/GPONPs increases linearly with increase in glycerol concentration. At high concentration of glycerol, a plateau current is perceived, depicting the feature of Michaelis–Menten kinetic process. The apparent Michaelis–Menten constant (Km), provided the evidence of enzyme substrate kinetics, which can be attained through the Lineweaver–Burk equation

$$\frac{I}{Iss} = \frac{I}{Imax} + \frac{Km}{ImaxC}$$

Here, Iss indicated steady-state current after addition of substrate, C represented the bulk concentration of the substrate, and Imax showed the maximum current sustained under saturated substrate conditions. The GKNPs/GPONPs/PG electrode exhibited a jmax of 7.76±0.012mA with app. Km of 0.1±0.01mM and showed high sensitivity of 7.24 $\mu A \cdot mM^{-1} \cdot cm^{-2}$at a potential of -0.3V. The high sensitivity of present biosensor was due to large surface area of enzyme nanoparticles, which lead to increase in interaction of glycerol molecules found in the biological samples.

Evaluation of glycerol biosensor

The biosensor offered a wide linear response between current (mA) and glycerol concentration ranges of 0.01mM-45mM, under optimized operational conditions (Fig. 2b). The limit of detection limit (LOD) of the present biosensor was 0.0001μM. The analytical recoveries of

added glycerol in sera at concentration of 0.5mM and 1.0mM were 96.01 and 98.73% respectively (Table 1). In addition, within and between batch coefficients of variation for working electrode were detected as 0.105% and 0.14%, respectively (Table 2).

Correlation of glycerol biosensor

The glycerol level detected by the present biosensor was also compared with the standard enzymic colorimetric kit method. A good correlation coefficient (R^2 = 0.99) was obtained between the present method and standard enzymic colorimetric kit method (Fig. 6a).

Application of glycerol biosensor

The quantity of sera glycerol in apparently healthy persons as detected by the current biosensor was in the range 0.05±0.01-0.1±0.02 (n=20)mM, which was in normal established range. The sera glycerol level in persons suffering from cardiogenic shocks was in the range 0.5±0.02-12±0.02 mM (n=20), which was significantly higher than the levels in apparently healthy persons ($p > 0.001$) (Table 3).

Interference study of glycerol biosensor

Interfering components like glucose, glutamic acid, uric acid, ascorbic acid and urea showed only 3.8%, 4.1%, 3.4%, 4.6% and 2.9% interference, respectively on the present glycerol biosensor response, at their physiological concentrations, under the standard assay conditions. The interference less than 5%, was considered as practically no interference on the present biosensor.

Storage stability of the GKNPs/GPONPs/PG electrode

The storage stability of working electrode was investigated by measuring the response of glycerol concentration (0.01mM) in 0.1 M PBS for 6 weeks (Fig. 6b). The biosensor showed no change in redox peaks, current and potential during 4 weeks, but in last 2 weeks it showed a drop in the value of the current without any change in redox peaks and potential. On that account, biosensor diminished 20% of its initial activity during 6 weeks of its regular uses, while being stored at 4°C. The biosensor fabrication reproducibility was checked for three electrodes by determining response of 0.01mM glycerol by cyclic voltammetry. The relative standard deviation (RSD) for the current response was 2.35%.

Discussion

In the present work, we prepared NPs of glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO), characterized and co-immobilized them onto PG electrode to fabricate an improved amperometric biosensor for determination of glycerol. The average size of GKNPs and GPONPs (20nm, range 5-100nm) (Fig. 3a) revealed the enhanced surface area of enzymes as compared to the native GK and GPO molecules. A strong absorbance peak of GKNPs and GPONPs at 280 nm (Fig. 3b), showed a high degree of absorption of aromatic amino acids of peptide chain/free enzyme following their NPs formation. The change of smooth and featureless morphology of PG electrode to globular structure after immobilization of GKNPs and GPONPs as seen in SEM images (Fig. 3c) was due to adsorption of GKNPs/GPONPs onto PG electrode. The porous electrode revealed larger and effective surface area after absorption of GKNPs/GPONPs. The increased R_{CT} value of GKNPs/GPONPs/PG electrode was due to high surface area and partial insulation of electrode due to enzyme nanoparticles. This increase in R_{CT} could be assigned to the reason that most biological molecules, including enzyme nanoparticles, have poor electrical conductivity at low frequencies (<10 kHz; applied voltage: -0.3V) and create obstacle to the transfer of electrons. However, GKNPs/GPONPs offer high surface to volume ratio and increase the electrocatalytic property of electrode for electro-oxidation of glycerol. At 10mM glycerol concentration, the current response of the GKNPs/GPONPs/PG electrode was 1.75mA, which is higher than that with native enzymes (GK/GPO) (0.14mA), which revealed that the hybrid ENPs had an enormous effect towards the analytical response of working electrode. The highest current of present biosensor was observed at 1.0mM ATP, which is lesser concentration than that of previously reported biosensor GK/GPOx/Horse Radish Peroxidase (1.5mM) [13]. Similarly for $MgCl_2$, the biosensor required 2.0mM concentration for optimum response,(Fig. 4d), which is also lower than GK/GPOx/Horse Radish Peroxidase (3.00mM) biosensor [13]. The maximum current was observed within 2.5s at -0.3V against Ag/AgCl, which is better than earlier biosensors (0.1V, 1.3V and 0.3V [13-15]. The app. Km of GKNPs/GPONPs/PG electrode for glycerol was 0.1±0.01mM. The small Km value means that the immobilized ENPs at PG electrode possess a high affinity to glycerol. The wide working range of the biosensor 0.01mM-45mM, under optimum conditions (Fig. 2b),is better than earlier biosensors(1μM-20μM, 1 to 25mM and 0.05 to 25.6mM [13-15].The LOD of the present biosensor (0.0001μM), was also better than earlier biosensors(0.4μM, 0.005mM and 0.05mM [13-15].The high recoveries of added glycerol in sera(96.01- 98.73%) (Table 1) demonstrated the reliability of the present

