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A novel solid-state $Ru(bpy)_3^{2+}$ electrochemiluminescence immunosensor based on poly(ethylenimine) and polyamidoamine dendrimers as co-reactants

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ABSTRACT

In this study, a novel solid-state $Ru(bpy)_3^{2+}$ electrochemiluminescence (ECL) sandwiched immunosensor for sensitive detection of α-fetoprotein (AFP) was constructed based on poly(ethylenimine) (PEI) functionalized reduced graphene oxide (PEI-rGO) and Au nanoparticles (AuNPs) decorated polyamidoamine (PAMAM) dendrimers. Both PEI and PAMAM are polymers with a lot of amino groups, which are able to serve as good co-reactant to remarkably enhance the ECL signal of $Ru(bpy)_3^{2+}$. For improving the poor conductivity of PAMAM, the AuNPs were decorated on the amino groups of PAMAM. Through Au-N bonds, the formed AuNPs-PAMAM was decorated on the PEI-rGO. The obtained AuNPs-PAMAM/PEI-rGO was introduced to immobilize the detection antibody (Ab_2). Then, the Ab_2 labeled AuNPs-PAMAM/PEI-rGO was modified onto the glass carbon electrode surface via sandwiched immunoreactions. The ECL substrate was prepared by mixing nafion and the complex (Ru-PtNPs) of Pt nanoparticles (PtNPs) and Ru $(bpy)_3^{2+}$, which could reduce the consumption of Ru complex, simplify the operation and enhance the ECL efficiency. The experimental results demonstrated that the proposed immunosensor had good response to AFP. The linear range was from 0.01 pg mL^{-1} to 10 ng mL^{-1} with a low detection limit of 3.3 fg mL^{-1}. Meanwhile, with satisfying stability, selectivity and reproducibility, the proposed sandwiched immunosensor was presented to possess good potential in clinical detection.

1. Introduction

Highly sensitive detection for tumor biomarker that is associated with a particular cancer is significant for early diagnosis and prognosis. Meanwhile, the demands for it are increasing rapidly [1]. Up to now, some methods have been developed for the detection of tumor biomarker, such as enzyme-linked immunoassay [2], fluorescent immunoassay [3], electrochemical immunoassay [4,5] and electrochemiluminescence immunoassay [6]. Among those methods, electrochemiluminescence (ECL), with high sensitivity, selectivity, low background signal and rapid response, has attracted much attention in recent years. And based on those distinct advantages, ECL will have wider application in immunoassay for tumor biomarkers [7–9]. In fact, commercial systems have started to use ECL in some important clinical detection (such as digoxin, thyrotopin, protein, steroidal hormones and various antibodies) [10,11]. Luminol, quantum dots and Ru complex are most common ECL reagents that are often used to construct biosensors [12–14]. Among these ECL systems, tris(2,2'-bipyridyl) ruthenium(II) ($Ru(bpy)_3^{2+}$) and its derivatives have special advantages such as high luminous efficiency, good stability in different pH and superior electrochemical reversibility [15,16]. However, the high price limits its application in clinical detection [12]. In order to solve the problem, $Ru(bpy)_3^{2+}$ was modified on the electrode surface to construct a solid-state ECL immunosensor, which could reduce the consumption of Ru complex, improve ECL efficiency and enhance the ECL signal.

According to the mechanisms, the co-reactants are important intermediates for ECL signal amplification [10,17]. The co-reactant ECL has high ECL efficiency, strong ECL signal and superior stability. Based on these advantages, the use of the co-reactant ECL has been increasing [17,18]. The previous reports have pointed out that many kinds of amine compounds could act as co-reactant to the ECL of $Ru(bpy)_3^{2+}$ [19]. Poly (ethylenimine) (PEI), with a lot of amino groups in its molecular structure, is a good co-reactant that can enhance the ECL

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signal [20,21]. In order to increase their immobilized amount when constructing a ECL sensor, the co-reactants are often immobilized on some nanomaterial, such as carbon nanotube, graphene and some metal nanomaterial [22]. Graphene, an atomical layer of sp^2 carbon atoms in a densely packed honeycomb two-dimensional lattice, has been paid great attention by both the experimental and theoretical scientific communities [23–25]. Due to its large specific surface area, superior electric conductivity and satisfactory stability, it is often used to fabricate biosensors. In this assay, graphene oxide was applied to immobilizing PEI. The formed PEI functionalized reduced graphene oxide (PEI-rGO) can enhance the ECL signal of $Ru(bpy)_3^{2+}$, improve the ECL efficiency and remarkably improve the electric conductivity of the proposed sensor. Moreover, the PEI-rGO can act as co-reactant and immobilized platform in the same time, which simplified the preparation of the sensor.

Polyamidoamine (PAMAM) dendrimers are hyper-branched and three-dimensional macromolecules with hundreds of functional groups at the periphery. It is well-known in designing biosensors due to its unique properties [26–28]. Aminated-terminated polyamidoamine (PAMAM, G4) dendrimers have many amino groups in their molecular backbones, which makes them serve as a co-reactant to the ECL of $Ru(bpy)_3^{2+}$. Therefore, they are introduced to construct the proposed sensor for further signal amplification and high sensitivity. However, the conductivity of PAMAM is poor, which limits its application in biosensors. In order to solve the problem, Au nanoparticles (AuNPs) were decorated on the surface of the PAMAM via Au-N bonds. The formed AuNPs-PAMAM can effectively immobilize the antibody and enhance the ECL signal.

