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Efficient streptavidin-functionalized nitrogen-doped graphene for the development of highly sensitive electrochemical immunosensor

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ABSTRACT

In this work, an efficient and universal streptavidin-functionalized nitrogen-doped graphene (NG) was for the first time proposed and used to develop a highly sensitive electrochemical immunosensor for the detection of tumor markers. Transmission electron microscopy, electrochemical impedance spectrum, static water contact measurement, and cyclic voltammetry were used to characterize the streptavidin-functionalized NG platform and immunosensor. The biofunctionalized NG showed excellent hydrophilicity, larger specific surface area, and high electrochemical activity. These properties of the platform enhanced the loading capacity of proteins, and retained the bioactivity of the immobilized proteins, and thus remarkably improved the sensitivity of the immunosensor. Using carcinoembryonic antigen (CEA) as model analyte, the proposed immunosensor demonstrated a wide linear range of 0.02–12 ng mL^{-1} with a low detection limit of 0.01 ng mL^{-1}. The CEA immunosensor could be applied to detect human serum samples with satisfactory results. The streptavidin-functionalized NG material provided an universal and promising platform for the electrochemical immunosensing applications.

1. Introduction

Electrochemical immunoassay as an ideal technique for tumor marker detection has attracted extensive attention due to its specific advantages such as high sensitivity, low cost, and easy handling (Du et al., 2010, 2011; Tang et al., 2011; Zhou et al., 2012; Lu et al., 2015). Recent researches for the development of electrochemical immunoassay have strongly emphasized the employment of advanced nanomaterials to improve their analytical performances (Li et al., 2015a, 2015b; Zhang et al., 2015; Wei et al., 2016). Nitrogen-doped graphene (NG), in comparison with graphene, has much larger surface area, higher electrochemical conductivity, good biocompatibility and catalytic activity, and has become a potential candidate in the construction of solar cell (Lin et al., 2014; Yu et al., 2016), batteries (Ji et al., 2015; Xu et al., 2015), and sensitive electrochemical biosensors (Xu et al., 2010; Sheng et al., 2012; Deng et al., 2013). Although NG has displayed the improved property, the further functionalization of NG has been expected to be excellent candidate for biosensor design (Wang et al., 2010; Balamurugan et al., 2016).

Recently, metal nanoparticles (NPs), such as AuNPs, PtNPs, and CuNPs, have been reported to functionalize NG for the biosensing applications (Luo et al., 2012; Yang et al., 2013, 2015; Gai et al., 2014). However, the development of an excellent biosensor relied heavily on highly efficient immobilization of the biomolecules on an advanced interface (Valsesia et al., 2008). Although the functionalization of NG with metal NPs improved the property of NG, this functionalization could not provide efficient immobilization of proteins on NG interface. Streptavidin, a glycoprotein purified from the bacterium *Streptomyces avidinii* (Lin et al., 2008), has an extraordinary affinity for biotin (Pérez-Luna et al., 1999). Moreover, one molecular streptavidin can combine with four molecules biotin, which exhibits an efficient streptavidin-biotin interaction (Yang et al., 2014). Therefore, biotin-streptavidin system has been widely used in immunohistochemistry (Moriarty and Unabia, 1982) and immunoassay (Ding et al., 2002; He et al., 2005; Yang et al., 2008; Zhu et al., 2015). Previous studies have shown that the biofunctionalization of nanomaterials with straptavidin could both improved their properties and enhanced the immobilization efficiency of proteins on materials modified interface (Yang et al., 2008; Lai et al., 2011; Zhao et al., 2014). To best of our knowledge, NG has not been reported to be functionalized with streptavidin for the biosensing applications.

Carcinoembryonic antigen (CEA) is a reliable tumor marker for screening and clinical diagnosis of breast tumors, colon tumors, ovarian carcinoma and cervical carcinomas (Wulfkuhle et al., 2003; Naghibalhossaini and Ebadi, 2006; Huang et al., 2010). The sensitive detection of CEA plays an very important role in clinical diagnosis

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applications (Liu and Ma, 2013; Gao et al., 2015). In this work, NG was for the first time biofunctionalized with streptavidin for the fabrication of highly efficient electrochemical immunosensor. The biofunctionalized NG showed larger specific surface area, excellent electrochemical property, and good biocompatibility, and high loading capacity of antibodies. The capture antibody could be efficiently immobilized on the sensing platform surface through the highly selective recognition of streptavidin to biotinylated antibody. The developed electrochemical CEA immunosensor showed a excellent analytical performance such as wide linear range, low detection limit, and high selectivity.

2. Experimental

2.1. Materials

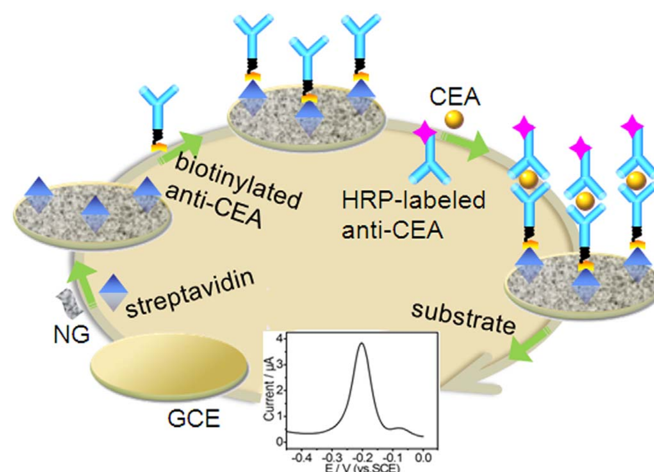
CEA ELISA reagent kit was purchased from CanAg Diagnostics, which consists of a series of CEA standard solutions from 0 to 75 ng mL^{-1}, the stock solutions of horseradish peroxidase (HRP)-labeled mouse monoclonal anti-CEA (HPR-anti-CEA) and biotinylated mouse monoclonal anti-CEA (biotin-anti-CEA). Graphite powder, pyrrole monomer, sulfuric acid (98%, H_2SO_4) and hydrogen peroxide (30%, H_2O_2) were bought from Sinopharm Chemical Reagent Co. Ltd. (China). Streptavidin, chitosan and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Thionin was purchased from Acros Organics. Phosphate buffer solution (PBS) was a mixture of 0.1 M Na_2HPO_4 and NaH_2PO_4 and its pH was adjusted with H_3PO_4 or NaOH solution. The clinical serum samples were supplied by Jiangsu Institute of Cancer Research. All other reagents were analytical grade.