biosensor. Very low values of within and between batches coefficient of variation for the added glycerol in sera measured by the present biosensor was 0.105% and 0.14%, respectively (Table 2). The excellent reproducibility exhibited by the biosensor is due to electrostatic interaction of GKNPs/GPONPs onto PG electrode. Furthermore, the working electrode displayed a correlation coefficient of R^2 = 0.99 between sera glycerol quantity detected with the standard enzymic colorimetric method and present biosensor method (Fig.6a), revealed the high accuracy of the present biosensor. The biosensor had good anti-interferent ability, which was owing to better operational conditions for instance low potential (-0.3V) and higher current. The interfering components lost their activity at such a low potential and current, which evidenced the high specificity of present biosensor. A <20% decrease in the activity of GKNPs/GPONPs/PG electrode at its everyday use during a period of 6 weeks , while being stored dry at 4°C, which showed higher storage stability of the enzyme electrode over earlier reported electrodes. It was due to the uniform immobilization of ENPs onto the PGE. Furthermore, graphite present in PGE could offer a biocompatible environment, which inhibits the denaturation of enzyme nanoparticles.

Conclusions

In the present study, the use of enzyme nanaoparticles (ENPs) has led to the better electroanalytical properties and improved analytical performance of glycerol biosensor as compared to those employing native enzymes. ENPs exhibited high surface to volume ratio, which allow a large electroactive surface of PGE. The large electroactive surface area of GKNPs/GPONPs/PGE has contributed in improved analytical performance of biosensor in terms of rapid response rate (2.5s), low detection limit (0.0001μM), wide linear response (0.01mM to 45mM) and prolonged storage stability (180 days). The improved analytical performance of biosensor can be accredited to the large surface area, good biocompatibility and high electrical conductivity of PGE. PGE allows the uniform immobilization of ENPs, retain their bioactivity and increases the direct electron kinetics from H_2O_2 [25]. Accordingly, enzyme nanoparticles modified PGE could be used to improve analytical performance of other biosensors also. The future research could be focused on the development microfluidic paper based glycrerol biosensing device, which can be used at bedside of the patients for real time monitoring of glycerol [26-27].

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Conflict of Interest: Authors have no conflict of interest.

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List of Figures

Figure: 1 Schematic representation of fabrication of GKNPs/ GPONPs/ PG electrode and electro-chemical reactions involved in its response measurement.

Figure: 2 (a) Standard curve of glycerol by glycerol biosensor based on GKNPs/GPONPs /PG electrode. (**b)** Effect of glycerol concentrations on current response of GKNPs/GPONPs/PG electrode. All readings are mean of five observations (n=5).

Figure: 3 (a) Transmission electron microscopic (TEM) images of GKNPs, and GPONPs. (**b)** UV absorbance of GKNPs and GPONPs. (**c)** Scanning electron microscopic (SEM) images of bare PG electrode and GKNPs/GPONPs /PG electrode. (**d)** Electrochemical Impedance Spectra (EIS) of a) bare electrode b) GPONPs /PG electrode.

Figure: 4 (a) Effect of applied potential on GKNPs/GPONPs /PG electrode containing, 1mM ATP, 2mM $MgCl_2$ and 0.1 mM glycerol. (**b)** Effect of scan rate on GKNPs/GPONPs /PG electrode containing, 1mM ATP, 2mM $MgCl_2$ and 0.1 mM glycerol. (**c)** Cyclic voltammetry of bare PGE, GKNPs/GPONPs/ PG electrode with or without substrate. (**d)** Effect of the ATP concentration in the buffer solution (containing 2mM $MgCl_2$) on the amperometric signal of 0.1 mM glycerol at GKNPs/GPONPs /PG electrode. **(e)** Effect of $MgCl_2$ concentration in the buffer solution (containing 1mM ATP) on the amperometric signal of 0.1 mM glycerol at GKNPs/GPONPs /PG electrode.

Figure:5 (a) Effect of pH on GKNPs/GPONPs /PG electrode containing, 1mM ATP, 2mM $MgCl_2$ and 0.1 mM glycerol. **(b)** Effect of temperature on GKNPs/GPONPs /PG electrode containing, 1mM ATP, 2mM $MgCl_2$ and 0.1 mM glycerol. (**c)** Effect of incubation time on GKNPs/GPONPs /PG electrode containing, 1mM ATP, 2mM $MgCl_2$ and 0.1 mM glycerol.

Figure: 6 (a) Correlation between serum glycerol level measured by enzo kit method (x axis) and the current method (y axis) employing the glycerol biosensor based on GKNPs/GPONPs /PG electrode. **(b**) Storage stability of GKNPs/GPONPs /PG biosensor at 4°C.

