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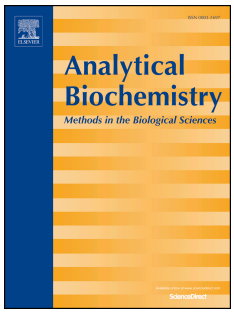

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C.S. Pundir, V. Aggarwal

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Amperometric triglyceride bionanosensor based on nanoparticles of lipase, glycerol kinase, glycerol-3-phosphate oxidase

C.S.Pundir*, V. Aggarwal

Department of Biochemistry, M.D. University, Rohtak-124001, Haryana, India

Running Title: Enzyme nanoparticles based triglyceride biosensor

Keywords**:** Triglyceride; Lipase nanoparticles; Glycerol kinase nanoparticles ; Glycerol phosphate oxidase nanoparticles ; Bionanosensor; Au electrode, Serum

*Corresponding author; Email:chandraspundir@gmail.com; Phone: +91-9416492413

A B S T R A C T

The nanoparticles (NPs) aggregates of lipase from porcine pancreas, glycerol kinase(GK) from Cellulomonas sp. and glycerol-3-phosphate oxidase(GPO) from Aerococcus viridanss were prepared by desolvation and glutaraldehyde crosslinking and functionalized by cysteamine. These enzyme nanoparticles (ENPs) were characterized by transmission electron microscopy (TEM) and Fourier transform infra red (FTIR) spectroscopy. The functionalzed ENPs aggregates were co-immobilized covalently onto polycrystalline Au electrode through thiolated bond. An improved amperometric triglyceride (TG) bionanosensor was constructed using this ENPs modified Au electrode as working electrode. Biosensor showed optimum current at 1.2V within 5s, at pH 6.5 and 35^0C .A linear relationship was obtained between current (mA) and triolein concentration in lower concentration range,10-100mg/dL and higher concentration range,100-500mg/dL. Limit of detection(LOD) of bionanosensor was 1.0 µg/ml . Percent analytical recovery of added trolein (50 and 100mg/dL) in serum was 95.2±0.5 and 96.0±0.17. Within and between batch coefficients of variation (CV) were 2.33% and 2.15% respectively. A good correlation (R^2=0.99) was obtained between TG values in sera measured by present biosensor and standard enzymic colorimetric method with the regression equation: y= (0.993x + 0.967). ENPs/Au electrode was used 180 times over a period of 3 months with 50% loss in its initial activity, when stored dry at 4ºC.

Introduction

Triglycerides (triacylglycerol, TG), an ester of glycerol and three molecules of fatty acids are major components of very-low-density lipoprotein (VLDL) and chylomicrons, which play an important role in metabolism as energy sources and act as transporters of dietary fat. In the human body, high level of triglycerides in the bloodstream has been linked to atherosclerosis and the risk of heart disease and stroke. The risk can be partly accounted for by a strong inverse relationship between triglyceride level and HDL-cholesterol level [1]. Serum TG level < 150 mg/dl is considered normal. If level lies between 150 to 199 mg/dl, it indicates hyperlipoproteinemias, while the level >500 mg/dl is related with high risk of pancreatitis .The level > 1000 mg/dl reveals hyperlipidemia and > 5000 mg/dl is associated with eruptive xanthoma, enlarged liver and spleen[2]. Among the various methods available for TG determination, immobilized enzyme based biosensors are comparatively simple, sensitive, rapid and does not require time consuming sample preparation[3]. There are two types of enzyme based biosensors- either lipase and NAD+ dependent glycerol dehydrogenase or lipase, glycerol kinase (GK) and glycerol-3-P oxidase (GPO) TG biosensors[4].The latter is considered better than former, as it does not require an electron mediator and its electron flow is irreversible. Nanoparticles have shown excellent biocompatibility with biomolecules possess unique structure and electronic, magnetic, optical and catalytical properties, which make them suitable for important applications in biosensors. Direct immobilization of native enzyme(s) onto bulk metal usually results in its denaturation and loss of activity, due to changes in its physicochemical, properties during this process. To overcome this problem, enzymes have been tried to crosslink within their self in controlled manner to make the cross linked enzyme nanoparticles (CLEN)[5].Enzyme nanoparticles(ENPs) are the aggregated form of enzyme molecules in nanometer scale. These ENPs not only have increased surface area but also show unique electronic, optical, electrical, thermal and catalytic properties[6]. These ENPs feature high retention of enzymatic activity and reusability. Few bionanosensors have been constructed based on direct immobilization of nanoparticles of horseradish peroxidise[5],glucose oxidase[7], Uricase [8],cholesterol esterase(ChE) and cholesterol oxidase [9,10] onto metal electrode to construct improved biosensors for determination of H_2O_2,glucose,uric acid, and total cholesterol respectively. To the best of our knowledge, there is no report available on preparation, characterization and co-immobilization of lipase, GK and GPO onto an electrode to construct a TG biosensor. The co-immobilized enzymes form a single step procedure rather than multi-steps reactions as in case of individual enzymes[11]. We describe herein the preparation of lipase NPs, GKNPs and GPONPs,

their characterization and co-immobilization onto Au electrode to construct an improved amperometric TG bionanosensor.

Materials and methods

Sources of chemicals and biochemicals

Lipase from porcine pancreas, glycerol kinase from *Cellulomonas*, glycerol-3-phosphate oxidase from *Aerococcus viridians*, horseradish peroxidase (HRP), 3,5-dichloro-2-hydroxybenzene sulfonate (DHBS) and 4–amino-phenazone were purchased from Sigma Aldrich Co. USA. Cysteamine dihydrochloride from Fluka, Mumbai, ethanol from Changshu Yangyuan Chemicals, China and glutaraldehyde (grade 25% solution), Folin Ciocalteu's phenol (F.C.) reagent, adenosine triphosphate, triolein, glycerol, ATP-sodium salt, $MgCl_2$, potassium ferrocyanide and Triton X-100 from SRL, Mumbai were used. All other chemicals were of AR grade. Double distilled water (DDW) was used throughout the experiments.

