Detection of glycated hemoglobin with voltammetric sensing amplified by 3D-structured nanocomposites

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ABSTRACT

Glycated hemoglobin (HbA1c), a marker for glycine level in blood, while detecting over a long period of time (up to 2–3 months) shows consistency. Therefore, HbA1c has been mostly used and indeed an established test for monitoring the glycemic control in persons suffering from diabetes. 3D-structured reduced graphene oxide (rGO), multiwalled carbon nanotubes (MWCNT) and platinum nanoparticles (PtNPs) composite (PtNPs/rGO–MWCNT) were synthesized and used as interface for the development of an electrochemical HbA1c biosensor. The network structure of rGO–MWCNT nanocomposite provides more active sites for Pt deposition and the synergistic effect of rGO, MWCNTs and PtNPs significantly improved the electrochemical performance of the working electrode. The structure of PtNPs/rGO–MWCNT nanocomposite was characterized by scanning electron microscopy (SEM), cyclic voltammetry (CV) and electrochemical impedance study (EIS). This biosensor exhibited a response time of less than 3 s, a wide linear concentration range of 0.05–1000 μM with detection limit of 0.1 μM, good repeatability and satisfactory reproducibility. The biosensor retained 50% of its initial response after 12 weeks at 25 °C. The proposed biosensor was successfully applied for the determination of HbA1c concentration in human blood samples with recoveries between 93.7 and 98.3%.

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1. Introduction

Nanocomposites have expectantly an intense research field mainly in biosensor fabrication in most recent years considering their potential applications for bioassays [1,2]. For utilization in the fabrication of biosensors, a wide range of nanomaterials such as gold, silver [3], carbon nanotubes (CNTs), thallium(III) oxide (Tl_2O_3) [4–6], aluminum oxide (Al_2O_3) [7,8], zinc oxide, iron oxide and their composites have been synthesized and characterized by various methods [9–11]. Graphene is a tremendously assorted material, and can be mixed with other elements (which includes gases and metals) to produce different materials with various advanced properties [12]. For instance, graphene oxide (GO) has been widely utilized in the development of various biosensors because of its exceptional properties including excellent water dispersibility, biocompatibility and large surface area [13,14]. Among a range of different nanomaterials, graphene possesses extraordinary structural, mechanical and physicochemical properties [15].

Another important carbon material is carbon nanotubes (CNTs), which are valuable for nanotechnology, electronics, optics and other fields of materials science and technology [16,17]. Structurally multi walled carbon nanotubes (MWCNTs) consist of several layers of graphite superimposed and they are rolled in on themselves to convert into a tubular shape [18]. The CNT and graphene with other metal nanoparticles gives an enhanced or synergetic effects in its properties [19]. Due to the higher stability and large surface area, graphene and CNT hybrid could be a better supporting material to attach the metal nanoparticles [20]. The chemical method is comparatively easy and inexpensive with negligible difficulties to place and align the consequential nanostructures in preferred configurations or patterns. Therefore, herein we utilized GO-MWCNT hybrid as a platform for the decoration of Pt nanoparticles via chemical reduction [21,22]. All these nanomaterials are used in the detection of glycated hemoglobin through enhanced electrochemistry.

Glycated hemoglobin (*hemoglobin A1c, A1C, HbA1c or Hb1c*; is also known as HGBA1C or HbA1c) is a form of hemoglobin which is considered primarily to identify the average plasma glucose concentration over extended or prolonged period of time [23]. It has been observed after it is formed in a non-enzymatic glycation pathway by hemoglobin's disclosure to plasma glucose. HbA1c is a computation of the beta-N-1-deoxy fructosyl component of hemoglobin [24]. HbA1c is defined as hemoglobin which is irretrievably glycated at one or both N-terminal valines of the beta

chains [25]. HbA1c has been the mostly used and accepted test for monitoring the glycemic control in individuals with diabetes. Once a hemoglobin molecule is glycated, it continues to remain in the red blood cell for the rest of its life span (120 days). HbA1c laboratory tests are used to check the control in diabetes mellitus.

In this work, by combining the advantages of PtNPs, rGO and MWCNT nanohybrids were designed and synthesized for the detection of HbA1c. A novel ultrasensitive electrochemical biosensor was fabricated and characterized for the selective detection of HbA1c using fructosyl amino oxidase (FAO) enzyme. The application is toward determination of HbA1c in whole blood samples. The study can provide a promising platform for fabricating nanohybrid bioelectrode.

2. Experimental

2.1. Chemicals and reagents

Fructosyl-amino acid oxidase (FAO, EC:1.5.3, extracted from recombinant *E. coli*), Bovine serum albumin (BSA) and glutaraldehyde (GA) were procured from Sigma–Aldrich, St. Louis, USA. Graphite powder, hexachloroplatinic (IV) acid hydrate ($H_2PtCl_6 \cdot xH_2O$), phenol, horseradish peroxidase, $NaNO_3$, H_2SO_4, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ from Sisco Research Laboratory, Mumbai, India and gold wire (1.5 cm × 0.05 cm) (23 carat) were purchased from nearby market. Carboxylated multi-walled carbon nanotubes (MWCNT or MWCNT) (metal content: nil, 12 walls, length 15–30 mm, purity 95%) from Intelligent Materials Pvt. Ltd., Panchkula (Haryana), India, were used.

