



A novel label-free electrochemical immunosensor based on functionalized nitrogen-doped graphene quantum dots for carcinoembryonic antigen detection



Yuying Yang^a, Qing Liu^{a,*}, Yan Liu^a, Jianjian Cui^a, Hui Liu^a, Ping Wang^a, Yueyun Li^a, Lei Chen^a, Zengdian Zhao^b, Yunhui Dong^{a,*}

^a School of Chemical Engineering, Shandong University of Technology, Zibo 255049, PR China

^b Analysis and Testing Center, Shandong University of Technology, Zibo 255049, PR China

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ABSTRACT

A novel and ultrasensitive label-free electrochemical immunosensor was fabricated for quantitative detection of carcino-embryonic antigen (CEA). The nitrogen-doped graphene quantum dots (N-GQDs) supported PtPd bimetallic nanoparticles (PtPd/N-GQDs) were synthesized by a simple and green hydrothermal procedure. Subsequently, PtPd/N-GQDs functionalized Au nanoparticles (PtPd/N-GQDs@Au) were prepared successfully via a self-assembly approach. Because of the synergetic effect present in PtPd/N-GQDs@Au, this novel nanocomposites has shown excellent electrocatalytic activity towards hydrogen peroxide (H₂O₂) reduction. Featuring good biocompatibility, excellent conductivity and large surface area, PtPd/N-GQDs@Au was applied as transducing materials to efficiently conjugate capture antibodies and amplify electrochemical signal. Under the optimal conditions, the proposed immunosensor was used for the detection of CEA with wide dynamic range in the range from 5 fg/mL to 50 ng/mL with a low detection limit of 2 fg/mL (S/N=3). Furthermore, this label-free immunosensor possesses high sensitivity, special selectivity and long-term stability, which shows promising application in bioassay analysis.

1. Introduction

Carcino-embryonic antigen (CEA), a highly glycosylated protein with molecular weight of approximately 200 kDa, is one of the most broad spectrum biological marker, and it is elevated in many malignancies, such as gastric cancer, colorectal cancer, breast cancer, liver cancer and pancreatic cancer (Thomas et al., 1995). In addition, the levels of CEA are extremely low in the colon tissue of adults at the range between 2.5 and 5.0 µg/L, but greatly elevated in patients with liver cancer (Zamcheck and Martin, 1981). The assay for CEA is commonly used during the tracking of patients having undergone curative resection (Wang et al., 1994). Therefore, a highly sensitive, selective and quantitative determination method for CEA is great demanded.

A series of methods have been used for the detection of low-abundance CEA such as electrochemiluminescent (Deng et al., 2013), electrochemical (Wen et al., 2016), surface-enhanced Raman scattering (Chon et al., 2009), fluorescent (Wang et al., 2014), mass spectrometric (Liu et al., 2011), chemiluminescent (Zong et al., 2012), and surface plasmon resonance techniques (Špringer et al., 2013). In comparisons,

electrochemical immunosensor has gained wide attentions because of its economical, sensitive, portable, easy-to-operate, and simple-to-construct properties (Ahirwal and Mitra, 2010; Zhao et al., 2014). As a significant branch of electrochemical immunosensors, the label-free immunosensors have an advantage over sandwich-type immunosensors because the sensor architecture is simpler (Joung et al., 2013; Li et al., 2016). Furthermore, the nonenzymatic label-free immunosensors have overcome some obstacles of enzyme-based biosensors, such as environmental instability, tedious immobilization procedures, and high cost (Gopalan et al., 2013).

In order to amplify the detection signal, various types of materials is used to fabricate the immunosensors, including nanoparticles (Han et al., 2016), quantum dots (Martín-Yerga et al., 2014), metal oxides (Ansari et al., 2008) and electroactive component-encapsulated nanoparticles (Viswanathan et al., 2009). Among the nanoparticles, the noble metal with well-defined, low-cost and controlled shape have attracted increasing attention and become a kind of promising materials in ultrasensitive chemical and biological molecules detections. For example, metal nanocatalysts, gold nanoparticles, platinum

* Corresponding authors.