Instuments

Potentiostat/Galvanostat PGSTAT 30 electrochemical workstation (Make: Autolab, model: AUT83785, manufactured by Eco Chemie, The Netherland) with GPES 4.9 software having a three electrode system composed of a Pt wire as an auxillary/counter electrode, a KCl saturated Ag/AgCl electrode as reference electrode and Lipase+GK+GPO ENPs/AuE as a working electrode. Transmission electron microscope (TEM) (JEOL 2100 F, Japan) and Scanning electron microscope (SEM) (Zeiss EV040) at Jawaharlal Nehru University, New Delhi. UV Spectrophotometer (Spectronic-20D, Thermo Scientific, U.S.A).

Dissolution of individual enzymes

Lipase, GK and GPO were dissolved individually in 0.01M sodium phosphate (PB) buffer, pH 7.0 at a concentration of 2 mg/ml and tested for their combined activity in a mixture of 100:50:20 unit ratio, as described above. Soluble protein in mixture was determined by Lowry method.

Combined assay of mixture of free lipase, GK and GPO

The combined assay of lipase, GK and GPO was carried out in a 15 ml conical flask wrapped in a black paper as described [12] with modification. The reaction mixture consisted 0.54 µmol $MgCl_2$, 0.63 µmol ATP, 1.0 µmol potassium ferrocyanide, 0.27 µmol 4-amino-phenazone, 1.53 µmol DHBS and 1.0 µmol of Triton X-100 per litre of 0.1 M sodium

phosphate buffer, pH 7.0. To this, 900 µl reaction mixture, 50 µl of mixture of enzymes was added and pre-incubated at 37 °C for 5 min. The reaction was started by adding 50 µl of triolein (650 mg/dl). After incubation at 37 °C for 15 min, A_{510} was read in Spectronic-20 (Thermo Scientific, U.S.A.) H_2O_2 generated in reaction was calculated from standard curve of H_2O_2 between A_{510} versus H_2O_2 concentration. The unit activity of mixture of enzyme was expressed as 1 µmol H_2O_2 /min/ ml.

Preparation of colour reagent

Colour reagent was prepared as described [12], which consisted of 0.7 mM adenosine-5-triphosphate, 0.3 mM 4-aminophenazone, 0.6 mM magnesium chloride, 1.7 mM DHBS and 1.0 µM Triton X-100 in 0.1 M phoshphate buffer, pH 7.0. It was stored in amber coloured bottle at 4°C and prepared fresh after one week.

Preparation of nanoparticles aggregates of individual enzymes

Nanoparticles of individual enzymes i.e. lipase, GK and GPO were prepared separately by desolvation method[5] using ethanol and glutaraldehyde cross-linking as follow:

To 3.0 ml solubilised lipase/GK/GPO in a 25 ml conical flask, 6 ml of desolvation agent i.e. ethanol was added dropwise under constant stirring at 500 rpm at room temperature, with a dropping rate of 0.1ml/min. After desolvation process, 1.8 ml of 2.5% glutaraldehyde (in DW) was added to this suspension and stirred for 24 hr, to ensure the formation of aggregates of ENPs. Then, 0.12g of cysteamine dihydrochloride was added to ENPs suspension and allowed for continuous stirring for 4-5 hr to add amide groups on the surface of prepared ENPs aggregates. The resulting ENPs were purified by one cycle of differential centrifugation (15,000 x g, 10 min) at 4^0 C and pellet was re-dispersed to the original volume in PB buffer pH 7.0. The redispersed ENPs aggregates were subjected to ultrasonication bath for over 5 min and was stored at 4^0 C.

Characterization of enzyme NPs aggregates:

The ENPs aggregates of lipase, GK and GPO were characterized individually by taking their images in transmission electron microscope (TEM)(TEM JEOL 2100 F,USA) at AIRF, JNU, New Delhi on commercial basis and recording their spectra in Fourier transform infra-red (FTIR) spectrometer (model Is10,Thermo Scientific, USA).

Assay of mixture of Lipase NPs, GKNPs and GPONPs

The assay of mixture of Lipase NPs, GKNPs and GPONPs was carried out as described for mixture free enzymes except that mixture of free enzymes was replaced by suspension of mixture of individual ENPs and the reaction mixture was stirred continuously during assay.

Co-immobilization of NPs of lipase, GK and GPO onto Au electrode/Preparation of ENPs electrode

Before modification, the surface of Au wire (2cm×1mm) was cleaned with piranha solution (H_2SO_4: H_2O_2 in 3:1 ratio) for 20 minutes and then rinsed thoroughly with DDW, then polished manually by silica gel followed by washing with DDW and then placed into ethanol for 10 minutes, sonicated to remove adsorbed particle and finally washed with DDW for 3-4 times. The cleaned polycrystalline Au electrode was dipped into suspension of mixture of Lipase NPs, GKNPs and GPONPs at 4^oC for 12 h with occasional stirring to immobilize self assembled layer of Lipase NPs, GKNPs and GPONPs onto Au electrode. The Lipase NPs, GKNPs and GPONPs modified gold electrode (ENPs/AuE) was rinsed with a 0.1 M of sodium phosphate buffer (PB, pH 7.0) carefully and stored at 4^oC, when not in use. The following reaction occoured during the covalent co-immobilization of ENPs onto polycrystalline Au electrode.

$$2Au \xrightarrow{H_2SO_4:\ H_2O_2} Au_n\text{-}AuO$$

$$\underset{\text{Polycrystalline AuE}}{Au_n\text{-}AuO} + \underset{\text{Functionalized ENPs mixture}}{2(ENPs)\text{-}SH} \longrightarrow \underset{\text{ENPs-AuE}}{Au_n(Au\text{-}S\text{-}ENPs)_2} + H_2O$$

Characterization of enzymeNPs/Au electrode

The fabricated ENPs/Au electrode was characterized by taking its image in scanning electron microscope (SEM) (Zeiss EV040) before and after immobilization of ENPs.