2.2. Preparation of substrate

The substrate for HbA1c i.e. fructosyl valine (FV) is not available commercially thus it was synthesized in our laboratory [26]. So 40 ml pyridine and 40 ml acetic acid were mixed together with L-valine (0.16 M, 1.88 g) and then stirred for 30 min at 25 °C. Afterwards, 4 g glucose (0.25 M) was added and purged by using nitrogen for 10 min and again stirred for 3 days at 25 °C. The dark colored product was separated out by filtering and the solvent was left for evaporation. By the process of re-crystallization in methanol, a yellowish amorphous product was obtained [26].

2.3. Preparation of graphene oxide

Graphene was synthesized by the modified Hummers method [27]. 3.33 g of natural graphite and 1.66 g of $NaNO_3$ were added to 76.66 ml of concentrated sulfuric acid under stirring in a flask immersed in an ice water bath. Then 10 g of $KMnO_4$ was added slowly and the mixture was stirred at 30 °C for 2 h. Next 153.33 ml of distilled water was added and the mixture was further stirred for 30 min at 95 °C. Finally, 313.33 ml of distilled water and 10 ml of H_2O_2 (5%) were subsequently added to terminate the reaction and the color of the solution turned from dark brown to yellow. The generated solid graphite oxide (GO) was separated by centrifugation then washed and lastly dried under vacuum [28]. This GO was further reduced to rGO by using hydrazine monohydrate.

2.4. Assembly of rGO–MWCNT hydrogel

The material was prepared by the addition of 2 mg of MWCNT to a 4 mg/ml homogenous rGO aqueous dispersion (the proportion of rGO to MWCNT was 2:1) under sonication for approximately 30 min. The mixture was subsequently sealed in a Teflon Lined Autoclave at 150 °C for 12 h. After the mixture was cooled in room temperature air with natural convection, a black gel like 3D cylinder substance was obtained. The size of the hydrogel could be freely adjusted by changing the volume of the rGO aqueous dispersion [29,30].

2.5. Biosensor fabrication

Piranha solution is prepared. It is a mixture which contains three parts of concentrated sulfuric acid and one part of 30% hydrogen peroxide solution in a ratio of 4:1. It should always be prepared by adding hydrogen peroxide to sulfuric acid very slowly, never in reverse [31]. Mixing the solution is extremely exothermic. For the preparation of rGO–MWCNT/Au electrode, 5.0 ml of rGO-MWCNT dispersion was used as electrolyte. A conventional three-electrode cell was used, including an Au as the working electrode, a Ag/AgCl electrode as the reference electrode, and platinum wire as the counter electrode. Cyclic voltammetry (CV) was performed by scanning between 0 and −1.0 V at a scan rate of 50 mV s^{-1} [32]. Then Au modified by rGO-MWCNT hybrids was taken off and rinsed with water three times lightly.

The electrochemical deposition of PtNPs was conducted using three-electrode system. The rGO-MWCNT/Au electrode was modified with PtNPs by using cyclic voltammogram with potential ranging from −0.2 to 0.7 V in a precursor solution consisting of 5 mM Potassium ferricyanide and K_2PtCl_6 for 10 cycles. The nanoparticles were controlled on the working electrode by changing the scan rate.

The glutaraldehyde (GA, 0.25 wt%), bovine serum albumin (BSA, 0.5 mgml^{-1}) and FAO (30 Uml^{-1}) were assorted together properly (all of these materials were dissolved in phosphate buffer, pH = 7.0), then they were allowed to drop onto an PtNPS/rGO–MWCNT/Au electrode with 5 μl of total amount. The biosensor will be achieved after the finish of cross-linking reaction during a period of stillness in a refrigerator at 4 °C (Scheme 1).

All the electrochemical analysis were performed by an potentiostat/galvanostat (Biologics, SP-150) with a typical three-electrode association composed of an Ag/AgCl (3 M KCl) reference electrode, a platinum auxiliary electrode and FAO/PtNPS/rGO–MWCNT/Au electrode as working electrode. We optimized all of parameters that robustly influenced the electrochemical performance of HbA1c. Simultaneously, the detection conditions like upper/lower limiting potential were also optimized.

2.6. The detection of HbA1c in whole blood samples through the fabricated biosensor

The healthy individuals and diabetes patients provided blood samples which were collected with EDTA as anticoagulant from Bio Diagnostics Laboratory, Rohini, New Delhi. In order to mix the blood cells, a mild inversion was applied. The mixing of 30 μl of whole blood in 300 μl of lysis buffer was carried out to check proper hemolysis and afterwards incubated for 10 min. Furthermore, the cells were lysed by using lysis buffer consisting 100 mM CHES (pH 8.7), 0.45% SDS, 1% Triton X-100 and 0.5 mM of Dess–Martin periodinane. A proteolytic digestion was performed of human blood samples by mixing of a solution including oxidizing agent (1 mM), 4 U/ml purified *Bacillus sp.* Proteases and 5 mM MES. This proteolytic digestion leads to the release of certain amino acids including glycated valines from the hemoglobin beta chains which then serves as substrates for the FAO and consequently produces H_2O_2 by combining 0.1 M sodium phosphate buffer (pH 7.5), 90 U/ml peroxidase and 0.8 mM of 4-amino anti pyrine which breaks N-terminal amino acids either histidine or valine [33,34]. The numbers of current produced for detection is principally based upon splitting of H_2O_2 released by the oxidation of substrate by immobilized enzyme (FAO), which is evidently proportional to the HbA1c concentration.