E-mail addresses: qingliu0315@163.com (Q. Liu), dyh651118@126.com (Y. Dong).

nanoparticles and palladium nanoparticles have been widely applied for the fabrication of electrochemical biosensors due to their well biocompatibility, excellent electrocatalytic activity, and high electrode conductivity (El-Deab and Ohsaka, 2002; Han et al., 2016; Roushani and Valipour, 2016). Compared with single metal nanoparticles, bimetallic nanoparticles combine the catalytic effect of their monometallic counterparts to generate distinctly unique characteristics (Cao et al., 2013). It is well-known that, in electrocatalysis and electrochemical immunosensor applications, metal nanoparticles are usually dispersed on supporting materials to obtain high conductivity, stability and large surface (Rajabzade et al., 2012). Because of their excellent properties, carbon materials have been widely used as catalyst supports, such as carbon nanotubes, ordered mesoporous carbon, graphene etc (Tang et al., 2011; Yang et al., 2016). Recently, graphene quantum dots (GQDs) have been applied as a robust substrate in electrocatalytic determination due to the advantages of large specific surface area, biocompatibility, low toxicity, excellent electrical conductivity and good thermal conductivity (Ju and Chen, 2015). According to the previous research, if doping heteroatoms into the π -conjugated system, the structure defects of GQDs may be manipulated (Li et al., 2011). For the nitrogen-doped graphene quantum dots (N-GQDs), the chemically bonded N atoms could drastically produce new phenomena and unexpected properties, for example, altering the electronic characteristics and increasing the number of anchoring sites for the adsorption of metal ions (Chizari et al., 2010; Li et al., 2011). At the same time, N-GQDs possess many oxygen-containing functional groups that can make it better water-solubility and easier to form a stable chemical bond with various materials. Therefore, N-GQDs supported bimetallic nanoparticles could have good biocompatibility and superior auxiliary catalytic activity. Herein, PtPd bimetallic nanoparticles (PtPd NPs) were supported on N-GQDs by hydrothermal coreduction of platinum and palladium using N-GQDs as both reducing agents and supports. The intimate electronic interactions between N-GQDs and PtPd NPs provided naked catalytic surface area and high electrical conductivity, which rendered them the highly active electrocatalysts for H_2O_2 reduction.

For the development of highly sensitive electrochemical immunosensor, Au nanoparticles (Au NPs) were considered as the most stable biocompatible nano-scaled materials (Mani et al., 2009; Zhuo et al., 2005). In this system, Au NPs, about 30 nm in diameter, were used to amplify electrocatalytic activity and link antibodies. N-GQDs supported PtPd NPs (PtPd/N-GQDs) were then functionalized by amino groups and finally self-assembled loaded on Au NPs according to the covalent bonding. Attributed to the effective immobilization of antibodies and the synergistic catalytic activity of PtPd NPs, Au NPs and N-GQDs towards the reduction of H_2O_2 , the as-prepared PtPd/N-GQDs@Au nanocomposites were applied as a signal amplification platform to fabricate a novel and ultrasensitive label-free immunosensor for the quantitative detection of CEA. The proposed novel immunosensor strategy showed high sensitivity and good stability for quantitative determination of CEA in human serum, which held a great potential in clinical and diagnostic applications.

2. Materials and methods

2.1. Reagents

The mixed Na_2HPO_4 and KH_2PO_4 stock solution were prepared to form phosphate buffered saline (PBS, pH=6.47) that used as the electrolyte in the process of electrochemical measurements. Chloroauric acid (HAuCl_4) was obtained from Sigma-Aldrich Co., Ltd. (Beijing, China). Bovine serum albumin (BSA, 96–99%) was purchased from Sigma reagent Co., Ltd. (St. Louis, MO, USA). Citric acid (CA) and dicyandiamide (DCD) were purchased from Bodi Chemical Reagent Co., Ltd. (Beijing, China). PdCl_2 and PtCl_2 were bought from Shanghai Macklin Biochemical Co., Ltd. $\text{K}_3[\text{Fe}(\text{CN})_6]$ was

bought from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). CEA and CEA antibody (anti-CEA) were purchased from Linc-Bio Science Co., Ltd (Shanghai, China). All the experimental processes used ultrapure water (18.25 $\text{M}\Omega$ cm, 24 °C). All solvents were of analytical grade. The prepared solutions were maintained at 4 °C prior to use.