Construction & cyclic voltammetric (CV) measurement of TG bionanosensor

To construct an amperometric TG bionanosensor, ENPs co-immobilzed Au electrode was used as working electrode, KCl saturated Ag/AgCl as reference electrode and Pt wire as auxillary/counter electrode were connected through potentiostat/galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785). The three electrode system was immersed into 25 ml 0.1 M phosphate buffer pH 7.0 containing 9.6 mg of ATP (sodium salt) and 3.0 mg $MgCl_2$. To start the reaction, triolein (100 µl, 200 mg/dl) was added into the reaction buffer

and the CV was recorded in the potential range -0.75 V to 1.75 Vat a scan rate of 20 mV/s. The following electrochemical reactions occurred during the cyclic response measurement of TG bio-nanosensor.

$$\text{Triolein} \xrightarrow{\text{Lipase}} \text{Fatty acid + Glycerol}$$

$$\text{Glycerol + ATP} \xrightarrow[Mg^{2+}]{\text{GK}} \text{Glycerol-3- phosphate + ADP}$$

$$\text{Glycerol-3- phosphate} \xrightarrow{\text{GPO}} \text{Dihydoxyacetone phosphate + } H_2O_2$$

$$H_2O_2 \xrightarrow{0.25V} 2H^+ + O_2 + 2e^- \text{ (current)}$$

The electrochemical measurements were carried out at room temperature.

Optimization of bionanosensor

The optimal working conditions of the bionanosensor were studied in terms of pH, temperature, response time and substrate (triolein) concentration. To determine optimal pH, the pH of reaction buffer (sodium phosphate buffer; 0.1 M) was varied from 6.0 to 8.0 at an interval of 0.5. Similarly optimum temperature was studied by incubating the reaction mixture at different temperature (15–50 °C) and time (1–40 s). The effect of triolein concentration on bionanosensor response was determined by varying its concentration from lower ranges (1.0-100 mg/dl) to higher ranges (100-500 mg/dl)).

Determination of triglyceride level in serum

Intravenous blood samples (1 ml each) from apparently healthy and diseased individuals suffering from heart disease, coronary heart disease (CAD) and hypolipoproteinemia both male and female, of different age groups were collected from local hospital, Pt. BDS University of Health Sciences and centrifuged at 5000 rpm for 5 min, and the supernatant (serum) was collected. The present bionanosensor was employed for determination of triglyceride level in the serum samples of apparently healthy adults and persons suffering from hypertriglycedemia. The determination of triglyceride level in serum samples were carried out in the same manner as described for CV measurement of triglyceride bionanosensor under optimal working conditions except that the triolein was replaced by serum. The concentrations of triglyceride level in sera were extrapolated from standard curve

between triolein concentration (50-500 mg/dl) and current in mA, prepared under optimal assay conditions of Lipase NPs+ GK NPs + GPO NPs modified Au electrode. The serum samples were stored at 4^{o}C before analysis.

Results and discussion

Characterization of lipase NPs, GK NPs and GPO NPs aggregates

By TEM

The size and shape of lipase NPs, GKNPs and GPONPs was measured by TEM (Fig. 1A, 1B and 1C respectively) The typical TEM images of these nanoparticles aggregates showed their spherical shape with an average size of 134nm (range 83-218nm) for lipase NPs, 221nm (range 144-329nm) for GKNPs and 45nm (range 22-68nm) for GPONPs.

By FTIR spectroscopy

FTIR spectra of both free/native form and nanoparticles of lipase, GK and GPO are shown in Fig. 1D, 1E and 1F respectively. The free form of all the three enzymes showed peaks at 3300cm^{-1}(due to H-O and –NH- stretching), 1630cm^{-1}(due to H-OH bending and amide bonding), which were increased in case of their NPs aggregates. However, an additional small peak at 1080cm^{-1} (due to –C-O stretching) was observed in case of lipase NPs and GPONPs compared to their free form but this peak was observed in case of both free and NPs form of GPO. FTIR spectra of mixture of ENPs was same as those of individual ENPs.

SEM studies of Au electrode after modification

The SEM image of the bare Au electrode showed a dark coloured smooth and featureless morphology (Fig. 2A),while the Lipase NPs+GKNPs+GPONPs composite film modified Au electrode (ENPs/AuE) exhibited bright coloured globular layers on the surface of Au electrode revealing the co-immobilization/attachment of ENPs onto Au electrode (Fig.2B).

Cyclic voltagramm/ response of Lipase NPs+GKNPs+GPONPs/Au electrode

Cyclic voltammogram (CV) for Lipase NPs+GKNPs+GPONPs/AuE in presence of 100µl triolein (200mg/dl) in PBS (0.1 M, pH 7.0) is shown in Fig. 3. No peak was observed in case of bare Au electrode. The ENPs/Au electrode showed two characteristic peak known as oxidation and reduction peaks between -0.75 to +1.75V in a conductive solution. Two

oxidation peaks at 0.193 V and 0.918 V and four reduction peaks at 1.103 V, 0.410 V, 0.163 V and -0.107 V were observed. Nanoparticles provided a conductive path for electron transfer and promoted electron transfer reaction (Fig. 3). The bionanosensor showed the maximum current response at 0.25 V.

Optimization of working conditions of TG bionanosensor

The combined kinetic properties of co-immobilized ENPs onto Au electrode were studied such as effect of pH, incubation temperature for maximum activity, response time, and effect of substrate (triolein) concentration to study the optimum working conditions of the bionanosensor. The optimal pH was obtained at pH 6.5 (Fig. 4A), which is comparable to earlier reported biosensors(Table 1). The optimal incubation temperature was 35 °C (Fig. 4B). Hence, the subsequent experiments were carried out at pH 6.5 and 35 °C. The bionanosensor showed maximum current response within 5s,which is lower than earlier biosensors (Table 1). There was a linear relationship of current response and triolein concentration when measured both at lower concentration (1.0-100 mg/dl) (Fig. 5A) and higher concentration (100-500 mg/dl) (Fig. 5B).

