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A novel electrochemiluminescent detection of protein biomarker using L-cysteine and in situ generating coreactant for signal amplification

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ABSTRACT

In this work, a novel peroxydisulfate electrochemiluminescence (ECL) strategy based on a sandwich-type immunosensor for sensitive detection of carcinoembryonic antigen (CEA) was developed. The primary antibody anti-CEA (Ab_1) was immobilized onto Au nanopractices (AuNPs) electrodeposited onto the electrode, which have large surface area and high electrical conductivity. Then, L-cysteine (L-Cys) and AuNPs functionalized multiwalled carbon nanotubes (MWCNTs) were synthesized to act as the platform for immobilization glucose oxidase (GOD), horseradish peroxidase (HRP) and secondary antibody (Ab_2). The CEA antigen and MWCNTS-L-Cys-AuNPs@GOD-HRP-labeled Ab_2 were then successively conjugated to form sandwich-type immunocomplexes through the specific interaction between antigen and antibody. The ECL signal amplification was significantly improved due to the synergistic effect of AuNPs and L-Cys. Furthermore, when proper amounts of D-glucose were added in the detection solution, GOD catalyzed the oxidation of glucose to generate H_2O_2, which could be further catalyzed by HRP to generate O_2 for the signal amplification. The developed ECL immunosensor exhibited high sensitivity and specificity for the detection of CEA and responded linearly to the clinically relevant concentration of CEA from 0.02 to 80 ng mL^{-1} with a low detection limit of 0.67 pg mL^{-1}. The present work provided a promising technique for highly sensitive bioassays applied in clinical detection.

1. Introduction

Carcinoembryonic antigen (CEA), a glycoprotein involved in cell adhesion, is normally produced during fetal development but the production stops before birth [1]. If the total CEA level is more than the normal average concentration in adult serum, people will suffer from some cancers, such as colorectal cancer, liver cancer and pancreatic cancer [2]. Thus, CEA is a useful tumor maker for the above-mentioned cancers. Therefore, the method for CEA evaluation should present not only large linear ranges but also high sensitivity to fulfill the need for the diagnosis and monitoring of those cancers in clinical analysis. Until now, a series of methods have been developed for its detection, including enzyme-linked immunosorbent assays (ELISA) [3,4], electrochemical immunoassay [5,6], fluorescence immunoassay (FIA) [7,8], chemiluminescence immunoassay (CLIA) [9], surface plasmon resonance immunoassay [10] and electrochemiluminescent immunoassay [11]. Among them, ECL-based immunosensors have attracted intensive and extensive research interests due to their important applications in clinical diagnosis with the promising advantages such as simplicity, high sensitivity, reproducibility, rapidity and low background.

Electrochemiluminescent immunoassays, with simple instrumentation and easy signal quantification, have become important analytical techniques for quantitative detection of biomolecules [12–14]. For sandwich-type immunoassays, signal amplification and noise reduction are crucial for obtaining low detection limits and high sensitivity in clinical immunoassays. Recently, much attention has been focused on signal amplification by using an enzyme, such as horseradish peroxidase (HRP) and alkaline phosphatase. Especially, using enzymatic reaction to generate coreactant in situ is a promising method in enhancing the sensitivity of ECL detection. In this way, the concentration of a coreactant could increase rapidly around the electrode surface, which achieves a high ECL amplification. However, reports concerning this amplification method to ECL system are still relatively less than others [11,15,16]. Therefore, we are endeavoring to design some novel ECL sensors concerning in situ generating coreactant.

Peroxydisulfate-O_2, as a novel ECL type, has been put forward to use in immunoassay recent years [17,18]. Due to its distinct advantages of simplicity, sensitivity, and cheapness, we believe that ECL

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of peroxydisulfate will gradually become an important and powerful analytical tool in many fields although reports concerning the ECL of peroxydisulfate solution are relatively scarce now. The possible mechanisms to the ECL of peroxydisulfate solution were raised by Yao et al. in 2008 [17].

$$S_2O_8^{2-} + e \rightarrow SO_4^{\bullet -} + SO_4^{2-} \quad (1)$$

$$SO_4^{\bullet -} + H_2O \rightarrow HO^{\bullet} + HSO_4^{2-} \quad (2)$$

$$HO^{\bullet} + H_2O \rightarrow HOO^{\bullet} + H_2 \quad (3)$$

$$O_2 + H_2O + e \rightarrow HOO^{\bullet} + HO^{-} \quad (4)$$

$$SO_4^{\bullet -} + HOO^{\bullet} \rightarrow HSO_4^{-} + {}^1(O_2)_2{}^* \quad (5)$$

$${}^1(O_2)_2{}^* \rightarrow 2\,{}^3O_2 + h\nu \quad (6)$$

According to the mechanisms, we know that the dissolved oxygen is a coreactant to the ECL of peroxydisulfate. Therefore, we conjecture that using enzymatic reaction to generate coreactant in situ is an effective way to enhance the sensitivity of peroxydisulfate system. Glucose oxidase (GOD) and Horseradish peroxidase (HRP) were used to catalyze D-glucose to generate O_2 for signal amplification.

