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ORIGINAL PAPER

Construction of an amperometric TG biosensor based on AuPPy nanocomposite and poly (indole-5-carboxylic acid) modified Au electrode

Jagriti Narang · Nidhi Chauhan · Poonam Rani · C. S. Pundir

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Abstract A method is described for construction of an amperometric triglyceride (TG) biosensor based on covalent co-immobilization of lipase, glycerol kinase and glycerol-3-phosphate oxidase onto gold polypyrrole nanocomposite decorated poly indole-5-carboxylic acid electrodeposited on the surface of a gold electrode. The enzyme electrode was characterized by transmission electron microscopy, scanning electron microscopy, electrochemical impedance studies, Fourier transform infrared spectroscopy and cyclic voltammetry. Biosensor showed optimum response within 4 s at pH 6.5 and 35 °C, when polarized at +0.1 V against Ag/AgCl. There was a linear relationship between sensor response and triolein concentration in the range 50–700 mg/dl. Biosensor was employed for determination of TG in serum. Detection limit of the biosensor was 20 mg/dl. Biosensor was evaluated with 91–95 % recovery of added triolein in sera and 4.14 and 5.85 % within and between batch coefficients of variation, respectively. There was a good correlation ($r = 0.99$) between sera TG values by standard method (Enzymic colorimetric) and the present method. The biosensor was unaffected by a number of serum substances at their physiological concentration. Biosensor lost 50 % of its initial activity after its 100 uses over 7 months, when stored at 4 °C.

Keywords Triglyceride · Lipase · Glycerol kinase · Glycerol-3-phosphate oxidase · Gold polypyrrole nanoparticles · Poly (indole-5-carboxylic acid)

Introduction

Triglycerides (TG) are main fat in our body. TG, as major components of very-low-density lipoprotein (VLDL) and chylomicrons, play an important role in metabolism as energy sources and transporters of dietary fat. TG contains twice energy (9 kcal/g or 38 kJ/g) than that of carbohydrates and proteins [1]. In human body, high levels of TG in the bloodstream are associated with atherosclerosis (hardening of the arteries) and, by extension, the risk of heart disease and stroke [2]. Among various methods available for determination of TG [3–7], including enzymatic methods [8], electrochemical sensors and biosensors have the advantage over the conventional methods, as these are simple, sensitive, rapid and require no sample preparation [9]. Amperometric TG biosensors based on enzymes immobilized onto artificial membranes such as collagen [10], cellulose acetate (CA) [11], polyvinylchloride (PVC) [12], polyvinyl alcohol (PVA) [13] and eggshell [14] membranes have been reported. A porous silicon-based potentiometric TG biosensor employing lipase has also been developed [15]. Direct modified electrodes have many advantages such as ease of construction, minimalism of usage, fast response time and stability. Due to the large surface-to-volume ratio and the increased surface activity, metal nanoparticles (NPs) of gold and other noble metals have been synthesized. These metal NPs possess unique physical and chemical characteristics, such as optical, electronic, magnetic, catalytic and electrochemical properties [16, 17]. TG biosensors were fabricated based on

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prussian blue nano-particles [18], iridium NPs [19], polyaniline/single-walled carbon nanotubes [20] and magnetic NPs using ion selective field effective transistor (ISFET) electrode [21]. An amperometric TG biosensor employing zinc oxide NPs has been reported from our laboratory [22].

Recently, composite materials consisting conducting polymers [23], nanomaterials [24–30] and nanoclusters [31] have been used for their combined properties of the individual components with a synergistic performance in biosensor fabrication.

The colloid of gold polypyrrole (AuPPy) composite NPs strongly adheres to the surface of Au electrodes and exhibits better electrocatalytical reduction than bare Au electrodes. In addition, the presence of gold in AuPPy composite NPs facilitates direct and fast electron transfer between enzymes and electrodes. We describe herein the construction of an amperometric TG biosensor based on covalent co-immobilization of enzymes onto AuPPy nanocomposite decorated poly (indole-5-carboxylic acid) modified Au electrode and its application for determination of serum TG.