Evaluation of proposed TG bionanosensor

The present TG bionanosensor was evaluated by determining its linearity, limit of detection (LOD), analytical recovery, precision and correlation.LOD was calculated using following formula: LOD=3.3xSD/S,where SD=Standard Deviation in current response; S=slope of calibration curve.

Linearity

There was a linear relationship between current (mA) and triolein concentration ranging from 1.0 to 100 mg/dl (lower concentration) and 100 to 500 mg/dl (higher concentrations), which is better than earlier reported biosensors (Table 1)

Limit of detection

The detection limit [S/N]=3 of the current bionanosensor was 0.1mg/dl (1.0µg/ml), which is also better than earlier reported TG biosensors (Table 1)

Analytical recovery

The mean analytical recovery of added triolein in serum samples (50 & 100 mg/dl) was 95.2% and 96.0% respectively, showing the high reliability of the bionanosensor .

Precision

To check the reproducibility and reliability of method, the triglyceride content of sample (within batch, five times on single day and between batch, five times again after their storage at -20°C/week). The result showed that the within and between batch CV were less than 1.25 % and 1.84 % respectively showing the high reproducibility and repeatability of the bionanosensor .

Amperometric determination of serum triglyceride

To study the accuracy of current method, triglyceride level in 15 serum samples of patients suffering from hyperlipidemia (atherosclerosis) was determined by the present bionanosensor method (y) and compared with those by standard enzymic colorimetric method (x). There was a good correlation (R^2=0.998) with the following regression equation y= 0.993x + 0.967 (Fig. 5C). These results show the high accuracy of present bionanosensor.

Amperometric determination of serum triglyceride

The TG level in sera of apparently healthy adults as measured by present bionanosensor was found in the range 70 to 190mg/dl for male 50 to 180mg/dl for female, which were in established normal range(40-190mg/dl). The TG level in sera of persons suffering from hyperlipidemia, by the present bionanosensor was in the range 220-600 mg/dl for male and 210-480 mg/dl in female.

Interference study

*T*he amperometric response was measured in the presence of potential interfering compounds such as glucose, fructose, uric acid, urea, ascorbic acid, ethanol, cholesterol, citric acid, lactic acid, alanine, leucine, creatinine, pyruvate and bilirubin at their physiological concentration. The results showed negligible interferences of these metabolites.

Reusability and storage stability of enzyme NPs/Au electrode

To reuse the working electrode, it was washed by dipping it in 0.1 M, sodium phosphate buffer, pH 7.0, 3-4 times at room temperature. The working electrode lost 50 % of its initial activity after its regular use for 180 times over a 3 months period, when enzyme electrode stored in a refrigerator at 4 °C in 0.1 M sodium phosphate buffer, pH 7.0. This storage

stability and reusability of the present bionanosensor is better than earlier TG biosensors (Table 1).

Conclusion

An improved amperometric TG bionanosensor was constructed by covalent immobilization of nanoparticles of Lipase, GK and GPO onto polycrystalline Au electrode. The bionanosensor showed improved analytical performance in terms of sensitivity, LOD, broader working range, good reproducibility and high storage stability. It is concluded that use of ENPs not only improves the analytical performance of biosensor but also simplify the construction of enzyme electrode.

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Table 1. A comparison of analytic characteristics of various triglycerides biosensors

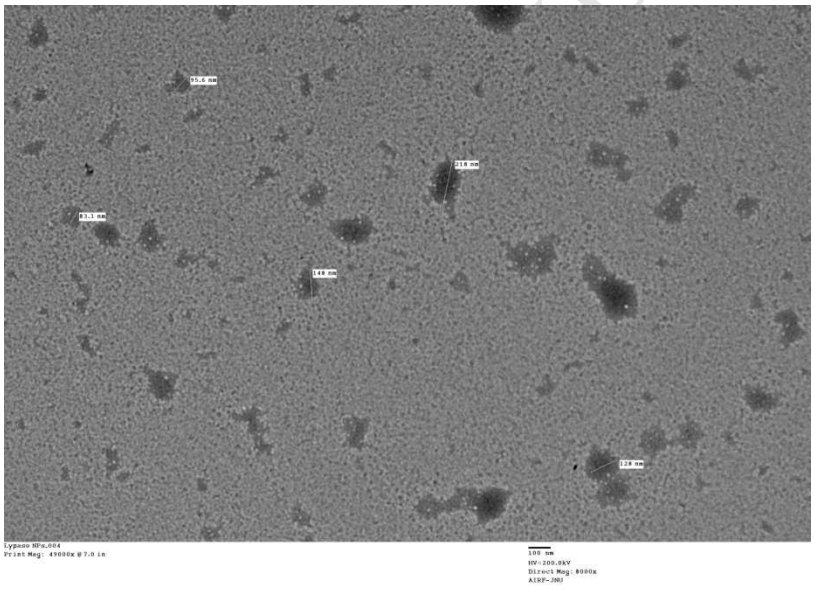
Type of biosensor	Support used for immobiliza-tion of enzymes	Method of immobilization	Type of electrode	Optimum pH	Linearity (mg/dl)	Detection limit (mg/dl)	Response time (seconds,s)	Storage stability at 4^0 C(% loss in days)	Reference
DO	PVC	Adsorption	DO	7.5	442.4-1770	30.97	NR	50 in 25	[13]
DO	Gelatin	Entrapment	DO	8.5	8.85-354	8.84	900	50 in 19	[14]
DO	NR	NR	DO	NR	NR	4.42	NR	NR	[15] [3]
Amperometric	Collagen memberane	NR	Rotating platinum electrode (RPE)	8.0	10-500	10	900	82 in 27	[16] [17]
Amperometric	AV	Covalent	Pt	8.5	0.17-88.5	0.044	NR	25in 30	[18]
Amperometric	CA	Adsorption	Pt	6.5	17.7- 310	17.7	40	50 in 25	[19]
Amperometric	PVC	Adsorption	Pt	7.5	49.55-199.11	9.73	30	50 in 40	[20]
Amperometric	PVA	Adsorption	Pt	7.0	49.55-199.11	18.58	2	50 in 70	[21]
Amperometric	Egg shell memberane	Covalent	Pt	7.0	49.55-199.11	24.77	NR	50 in 70	[22]
Amperometric	NPG	Physical adsorption	Glassy carbon	7.0	50-250 (tributyrin)	2.68	NR	5 in 55	[23]
Amperometric	CeO_2-SiO_2	Physical adsorption	ITO	-	50-400	-	-	NR	[24]

