Biochemical Engineering Journal 125 (2017) 238–245

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

A contrivance based on electrochemical integration of graphene oxide nanoparticles/nickel nanoparticles for bilirubin biosensing

Rachna Rawal [a,*], Nidhi Chauhan [b], Monika Tomar [c], Vinay Gupta [a]

[a] Department of Physics and Astrophysics, University of Delhi, Delhi 110007, India
[b] Amity Institute of Nanotechnology, Amity University, Noida 201303, Uttar Pradesh, India
[c] Physics Department, Miranda House, University of Delhi, Delhi 110007, India

ARTICLE INFO

Article history:
Received 11 April 2017
Received in revised form 26 May 2017
Accepted 10 June 2017
Available online 10 June 2017

Keywords:
Bilirubin
Bilirubin oxidase
Graphene oxide
Nickel
Indium tin oxide coated glass plate

ABSTRACT

An amperometric bilirubin biosensor was fabricated by immobilization of bilirubin oxidase (BOx) on graphene oxide nanoparticles (GrONPs) that was decorated with a thin layer of nickel nanoparticles (NiNPs), and electrodeposited onto the surface of ITO electrode. The enzyme electrode was characterized by scanning electron microscopy (SEM), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) study. BOx/GrONP/NiNPs/ITO/glass was chosen as the bioelectrodes for amperometric detection of bilirubin. The biosensor showed an optimum response at a pH of 8.0 and 35 °C within 2 s when polarized at +0.2 V vs Ag/AgCl. The observed electrocatalytic response showed a linear dependence on bilirubin with concentration varying from 0.01 to 600 μM. The biosensor retained about 80% of its initial activity on being repeatedly utilized over a period of 180 days for about 100 times. The biosensor measured bilirubin levels in sera of apparently healthy and persons suffering from jaundice. The results have been correlated very well with those measured using a standard colorimetric technique (r = 0.99).

1. Introduction

Bilirubin is a tetrapyrrole cytotoxic yellow pigment synthesized by metabolism of heme and is present in the blood as in the form of unconjugated and conjugated fraction. Bilirubin oxidase (BOx) catalyzes the oxidation of bilirubin to biliverdin with the concurrent reduction of oxygen to hydrogen peroxide [1]. It is categorized under multi-copper oxidases such as laccase, ascorbate oxidase and ceruloplasmin. The total serum bilirubin concentration in healthy human beings is 5.13–20.52 and it exists in two forms such as conjugated bilirubin: 0–3.42 μmol/L and unconjugated bilirubin: 3.42–17.1 μmol/L [2]. Serum bilirubin is an essential diagnostic marker for assessing liver function and differentiating various disorders e.g. hepatitis, cirrhosis, gilbert syndrome, thalassemia, sphaerocytosis, heamolytic uremic syndrome and sickle cell deficiency [3]. In this field various methods have been used for the estimation of bilirubin while the most widely recognized methods are the direct spectroscopic estimation and diazo reaction [4,5]. Direct spectroscopic estimation of bilirubin is directly related to the heme proteins while the diazo reaction is pH based [6]. Other investigative techniques for bilirubin determination are polarography [7], enzymatic measure [8], fluorimetric strategy [9], capillary electrophoresis [10], high-pressure liquid chromatography (HPLC) [11], chemiluminescence [12] and piezoelectric effects [13]. These techniques entail expensive apparatus, tedious specimen preparation and skilled manpower to work on these equipments and therefore are not user friendly.

Biosensors are sensitive, simple, fast and require no complicated prior sample preparation. Various amperometric electrochemical bilirubin biosensors have been reported based on the decreased level of O_2 [14], H_2O_2 oxidation [1], Mn(II) mediated electron transfer by utilizing a conductive poly-terthiophene-Mn(II)complex [15], zirconia coated silica nanoparticles/chitosan hybrid film [16] and polypyrrole nanoparticles and polyaniline composite [17]. These biosensors have certain drawbacks including poor electron transfer characteristics and decreased shelf life.

