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Ultrasensitive simultaneous detection of four biomarkers based on hybridization chain reaction and biotin–streptavidin signal amplification strategy

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ABSTRACT

A sandwich-type electrochemical immunosensor based on redox probe tags identification technology for ultrasensitive simultaneous detection of four antigens was proposed. In this project, well-distributed graphene/gold (GR–Au) hybrid film was acquired through one-step codeposition in an electrode surface and served as the base substrate for immobilizing capture antibodies (Ab1). Hybridization chain reaction (HCR) and biotin/streptavidin (B/SA), combining with gold magnetic nanoparticles were applied to increase the immobilization amount of signal tags in detection antibody (Ab2) bioconjugates. To verify this strategy, four representative biomarkers, a-fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen (CA)125 and prostate special antigen (PSA), were used as model analytes. The resulting immunosensor could simultaneously detect four antigens in single-pass differential pulse voltammetry (DPV) scan, and exhibited obviously improved senstivity compared to previous similar immunosensors, displayed good linear relationships in the ranges from 0.2 to 800 pg/mL for AFP, 0.2 to 600 pg/mL for CEA, 0.2 to 1000 pg/mL for CA125, 0.2 to 800 pg/mL for PSA and with detection limits of 62, 48, 77 and 60 fg/mL, respectively.

1. Introduction

Biomarkers initially are considered for cancer staging or grading and cancer screening or risk assessment (Ludwig and Weinstein, 2005). The ideal marker would be a "blood test" for cancer in which a positive result would occur only in patients with malignancy, and correlate with stage and response to treatment. However, no single tumor marker is sensitive and specific enough to meet the strict diagnostic criteria (Maruvada et al., 2005; Li et al., 2011). Instead, using multiple biomarkers to estimate one kind of cancer has been widely accepted in practice.

To acquire independent signals for each biomarker in multi-analytes sample, electrochemical researchers have validated different types of tags as nanowires (Walton et al., 2002; Tok et al., 2006), barcodes (Nicewarner-Peña et al., 2001; Sha et al., 2006; Stoeva et al., 2006; Pregibon et al., 2007), enzyme (Kojim et al., 2003; Wilson and Nie, 2006a; Wilson and Nie, 2006b; Fu et al., 2007), quantum dots (QD) (Han et al., 2001; Allen et al., 2007; Liu et al., 2007), dye-doped nanoparticles (Yan et al., 2007), metal ion (Hayes et al., 1994) and redox-probe (Song et al., 2010) etc. Redox probes could fast transmit electron, provide obvious redox peaks in electrochemical scan and have widely used in electrochemical sensors. Recent years, some researchers attempted to apply them in simultaneous multi-analytes immunoassay, several redox probes based immunosensors for simultaneous detection of two (Bai et al., 2012; Xiang et al., 2011; Zhu et al., 2013a; Tang et al., 2011), three (Li et al., 2012) and four (Zhu et al., 2013b) analytes have been presented. Even so, as several immunoreactions took place in same electrode surface, insufficient reaction space may lead to low signal intensity and detection limit compared to conventional single analyte detection. Thus, further improvement of the detection performance was an important research target. Here, we attempt to design a highly sensitive immunosensor for simultaneous detection of four analytes.

Nano-materials usually played key role in sensors construction and performance improvement (Tang and Ren, 2008). Recently, one-step codeposition of GR (Guo et al., 2009) and GR–Au (Liu et al., 2011) methods have attracted much attention due to remarkable traits of simple, fast, and green (need not any reductive

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agent or tedious treating process). Owning to the presence of oxygen functionalities on GR surface provide reactive sites for the nucleation, AuNPs could uniformly grow at GR surfaces without agglomeration (Goncalves et al., 2009). In this work, GR–Au composite was codeposited on GCE surface as substrate for immobilization of bioactive materials,

By addition of an initiator strand of DNA in two species matched DNA, hybridization chain reaction (HCR) events could be triggered and resulted to form nicked double helices analogous (dsDNA) (Dirks and Pierce, 2004). HCR has been used to increase the amount of signal tags in various sensors (Xia et al., 2010). In this work, GR–Au nanomaterial, HCR and biotin/streptavidin (B/SA) signal amplification methods were adopted to synergistically improve the response performance of the multi-analytes immunosensor.