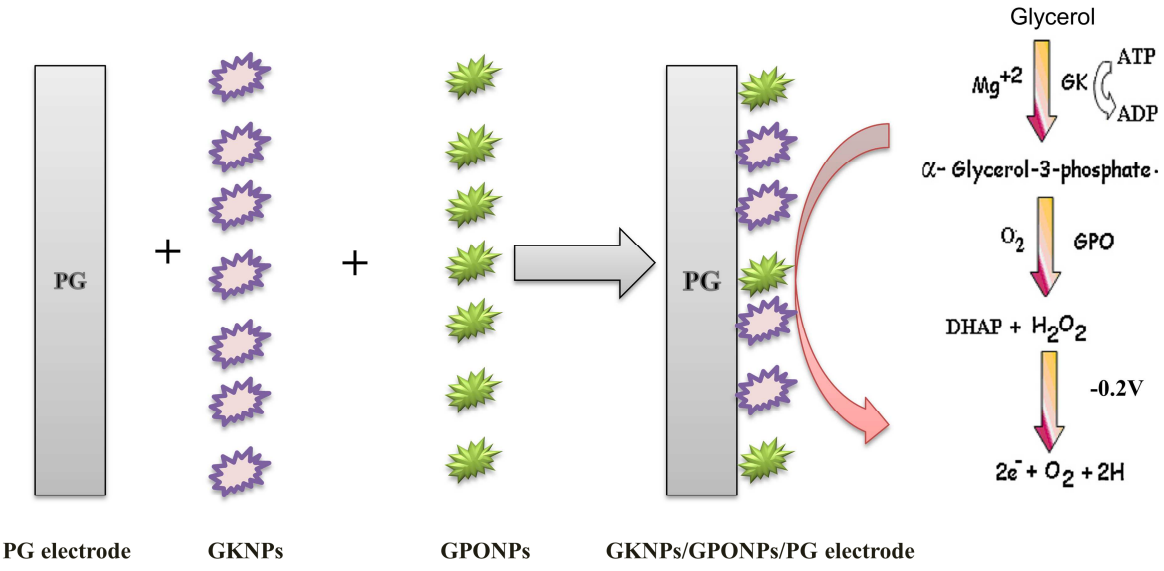

Fig. 1

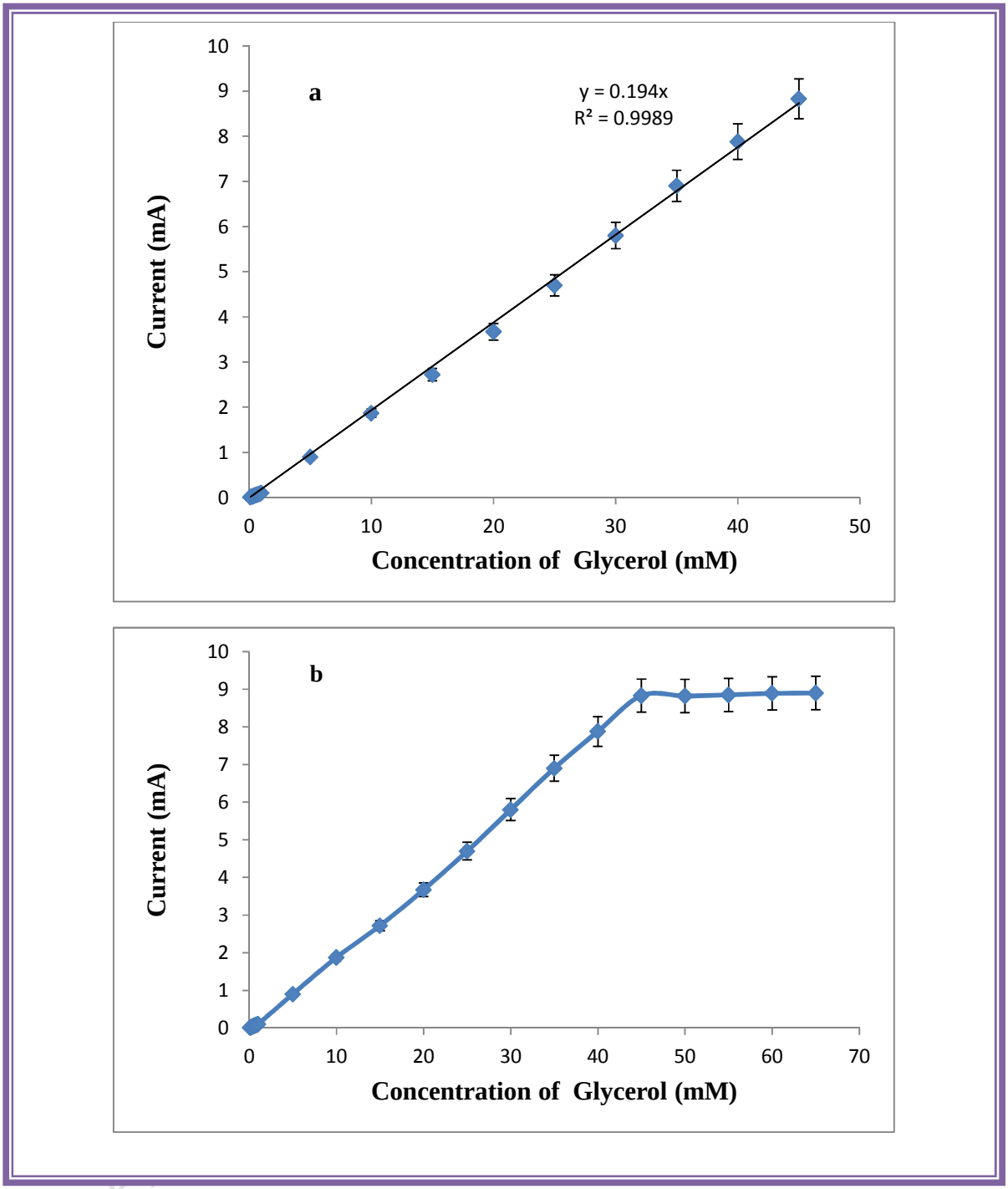
a
y = 0.194x
R² = 0.9989
Current (mA)
Concentration of Glycerol (mM)
b
Current (mA)
Concentration of Glycerol (mM)