Experimental

Reagents and materials

Lipase from porcine pancreas (40–70 U/mg), glycerol kinase (GK) from *Cellulomonas species* (25–75 U/mg), glycerol-3-phosphate oxidase (GPO) from *Aerococcus viridans* (113 U/mg), Triton X-100, indole-5-carboxylic acid from Sigma Aldrich Co, St. Louis USA; 3,5-dichloro-2-hydroxy benzene sulphonic acid (DHBS) (98 %), triolein (99 %), ATP (98 %), $HAuCl_4$ (~49 % Au), cetyltrimethylammonium bromide (CTAB) (Extra pure), NAD^+, pyrrole (98 %) and zinc nitrate ($Zn(NO_3)_2.4H_2O$) (99 %) from Sisco Research Laboratory Pvt. Ltd., Mumbai, India and kit for enzymic colorimetric method (Enzo kit) for TG from Erba Transasia, Daman, India were used. All other chemicals were of AR grade. Double-distilled water (DW) was used for experimental studies.

Apparatus used

Cyclic voltammetry (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat/Galvanostat (Autolab, Eco Chemie, The Netherland. Model: AUT83785) with a three-electrode system. All the electrochemical experiments were performed at an ambient temperature (25 °C).

Preparation of triolein solution

Triolein was used as a substrate for lipase, GK and GPO. Triolein emulsion was prepared as described [32]. Solutions of different concentrations of triolein ranging from 10 to 800 mg/dl were prepared in 0.1 M sodium phosphate buffer (pH 7.0) and stored at 4 °C until use.

Preparation of AuPPy nanocomposite

AuPPy composite NPs were prepared by direct redox reaction between pyrrole monomers and $HAuCl_4$. Typically, 0.01 µl pyrrole was mixed in 2 ml 0.5 M H_2SO_4 containing 10 mM CTAB. Then, 10 µl 2 mM $HAuCl_4$ aqueous solution was added to it drop wise. The solution turned transparent light yellow overnight, which resulted into a stable dispersion of AuPPy colloid [33].