2.2. Apparatus

Electrochemical measurements were recorded using a CHI760E electrochemical workstation by using a conventional three-electrode system consisted of a glassy carbon electrode (GCE) as working electrode, a saturated calomel electrode (SCE) as the reference electrode, and the platinum wire electrode as the counter electrode (Chenhua Instrument Shanghai Co., Ltd, China). Transmission electron microscope (TEM) images and energy-dispersive X-ray spectroscopy (EDX) analysis were collected using a Tecnai G² F20 (America).

2.3. Preparation of the N-GQDs

N-GQDs were prepared by following a literature procedure (Ju and Chen, 2015). Briefly, 2.0g of CA and 1.0g of DCD were mixed with ultrapure water (5 mL). Then, the mixture was transferred into a 25 mL Teflon-lined autoclave and heated for 12 h at 180 °C. Eventually, the product was dispersed in 100 mL of ultrapure water, and the N-GQDs were collected by removing the large dots through centrifugation at 10,000 rpm for 10 min

2.4. Preparation of the Au NPs

According to the classical method, seed Au NPs were prepared using sodium citrate to reduce AuCl_4^- ions (Frens, 1973). In brief, HAuCl_4 (0.01 wt%, 100 mL) was refluxed and heated to boiling. Then sodium citrate (2.5 mL, 10 mg/mL^{-1}) was rapidly added into the solution and stirred for another 15 min. The resultant wine-red solution was cooled down to room temperature and stored at 4 °C for further usage.

These seed Au NPs were then used for the synthesis of Au NPs with an average diameter of 50 nm by the following procedure (Brown et al., 2000). The prepared seed Au NPs (1 mL) and $\text{NH}_2\text{OH}\cdot\text{HCl}$ (100 μL , 0.2 M) were mixed to ultrapure water (25 mL) and was stirred vigorously at room temperature. By adding HAuCl_4 (3.0 mL, 0.1 wt %) drop-wise, a crimson solution was obtained and stored at 4 °C.

2.5. Preparation of N-GQDs-supported PtPd Bimetallic Nanoparticles (PtPd/N-GQDs)

PtPd/N-GQDs were synthesized through a simple hydrothermal method (He et al., 2013; Peng et al., 2012; Song and Chen, 2014). Typically, PtCl_2 (6.65 mg, 0.025 mmol) and PdCl_2 (5.75 mg, 0.025 mmol) were dissolved in 1 mL of hydrochloric acid under heating. The solution was then added into a 15 mL aqueous solution with 20 mg of N-GQDs. The pH was adjusted to 10 with NaOH (0.1 mg/mL), and the mixture was transferred to Teflon-lined autoclave (160 kPa) and hydrothermally treated at 160 °C for 6 h. Black precipitates appeared in the flask as a result of thermolytic reduction of Pt, Pd on N-GQDs to form PtPd/N-GQDs NPs. The precipitates were collected, washed extensively with Nanopure water and ethanol, and dried in a vacuum oven at room temperature for 12 h.

2.6. Preparation of N-GQDs-supported PtPd NPs loaded on Au Nanoparticles (PtPd/N-GQDs@Au)

To obtain the amino-functionalized PtPd/N-GQDs, 1.0 mL PtPd/N-GQDs and 2.0 mL ethylenediamine was first dispersed in 5 mL ultrapure water by sonication (240 W) for 1 h. After stirring for 5 h, the suspension was centrifuged and washed with ultrapure water for

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