Two-dimensional (2D) materials have traditionally being amongst the foremost extensively studied categories of materials because of their novel physical, chemical and potential applications. 2D network increases the surface area of the electrode and hence enhancing the enzyme loading. Amongst the 2D materials, graphene based matrices are being used recently for constructing nano-scale biosensors. Graphene oxide nanoparticles (GrONPs) have currently emerged as a new carbon – based nanoscale particles that provide an alternate to graphene due to their extraordinary properties like large specific surface area to volume ratio,

* Corresponding author.
E-mail address: rachu_biochem@yahoo.com (R. Rawal).

http://dx.doi.org/10.1016/j.bej.2017.06.006
1369-703X/

low production cost, minimum detection limit and long shelf life [18]. In modern years, a range of metal (Mn, Au, Ag, Pt, Ni, Mg) and metal oxide (CuO, NiO, Co_3O_4) nanoparticles are explored widely in biosensing due to their distinctive properties such as wider surface area, quick mass transport and remarkable chemical action. Amongst, non-noble metals, Ni based nanomaterials display biocompatible properties including excellent durability, electrochemical stability to detect bilirubin efficiently[19].

The present work describes the possibility of exploring the bionanocomposites based on GrONPs, nickel nanoparticles (NiNPs) for the sensor platform fabrication and BOx as the specific enzyme for bilirubin detection. The efforts have also been made to determine the bilirubin content in real serum samples using the fabricated BOx/GrONP/NiNPs/ITO/glass or enzymatic bioelectrode.

2. Materials and methods

2.1. Chemicals and reagents

BOx; 15 IU/mg (*Myrothecium verrucaria*), glutaraldehyde (GA), tris (hydroxymethyl) aminomethane (Tris HCl) and 4-aminophenazone were obtained from Sigma–Aldrich, USA. Horseradish peroxidase (HRP), bilirubin, phenol, hydrazine hydrate solution ($N_2H_4H_2O$), nickel hydrazine (NiN_2H_4), nickel(II) chloride hexahydrate ($NiCl_26H_2O$), potassium ferricyanide $K_3Fe(CN)_6$, potassium ferrocyanide $K_4Fe(CN)_6$ and cysteamine were obtained from Sisco Research Laboratory (SRL), Mumbai, India. All chemical utilized were of analytical reagent grade.

2.2. Device

An Autolab PGSTAT 30 electrochemical workstation (Eco Chemie BV Utrecht, The Netherlands) with the GPES 4.9 programming was used to perform all electrochemical analysis. In all electrochemical investigations ITO/glass, Ag/AgCl and Pt were utilized as the working, reference and counter electrodes respectively. Transmission Electron Microscope images (TEM) and Scanning electron microscope images (SEM) of the prepared sample were obtained using the JEOL 21001 and ZEISS EVO® HD microscopes respectively.

2.3. Assay of free BO_X

BOx assay as explained by Satyapal and Pundir was performed where H_2O_2 produced from bilirubin oxidation with BOx developing a colour reaction comprising of 4-aminophenazone, phenol and horseradish peroxidase (HRP) as the chromogenic system [20]. 0.7 mL of Tris HCl buffer pH 8.5 (0.2 M), 0.1 mL of bilirubin solution (34.21 μM), 0.1 mL of BOx solution (5 U/mL) reaction mixture was incubated at 37 °C for 10 min. For colour production 1 mL of colour reagent was added and incubated in dark at 37 °C for 15 min. A_{520} was studied and from its standard curve, H_2O_2 concentration was calculated.

Following the method reported by Bais et al. [21], colorimetric reagent was prepared by mixing 50 mg of 4-aminophenazone, 100 mg phenol and 1 mg horseradish peroxidase (HRP) in 100 mL of 0.4M sodium phosphate buffer, pH 7.0, and stored in a dark bottle at 4 °C.