Preparation of nanocomposites of AuPPy and Pin5COOH

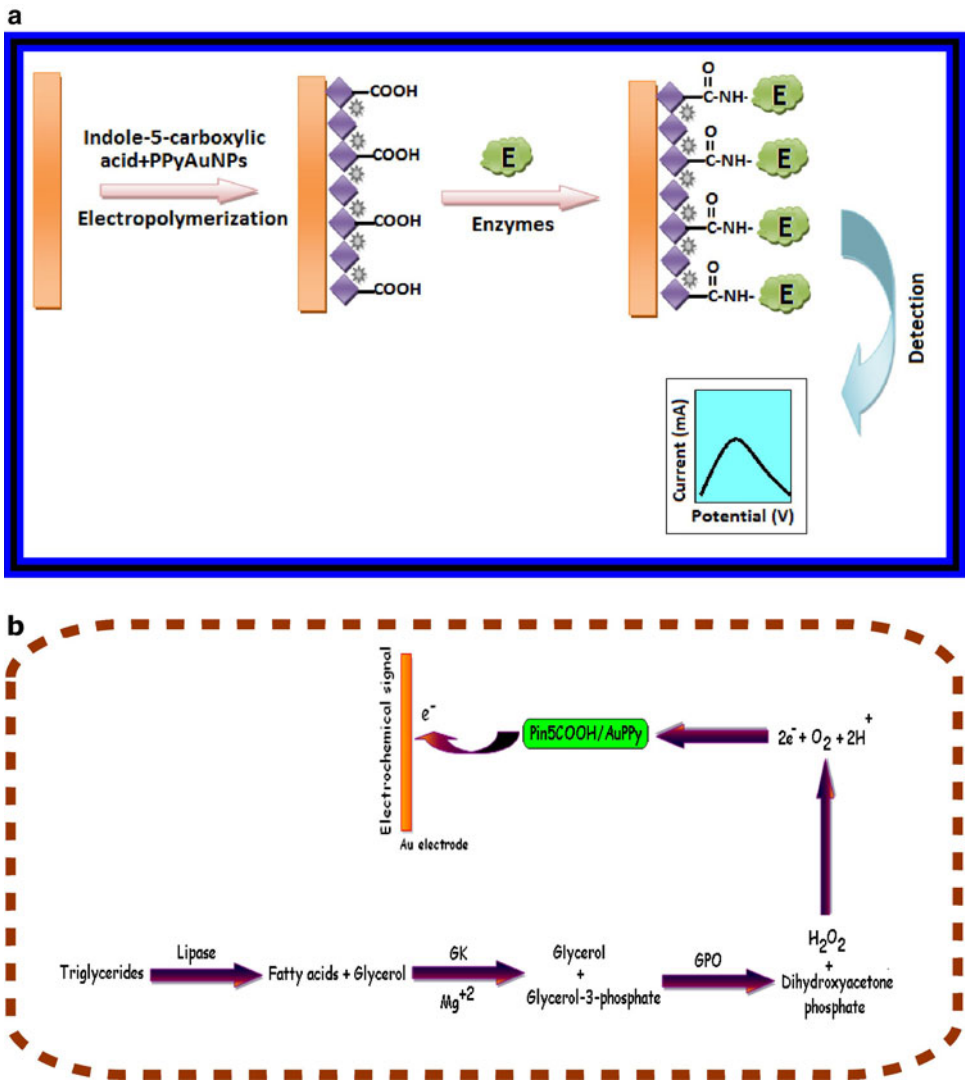
Nanocomposites of AuPPy and poly indole-5-carboxylic acid (Pin5COOH) were prepared as described [34] with modification. $FeCl_3$ (4.5 g) was added to a mixture of 1.0 ml AuPPy suspension and 1.0 ml indole monomers (5.0 mg in 20.0 ml DW) under continuous stirring for 2 h. The particles of nanocomposite were washed by DW, filtered, extracted (in ethanol) for 10 h and then dried at 50 °C.

Construction of enzyme electrode (enzymes/Pin5COOH/AuPPy/AuE)

Electropolymerization of nanocomposite was carried out on Au electrode from acetonitrile solution containing nanocomposite [35]. To achieve it, an Au (1.5 cm × 0.05 cm) electrode was polished with alumina powder followed by cyclic voltammetry in the potential range, from 0.0 V to +0.6 V versus Ag/AgCl at a scan rate 20 mV/s. The cleaned Au electrode was immersed into 25 ml acetonitrile containing 0.1 g nanocomposite and then applied 10 polymerization cycles in the potential range from 0.0 V to +0.6 V and back to 0.0 V at a scan rate of 20 mV/s. After electrodeposition, the electrode was washed carefully with acetonitrile and finally with DW. Enzymes solution (20 µl) was added on the surface of this modified electrode. After 10 min, the electrode was washed with DW and used for electrochemical measurements (Scheme 1a).

Construction and response measurement of TG biosensor

An amperometric TG biosensor was constructed by connecting modified Au electrode (enzymes/Pin5COOH/AuPPy/AuE) as working electrode, Ag/AgCl as reference electrode and Pt wire as an auxiliary electrode through



Scheme 1 **a** Schematic illustration of the stepwise amperometric biosensor fabrication process and immobilization of enzymes. **b** Electrochemical response studies of enzymes/Pin5COOH/AuNPPPy/Au electrode as function of triolein concentration using CV

Potentiostat/Galvanostat. Prior to response measurements, the steady-state current was achieved by polarizing the working electrode at +0.1 V versus Ag/AgCl in 0.1 M phosphate buffer saline (PBS, 50 mM, pH 6.5, 0.9 % NaCl) containing 5 mM $[Fe(CN_6)^{3-/4-}$ as a redox probe. The reaction was started by adding 1.0 ml triolein. The electrochemical reactions involved in measuring the TG by the present biosensor are given in Scheme 1b. The enzymatically produced H_2O_2 from TG was split into $2H^+ + O_2$ and $2e^-$, flow of these e^- under potential difference produces current, which is sensed and monitored by an electrochemical sensor. The current was measured in mA at an applied voltage of 0.1 V versus Ag/AgCl by the square wave voltammetry.