Fig. 2 a and b

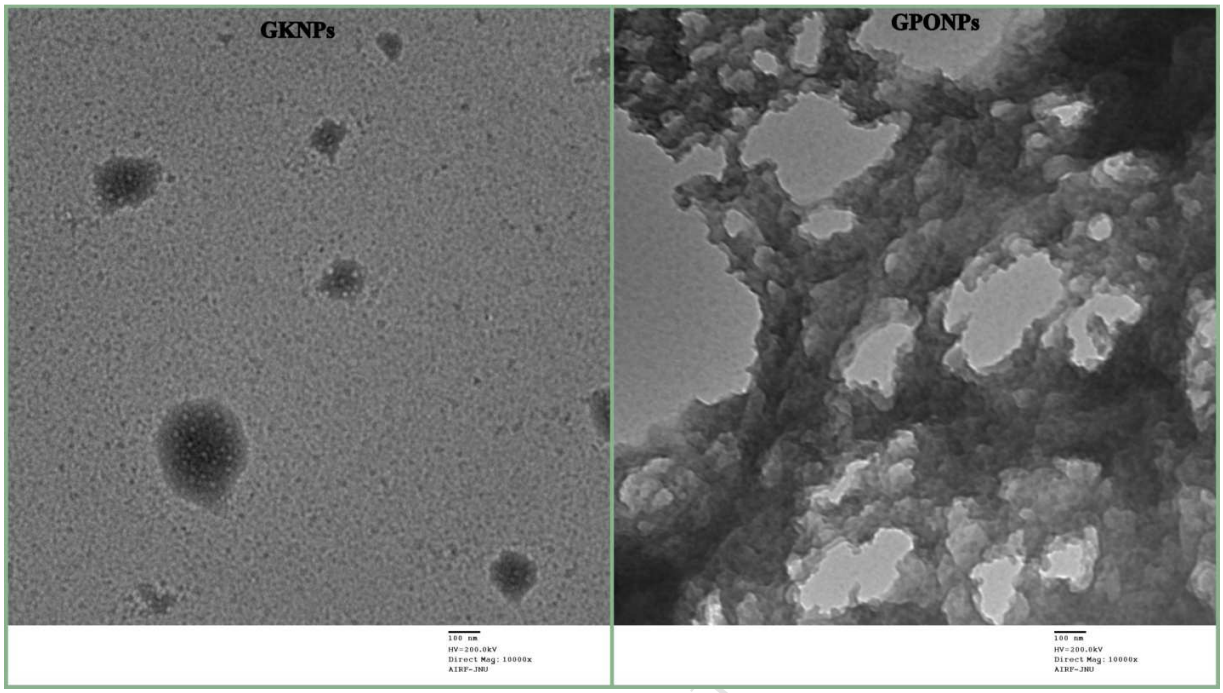

Fig. 3a

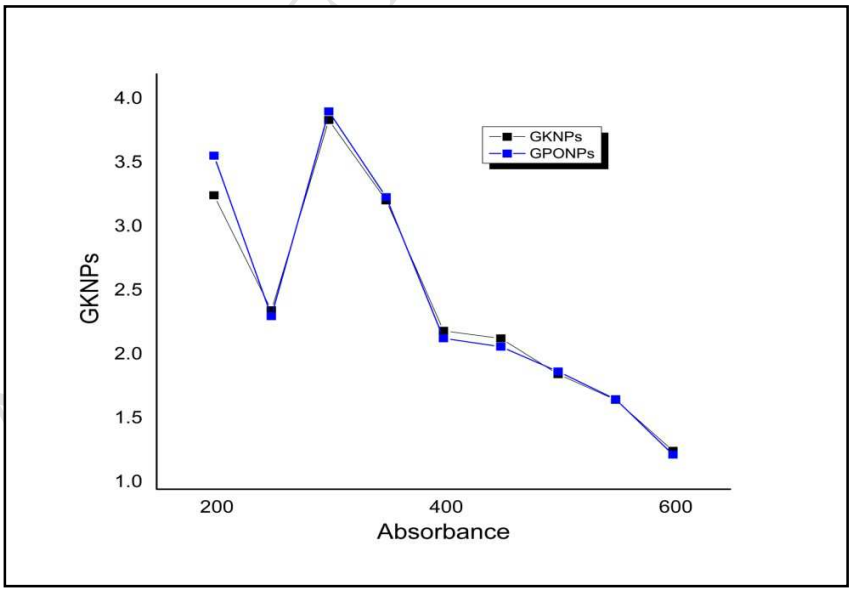

Figure 3b

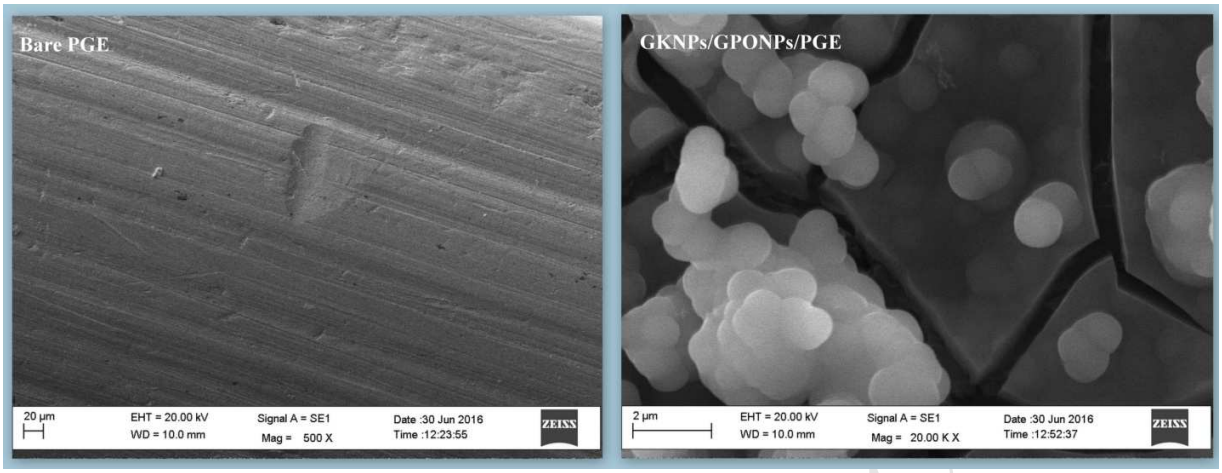
Bare PGE
20 µm
EHT = 20.00 kV
WD = 10.0 mm
Signal A = SE1
Mag = 500 X
Date :30 Jun 2016
Time :12:23:55
ZEISS
GKNPs/GPONPs/PGE
2 µm
EHT = 20.00 kV
WD = 10.0 mm
Signal A = SE1
Mag = 20.00 K X
Date :30 Jun 2016
Time :12:52:37
ZEISS