Amperometric	ERGO	Covalent	ITO	7.8	50-300 (tributyrin)	50	12	NR	[25]
Amperometric	Gelatine membarane	Entrapment	Glassy carbon	7.0	12.4-99.5	12.4	NR	39 in 30	[26]
Electrochemical	CHIT-nano-ZrO_2	Electrodeposition	ITO coated glass substrate	7.4	25-400	1.5l	45	85 in 77	[27]
Electrochemical	Cysteamine SAM	Electrodeposition and cross linking	Au	7.2	15-200	15	30	NR	[28]
Electrochemical	MNP/CHIT/ZnO-ZnHCF film	Covalent	Glassy carbon	7.0	10-1000	10	-	-	[29]
Electrochemical	Polyaniline/ single walled CNT	Covalent cross linking	ITO	7.4	50-400	50	12	84 days	[30]
Electrochemical	Iridium NPs		Carbon	NR	50-400	NR	NR	Disposable	[31]
Electrochemical	Cerium oxide NPs	Physical Electrostatic	ITO	6.5	0-885	32.8	20	55 days	
Electrochemical	MWCNT-Cerium oxide NPs	interaction Physical adsorption	Glassy carbon	6.4	1-100	0.5	25	>55 days	[32]
Electrochemical	Zinc oxide NPs	Covalent cross linking	Pt	7.5	50-650	20	6	50 in 213	[33]
Electrochemical	Gold polypyrrole	Covalent	Au	6.5	50-700	20	4	50 in 213	[34]
Potentiometric	pH-FET's	Physical adsorption	pH-FET's	NR	8849.55-35398.23	0.9	120	18 in 44	[35]
Potentiometric	Porous silicon	Physical adsorption	NR	NR	522.12-1858.40	522.12	900	82 days	[36]
Potentiometric	Porous silicon	Physical adsorption	EISCAP		NR	NR	NR	NR	[37]
Potentiometric	Mesoporous Si matrix	Physical adsorption	Psi sample	NR	<150	NR	NR	NR	[38]

Potentiometric	Silica gel beads	Physical adsorption	ISFET	NR	Upto 2654.86	NR	NR	60 days	[39]
Potentiometric	gate surface of FET	Physical adsorption	ISFET	NR	442.47-2654.86	NR	<300	40 days	[40]
Potentiometric	Silicon nitride layer of the EISCAP	Physical adsorption	EISCAP	NR	88.49-619.46	0.44	2700	45 in 14	[41]
Microcantilever sensor	Cantilever beams	Physical adsorption	Microcantilever	NR	2123.89-42477.87	0.0885	1200	NR	[27]
Electrochemical	Au	Covalent	Au wire	6.5	10-100	0.1	5	50 in 90	Present

NR=Not reported

List of Figures



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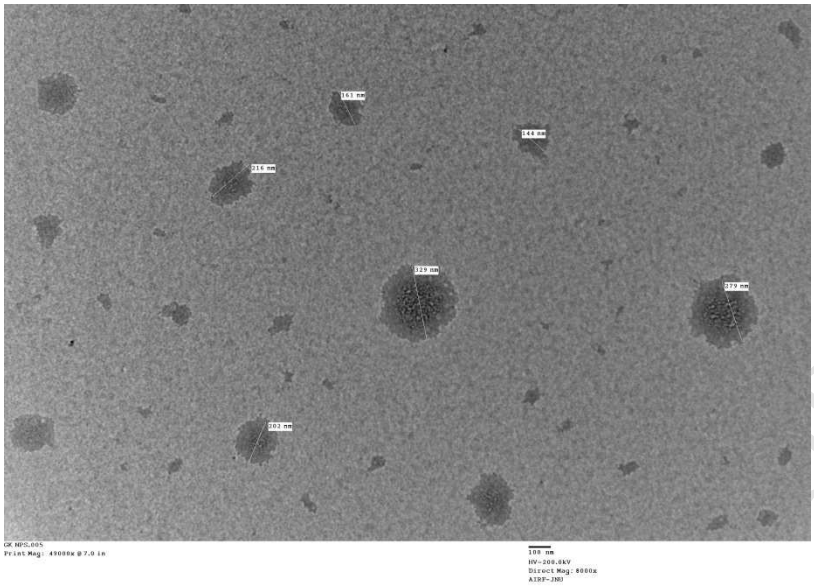

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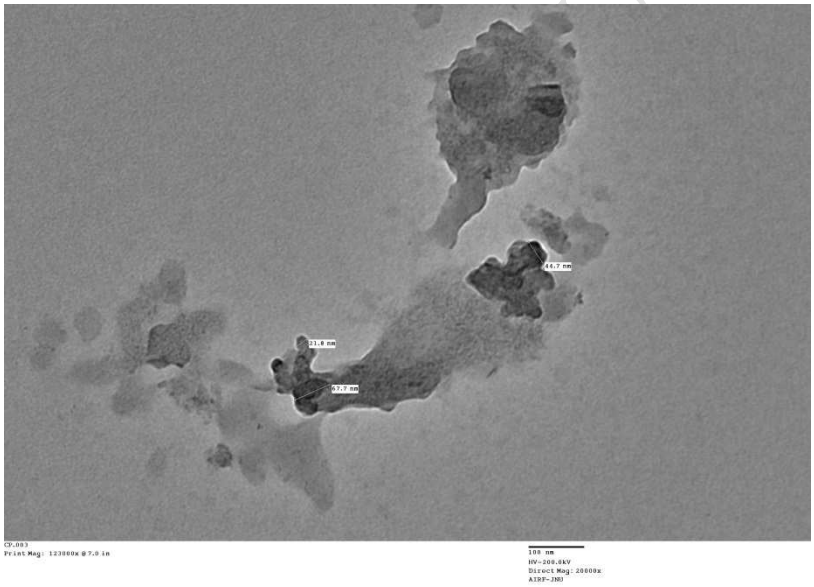