Inspired by all those perspectives, we constructed a new sensitive solid-state $Ru(bpy)_3^{2+}$ ECL sandwiched immunosensor with two co-reactants labeled detection antibody (Ab_2). The capture antibody (Ab_1) was assembled on the Nafion-Ru-PtNPs layer which was modified on the bare glass carbon electrode (GCE). Simultaneously, the AuNPs-PAMAM was decorated on the PEI-rGO to obtain AuNPs-PAMAM/PEI-rGO which was used to immobilize the Ab_2. With two different co-reactants in the AuNPs-PAMAM/PEI-rGO, the ECL signal of the immunosensor was drastically amplified and the detection sensitivity was accordingly improved. Moreover, based on this method, the preparation of the sensor was significantly simplified and the proposed sensor showed a new promising platform to clinical immunoassay.

2. Experimental

2.1. Reagents and material

AFP antibody (Anti-AFP), antigen (AFP), antigen (CEA) and antigen (PSA) standard solutions were purchased from Biocell (Zhengzhou, China). Phosphate buffer solutions (PBS) with pH 7.4 were prepared by mixing standard stock solutions of 0.1 M K_2HPO_4, 0.1 M NaH_2PO_4, and 0.1 M KCl and adjusting the pH with 0.1 M HCl or NaOH, then diluting with double distilled water. Gold chloride ($HAuCl_4 \cdot 4H_2O$) and Albumin from bovine serum (BSA) (96–99%) were obtained from Shanghai fine chemical materials institute. Polyamidoamine (PAMAM) dendrimers were purchased from Weihai CY Dendrimer Technology Co., Ltd. Poly(ethylenimine) (PEI), 3-thiophenemalonic acid (TA), $Ru(bpy)_3Cl_2 \cdot 6H_2O$, Nafion (5%, *V*/*V*), $H_2Pt_2Cl_6 \cdot 6H_2O$, $H_2AuCl_6 \cdot 4H_2O$ and sodium citrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Graphene Oxide was received from Nanjing Xianfeng Nano Go. (Nanjing, China). All chemicals were of analytical grade and used without further purification. All solutions were prepared with double distilled water and stored in the refrigerator (4 °C).

2.2. Apparatus

The ECL emission was monitored by a model MPI-A electrochemiluminescence analyzer (Xi'An Remax Electronic Science &Technology Co. Ltd., Xi'An, China). Electrochemical impedance spectroscopy (EIS) measurements were carried out with a CHI 610A electrochemistry workstation (Shanghai CH Instruments, China). A three-electrode electrochemical cell was composed of a modified glass carbon electrode (GCE, Φ=4 mm) as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (sat. KCl) as the reference electrode. GCE, platinum wire and AgCl electrode were purchased from Tianjin Aidahengsheng technology Co. Ltd., China. Centrifuge was purchased from Hunan Xiangyi Centrifuge Instrument Co. Ltd., China. The morphologies of different nanomaterial were characterized by scanning electronmicroscopy (SEM, S-4800, Hitachi, Tokyo, Japan) at an acceleration voltage of 20 kV and transmission electron microscope (TEM, TECNAI 10, Philips Fei Co., Hillsboro, OR).

2.3. Preparation of Nafion-Ru-PtNPs

Nafion-Ru-PtNPs were prepared by a simple method. Firstly, Ru-PtNPs were prepared by mixing the PtNPs solution with Ru $(bpy)_3^{2+}$ aqueous solution according to a previous literature with a little modification [29]. Briefly, 2 mL of 0.019 M H_2PtCl_6 aqueous solution was diluted to 50 mL with water, and then 0.5 mL of 0.3 M TA was added into the solution. The solution was heated at 100 °C for 20 min. When it turned to be a dark brown solution, Pt nanoparticles (PtNPs) were well formed. Then, 1 mL of 0.038 M $Ru(bpy)_3Cl_2$ aqueous solution was added into 50 mL of PtNPs solution with vigorous stirring. Several minutes later, Ru-PtNPs were formed. In order to improve the film forming ability of Ru-PtNPs, 1 mL Nafion solution (5%, *wt*/*wt*) was added into Ru-PtNPs under stirring and Nafion-Ru-PtNPs were prepared successfully.

2.4. Preparation of PEI-rGO

The PEI-rGO was prepared according to literature with a little modification [30]. A stable dispersion of exfoliated GO sheets (1 mg mL^{-1}, 10 mL) was mixed with PEI (3%, 1 mL) and heated under refluxing at 135 °C for about 3 h. The PEI here served as reductant to reduce graphene oxide sheets, which was immobilized on the graphene in the same time.

2.5. Preparation of Au nanoparticles

Au nanoparticles were prepared according to a previous literature with a little modification [31]. In brief, 2.5 mL of 1% sodium citrate was added into 100 mL of boiling 0.01% $HAuCl_4$ solution with vigorous stirring. When the color changed from yellow to red violet, the mixture was boiled continually for 10 min under stirring. Then, it was cooled in room temperature and Au nanoparticles were well formed.

2.6. Preparation of Ab_2/AuNPs-PAMAM/PEI-rGO (Ab_2 bioconjugate)

The Ab_2/AuNPs-PAMAM/PEI-rGO was synthesized by the following steps (Scheme 1A). Firstly, PAMAM (0.07 mM) was dissolved in 1 mL AuNPs solution which had been prepared before the step and stirred for 6 h in room temperature. Then, 1 mL PEI-rGO, which had been prepared before the step, was added into the mixture and stirred for another 6 h. Subsequently, the Ab_2 was added into the as-prepared AuNPs-PAMAM/PEI-rGO solution, and the mixture of them was slightly stirred for 12 h at 4 °C to conjugate Ab_2 to the complex. After that, 100 μL BSA solution (1%, *wt*/*wt*) was implemented to block the non-specific adsorption