Optimization of working conditions of TG biosensor

Various kinetic properties of enzymes/Pin5COOH/AuPPy/Au electrode such as optimum pH, incubation temperature, response time and effect of substrate (triolein) concentration were studied amperometrically to optimize the working conditions of the biosensor.

Amperometric determination of TG in serum

Blood samples (1 ml each) from apparently healthy males and females (25 each) and persons suffering from hypertriglyceridemia of various categories such as peripheral vascular disease and pancreatitis were collected from local Pt. BDS PGIMS, Rohtak hospital and centrifuged at $5{,}000 \times g$ for 5 min and their supernatant (serum) was collected. TG content in serum was determined by the present biosensor in the similar manner as described above for its response measurement, under its optimal working conditions except that triolein was replaced by serum. The current (mA) was measured and the amount of TG in serum was interpolated from calibration curve between triolein

concentrations and current (in mA) prepared under optimal working conditions.

Evaluation of TG biosensor

The biosensor was evaluated by studying analytic recovery, precision and correlation. The effect of various possible interfering substances found in blood such as urea, uric acid, glucose, fructose, ethanol, cholesterol, citric acid, ascorbic acid, lactic acid, malic acid, tartaric acid, alanine, leucine, bilirubin, creatinine and pyruvic acid was also studied at their physiological concentrations.

Reusability and storage stability of enzymes/Pin5COOH/AuPPy modified Au electrode

The reusability and long-term storage stability of the biosensor were investigated by measuring its activity up to 7 months at a regular interval of 1 week, during its storage at 4 °C.

Results and discussion

Characterization of AuPPy nanocomposite

Transmission electron microscopy (TEM) image of AuPPy nanocomposite (Fig. 1) shows the presence of spherical particles, with a diameter of 20 nm. In addition to small submicron particles dispersed on the surface, large agglomerated particles were also observed in the image. These observations confirm formation of AuPPy nanocomposite.

