Analytical Biochemistry 540–541 (2018) 1–8

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Label-free electrochemical immunoassay for neuron specific enolase based on 3D macroporous reduced graphene oxide/polyaniline film

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ARTICLE INFO

Keywords:
Label-free
Three dimensionally macroporous materials
Reduced graphene oxide/polyaniline film
Neuron specific enolase

ABSTRACT

The content of neuron specific enolase (NSE) in serum is considered to be an essential indicator of small cell lung cancer (SCLC). Here, a novel label-free electrochemical immunoassay for the detection of NSE based on the three dimensionally macroporous reduced graphene oxide/polyaniline (3DM rGO/PANI) film has been proposed. The 3DM rGO/PANI film was constructed by electrochemical co-deposition of GO and aniline into the interspaces of a sacrificial silica opal template modified Au slice. During the co-deposition, GO was successfully reduced by aniline and PANI could be deposited on the surfaces of rGO sheets. The ratio of rGO and PANI in the composite was also optimized to achieve the maximum electrochemical performance. The 3DM rGO/PANI composite provided larger specific surface area for the antibody immobilization, exhibited enhanced conductivity for electron transfer, and more important was that PANI acted as the electroactive probe for indicating the NSE concentration. Under the optimal conditions, a linear current response of PANI to NSE concentration was obtained over 0.5 pg mL^{-1}-10.0 ng mL^{-1} with a detection limit of 0.1 pg mL^{-1}. Moreover, the immunosensor showed excellent selectivity, good stability, satisfactory reproducibility and regeneration, and was employed to detect NSE in clinical serum specimens.

Introduction

Lung cancer is the most frequent cancer in the world, both in terms of incidence and mortality. Reflecting different clinical behavior and sensitivity to chemo- and radiotherapy, lung cancers can be grouped in two major histological types, i.e. non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [1]. About 20% of all lung cancer patients suffer from SCLC, which is diagnosed in about half of the patients at stage of limited disease and extended disease. Recent clinical trials employing concurrent chemoradiotherapy showed relatively high long-term survival rates in patients with limited disease-SCLC [2]. Accordingly, sensitive and reliable tumor makers will provide useful information for effective treatment of SCLC [3]. Neuron-specific enolase (NSE) is a sensitive, specific, and reliable tumor marker for SCLC at the time of diagnosis [4–6]. Therefore, the determination of NSE level is of great importance to process the early diagnostic and prognostic values for monitoring the SCLC state.

Electrochemical immunosensors, based on the specific antigen–antibody recognition, have gained wide interest in detection and quantification of tumor markers [7,8]. Recently, increasing interests have been focused on label-free electrochemical immunosensor due to their rapid recognition, simple fabrication and operation [9–12]. As for the construction of a label-free immunosensor, the crucial step is the efficient and effective immobilization of antibodies onto the electrode surface [13]. In recent years, nanostructured materials have been developed rapidly to promote the progress of immunosensors due to their good biocompatibility, large surface area, excellent electrocatalytic activity, fascinating conductivity [14–16].

As a two-dimensional single atom thick of carbon material, graphene has become a material of interest for electrode surface modification due to its outstanding electrical conductivity, high mechanical strength and large surface area. Zhao et al. developed an electrochemical immunosensor based on ionic liquid functionalized graphene was used to anchor primary carbohydrate antigen 15-3 antibody [17]. Ozcan et al. designed a label free immunosensor for determination of HSP70 (heat shock protein 70), and AntiHSP70 as a biorecognition element of the biosensor was covalently immobilized onto the graphene oxide layer by using EDC/NHS chemistry [18]. Comparing with the

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http://dx.doi.org/10.1016/j.ab.2017.10.009
Received 18 April 2017; Received in revised form 24 August 2017; Accepted 11 October 2017
Available online 04 November 2017
0003-2697/

two-dimensional graphene sheet, three dimensionally macroporous reduced graphene oxide (3DM rGO) has become an ideal material for modification of electrode because of its excellent physical and chemical properties, such as large specific surface area, excellent conductivity, and good mechanical performances. He et al. described the use of 3D graphene networks, loaded with MnO_2 by electrodeposition, as the electrodes of a flexible supercapacitor [19].

One problem associated with graphene-based electrode modification is the potential for self-agglomeration of pure graphene on the electrode surface. One solution is use of a nanocomposite consisting of conducting polymer and graphene to increase the distribution of graphene [20]. Polyaniline (PANI) is a promising conducting polymer for antibody immobilization owing to its facile synthesis, high conductivity, low toxicity, high environmental stability and reversible redox behavior [21,22]. Lin et al. presented an electrochemical immunosensor by employing Fe_3O_4/polyaniline layer as sensor platform. Anti-PAH binding occurred through electrostatic interaction between oppositely charged amino groups on PANI and carboxylic groups on antibodies [23]. When PANI is combined with graphene, the composites exhibit enhanced high performance, such as electrical conductivity and electrochemical stability, compared with pure PANI [24]. The composite materials of graphene and PANI could overcome the drawbacks of graphene and PANI, and lead to good performance in immunosensors. Graphene/PANI composite can act as a suitable matrix for immobilization of biomolecules and mediator for redox reactions, which exhibits impressive signal amplification and antifouling properties [25,26]. In previous studies, graphene/PANI nanocomposites with different morphologies have been used for various electrochemical applications [27–31]. Dong et al. reported a free-standing and flexible supercapacitor electrode prepared by in-situ polymerization of aniline monomers on 3D graphene foam. Such 3D graphene/PANI hybrid exhibited a high specific capacitance [32]. The 3DM rGO/PANI film nanostructure showed a well-defined and cross-linked 3D porous structure and a remarkably high specific surface area. And 3D structure provides a highly conductive network for charge transfer due to the high intrinsic conductivity. However, there are still no reports related to the 3DM rGO/PANI nanocomposites for the construction of electrochemical immunosensor.

