Sensors and Actuators B 258 (2018) 141–147

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

A novel immunoprobe composed of reduced graphene oxide-hemin-thionin-Au nanohybrid for ultrasensitive detection of tumor marker

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ARTICLE INFO

Article history:
Received 28 June 2017
Received in revised form 24 October 2017
Accepted 17 November 2017
Available online 21 November 2017

Keywords:
Neuron specific enolase (NSE)
Electrochemical immunosensor
Biomarker
Thionin
Hemin

ABSTRACT

An ultrasensitive electrochemical immunosensor was fabricated by employing reduced graphene oxide-thionin-hemin-Au (H-rGO-Thi-Au) nanohybrid as probe to measure neuron specific enolase (NSE), a lung cancer biomarker. In this work, the reduced graphene oxide (rGO) was used as support material to immobilize hemin and thionin through aromatic π-π interaction. Then Au nanoparticles were reduced by thionin in situ on the probe. The immunoprobe offers several advantages as follows: (1) thionin can be enriched for signal amplification; (2) the catalysis ability of hemin can be enhanced by preventing the molecules aggregation; (3) the reduced graphene oxide-hemin (H-rGO) acts as signal amplifier to accelerate the redox cycling with H_2O_2; (4) the composite with good conductivity is beneficial to improve response current. Apart from that, polyaniline hydrogel as substrate prepared by electrochemical polymerization on the glassy carbon electrode showed excellent conductivity which can further improve the performance of immunosensor. Thus, the proposed immunosensor showed excellent analytical performance for the detection of NSE with wide linear range from 0.1 pg mL^{-1} to 100 ng mL^{-1} and low detection limit of 0.026 pg mL^{-1}. It is also of note that human serum samples were determined with satisfactory results, indicating its value on clinical application.

1. Introduction

Tumor markers can give information about the stages of the corresponding tumors and it is useful in the assessment of early diagnosis and treatment of cancers [1,2]. For example, neuron specific enolase (NSE) has been identified as the indicator of small cell lung cancer and has been used for early detection, diagnosis and monitoring of cancer [3–5]. Thus, sensitive measurement of tumor markers is a critical requirement. Recently, considerable efforts have been made to develop a sensitive and reliable detection methodology for exact quantitative analysis of tumor markers such as enzyme linked immunosorbent assay, electrochemiluminescence immunoassay, photoelectrochemical immunosensor and electrochemical immunosensor [6–8]. Among these methods, the sandwich-type electrochemical immunosensor has been widely applied for its simple pretreatment, ease operation, high specificity and sensitivity [9–11]. For sandwich-type immunosensor, the performance was tightly relied on the immunoprobes which can generate signals. In this case, the development of novel immunoprobes with outstanding performance to sensitively detect tumor markers was essentially urgent.

Such immunoprobes can be designed with the following considerations: (1) good conductivity for quick electron transfer; (2) high concentration of redox species on the electrode surface [12,13]; (3) appropriate catalysis for signal amplification [14]. For the purpose of these instructions, varieties of nanomaterials with catalytic performance have been used to enhance the performance of immunoprobes, such as carbon-based nanomaterials (NPs), metal oxides, noble metal [15–17]. Taking the advantage of large specific surface area and favorable electronic properties, the reduced graphene oxide (rGO) have led to much excitement in the construction of immunoprobes [18]. For example, rGO can act as carrier to immobilize redox species to amplify the signal; the excellent electron transfer ability can be used to improve the conductivity of the probe [19]; it can be used to improve the stability and performance of nanomaterial and even produce new functions [20]. In addition, the multistep modification based on rGO can be achieved easily by interfacing with functional aromatic molecules through noncovalent functionalization such as thionin, hemin, methylene blue and so on [21,22]. Thus, the rGO was introduced into the novel immuno-

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https://doi.org/10.1016/j.snb.2017.11.085
0925-4005/

probe with multiple functions for ultrasensitive detection of tumor markers.

In this work, a novel multifunctional-probe composed of reduced graphene oxide-hemin-thionin-Au nanohybrid was farbricated simply. The advantages of as-prepared probe are as follows: (1) rGO and AuNPs with excellent conductivity is helpful for electron conduction; (2) large numbers of redox species can be easily immobilized on rGO; (3) antibody can be captured directly by AuNPs; (4) the catalytic ability of hemin greatly enhanced by rGO [23]; (5) the peak current can be amplified significantly for the prefect catalytic activity of the probe. A sandwich type immunosensor was fabricated based on the proposed immunoprobe and the polyaniline hydrogel (PANI) subustrate for NSE. Under the optimum conditions, the proposed immunosensor showed excellent analytical performance for the detection of NSE with wide linear range from 0.1 pg mL^{-1} to 100 ng mL^{-1} and low detection limit of 0.026 pg mL^{-1} (S/N = 3). Moreover, human serum samples were evaluated by the developed immunosensor with a satisfactory result, indicating its value on clinical application.