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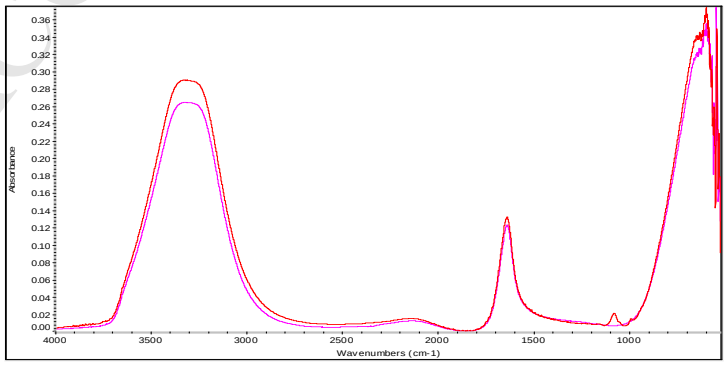

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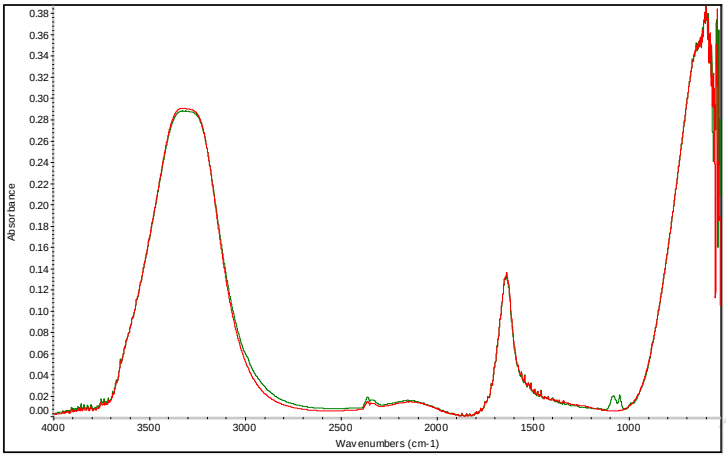

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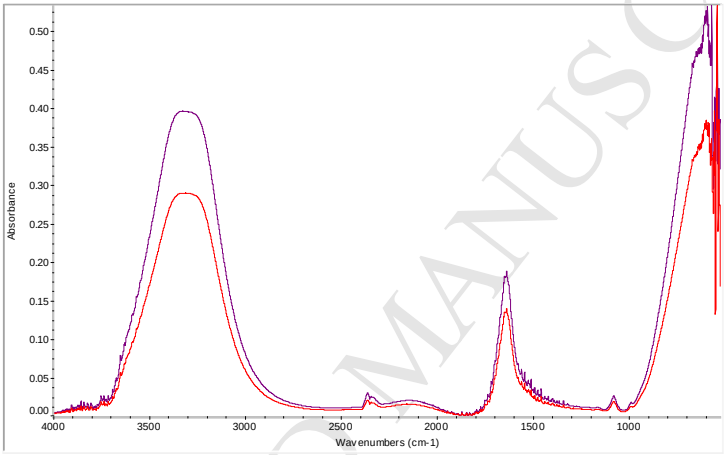

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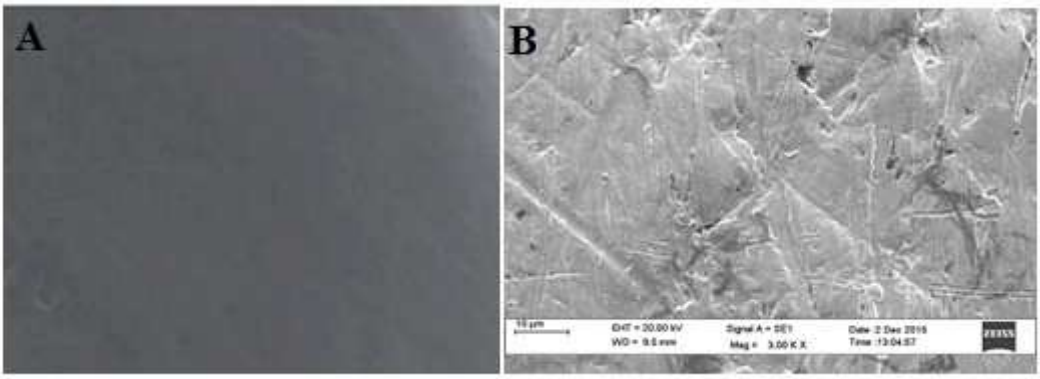
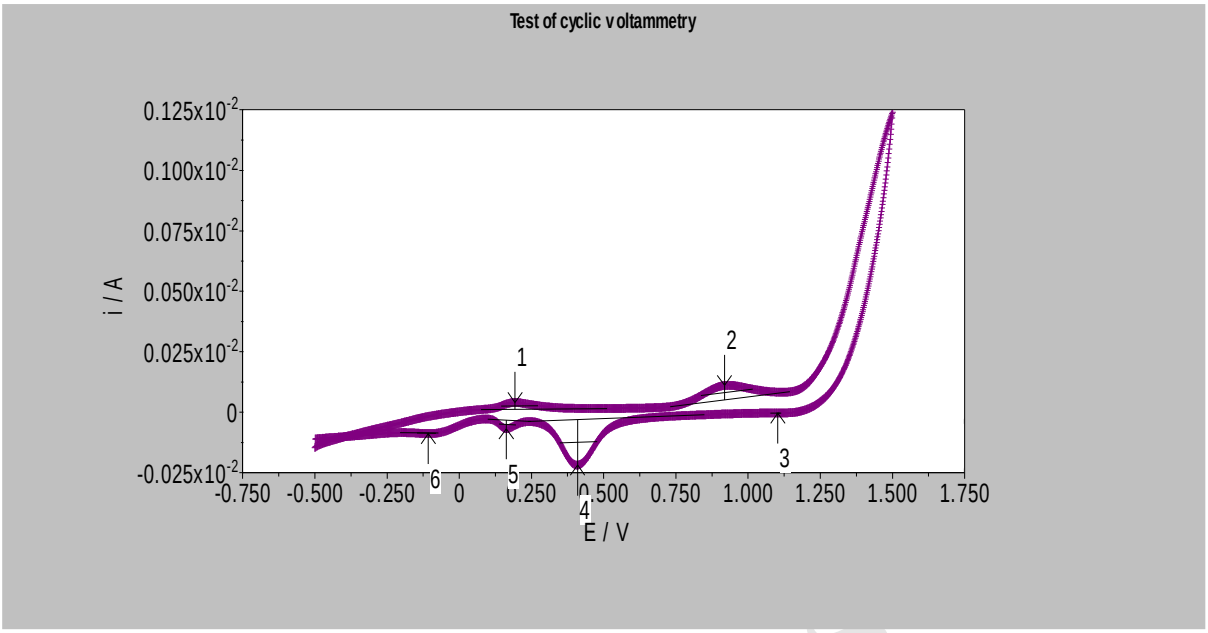