2.2. Apparatus

Electrochemical measurements were performed on a CHI852C electrochemistry workstation (Shanghai CH Instruments Co., China) with a conventional three-electrode system. Raman spectra were recorded using a Renishaw InVia microRaman system. X-ray photoelectron spectroscopic (XPS) spectrum was obtained with an ESCALAB 250Xi spectrometer (USA). Scanning electron micrographs (SEM) were obtained by a scanning electron microscope (Hitachi S-4800, Japan) at an accelerating voltage of 15 kV. Transmission electron micrographs (TEM) were obtained on Philips Tecnai-12 transmission electron microscope using an accelerating voltage of 120 kV. High-resolution transmission electron mcirographs (HRTEM) were obtained with a FEI Tecnai G2 F30 S-TWIN field-emission transmission electron microscopy (USA) at an acceleration voltage of 300 kV. The electrochemical impedance spectroscopy (EIS) analysis was performed on an Autolab PGSTAT30 (The Netherlands) using a solution of 0.10 M KCl containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The amplitude of the applied sine wave potential was 5 mV and the frequency range was from 0.1 to 10 kHz at a bias potential of 190 mV. The static water contact angle measurements were done at 25 °C using a contact angle meter (Rame-Hart-100).

2.3. Preparation of graphene oxide

Graphene oxide (GO) was synthesized according to a typical Hummers synthesis method (Hummers and Offeman, 1958). Firstly, 5g of graphite and 2.5g of $NaNO_3$ were mixed into 15 mL of 98% H_2SO_4, and the mixture was kept 0 °C by ice bath. Afterwards, 15g of $KMnO_4$ was very slowly added into the mixture so as to keep its temperature within 20 °C. Then, the mixture was taken away from the ice bath, and stirred for 30 min at a controlled temperature of 35 °C. Next, 230 mL water was added into above mixture, and its temperature increased to 98 °C. After keeping a reaction temperature at 98 °C for 15 min, 700 mL water and 3% H_2O_2 were added to reduce the residual permanganate and manganese dioxide. The obtained yellowish-brown product was filtered and washed with warm water, and then treated with resinous anion and cation exchangers. After several centrifugations the final dry GO products were obtained in vacuum at 40 °C.



Scheme 1. Schematic illustration for the fabrication of CEA immunosensor and immunoassay procedure for CEA.

2.4. Preparation of nitrogen doped graphene (NG)

NG was prepared according to a reported method with some modifications (Ma et al., 2011). 200 mg of GO was dispersed in 200 mL of water and sonicated until there was no granular substances. Then 2 mL of hydrazine was added to the solution, and refluxed at 100 °C for 24 h. Black powders of reduced graphene oxide (rGO) was finally obtained by filtration and dried in vacuum. Next, 40 mg of rGO was dispersed in 40 mL deionized water, and then 0.05 mL of pyrrole monomer was added into the rGO solution and magnetically stirred at room temperature for 24 h. Subsequently, 50 mL ammonium peroxydisulfate solution (3.79 mg mL^{-1}) was added into the solution to trigger the polymerization reaction in an ice-bath for 24 h. After the polymerization reaction, the solid product was filtered and washed with ethanol and deionized water several times. Finally, 0.1 g of PPy/rGO was transferred in a quartz tube furnace, and heated from room temperature to 600 °C at a rate of 1 °C min^{-1} under Ar atmosphere, and then stayed there for 2 h. After the furnace cooled to room temperature, a black powder of NG was obtained.

2.5. Preparation of electrochemical CEA immunosensor

The schematic illustration of the fabrication of the proposed electrochemical immunosensor was shown in Scheme 1. The glassy carbon electrode (GCE) was polished with 0.03 and 0.05 mm alumina slurry (Beuhler), followed by sonication in ethanol solution and deionized water successively, and dried under a stream of nitrogen. 2.0 mg of NG was firstly dispersed in 2.0 wt% chitosan solution with sonication. Then chitosan-dispersed NG solution (2.0 mg mL^{-1}) was mixed with streptavidin solution (100 μg mL^{-1}) at 1:1 ratio under stirring for 2 h. Subsequently, 5.0 μL of the resulting mixture was dropped on the pretreated GCE, and allowed to dry at 4 °C overnight. 10 μL of 2 μg mL^{-1} biotin-anti-CEA was dropped onto streptavidin-NG modified GCE for 30 min at room temperature, followed by washing with buffer solution to remove physically adsorption. Finally, the antibodies modified electrode was incubated with 1% BSA solution for 1 h to block nonspecific binding sites. After washing with PBS for three times, the prepared immunosensor was stored at 4 °C prior to use.

2.6. Immunoassay procedure for the detection of CEA

The one-step sandwich electrochemical immunoassay of CEA was

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