Fig.3c

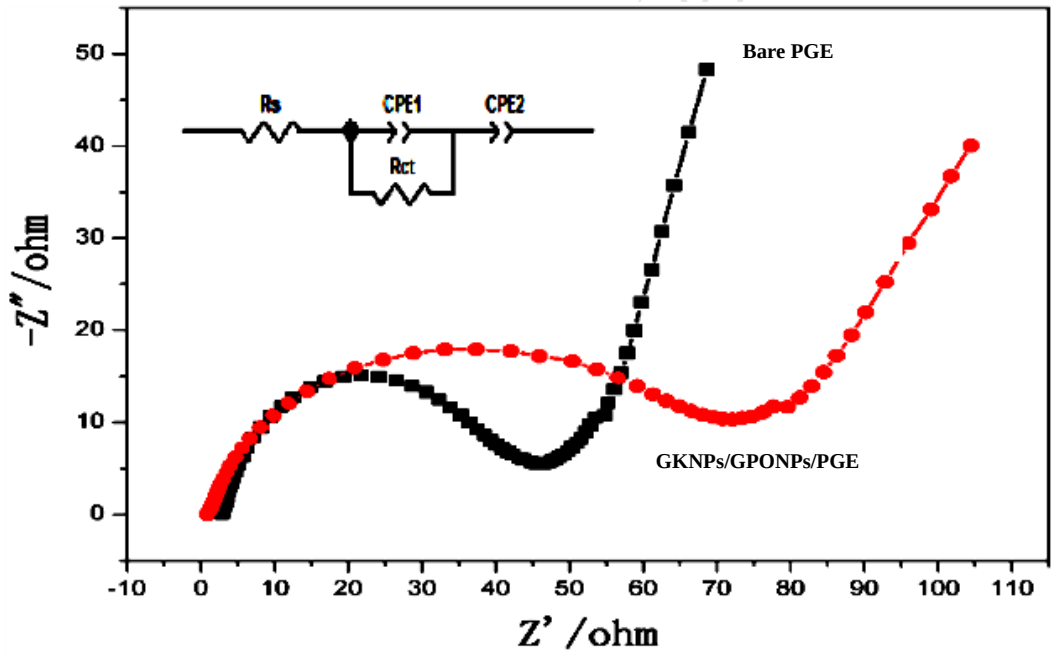
Rs
CPE1
CPE2
Rct
Bare PGE
GKNPs/GPONPs/PGE
-Z'' /ohm
Z' /ohm
50
40
30
20
10
0
-10
0
10
20
30
40
50
60
70
80
90
100
110