In recent years, tremendous interests are focused on nanoparticles because of their combination of unique inherent properties and high potential of applications [19,20]. Among various nanoparticles, multiwalled carbon nanotubes (MWCNTs) and Au nanopractices (AuNPs) are the most widely applied nanomaterials due to their large specific surface area, promising catalytic properties, excellent conductivity [21,22] and good biocompatibility of AuNPs [23,24]. Furthermore, it is worthwhile to note that the decorated carbon nanotubes with metal nanoparticles exhibit the integrated properties of two components with better catalytic activity and enhanced electrical conductivity [25,26]. L-cysteine (L-Cys) that simultaneously posses —SH and —NH_2 is a good linking reagent between carboxyl functionalized MWCNTs and AuNPs. Additionally, according to our previous work, we found that L-Cys was a coreactant of the peroxydisulfate-O_2 system [15]. Therefore, we constructed a platform to immobilize the enzyme and the secondary antibody (Ab_2) by using L-Cys and AuNPs to decorate the MWCNTs dispersed by 3,4,9,10-perylene tetracarboxylic acid (PTCA) which possessed abundant carboxyl and excellent dispersity.

Herein, we report an effective sandwich-type ECL immunosensor for sensitive quantification of CEA. AuNPs electrodeposited onto the electrode were initially employed as a nanoscale anchorage substrate to load a large amount of primary antibody (Ab_1). Then L-Cys and AuNPs decorated MWCNTs were used to enhance the ECL response signal and label Ab_2. After incubation with CEA samples, an ECL signal with great amplification could be achieved by the synergistic function of MWCNTs, AuNPs and L-Cys. Moreover, when proper amounts of glucose were added in the detection solution, GOD catalyzed the oxidation of D-glucose to generate gluconic acid accompanying with the generation of H_2O_2, which could be further catalyzed by HRP to generate O_2 for further signal amplification. With the several amplification factors mentioned above, the designed sandwich-type immunosensor have obtained a wide linear range and a relatively low detection limit for CEA.

2. Experimental

2.1. Reagents

Multi wall carbon nanotubes (MWCNTs, >95% purity) were purchased from Chengdu Organic Chemicals Co. Ltd. of the Chinese Academy of Science. Gold chloride ($HAuCl_4$) and L-cysteine (L-Cys) were purchased from Kangda Amino Acid company (Shanghai, China). $K_2S_2O_8$ was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Carcinoembryonic antigen antibody (Anti-CEA), antigen (CEA), prostate specific antigen (PSA) and α-1-fetoprotein (AFP) were purchased from Biocell (Zhengzhou, China). Bovine serum albumin (BSA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimidehydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3,4,9,10-Perylene tetracarboxylic acid (PTCA) was received from Lian Gang Dyestuff Chemical Industry Co. Ltd. (Liaoning, China). Glucose oxidase (GOD) and horseradish peroxidase (HRP) were obtained from the Shanghai Biochemical Co. (China). The serum specimens were obtained from native hospital. Phosphate-buffered solution (PBS) (pH 7.4, 0.1 M) was prepared with 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4 and 0.1 M KCl. Double distilled water was used throughout this study.

2.2. Apparatus

Depositions were carried out with a CHI 660A electrochemical workstation (Shanghai Chenhua Instrument, China). The ECL emission was monitored with a model MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) with the voltage of the photomultiplier tube (PTM) set at 800 V and the potential scan from 0 to −2.0 V in the process of detection. The experiment was performed with a conventional three-electrode system, in which the modified glassy carbon electrode (GCE) was the working electrode, a platinum wire was the counter electrode and an Ag/AgCl (sat. KCl) was the reference electrode. The morphologie of bioconjugate was tracked by a transmission electron microscope (TEM, TECNAI 10, Philips Fei Co., Hillsboro, OR).

2.3. Preparation of MWCNTS-L-Cys-AuNPs@Ab_2-GOD-HRP bioconjugate

The process of the preparation of MWCNTS-L-Cys-AuNPs@Ab_2-GOD-HRP bioconjugate was shown in Scheme 1. Firstly, 1 mg MWCNTs was added into 2 mL PTCA, followed by stirring for about 12 h. The resulting PTCA functionalized MWCNTs possessed abundant carboxyl on its surface due to the presence of PTCA. Then, carboxyl functionalized MWCNTs was surface-chemically modified by L-Cys through the cross-linking effect of EDC and NHS. The resulting MWCNTs-L-Cys compound possessed abundant sulfydryl on its surface, which could interact with Au nanopractices (AuNPs) to form MWCNTs-L-Cys-AuNPs nanocomposites. After that, immobilization of HRP, GOD and Ab_2 onto the as-prepared MWCNTs-L-Cys-AuNPs nanocomposites was completed according to the following steps: firstly, the Ab_2 was connected with MWCNTs-L-Cys-AuNPs by slowly adding 0.2 mL Ab_2 to 1 mL prepared (L-Cys-HPtPd)$_n$ suspension under softly stirring and incubated for 8 h at 4 °C, followed by centrifugation at 9000 rpm for 15 min at 4 °C to discard excess reagents. The as-prepared nanocomposite was characterized by TEM (Fig. 1). As shown Fig. 1, multiple spheres adsorbed tubular structures were clearly observed, which suggested that the nanocomposite was prepared successfully. Subsequently, 1 mL of GOD and HRP (1 mg mL^{-1}) was added into the residual complexes under gently stirring for about 4 h at 4 °C to block the remaining active sites. At last, the MWCNTS-L-Cys-AuNPs@Ab_2-GOD-HRP bioconjugate was collected by centrifugation and redispersed in 1 mL PBS (pH 7.4) and then stored at 4 °C when not use.