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Enzyme modified peptide nanowire as label for the fabrication of electrochemical immunosensor

Jianglin Zhang[a], Xiang Chen[a,*], Minghui Yang[a,b,**]

[a] Department of Dermatology, XiangYa Hospital, Central South University, Changsha 410008, China
[b] College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China

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ABSTRACT

Ferrocene (Fc) functionalized self-assembled peptide nanowire (Fc-PNW) was synthesized, functionalized and used as label for the fabrication of electrochemical immunosensors to detect substance P (SP). To prepare the label, gold nanoparticles (AuNPs) were immobilized onto the Fc-PNW for the following adsorption of horseradish peroxidase (HRP) and secondary anti-SP antibody (Ab_2, Fc-PNW-HRP-Ab_2). The role of Fc-PNW in the electrochemical label was not only as supporting matrix for HRP and Ab_2, but also as the mediator for HRP to enhance the sensitivity of HRP toward H_2O_2 detection. The immunosensor was fabricated based on the traditional sandwich protocol with primary anti-SP antibody (Ab_1) immobilized onto graphene surface. The catalytic current of HRP toward H_2O_2 in the presence of mediator Fc was used as signal of the immunosensor. Different parameters, such as the weight ratio of HRP to Ab_2 on the Fc-PNW and the antibody–antigen incubation time were optimized to improve the performance of the immunosensor. The resulting immunosensor has high sensitivity, wide linear range (0.01–50 ng/mL) and low detection limit (5 pg/mL).

1. Introduction

Substance P (SP) is a neuropeptide that functions as a neurotransmitter and neuromodulator [1]. SP is discovered around 1931, which exist in the brain and spinal cord, and is associated with inflammatory processes and pain [2,3]. For example, SP regulates the inflammatory response by stimulating mononuclear and polymorphonuclear leukocytes and regulates the release of inflammatory mediators [4]. The increased SP level in human fluidics is related to different physical and mental disorders, including inflammatory bowel disease, sickle cell crisis, rheumatological diseases, stress and anxiety, as well as cancer [5]. So the precise detection of SP is of great importance for clinical diagnosis and biomedical research.

Recently, peptide diphenylalanine (Phe-Phe, FF) based self-assembled nanomaterials have received great interest due to their low cost, simple self-assemble process and mild assemble condition [6,7]. FF, which is composed of two aromatic phenylalanine residues, is the recognition motif of the amyloid-β protein that associated with Alzheimer's disease (AD) [8]. Reches and Gazit have reported FF can be self-assembled into nanotube structure when dissolved in organic/water solution [9]. The assembled peptide nanomaterials are biocompatible, have good chemical and thermal stability, and can be easily functionalized. These interesting properties make them widely applied in different areas, such as biosensing, catalyst, drug delivery and electronic circuits [10–13].

Electrochemical sensors and biosensors, due to their low cost, high sensitivity and simple instrumentation have been widely used in different areas, such as environmental monitoring, industrial quality control and clinical diagnosis [14–17]. For the fabrication of electrochemical immunosensors, great efforts have been devoted to enhance the sensitivity and decrease the detection limit as for some analytes, their concentrations in the samples if of ultralow. For example, horseradish peroxidase (HRP) is one of the most widely used electrochemical labels for electrochemical immunosensors due to its high catalytic activity toward H_2O_2 and good stability [18–20]. Different nanoparticles have been chosen as matrix to immobilize HRP for the increase of HRP loading, such as magnetically nanoparticles and carbon nanoparticles [21,22].

In this work, we utilize ferrocene (Fc) functionalized peptide nanowire (Fc-PNW) as electrochemical label for the fabrication of immunosensors to detect SP. To synthesize Fc-PNW, Fc was first covalently linked to FF, and then the modified FF molecules were assembled into nanowire structure. For the fabrication

* Corresponding author. Tel.: +86 731 84328478; fax: +86 731 84328478.
** Corresponding author at: Department of Dermatology, XiangYa Hospital, Central South University, Changsha 410008, China.
E-mail addresses: chenxiangck@gmail.com (X. Chen), yangminghui@csu.edu.cn (M. Yang).

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of the electrochemical label, gold nanoparticles (AuNPs) were attached onto the Fc-PNW for the following adsorption of HRP and secondary anti-SP antibody (Ab_2, Fc-PNW-HRP-Ab_2). With the immobilization of primary anti-SP antibody (Ab_1) onto graphene surface, the resulting immunosensor displays high sensitivity, wide linear range and low detection limit toward SP detection. In addition, the immunosensor was applied for the detection of SP in clinical serum samples with satisfactory results.

2. Materials and methods

2.1. Apparatus and reagents

Substance P (SP) and goat monoclonal anti-SP antibody were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Poly(diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW = 200,000–350,000) was obtained from Sigma–Aldrich. A 0.1 M phosphate buffer solutions (PBS) were prepared by mixing 0.1 M NaH_2PO_4 and Na_2HPO_4. All other reagents were of analytical grade and deionized water was used throughout the study. The study was under ethic approval.

All electrochemical measurements were performed on a CHI 650D electrochemical workstation (Shanghai CH Instruments Co., China). A conventional three-electrode system was used for all electrochemical measurements: a glassy carbon electrode (GC, 3 mm in diameter) as the working electrode, a saturated calomel electrode as the reference electrode, and a platinum wire electrode as the counter electrode. Scanning electron microscope (SEM) images were obtained from Nova NanoSEM230 (FEI, USA).