Fig. 3d

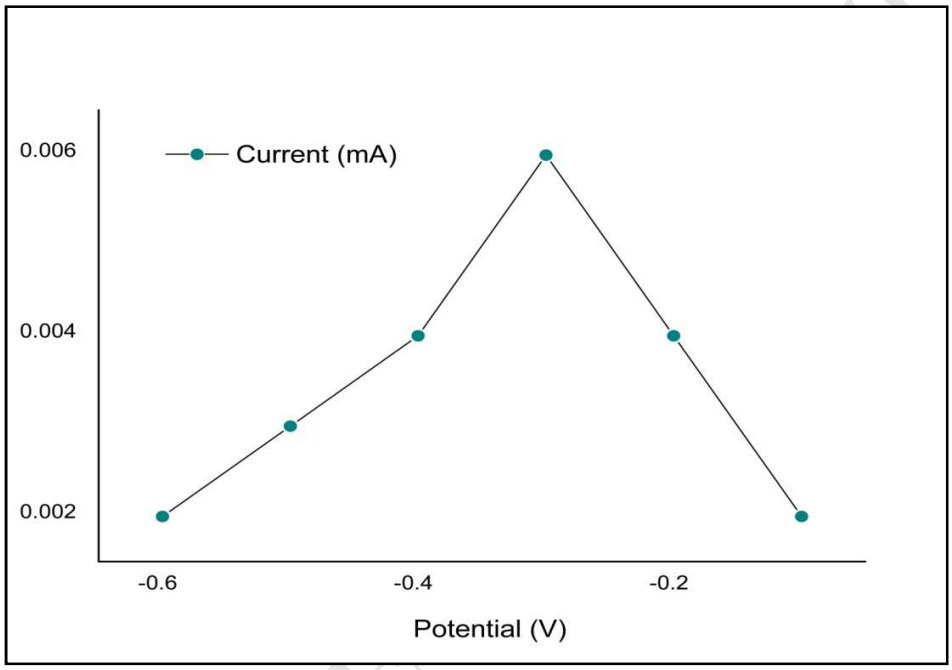

Fig. 4a

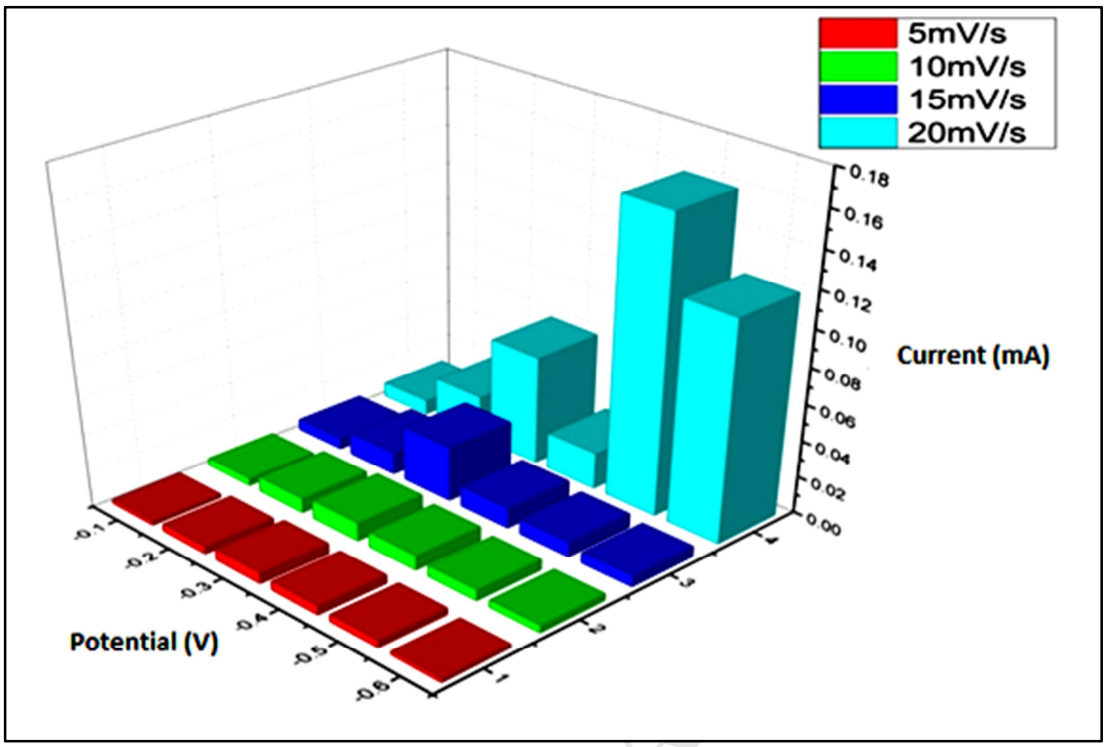
5mV/s
10mV/s
15mV/s
20mV/s
Current (mA)
Potential (V)

Fig. 4b

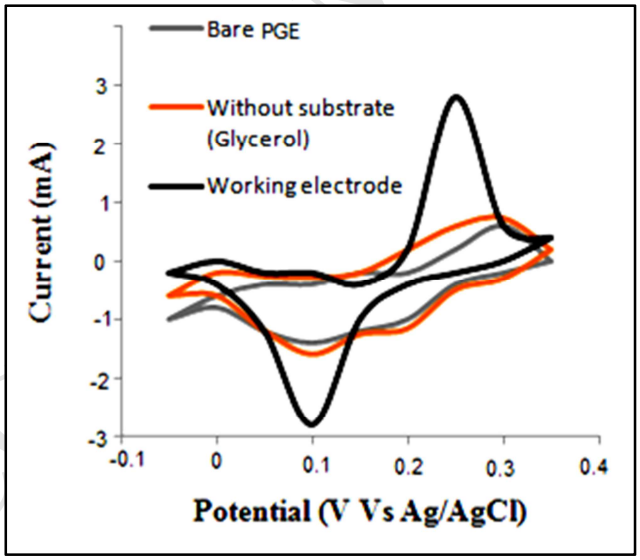
Bare PGE
Without substrate
(Glycerol)
Working electrode
Current (mA)
Potential (V Vs Ag/AgCl)

Fig. 4c

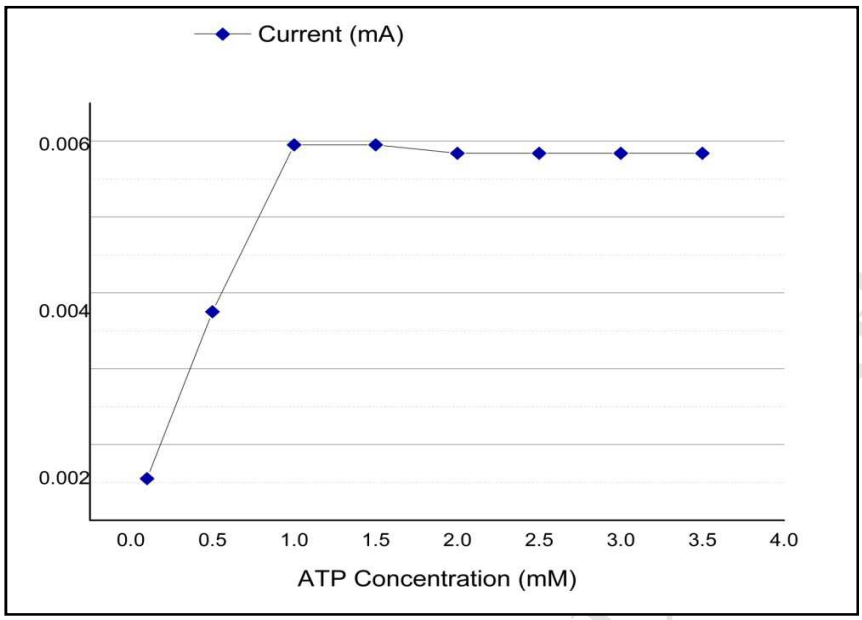
Current (mA)
0.006
0.004
0.002
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
ATP Concentration (mM)