Fig. 2



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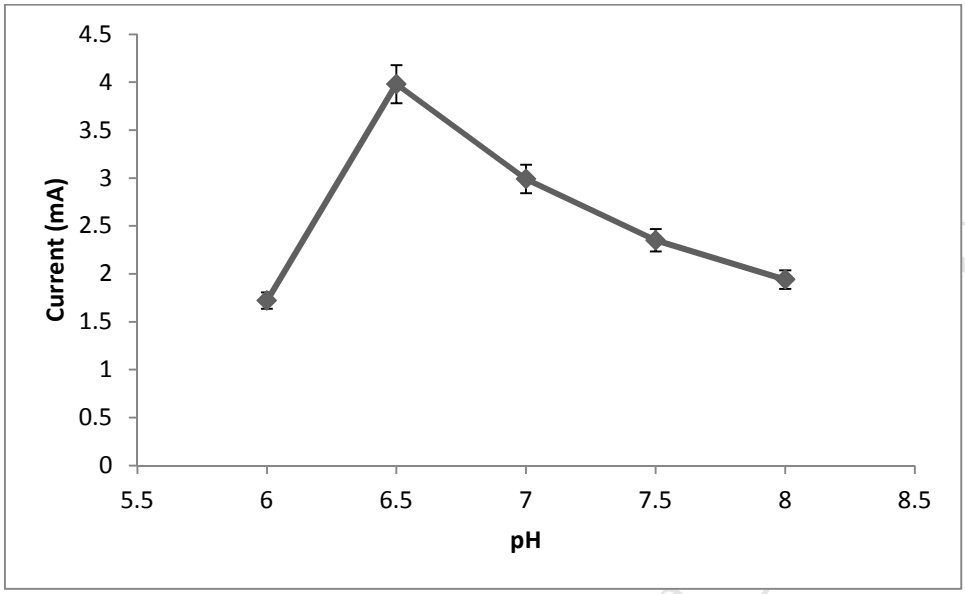

Current (mA)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
5.5
6
6.5
7
7.5
8
8.5
pH

Fig.4A

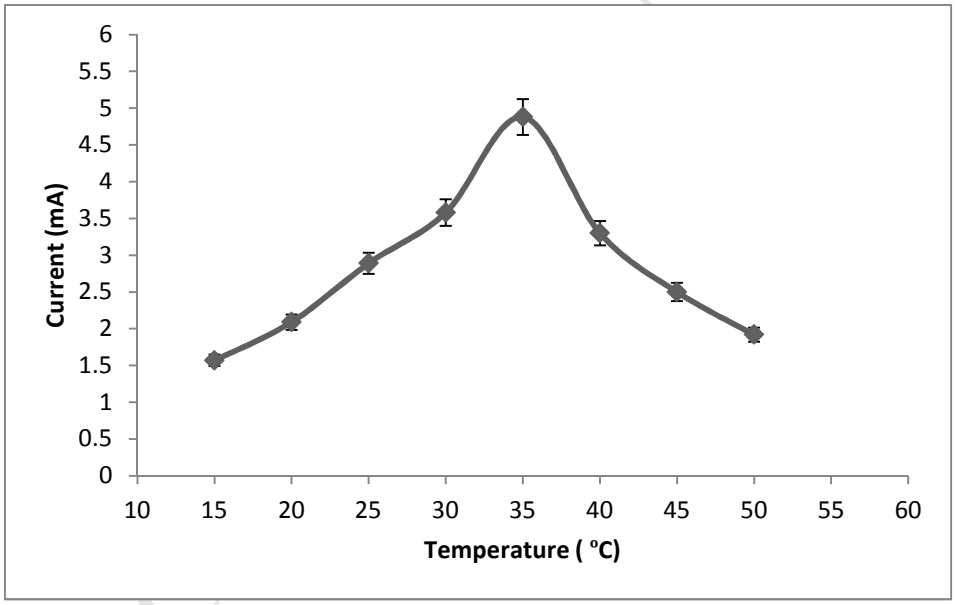
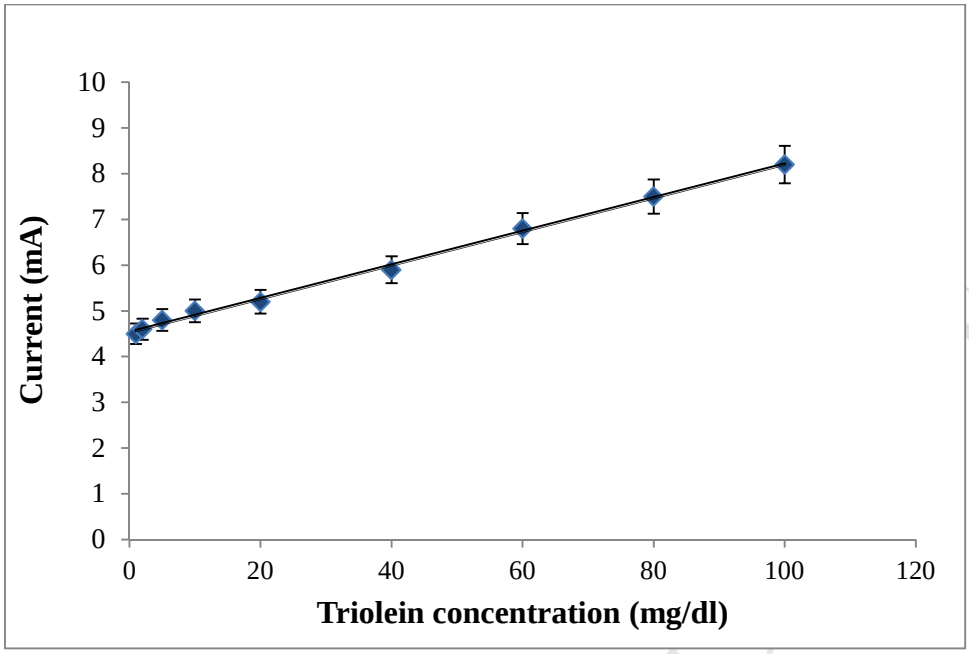

