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Signal amplification for DNA detection based on the HRP-functionalized Fe_3O_4 nanoparticles

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ABSTRACT

An electrochemical approach for the sensitive detection of sequence-specific DNA has been developed. Horseradish peroxidase (HRP) assembled on the Fe_3O_4 nanoparticles (NPs) were utilized as signal amplification sources. High-content HRP was adsorbed on the Fe_3O_4 NPs via layer-by-layer (LbL) technique to prepare HRP-functionalized Fe_3O_4 NPs. Signal probe and diluting probe were then immobilized on the HRP-functionalized Fe_3O_4 NPs through the bridge of Au NPs. Thereafter, the resulting DNA–Au–HRP–Fe_3O_4 (DAHF) bioconjugates were successfully anchored to the gold nanofilm (GNF) modified electrode surface for the construction of sandwich-type electrochemical DNA biosensor. The electrochemical behaviors of the prepared biosensor had been investigated by the cyclic voltammetry (CV), chronoamperometry (*i*–*t*), and electrochemical impedance spectroscopy (EIS). Under optimal conditions, the proposed strategy could detect the target DNA down to the level of 0.7 fmol with a dynamic range spanning 4 orders of magnitude and exhibited excellent discrimination to two-base mismatched DNA and non-complementary DNA sequences.

1. Introduction

The development of highly sensitive and selective DNA sensors is a challenging subject because of its great importance in various areas such as clinical diagnosis, environmental monitoring, biological research, forensic analysis and antibioterrorism [1,2]. It also offers opportunities in understanding fundamental biological processes involved in disease development and progression and in monitoring patient responses to selected therapy methods [3]. Since the conventional methods could not meet the requirements of detecting DNA at the trace level, considerable efforts have been made towards the amplification techniques for the goal.

Recently, amplification of the sample based on polymerase chain reaction had been proved as an important strategy for sensitive DNA or protein assays [4–6]. However, it required thermal cycle process and strict laboratory conditions to avoid contamination or false results. Strategies based on the amplification of signal produced by hybridization events have demonstrated especially great potential for the direct detection of small amounts of biomolecule with impressive limits of detection. Successful signal amplification strategies include applying new redox-active probes, coupling amplification-by-polymerization concepts with electrochemical detection, integrating nanomaterials to increase loading of tags, and incorporating enzyme-assisted signal amplification processes, etc. [7–19].

Among these strategies, the enzyme amplification technology had attracted special interests due to the outstanding catalytic property and biocompatible performance of enzyme. For example, amplification of electrochemical signals has been demonstrated in stem-loop capture probe system with streptavidin–biotin chimerism for the enzyme binding [13]. With streptavidin–alkaline phosphatase as reporter molecule, enzyme-amplified electrochemical biosensor was used to detect nucleic acid sequences specific of *Legionella pneumophila* [20]. Fan and co-workers reported a stem-loop probe dually labeled with biotin and digoxigenin (DIG) to bind HRP linked-anti-DIG antibody for enzymatically amplifying the electrochemical current signal [14]. However, almost all of the enzyme amplification DNA biosensors were based on hybridization target DNA with signal probe labeled biotin, followed by conjugate with streptavidin labeled enzyme. The amount of labeled enzyme was limited via ligand–receptor interaction events, which would limit the sensitivity level of detection.

Aiming at further improving the efficiency of the enzyme amplification technology, it is anticipated that if more amount of HRP could be introduced in the detection system, the sensitivity and detection limit of DNA biosensor would be enhanced significantly. Herein, we proposed a highly efficient enzyme amplification method using DNA–Au–HRP–Fe_3O_4 (DAHF) bioconjugates as amplification label to construct the sandwich-type DNA biosensor. Fe_3O_4 nanoparticles were utilized as matrix for loading a large

