Sensors and Actuators B 197 (2014) 164–171

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Immobilizing haemoglobin on gold/graphene–chitosan nanocomposite as efficient hydrogen peroxide biosensor

Lingling Zhang, Guangqiang Han, Yun Liu, Jian Tang, Weihua Tang*

Key Laboratory of Soft Chemistry and Functional Materials, Ministry of Education, Nanjing University of Science and Technology, Nanjing 210094, PR China

ARTICLE INFO

Article history:
Received 18 January 2014
Received in revised form 18 February 2014
Accepted 20 February 2014
Available online 2 March 2014

Keywords:
Haemoglobin
Graphene
Chitosan
Gold nanoparticles
Direct electron transfer

ABSTRACT

A method is reported for the preparation of novel electrode by layer-by-layer assembly of haemoglobin (Hb), gold (Au) nanoparticles, chitosan (CS) and graphene (GR) onto glassy carbon electrode (GCE). The Au/GR–CS substrate shows an obvious promotion for the direct electron transfer between Hb and GCE. The surface concentration (Γ^*) of Hb on the as-modified electrode can be as high as 4.33×10^{-9} mol/cm^2, which is 228 times higher than the theoretical value for Hb monolayer on bare GCE. The Au/GR–CS/GCE shows good electrocatalytic performance for the reduction of hydrogen peroxide within a linear range from 2 to 935 μM, a low detection limit of 0.35 μM (S/N = 3) and high sensitivity of 347.1 mA cm^{-2} M^{-1}. The Au/GR–CS nanocomposite demonstrates as a versatile matrix for immobilizing redox protein to realize direct electrochemistry and hence prepare efficient and robust biosensor.

1. Introduction

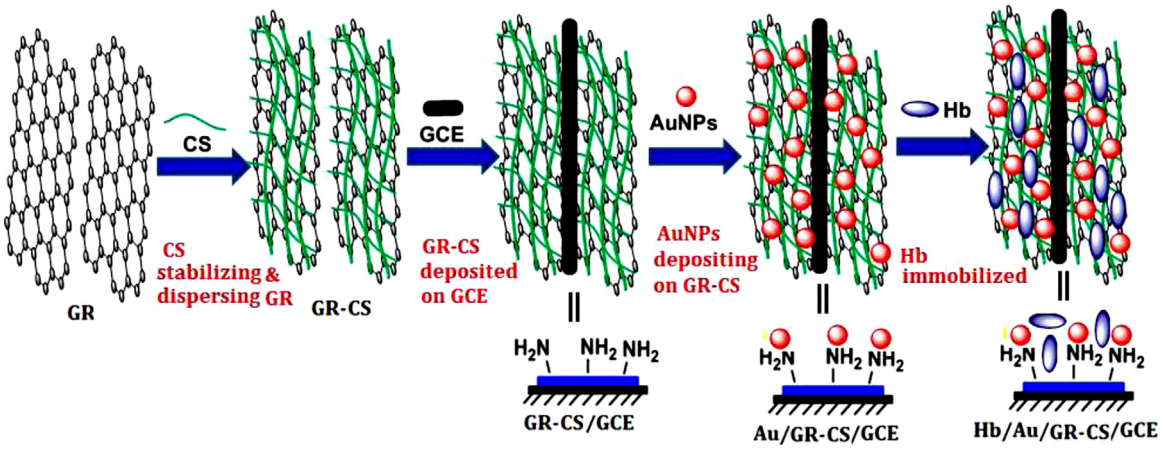
The catalytic process and chemical properties of redox proteins with mediator are very complex, hence the investigation about the direct electron transfer (DET) of redox proteins has attracted much interest in recent years [1]. However, the embedment of the electroactive centres within protein structure makes it very difficult to detect a protein when the denaturalization or the unfavourable orientation of proteins occurs on electrode interface [2,3]. As a well-characterized protein, haemoglobin (Hb) has intrinsic peroxidase-like activity and could be an alternate for horseradish peroxidase, specifically for improving the biosensor performance and reducing the fabrication cost. Hb has thus been used extensively as electron transfer in studying the DET of redox proteins [4]. In order to enhance the Hb-based biosensors' performance, Hb was generally immobilized onto various conductive matrices [4–11].