In this paper, NSE was chosen as a tumor marker model to evaluate the electrochemical immunoassay. We fabricated a lable-free immunosensor for the determination of NSE, based on 3DM rGO/PANI film as the sensor platform. Here, 3DM rGO/PANI film was constructed by one-step electrochemical deposition in a HCl solution containing aniline (AN) and graphene oxide (GO). The proposed immunosensor has three advantages over other routine methods: (1) the film with 3DM structure provided a large accessible surface area; (2) the high intensity of amino group in PANI and carboxyl group in rGO ensured sufficient antibodies immobilized onto its surface; (3) the streptavidin (SA)-biotin (bio) complex could further enhance the coupled amount of antibodies on the electrode surface.

Experimental section

Reagents

Graphite was purchased from Sigma-Aldrich (Shanghai, China). Au electrodes were provided by Shanghai Institute of Microsystem And Information Technology (Shanghai, China). Biotinylated antibody (Bio-Ab) and NSE were supplied by Shanghai Yuanmu Bio-tech Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA, 99%) and SA were purchased from Baoman Bio-tech Co., Ltd. (Shanghai, China). The monodispersed silica spheres with the diameter of 500 nm were obtained from Alfa Asear. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were from Sigma-Aldrich. AN was distilled twice under reduced pressure and stored in the dark at low temperature before use. Phosphate buffer saline (PBS, 0.1 M) with various pH values was prepared by mixing a stock standard solution of NaH_2PO_4 and Na_2HPO_4, which was used as electrolyte for all electrochemistry measurement. The washing buffer (PBST) was PBS (0.1 M, pH 7.5) containing 0.05% (w/v) Tween 20. The supporting electrolyte solution of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements was 0.1 M KCl solution containing 2 mM $[Fe(CN)_6]^{4-/3-}$(1:1). All other chemicals were of analytical reagents grade and used without further purification. Double distilled water was used throughout the experiments.

Apparatus

Electrochemical experiments, including cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) were performed with a CHI 660D electrochemical workstation (Shanghai CH Instruments Co.). A conventional three-electrode system comprised a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and a 3DM rGO/PANI film modified working electrode. All potentials herein are referenced to the SCE. The morphologies of 3DM rGO/PANI film were characterized by scanning electron microscopy (SEM, Hitachi S-4800). Fourier-transform infrared (FTIR) spectra were recorded on a Nicolet iS 10 FT-IR spectrophotometer (Thermo Fisher Scientific Inc., USA). X ray diffraction (XRD) patterns of the samples were taken by MiniFlex 600 (Rigaku, Japan).

Preparation of graphene oxide

The GO was synthesized from graphite by the modified Hummers method [33]. 3 g of graphite powder was added to a 13 mL mixture of concentrated H_2SO_4, 2.5 g of $K_2S_2O_8$, and 2.5 g of P_2O_5 at 80 °C. The mixture was reacted for 5 h and then diluted with 500 mL of water. After filtration using a 0.2 μm nylon Millipore filter, it was dried in air overnight. The oxidized graphite was added with 40 mL of concentrated H_2SO_4 and meanwhile chilled to 0 °C using an ice bath. Then 5 g of $KMnO_4$ was slowly added with temperature controlled below 10 °C. After this mixture was allowed to react at 35 °C for 8 h, 80 mL of distilled water was slowly added at a temperature below 50 °C for further reaction for 2 h. Water (230 mL) and 30% H_2O_2 (3 mL) were finally added to produce a brilliant yellow color along with bubbling. The resultant solution was stirred for 30 min and then allowed to settle down for 24 h; after that, the supernatant was decanted. The resultant yellow slurry was centrifuged and then washed with 10% HCl till bisulfate ions were removed and then with copious amount of distilled water till the solution became neutral. Subsequently, the GO was gotten by centrifuging, and was dried under vacuum at 40 °C for 48 h. The dry process for GO must be carried out at low temperatures because it slowly decomposes (deoxygenates) above 60 °C [34].

Fabrication of the immunosensor

Before modification, the Au electrode with controlled area (Φ = 3 mm) was cleaned with acetone, ethanol and water in turn, and then dried under a stream of nitrogen. 3D silica close-packed colloidal crystal modified Au electrode was prepared according to the literature [35]. Then the rGO/PANI film was electrochemically deposited into the template interspaces. In a typical synthesis, a 3D silica modified Au electrode was immersed in a 1 M HCl solution containing 103 μL AN and 0.045 g GO, followed by cycling the potential between −1.5 and 0.9 V at a rate of 50 mV/s for a certain charge. An ordered pore array was finally obtained by removing the template in aqueous HF (5%) for about 60 s after electrodeposition. The fabrication process of the electrochemical immunosensor was illustrated in Scheme 1. Prior to the experiment, the 3DM rGO/PANI film electrode was electrochemically cleaned by cyclic scanning with a potential range of 0–1.5 V in 0.1 M H_2SO_4 at a scan rate of 100 mVs^{-1} until a reproducible cyclic