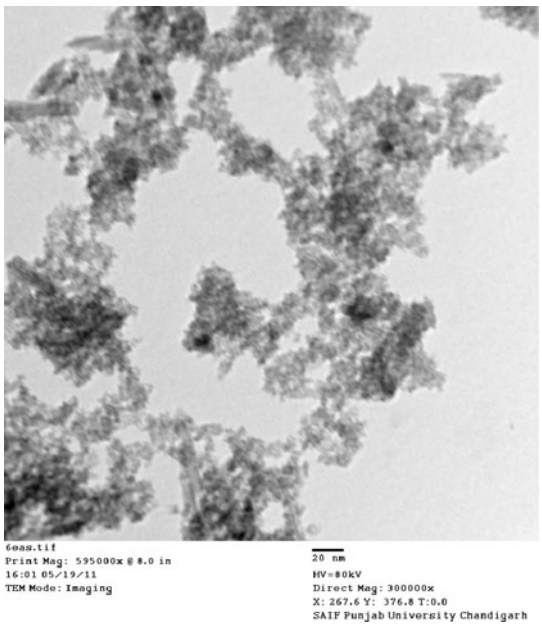


Fig. 1 Transmission electron microscopy of AuPPy nanocomposite

Evidences for immobilization of enzymes

To confirm enzyme immobilization on the Pin5COOH film, the surface morphologies of bare electrode (a), Pin5COOH/AuPPy/Au electrode (b) and enzymes/Pin5COOH/AuPPy/Au bioelectrode (c) were investigated by scanning electron microscopy (SEM) (Fig. 2). The bare electrode showed smooth surface (Fig. 2). The granular morphology with heterogeneous roughness of Pin5COOH/AuPPy/Au electrode showed the dispersed NPs in Pin5COOH matrix (b). Upon immobilization of enzymes, the granular morphology of Pin5COOH/AuPPy changed into the regular form, due to the interaction between Pin5COOH-AuPPy and enzymes (c). Figure 3 shows electrochemical impedance spectra (EIS) of AuPPy/Au electrode (a), Pin5COOH/AuPPy/Au electrode (b) and enzymes/Pin5COOH/AuPPy/Au electrode (c). The value of the electron transfer resistance (semicircle diameter) (Rct) depends on the dielectric and insulating features at the electrode/electrolyte interface. The Rct values for the AuPPy/Au electrode, Pin5COOH/AuPPy/Au electrode and enzymes/Pin5COOH/AuPPy/Au electrode were obtained as 90, 60 and 225 Ω, respectively. These results showed that the electron transfer via redox couple was hindered by the presence of enzymes on electrode surface. The increased Rct value of enzymes/Pin5COOH/AuPPy/Au electrode was due to the immobilization of enzymes onto Pin5COOH/AuPPy/Au surface. This increase in Rct is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer. Figure 4 shows Fourier transform infrared (FTIR) spectra for Pin5COOH/AuPPy/Au electrode (curve a) and enzymes/Pin5COOH/AuPPy/Au electrode (curve b). The spectrum associated with the oxidized Pin are characterized by a very large adsorption band located in the spectral domain between 3,700 and 3,100/cm, which is a characteristic of –OH groups belonging to residual water molecules trapped in the polymer matrix as well as water molecules absorbed in Pin. The peaks at 3,401, 2,924, 1,593 and 1,312/cm could be attributed to –N–H stretching, –C–H (aromatic) stretching, C–C stretching and C–N stretching (between two indole units), respectively. A sharp absorption peak was found at 1,690 /cm, which might be due to the attachment of –COOH group onto the indole. In the FTIR spectrum of Enzymes/Pin5COOH/AuPPy/Au (curve b), enzyme binding is indicated by the appearance of additional absorption bands at 1,641 and 1,539/cm assigned to the carbonyl stretch (amide I band) and –N–H bending (amide II band), respectively.

Voltammetric response of the enzymes/Pin5COOH/AuPPy modified Au electrode

Figure 5 shows cyclic voltammogrammes for AuPPy/Au electrode, Pin5COOH/AuPPy/Au electrode and

