Analytical Biochemistry 517 (2017) 56–63

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

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

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ARTICLE INFO

Article history:
Received 14 October 2016
Received in revised form
11 November 2016
Accepted 17 November 2016
Available online 19 November 2016

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

ABSTRACT

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 1.2 V within 5s, at pH 6.5 and 35 °C.A linear relationship was obtained between current (mA) and triolein concentration in lower concentration range,10–100 mg/dL and higher concentration range, 100–500 mg/dL. Limit of detection (LOD) of bionanosensor was 1.0 μg/ml. Percent analytical recovery of added trolein (50 and 100 mg/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 4 °C.

1. 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 and 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],

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http://dx.doi.org/10.1016/j.ab.2016.11.013
0003-2697/

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.

2. Materials and methods

2.1. 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.

2.2. Instruments

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).

2.3. 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.

2.4. 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.

2.5. 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.

2.6. 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 0.1 ml/min. After desolvation process, 1.8 ml of 2.5% glutaraldehyde (in DW) was added to this suspension and stirred for 24 h, to ensure the formation of aggregates of ENPs. Then, 0.12 g of cysteamine dihydrochloride was added to ENPs suspension and allowed for continuous stirring for 4—5 h to add amide groups on the surface of prepared ENPs aggregates. The resulting ENPs were purified by one cycle of differential centrifugation (15,000×*g*, 10 min) at 4 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 C.

2.7. 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).

2.8. 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.

2.9. Co-immobilization of NPs of lipase, GK and GPO onto Au electrode/preparation of ENPs electrode

Before modification, the surface of Au wire (2 cm × 1 mm) was cleaned with piranha solution (H_2SO_4: H_2O_2 in 3:1 ratio) for 20 min and then rinsed thoroughly with DDW, then polished manually by silica gel followed by washing with DDW and then placed into ethanol for 10 min, 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 °C 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 °C, when not in use. The following reaction occured during the covalent co-immobilization of ENPs onto polycrystalline Au electrode.

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