Fig.4d

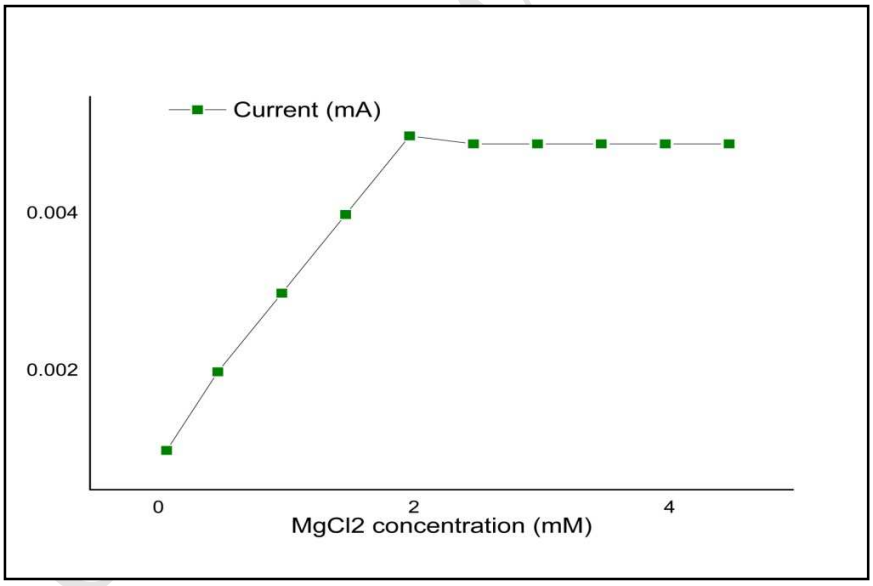
Current (mA)
0.004
0.002
0
2
4
MgCl2 concentration (mM)

Fig. 4e

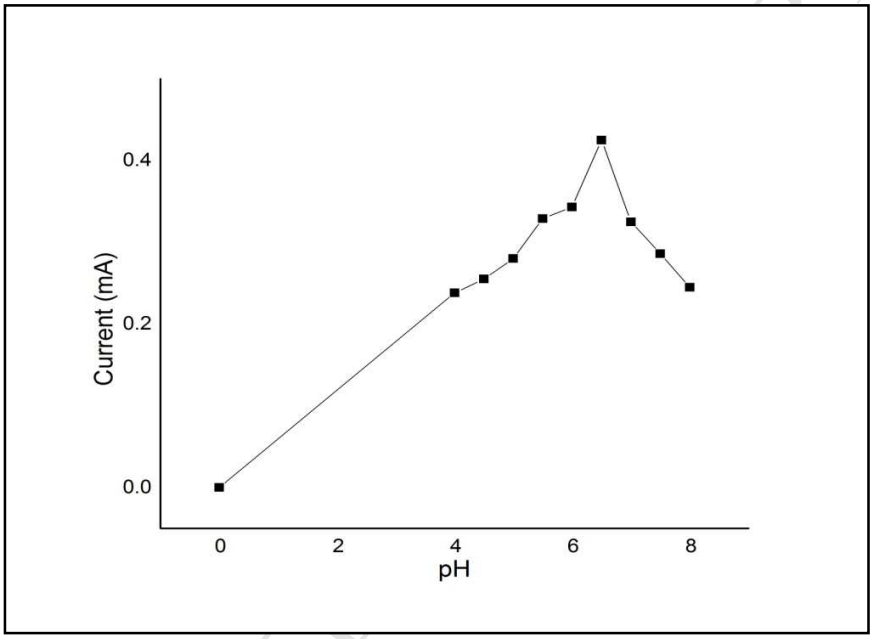

Fig. 5a

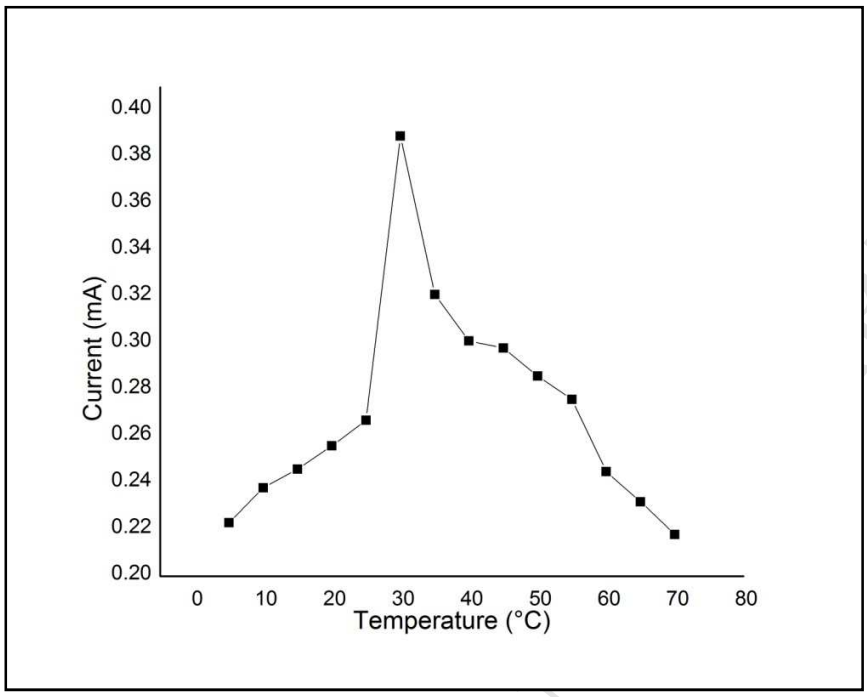
Current (mA)
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0
10
20
30
40
50
60
70
80
Temperature (°C)