2. Experimental

2.1. Materials and reagents

Thionine acetate, ascorbic acid (AA), hemin (ferriprotoporphyrin IX chloride, 98 wt%) and hydrogen tetrachloroaurate hydrate ($HAuCl_4 \bullet XH_2O$, 99.9%) were purchased from Alfa Aesar. Graphene oxide was obtained from JCNANO (Nanjing, China). Neuron specific enolase (NSE), NSE antibody, carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) were obtained from Shanghai Linc-Bio Science Co. Human immunoglobulin G (IgG) was obtained from Chengwen Biological Company (Beijing, China). Bovine serum albumin (BSA) was purchased from BJXJKSW. Human serum samples were obtained from Beijing GENIA Biotechnology Co. Ltd. Human serum albumin (HSA) was purchased from Sigma. Urea acid (UA), glucose (Glc) and dopamine (DA) were purchased from Beijing Chemical Reagents Company (Beijing, China). Ultrapure Water (18.25 MΩ) was used throughout the experiments.

2.2. Apparatus

All electrochemical measurements were performed on a CHI832 electrochemical workstation (Chenhua Instruments Co., Shanghai, China). A three-electrode system was used for electrochemical measurements: a glass carbon electrode as working electrode, an Ag/AgCl electrode as reference electrode and a platinum wire as counter electrode. X-ray photoelectron spectroscopy (XPS) was obtained on an Escalab 250 X-ray Photoelectron Spectroscope (Thermofisher, American).Scanning electron microscope (SEM) images and energy disperse spectroscopy (EDS) were carried out with a Hitachi SU8010 SEM. The transmission electron microscopy (TEM) images were obtained with a Hitachi (H7650, 80 kV) TEM. The Raman spectrometer (RenishawInVia) was calibrated by a silicon wafer at 520 cm^{-1} Raman shift before SERS measurement. The water used was purified through an Olst ultrapure K8 apparatus (Olst, Ltd., resistivity = 18.2 MΩ).

2.3. Synthesis of the reduced graphene oxide-hemin

One-step green microwave synthetic approach was developed for the synthesis of hemin modified reduced graphene oxide (H-rGO) [24]. Briefly, graphene nanosheets dispersion was prepared by ultrasonic dispersion for 30 min. 2 mL of the homogeneous graphene oxide dispersion (1 mg mL^{-1}) was mixed with 2 mL of 0.5 mg mL^{-1} hemin aqueous solution and 20 μL of 0.1 M NaOH were introduced to this solution and ultrasonic dispersion for another 30 min following irradiated under microwave for15 min. The H-rGO nanohybrids can easily be further processed by centrifuged at 16000 rpm for 15 min. Then it was dispersed in the PBS to reconstruct a stable and uniform emulsion for further experiments.

2.4. Synthesis of the H-rGO-Thi –Au nanoparticles

Different amount of $HAuCl_4$ solutions were added into the mixture of rGO-HN nanohybrids and thionin (1.0 mM). The obtained mixture was stirred slowly for 3 h at 40 °C. Then the nanohybrids were collected by centrifugation at 6000 rpm. Finally, the obtained precipitation was dispersed in PBS and stored at 4 °C.

2.5. Synthesis of the H-rGO-Thi-Au-Ab_2 labels

Briefly, the diluted Ab2 was added into H-rGO-Thi-Au suspension and stirring carefully for 12 h at 4 °C. Then the obtained anti-NSE-H-rGO-Thi-Au was dispersed in 1% BSA solution for 1 h at room temperature to block possible remaining active sites of the AuNPs and avoid the nonspecific adsorption of the AuNPs [25].

2.6. Fabrication of the immunosensor

Firstly, the glassy carbon electrode (GCE) with a diameter of 4 mm was polished carefully and then washed thoroughly to get a mirror-like surface. The electrochemical polymerization of PANI hydrogel on the clean surface of bare glass carbon electrode can be achieved by applying a constant potential at 1.0 V for 40 s. The electrolyte was prepared by mixing 0.5 mL phytic acid, 0.3 mL aniline and 19.2 mL KCl solution. After being washed several times, AuNPs were electroplated on the surface of the modified electrode by applying potential cycling from −1.0 V to 0.2 V at a scan rate of 50 mV s^{-1} in1 mM $HAuCl_4$ solution (containing 0.1 mM KCl). After deposition, the modified electrode was rinsed with deionized water. Subsequently, Ab_1 (200 μg mL^{-1}) was added on to the modified electrode and incubated in the refrigerator at 4 °C overnight. BSA was used as blocking buffer to prevent nonspecific bonding effects. Afterwards, the immunosensor was incubated with NSE samples with different concentration or different human serum samples (80 μL) for 45 min at 37 °C. Subsequently, 40 μL of the prepared immunoprobes were added onto the substrate and put the immunosensor in an incubator for immune reaction at 37 °C. The electrochemical signal was measured by an electrochemical workstation in PBS (pH 7.0) with 1 mM H_2O_2, which gave the quantitative criteria for electrochemical detection of NSE.

3. Results and discussion

3.1. Choice of materials

In sandwich-type amperometric immunosensors, the signal can be amplified by improving conductivity, enriching redox species, introducing appropriate catalysis or enlarging the surface area. Based on these considerations, the graphene oxide was taken as the supporting material for its high conductivity, large special surface and two-dimensional planar structure. Hemin, with peroxidase-like activity, was chosen as the catalyst. Thioin was selected as signal species for its excellent good conductivity and high electrochemical signal [26]. This arrangement was made with the following concern: (1) both of thionin and hemin are aromatic molecules that can be loaded on the surface of rGO based on π-stacking interaction; (2) the enrichment of thionin can be achieved due to strong interaction with the graphene; (3) the current response of thionin can be significantly increased due to the catalytic amplification of hemin; (4) the catalytic ability of hemin for H_2O_2 can be enhanced due to immobilization of rGO which