In detailed, biotin labeled Ab2 (bio-Ab2) was immobilized by gold magnetic nanomaterials ($Au/SiO_2–Fe_3O_4$), then using SA as "bridge" to connect bio-Ab2 and bio-S1, next, S2 and bio-S3 were added to trigger HCR so as to form long dsDNA containing abundant biotins and reacted with redox probes labeled SA. The resulting Ab2 bioconjugates contained abundant redox probes, thus could acquire big response signal intensity. This project has as following advantages. Firstly, as one SA could combine with four biotins, using of B-SA-B "bridge" could increase the immobilization amount of bio-S1. Secondly, all of the four kinds of redox probe tags were integrated with SA by amidation and linked with dsDNA through B/SA reaction, this combination method was very simple and highly efficient. Thirdly, using one-step codeposition of GR–Au as the base substrate was simple, fast and green. In addition, magnetic nanomaterials provided great convenience in the preparation of Ab2 bionconjugates through magnet separation. When using four kinds of classic biomarkers (AFP, CEA, PSA and CA125) as model, the resulted immunosensor exhibited wide detection range and ultralow detection limit, which were obviously improved compared with previous reported similar works (detailed comparison of the parameters were presented in Table S1).

2. Experiment

2.1. Reagents

Anti-AFP, anti-CEA, anti-CA125, anti-PSA (monoclone, Ab1); biotin labeled detection antibodies (Ab2); AFP, CEA, CA125 and PSA (antigen) were purchased from Biocell Co. (Zhengzhou, China), graphene oxide (GO) was obtained from Nanjing Xianfeng Nano Co. (Nanjing, China), tetraethyl orthosilicate (TEOS) was purchased from Tiantai Chemical Co. (Tianjin, China), gold chloride tetrahydrate ($HAuCl_4$), poly(diallydimethlammonium chloride) (PDDA), anthraquinone 2-carboxylic acid (Aq), ferrocenecarboxylic acid (Fc), thionine (Thi), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC) and N-hydroxy succinimide (NHS) were from Sigma-Aldrich Co. (USA). Tris(2,2′-bipyridine-4,4′-dicarboxylic acid) cobalt(III) ($Co(bpy)_3^{3+}$, expressed as "Co" for short) was prepared according to literature with slight modification (Burstall and Nyholm, 1952), Gold colloidal nanoparticles (16 nm) were prepared by reduction of gold chloride tetrahydrate. Serum specimens were provided by Xinqiao Hospital of Third Military Medical University (Chongqing, China). Streptavidin (SA) and oligonucleotides were from Biosynthesis Biotechnology Co. Ltd. (Beijing, China), the sequences of these oligonucleotides were as follows:

S1: 5′-Biotin-ACG AAA GAT AGC CAC TCG TAT TCA TCA CTG GAC CGA TAC G***CG ACA TAT CGT GCC AAT TAG***-3′
S2: 5′- Biotin -TGA CAT TTG CTC GAT TCC TAT ACG AGT GGC TAT CTT TCG T***CT AAT TGG CAC GAT ATG TCG***-3′
S3: 5′-ACG AAA GAT AGC CAC TCG TAT TCA TCA CTG GAC CGA TAC G***CG ACA TAT CGT GCC AAT TAG***-3′

All of the chemicals used were of analytical grade and solutions were prepared using ultrapure water (specific resistance of 18 MΩ/cm)