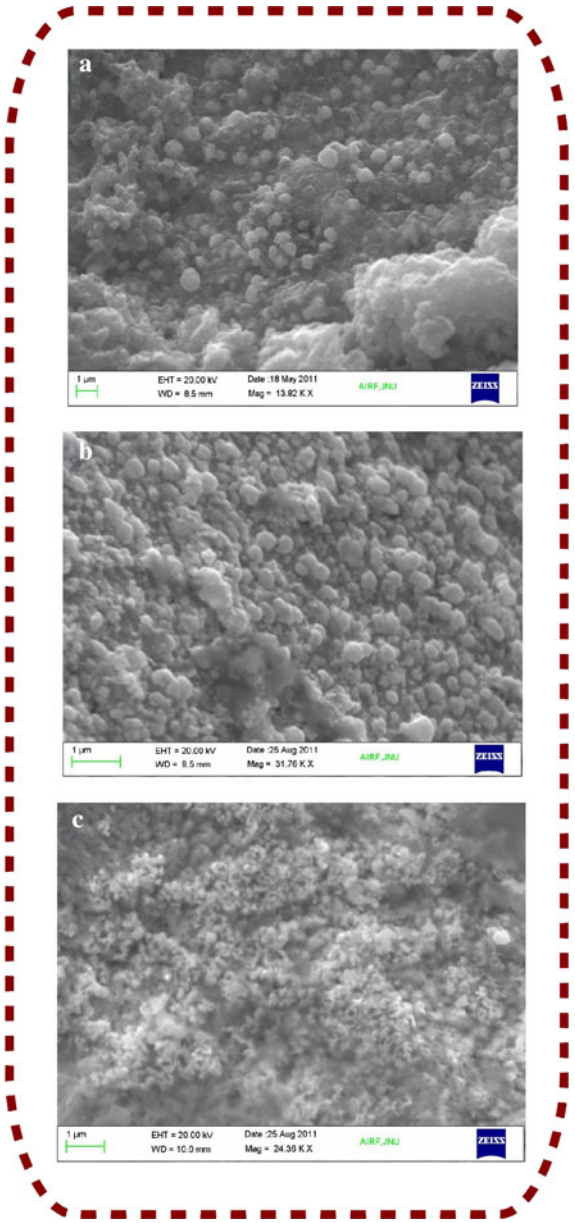


Fig. 2 SEM of **a** bare electrode, **b** Pin5COOH/AuPPy/Au electrode and **c** enzymes/Pin5COOH/AuPPy/Au electrode

enzymes/Pin5COOH/AuPPy/Au electrode in the presence of 20 mM triolein in PBS (0.1 M, pH 7.5). No peak was observed in case of unmodified electrode. The polymer exhibited two characteristic oxidation and reduction peaks in acetonitrile solution. This process could be ascribed to the creation of radical cations and the formation of a very stable polycationic intermediate form [36]. The process, accompanied by deprotonation of the intermediate state, leads to a fully oxidized polymer form [37] and showed that the polymer was stable, conducting and electroactive in aqueous solution in the broad pH range. Nanocomposite provided a conductive path for electron transfer and

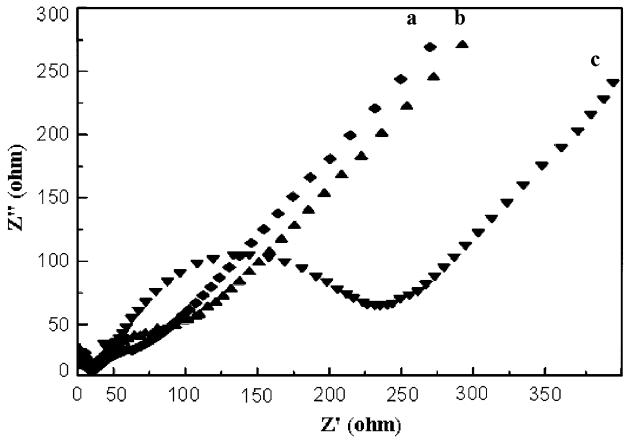


Fig. 3 Electrochemical impedance spectra of *a* AuPPy/Au electrode, *b* Pin5COOH/AuPPy/Au electrode and *c* enzymes/Pin5COOH/AuPPy/Au electrode in PBS (50 mM, pH 6.5, 0.9 % NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. Frequency range: 0.01 Hz–10 kHz

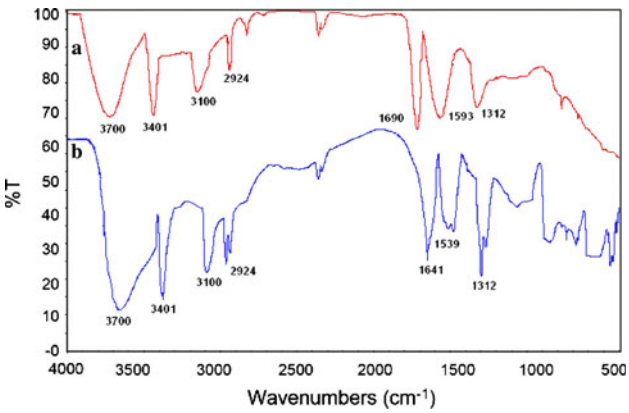


Fig. 4 FTIR spectra of Pin5COOH/AuPPy/Au electrode (*curve a*) and enzymes/Pin5COOH/AuPPy/Au electrode (*curve b*)