Graphene (GR), a two-dimensional single-atom-thick carbon network, has attracted intensive interest recently due to its unique structure and properties, such as large surface area, high electrical conductivity and mechanical strength [12,13]. GR has found ever-increasing application in electrochemical biosensors [14,15], featuring high sensitivity with effective GR-promoted electron transfer of redox enzymes on electrodes [16–18]. GR can be prepared by both physical and chemical methods, with the former including micromechanical cleavage and liquid-phase exfoliation while the latter including chemical vapour deposition, epitaxial growth and reduction of graphene oxide (GO). The GO reduction approach was beneficial to introduce special functionalities onto the GR nanosheets. However, the reduced GO tends to aggregate and even restack to form graphite due to π–π stacking interactions and van der Waals [19]. To minimize or even inhibit the aggregation of GR nanosheets, polymers [5–8] or nanomaterials [9–11] were used to form GR hybrid composites. As a naturally occurring polymer, chitosan (CS) has been universally used to disperse nanomaterials and immobilize biomolecules for constructing biosensors owing to its biocompatibility, nontoxicity, good water permeability and excellent film-forming ability [20]. The GR–CS hybrid films were prepared to immobilize Hb for DET [21]. The GR–CS film was used to fabricate glucose sensor by immobilizing glucose oxidase [22]. When used to disperse GR, CS can provide a good biocompatible microenvironment for proteins or enzymes [23,24]. As a widely used nanomaterial, gold nanoparticles (AuNPs) are advantageous to fabricate electrochemical biosensors because of their unique properties in facilitating DET between redox-active proteins and electrode and providing benign microenvironments for immobilizing biomacromolecules [11]. By depositing a layer of gold nanoparticles (AuNPs) onto GR-NH_2/electrode, the AuNPs/GR-NH_2 nanocomposite enhanced the DET from catalase to electrode [25]. The AuNPs/GR electrode can thus be used as biosensor to detect the electrocatalytic response to the reduction of H_2O_2 [26].

Inspired by the above-mentioned research, it is wondering to us whether we can prepare Au/GR–CS ternary nanocomposites to enhance the DET in electrochemical process for ultrasensitive

* Corresponding author. Tel.: +86 25 8431 7311; fax: +86 25 8431 7311.
E-mail address: whtang@njust.edu.cn (W. Tang).

http://dx.doi.org/10.1016/j.snb.2014.02.077
0925-4005/



Scheme 1. Lay-by-layer assembly preparation of Hb/Au/GR–CS/GCE.

biosensors. By stabilizing GR nanosheets and absorbing AuNPs with the abundant positive charge of $-NH_3^+$ groups in CS [27], a novel Au/GR–CS ternary composite modified glassy carbon electrode (GCE) is herein constructed to demonstrate the direct electrochemistry by taking full advantage of the synergetic interactions between GR, CS and AuNPs (Scheme 1). By immobilizing Hb on Au/GR–CS/GCE, the as-prepared Hb/Au/GR–CS/GCE demonstrated excellent electrocatalytic ability to H_2O_2 with the larger surface concentration and wider linear range than the values obtained from those Hb-based biosensors in literature. The Au/GR–CS nanocomposite film thus demonstrates as a promising platform for the development of robust high-efficiency electrochemical biosensors.

2. Experimental

2.1. Materials

Hb (M_w = 68,000) was purchased from Shanghai Jinsui Biotechnology Co. Ltd (Shanghai, China) and used without further purification. Graphite powder and CS were purchased from Sinopharm Chemical Reagent Co., Ltd. $HAuCl_4{\cdot}4H_2O$ and 30% H_2O_2 solution were purchased Nanjing Chemical Reagent Co., Ltd. All other chemicals were of analytical grade, and distilled water was used in all experiments. Phosphate buffer solution (PBS, 0.1 M, pH 7.0) was used as supporting electrolyte.