2.2. Synthesis of Fc-PNW

The synthesis of the Fc-PNW was according to our previous report [23]. Briefly, Boc-Phe-COOH (4 mM) and HBTU/HOBt (4.4 mM) were dissolved in DCM (50 mL), Et_3N was then added dropwise to activate the carboxyl group for 1 h at 0 °C. After that, H-Phe-OMe·HCl (4.4 mL) was added and the reaction mixture was stirred overnight, followed by washing with saturated aqueous solutions of $NaHCO_3$, HCl (10%), and water, then dried over Na_2SO_4 under reduced pressure. The crude product was purified by flash column chromatography (DCM: EtOAc = 2:1, v/v), then evaporated under reduced pressure in a rotovap to a white oil. The oil was dissolved in dimethyl sulfoxide (DMSO) and dried in freeze dryer for overnight, resulting in a white crystalline, which is Boc-Phe-Phe-OMe.

The synthesized Boc-Phe-Phe-OMe was then dissolved in the mixture of DCM (20 mL) and trifluoroacetic acid (TFA, 10 mL). The reaction mixture was stirred for 30 min, and then CH_2Cl_2 and TFA were subsequently removed. The resulting H-Phe-Phe-OMe was treated with Et_3N in DCM (2 mL, pH ~ 8). The solution was diluted in DCM (20 mL) and mixed with Fc-OBt (2.2 mM) that was obtained by using standard HBTU/HOBt method in solution of activating Fc-OH. The reaction mixture was then stirred for 1 h, followed by washing with saturated aqueous solutions of $NaHCO_3$, HCl (10%), and water, then dried. The crude product was purified by flash column chromatography (DCM:EtOAc = 3:1, v/v), then evaporated under reduced pressure in a rotovap to a yellow oil. The oil was dissolved in DMSO and dried in freeze dryer for overnight, resulting in a yellow needle crystalline, which is Fc-Phe-Phe-OMe.

For the preparation of Fc-PNW, We dissolved the synthesized compound Fc-Phe-Phe-OMe into certain amount of 1,1,1,3,3,3-hexafluoro-2-propanol to get the stock solution (100 mg/mL). The peptides stock solution was then diluted by MeOH to reach a concentration of 2 mg/mL. After the evaporation of solvent, Fc-Phe-Phe-OMe was self-assembled into Fc-PNW.

2.3. Preparation of Fc-PNW-HRP-Ab_2

The procedure for the preparation of Fc-PNW-HRP-Ab_2 was shown in Fig. 1A. First, AuNPs of 13-nm diameter were prepared according to the previous report method [24]. The synthesized Fc-PNW (1 mg) was dispersed into 1 mL of 0.5% PDDA aqueous solution containing 0.5 M NaCl and stirred for 30 min. The solution was centrifuged and washed twice with water to remove the residual PDDA and obtain PDDA wrapped Fc-PNW. Then, the PDDA functionalized Fc-PNW was dispersed into as-prepared colloidal AuNPs and stirred for 30 min to adsorb AuNPs onto Fc-PNW surface. Finally, the modified Fc-PNW was added into solution containing 2 mg/mL of HRP and 0.02 mg/mL of Ab_2. The mixture was gently mixed for 3 h and centrifuged. The obtained nanostructure (Fc-PNW-HRP-Ab_2) was stored at 4 °C before use.

2.4. Preparation of the immunosensor

To fabricate the immunosensor, Ab_1 was immobilized onto graphene surface through an amidation reaction between the carboxylic acid groups on graphene and the available amine groups of Ab_1. Graphene was prepared from graphite oxide through a thermal exfoliation method. To immobilize Ab_1 onto graphene, typically, into 1 mL of graphene solution (2 mg/mL), EDC and NHS (100 mM) were added. The mixture was stirred for 4 h and after that, 1 mL of Ab_1 solution (100 μg/mL) was added into the mixture. After another 12 h of reaction, the GS solution was centrifuged and washed. The resulting graphene-Ab_1 conjugates were stored at 4 °C in phosphate buffer solution before use.

Fig. 1B displays the procedure for the fabrication of the immunosensor. Onto the GC electrode, 5 μL of graphene-Ab_1 buffer solution was added. The electrode was dried and washed with buffer, then it was incubated in 1 wt% BSA solution for 30 min to eliminate nonspecific binding between the antigen and the electrode surface. Subsequently, SP buffer solution with a varying concentration was added onto the electrode surface and incubated for 1 h at room temperature, and then the electrode was washed extensively. Finally, the prepared Fc-PNW-HRP-Ab_2 buffer solution

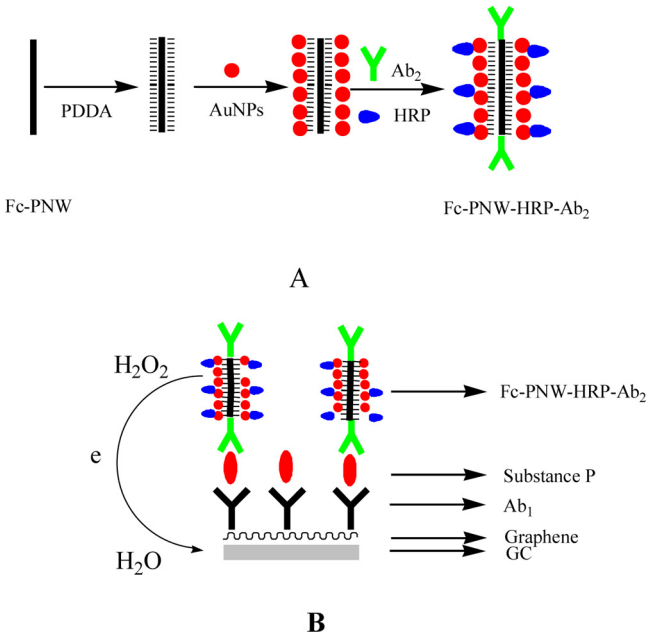

Fig. 1. Schematic representation for the preparation of the Fc-PNW-HRP-Ab_2 label (A) and the immunosensor (B).