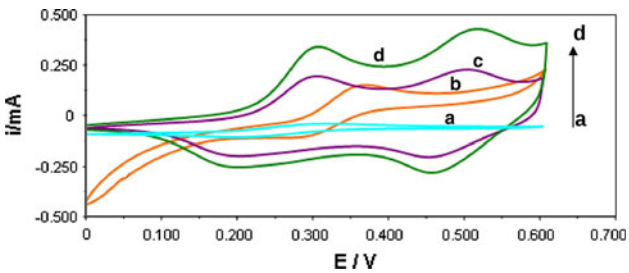


Fig. 5 Cyclic voltammograms for *a* bare Au electrode, *b* AuPPy/Au electrode, *c* Pin5COOH/AuPPy/Au electrode and *d* enzymes/Pin5-COOH/AuPPy/Au electrode in the presence of 20 mM triolein in PBS (0.1 M, pH 7.5)

promoted electron transfer reactions at a lower potential. These results indicated that a slow redox process occurred at the electrode surface due to slow electron transfer from the surface showing the immobilization of enzyme.

Current response measurement of enzymes/Pin5COOH/AuPPy/Au electrode

The electrochemical responses of the enzymes/Pin5COOH/AuPPy/Au electrode were investigated as a function of triolein concentration (50–700 mg/dl) using CV technique at 50 mV/s scan rate in PBS (50 mM, pH 6.5, 0.9 % NaCl) containing 5 mM $Fe(CN_6)^{3-/4-}$. Proposed biochemical reaction at the enzymes/Pin5COOH/AuPPy/Au during triolein detection is shown in Scheme 1b. The generated electrons come via a mediator that transfers to electrode surface through modified electrode and observed response correlated well to response obtained as function of substrate concentrations.

Optimization of the biosensor

Effect of working potential, response time, pH and temperature on the biosensor was observed. The influence of applied potential on current response of enzymes/AuPPy/Pin5COOH/Au modified electrode was studied ranging from 0.0 to +0.5 V versus Ag/AgCl. The optimum response was obtained at +0.1 V versus Ag/AgCl, which ensures a minimizing of interference effects, when the electrode is used in real and complex matrices and oxygen reduction also does not occur. When, the current response was measured from 2 to 12 s at an interval of 2 s, it showed maximum response at 4 s. The effect of pH (ranging from 6.0 to 8.0) onto enzyme electrode in PBS (50 mM) containing 5 mM Fe $(CN_6)^{3-/4-}$ at scan rate of 50 mV/s was studied. The sensor response was found optimum at pH 6.5. The effect of temperature on the biosensor was examined between 20 and 60 °C. The current response of the biosensor increased with increasing temperature and reached a maximum at approximately 35 °C and then went down as the temperature turned higher (figure not given). Supplementary Fig. 1 shows square wave voltammogram of enzyme electrode for different concentration of triolein (50–700 mg/dl). The magnitude of the current response increased with increasing concentration of triolein up to 700 mg/dl. The detection limit of the biosensor was 20 mg/dl ($S/N = 3$). The precision and recovery of the present method were determined over 7 days at −20 °C. Each day seven replicate quality control serum samples were analyzed for TG content. The within- and between-batch precision were 4.14 and 5.85 %, respectively, and mean analytical recoveries of added triolein (10 and 20 mM) in serum sample were 94.3 and 91.3 %. These observations demonstrated that the present method was reliable and reproducible.

To study the accuracy of the present method, TG level in 30 serum samples were determined by both the commercial enzymic colorimetric method using free enzymes (x) and the present method (y). The values obtained by both the methods were correlated with each other. The correlation coefficient (r) was calculated using regression equation ($y = 1.10233x - 0.0639$) and found to be 0.99, showing a good correlation. This correlation is better than those for earlier CA membrane-bound enzyme biosensor (0.91) [38], PVC membrane-bound enzyme biosensor (0.91) [12] and DO metric biosensor (0.97) [39].