2.4. Preparation of GrO

GrO were prepared by Hummer's method with slight alterations [22]. Graphite rod obtained from the HB pencil was crushed to make a fine powder in a pestle- mortar. 0.5 g of graphite powder was first mixed in 23 mL of H_2SO_4 at 0 °C and 0.5 g of $NaNO_3$ followed by adding 10 mL of $KMnO_4$ (2 mM) dropwise. In a water bath, slurry was mixed for 1 h at 35 °C. To this mixture 140 mL of distilled water (DW) was added and kept at 90 °C for 15 min, a light brown colour was developed by infusion of 3 mL of H_2O_2. After centrifugation at 1792 × *g* GrO was obtained.

2.5. Decoration of GrONPs with NiNPs

GrONPs and nickel hydrazine complex using hydrazine hydrate under in situ reducing NiNPs/GrONPs composites were prepared. GrONPs suspension was prepared by the addition of 100 mg of GrO into 50 mL of deionized (DI) water with ultrasonication for 1 h. Ni–hydrazine complex suspension was obtained by adding 20 mL of an aqueous $NiCl_2$ $6H_2O$ solution (0.07 M) and 40 mL of $N_2H_4H_2O$ with vigorous mixing. GrONPs solution was added to this suspension and stirred for 30 min. Afterwards, 20 mL of hydrazine hydrate and 40 mg of NaOH were added into the miscellaneous solution, followed by ultrasonication for 15 min. The mixture was refluxed at 100 °C for 5 h. After cooling at room temperature, the as-synthesized mixture was collected by vacuum filtration, washed with DW and ethanol for three times. The NiNPs/GrONPs nanocomposites were obtained by drying in a vacuum oven at 60 °C for 24 h [23].

2.6. Preparation of GrONPs/NiNPs/ITO/glass bioelectrodes

ITO/glass platform were thoroughly cleaned in piranha solution (H_2SO_4:H_2O_2 = 3:1) for 20 min, followed by rinsing with DW. GrONPs/NiNPs were electrochemically deposited onto ITO/glass platform using cyclic voltammetry (CV) technique by applying potential between −0.2 to +0.6 V (vs. Ag/AgCl) for 25 cycles at a scan rate of 0.02 $V\,s^{-1}$ and immersing the ITO/glass platform into a mixture of 23 mL of 2.5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) and 2 mL of GrONPs/NiNPs solution. The GrONPs/NiNPs/ITO/glass was rinsed thoroughly with DI water and dried in blow of nitrogen air. Amine-terminated self-assembled monolayer (SAM) of cysteamine (10 mM) was created on the surface of GrONPs/NiNPs/ITO/glass by immersing it in one mM ethanol solution of cysteamine for 10 h at room temperature. The sulfur atoms of the molecules were bound to the metallic nanoparticles (NiNPs) surface while the amino groups were employed for the attachment of CHO group of the glutaraldehyde. The resultant monolayer-changed electrode was rinsed totally with DW to get rid of the physically absorbable cysteamine and dried in air [24].

2.7. Preparation of enzyme bioelectrode (BOx/GrONPs/NiNPs/ITO/glass)

The GrONPs/NiNPs/ITO/glass was immersed in 5% glutaraldehyde solution for 1 h to activate the surface of electrodes followed by washing with DW and dried in air. The GrONPs/NiNPs/ITO/glass was dipped in 2.5 mL solution of phosphate buffer (0.1 M, pH 7.0) containing 10 μL of BOx solution (2 mg/mL) of enzyme for 24 h. This resulted in the formation of an amide bond between free $-NH_2$ groups on the surface of enzymes molecule and the free–CHO group of glutaraldehyde. The enzyme bioelectrode was rinsed with phosphate buffer (0.1 M, pH 7.0) after the immobilization of enzyme on cysteamine monolayer. The resulting bioelectrode, was kept at 4 °C when not in use (Scheme 1).

2.8. Construction and response measurement of enzyme bioelectrode (BOx/GrONPs/NiNPs/ITO/glass)

The BOx/GrONP/NiNPs/ITO/glass as the working electrode, together with Ag/AgCl as the reference and Pt as the auxiliary electrode, was associated through a potentiostat/galvanostat to