2.2. Apparatus

Electrochemical station (CHI 660C, Shanghai Chenhua Instrument, China) was adopted for CV and DPV scan in conventional three-electrode system consists of a modified glass carbon electrode (GCE, Φ=4 mm) as work electrode, a platinum wire as counter electrode and a saturated calomel electrode (SCE) as reference electrode. Scanning electron microscope (SEM, S-4800, Hitachi, Japan) was adopted to character the morphology of prepared nano-composites. Serum sample comparation test was carried out with an electrochemiluminescence (ECL) equipment (cobas e 411 analyzer, Roche, German) in Xinqiao Hospital of Third Military Medical University (Chongqing, China)

2.3. Preparation of gold magnetic materials

The nano-sized Fe_3O_4 was prepared according to the literature (Kim et al., 2003) with slight modification. 5 mL aqueous solution containing 0.1 M Fe^{2+}($FeCl_2$) and 0.2 M Fe^{3+}($FeCl_3 \cdot 6H_2O$) were droped into 50 mL NaOH (2 M, 80 °C) under vigorous mechanical stirring for 30 min The black precipitate was rinsd to neutral and collected.

The $Fe_3O_4–SiO_2$ composites were obtained through the alkaline hydrolysis of TEOS based on the method reported previously (Girginova et al., 2010). 10 mg $SiO_2–Fe_3O_4$ composites were dispersed in 20 mL PDDA (0.20%) aqueous solution by ultrasonic, after the procedure of centrifugation, the sediments (PDDA/$SiO_2–Fe_3O_4$) were washed and collected. Then, 1 mg PDDA/$SiO_2–Fe_3O_4$ complexes were dispersed in 10 mL AuNPs solution (16 nm) and gently mechanically stirred for 20 min, and collected and redispersed in 2.5 mL PBS (0.1 M, pH 7.4), the SEM morphology of $Au/SiO_2–Fe_3O_4$ was shown in Fig. 1.

2.4. Preparation of Ab2 bioconjugates

The Ab2 bioconjugates contained four kinds of Ab2 (anti-AFP, anti-CEA, anti-CA125 and anti-PSA), they were separately integrated with signal amplification materials in advance. For example, the preparation procedure of anti-AFP bioconjugate can be described as: 100 μL 200 ng/mL biotin labeled anti-AFP solution was added to 1 mL $Au/SiO_2–Fe_3O_4$ solution and stirred gently for 12 h at 4 °C, the resulting complexes (bio-anti-AFP/$Au/SiO_2–Fe_3O_4$) were washed and collected, and dispersed in 1 mL PBS (0.1 M, pH 7.4), then 100 μL 1 mg/mL SA was added and stirred for 30 min, and separation and washing, and redispered in 500 μL PBS (SA/bio-anti-AFP/$Au/SiO_2–Fe_3O_4$), next, 100 μL 2.5 μM/mL initiator bio-S1 was added and gently stired for 30 min to form bio-S1/SA/bio-anti-AFP/$Au/SiO_2–Fe_3O_4$. After washing, a mixture of 100 μL 2.5 μM/mL S2 and 100 μL 2.5 μM/mL bio-S3 were added and incubated for 2 h to form bio-dsDNA/SA/bio-anti-AFP/$Au/SiO_2–Fe_3O_4$ through HCR, after separation and redisperse, 100 μL Aq-SA solution (Aq was integrated with SA through amide reaction in advance) was added and stired for another 30 min to form Aq-SA/bio-dsDNA/SA/bio-anti-AFP/$Au/SiO_2–Fe_3O_4$ composite, after carelly washing, the rusulted complex was dispersed in 1 mL PBS (0.1 M, pH 7.4) for use. The shematic diagram of preparation procedure is shown in Fig. S1. With similar procedures, we also prepared Thi-SA/bio-dsDNA/SA/bio-anti-CEA/$Au/SiO_2–Fe_3O_4$, Co-SA/bio-dsDNA/SA/bio- anti-CA125/$Au/SiO_2–Fe_3O_4$ and Fc-SA/bio-dsDNA/SA/bio-anti-PSA/$Au/SiO_2–Fe_3O_4$ complexes.