The addition of the following serum substances such as urea (1.33 mM), uric acid (0.35 mM), glucose (22.2 mM), fructose (60 mM), ethanol (70 mM), cholesterol (5.71 mM), citric acid (100 mM), ascorbic acid (4.8 mM), lactic acid (0.55 mM), malic acid (0.1 mM), tartaric acid (0.1 mM), alanine (0.1 mM), leucine (0.1 mM), bilirubin (0.25 mM), creatinine (0.08 mM) and pyruvic acid (0.56 mM) evoked a negligible interference (a decrease of only 5 % for urea, 8.2 % for uric acid, 6 % for glucose, 3 % for fructose, 4 % ethanol, 9.5 % for cholesterol, 12 % for citric acid, 2 % for ascorbic acid, 7.1 % for lactic acid,

Table 1 Serum triglyceride level of apparently healthy and diseased individuals as measured by TG amperometric biosensor based on AuPPy composite nanoparticles and poly (indole-5-carboxylic acid) modified Au electrode

Age group ($n = 08$)	Sex	Serum triglyceride (mg/dl) (mean ± SD)	
		Healthy persons	Diseased persons
11–20	M	90.0 ± 10.4	230.0 ± 15.2
	f	88.3 ± 11.6	210.0 ± 10.1
21–30	M	129.2 ± 43.6	394.1 ± 39.5
	f	105.2 ± 22.3	390.0 ± 57.1
31–40	M	136.2 ± 51.2	422.0 ± 68.1
	f	110.3 ± 30.2	420.0 ± 57.2
41–50	M	164.2 ± 54.6	572.8 ± 51.3
	f	142.1 ± 38.9	413.0 ± 35.4
51 and above	M	195.0 ± 43.5	591.6 ± 54.5
	f	184.2 ± 26.1	495.6 ± 58.3
Range	M	90.0–195.0 mg/dl	230.0–591.6 mg/dl
	f	88.3–184.2 mg/dl	210.0–495.6 mg/dl

Table 2 A comparison of various TG amperometric biosensors based on AuPPy composite nanoparticles and poly (indole-5-carboxylic acid)

Type of support for immobilization	Type of electrode	Method of immobilization	Optimum pH	Potential (V) for max. current	Detection limit	Linear range	Response time	Storage stability	References
Porous silicon		Physical	7.0	NR	5.9 mM	5.9–21 mM	15 min	6 months	[15]
Iridium nanoparticles	Carbon	Physical	NR	+0.15	NR	0–10 mM	NR	Disposable	[19]
Polyaniline/ single-walled carbon nanotubes	ITO	Covalent cross-linking	7.4	NR	50 mg/dl	50–400 mg/dl	12 s	3 months	[20]
Gate surface of a FET	ISFET	NR	NR	NR	NR	5–30 mM	<5 min	45 % loss of activity after 2 weeks	[21]
Enzymes/ Pin5COOH/ AuPPy/Au	Au	Covalent immobilization	6.5	+0.1	20 mg/dl	50–700 mg/dl	4 s	7 months	Present

NR not reported

5 % for malic acid, 9 % for tartaric acid, 3 % alanine, 5 % leucine, 4 % for bilirubin, 12 % for creatinine and 8.3 % for pyruvic acid on the biosensor response), comparable to earlier reports [12, 38, 39].

The electrode lost about 50 % of its initial activity after, its 100 uses over 7 months, when stored at 4 °C, which is higher than earlier reported TG biosensors.

Determination of serum TG

TG serum level in apparently healthy adults as measured by the present biosensor ranged from 88.3 to 195.0 mg/dl, which is in established normal range (40–190 mg/dl). The TG level in the serum of persons suffering from hypertriglyceridemia was in the range 210.0–591.6 mg/dl (Table 1).

A comparison of analytical characteristics of recent amperometric TG biosensors with the present one is summarized in Table 2.

Conclusion

An improved amperometric TG biosensor was constructed by covalent co-immobilization of lipase, GK and GPO on AuPPy/Pin5COOH modified Au electrode. The biosensor was highly specific, more rapid (response time 4 s), sensitive (detection limit, 20 mg/dl) and stable (7 months at 4 °C) than earlier biosensors. The electrode was unaffected by a number of serum substances. The improved TG biosensor could be employed for direct measurement of TG in biological materials other than serum. Such an electrode could also be used for improvement of other biosensors.

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