Fig. 5b

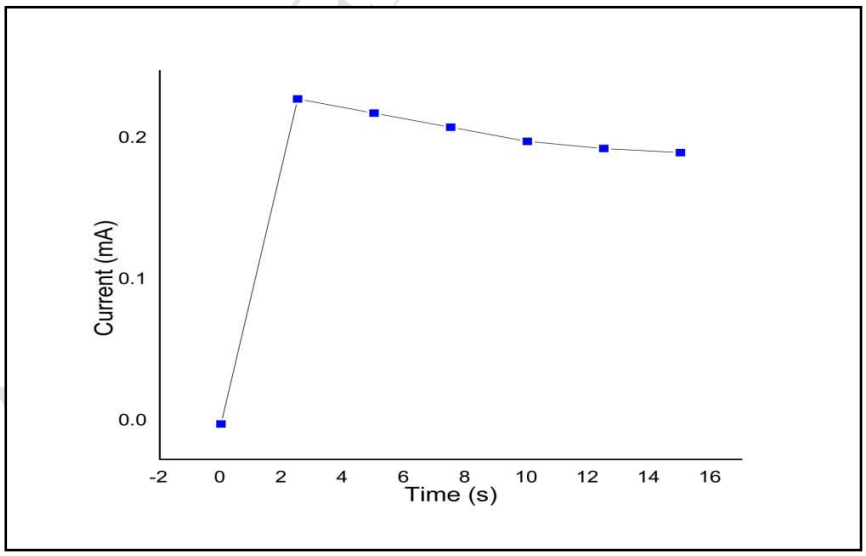
Current (mA)
0.2
0.1
0.0
-2
0
2
4
6
8
10
12
14
16
Time (s)

Fig. 5c

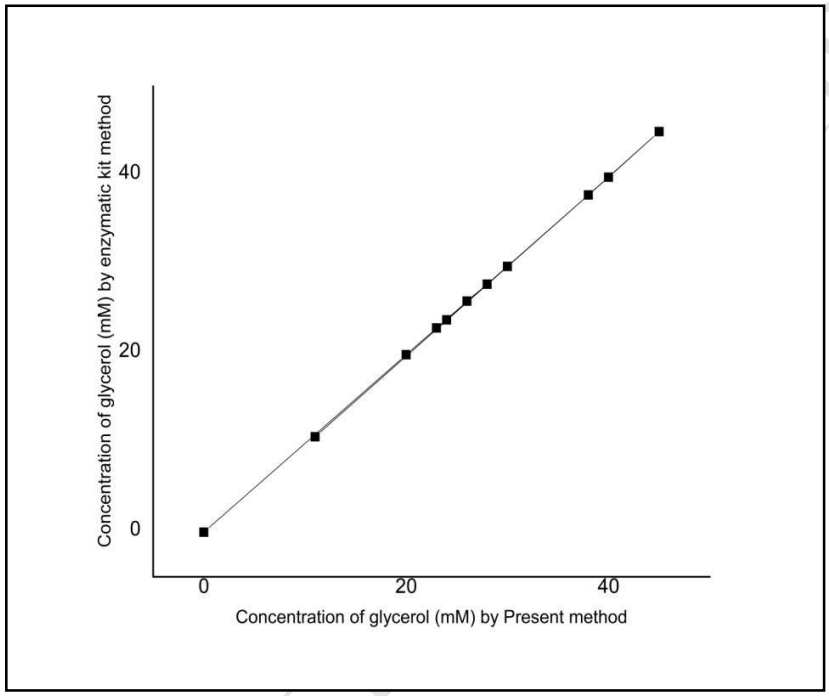
Concentration of glycerol (mM) by enzymatic kit method
0
20
40
0
20
40
Concentration of glycerol (mM) by Present method

Fig. 6a

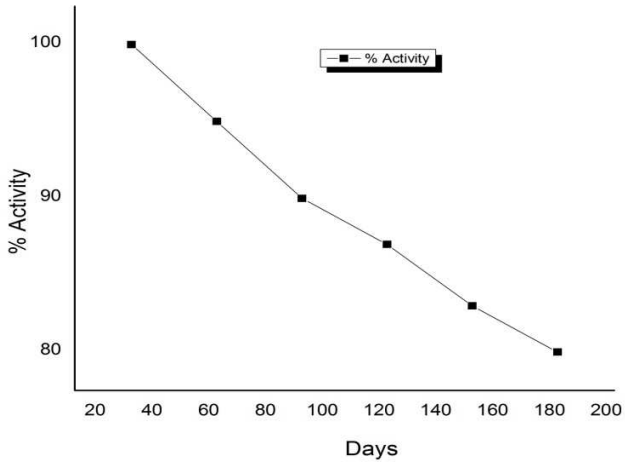
% Activity
100
90
80
% Activity
20
40
60
80
100
120
140
160
180
200
Days

Fig. 6b

Highlights

- Constructed an improved amperometric glycerol biosensor based on co-immobilization of glycerol kinase NPs, glycerol-3-phosphate oxidase NPs onto pencil graphite (PG) electrode.
- Biosensor showed optimum current within 2.5 s, pH 7.0 and temp 30ºC.
- The limit of detection and working range of biosensor were 0.0001 μM and 0.01-45mM respectively.
- The enzyme electrode lost 20% of its initial activity following 180 days of its normal uses, when stored at 4^{o}C.
- Biosensor measured TG level in sera of apparently healthy adults and persons experiencing cardiogenic shocks.