Current (mA)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
10
15
20
25
30
35
40
45
50
55
60
Temperature (°C)

Fig.4B


Current (mA)
Triolein concentration (mg/dl)
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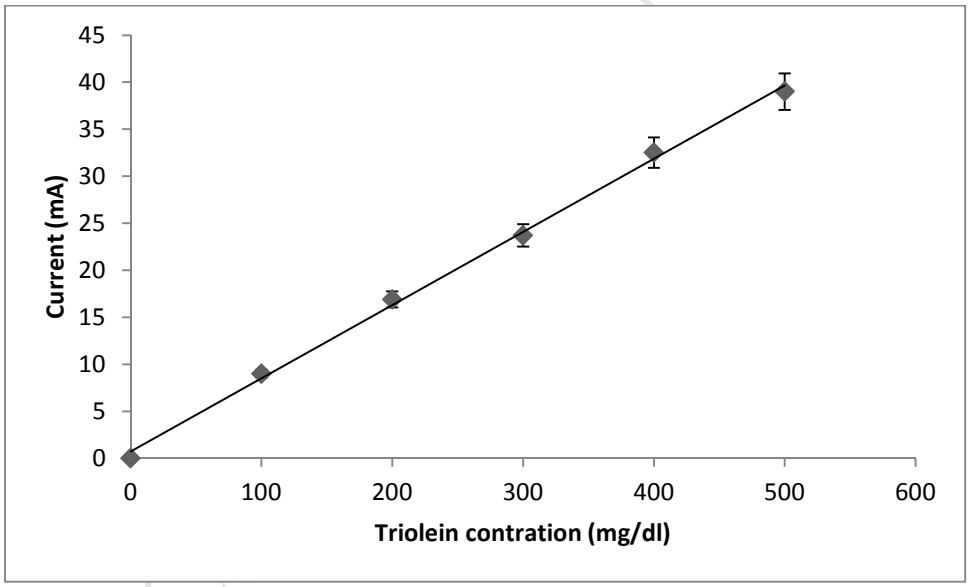
3
4
5
6
7
8
9
10
0
20
40
60
80
100
120

Fig 5A


Current (mA)
Triolein contration (mg/dl)
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10
15
20
25
30
35
40
45
0
100
200
300
400
500
600

Fig.5B

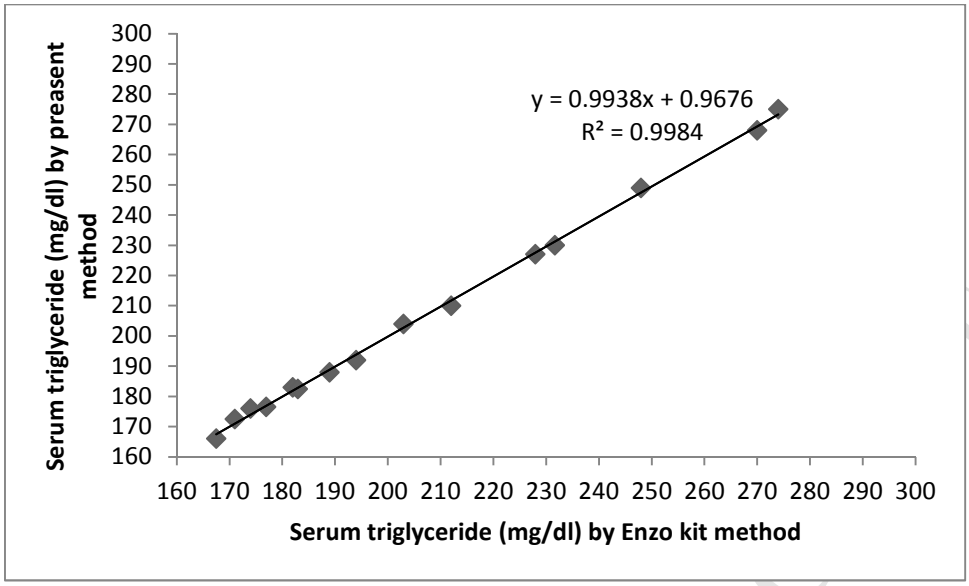

Fig 5C