2.2. Preparation of GR and AuNPs

GR was prepared with the reduction of GO according to the reported methods [28]. Graphite powder (5 g) was added to a mixture of concentrated H_2SO_4 (12 mL), $K_2S_2O_8$ (2.5 g) and P_2O_5 (2.5 g), then the mixture was heated to 80 °C and kept to react for 6 h. After cooling to room temperature, the resulting mixture was diluted with water. The pre-oxidized graphite was obtained after filtration. The pre-oxidized graphite (2 g) was re-oxidized by putting into 0 °C concentrated H_2SO_4 (46 mL) with gradually addition of $KMnO_4$ (6 g) and kept the temperature under 20 °C using ice bath. The mixture was kept to react at 35 °C for about 2 h and diluted slowly with water (250 mL) and H_2O_2 (4 mL). After filtration and washing with aq. HCl (10 wt%) and water, the as-obtained GO powder was dried in vacuum at 60 °C for overnight. The suspension of GO (0.1 g) in water (200 mL) was added with hydrazine aq. solution (1 mL, 85 wt%) and stirred at 80 °C for 24 h. The black precipitates were filtered and washed with water before drying to obtain GR.

AuNPs were prepared by the citrate reduction method as previously reported [29]. Briefly, $HAuCl_4$ solution (2 mL, 1%) was diluted in water (200 mL) and then heated to boiling with continuous stirring. After the addition of trisodium citrate solution (4 mL, 1%), the solution was kept continually boiling for another 20 min to give a wine-red solution. The mixture was cooled to room temperature under continuous stirring. The solution was then stored in refrigerator at 4 °C for use. The average size of the as-prepared AuNPs was measured as about 20 nm.

2.3. Fabrication of modified electrodes

Polished to a mirror-like surface with alumina slurry (0.3 and 0.05 μm) and sonicated in water and acetone bath, the GCEs were dried at room temperature for use. The Au/GR–CS/GCE was fabricated using a layer-by-layer deposition method. CS aq. solution (0.2 wt%) was prepared by dissolving CS (0.2 g) in acetic acid (100 mL, 1 wt%) with its pH adjusted to 5.0 using concentrated NaOH. The CS-dispersed GR suspension was prepared by dispersing GR (5 mg) in CS solution (10 mL) under ultrasonication. Hb (10 mg/mL) solution was prepared with PBS (50 mM). The GR–CS/GCE was first prepared by dropping the CS-dispersed GR suspension (5 μL) onto GCE surface and drying in air. The electrode was then immersed in Au solution for 4 h to capture Au. The as-prepared Au/GR–CS/GCE was thus washed with water and dried in air. The Au/GR–CS/GCE was further surface-modified by dropping with Hb solution (5 μL) and dried at 4 °C. The as-modified electrode was then immersed in PBS to remove the un-bounded Hb to obtain the *title* Hb/Au/GR–CS/GCE.

For comparison, Hb/GR–CS/GCE, Hb/GCE, Au/GR–CS/GCE and GR–CS/GCE were prepared with the same procedures. The CS/GCE was also prepared by dropping CS solution (5 μL) on the surface of bare GCE. The as-fabricated modified electrodes were stored at 4 °C in refrigerator when not in use.

2.4. Characterization

UV–visible spectra were obtained on a UV-1800 spectrophotometer (Shimadzu). Fourier transform-infrared spectra (FT-IR) were recorded on KBr disc using an IR Tensor 27 spectrometer (Bruker, Germany). Field emission gun scanning electron microscopy (FE-SEM) analyses were conducted on a Hitachi S4800 FESEM at an accelerating voltage of 15 kV. The transmission electron microscopy (TEM) analyses were taken with a JEOL JEM-2100 microscope.

Electrochemical experiments were carried out with a CHI660D electrochemical workstation (Chenhua, China) in a conventional three-electrode cell. The three-electrode system was composed of