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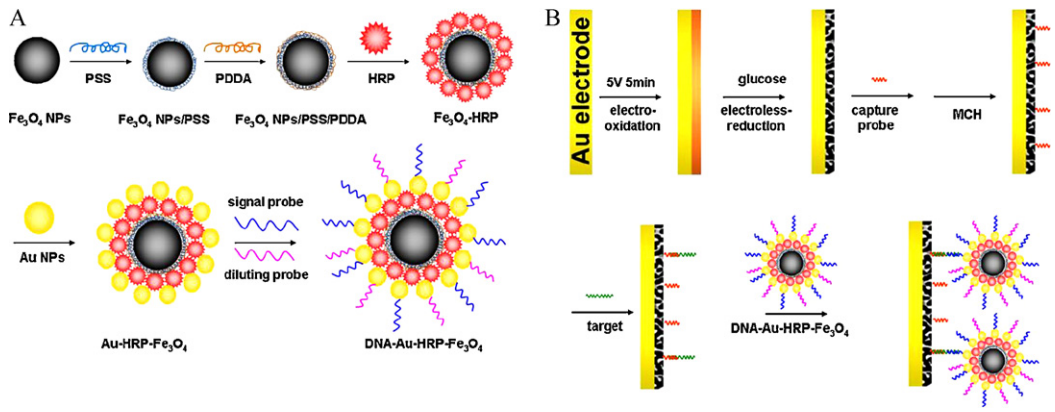

Scheme 1. Schematic for preparation of DAHF bioconjugates (A); preparation procedure of DNA sensor and the sandwich type detection strategy (B).

quantity of HRP through LbL technique, gold nanofilm (GNF) of the electrode surface with unique properties [21–25] was employed as substrate to anchor capture probe. In presence of the target DNA, hybridization would ensure the attachment of the DAHF bioconjugates onto the GNF, followed by the enzyme catalyze oxidization of TMB substrate for the amplification of electrochemical signal. Since the DAHF bioconjugates carried large quantities of HRP, the approach proposed here would provide a more sensitive method for DNA detection.

2. Experimental

2.1. Materials and reagents

Hydrogen tetrachloroaurate ($HAuCl_4$), trisodium citrate, β-d-glucose, horseradish peroxidase (HRP, MW 44,000, ~250 units/mg protein), ferric chloride hexahydrate ($FeCl_3{\bullet}6H_2O$ > 99%), ferrous chloride tetrahydrate ($FeCl_2{\bullet}4H_2O$ > 99%), sodiumdodecylsulfate (SDS), sodium hydroxide, hydrochloric acid, poly(dimethyldiallyl ammonium chloride) (PDDA), poly(sodium 4-styrenesulfonate) (PSS), tri(2-carboxyethyl) phosphinehydrochloride (TCEP), streptavidin labeled horseradish peroxidase, albumin bovine serum (BSA), 6-mercapto-1-hexanol (MCH), hexaammineruthenium(III) chloride (RuHex) were obtained from Sigma–Aldrich (St. Louis, MO). 3,3′,5,5′ tetramethylbenzidine (TMB) was purchased from Neogen (Lexington, KY) in the format of a ready-to-use reagent (K-blue low-activity substrate, H_2O_2 included). All of the synthetic oligonucleotides were purchased from Shanghai Sangong biotechnology Co. Their base sequences are as follows:

Capture probe sequence, 5′-TGG AAA ATC TCT AGC AGT CGT–$(CH_2)_6$–SH-3′.
Target DNA sequence, 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA GGG TCT GAG GGA-3′.
Signal probe sequence, 5′-SH–$(CH_2)_6$–ATG TCC CTC AGA CCC TTT-3′.
Diluting probe sequence, 5′-SH–$(CH_2)_6$–GTC GCG CGA ACC GTA TAG-3′.
Two-base mismatched DNA sequences, 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA GCG TCT GTG GGA-3′.
Non-complementary DNA sequences, 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA CTT CAA CAG TGC CCC-3′.
Biotinylated probe sequences, 5′-biotin–$(CH_2)_6$–ATG TCC CTC AGA CCC TTT-3′.

The other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using ultra-pure water (Milli-Q, Millipore). The buffers involved in this work were as follows: DNA immobilization buffer, 10 mM Tris–HCl, 1.0 mM EDTA, 0.3 M NaCl, and 1.0 mM TCEP (pH 8.0); DNA hybridization buffer, 10 mM Tris–HCl, 1.0 mM EDTA, and 0.25 M NaCl (pH 8.0); Washing buffer, 10 mM phosphate buffer (PBS), 0.1% SDS and 0.1 M NaCl (pH 8.0).

2.2. Apparatus

The electrochemical measurements for cyclic voltammetry (CV), chronocoulometry (CC) and chronoamperometry (*i*–*t*) were performed on a CHI 660C electrochemical workstation (CH Instrument Co.) Electrochemical impedance spectroscopy (EIS) experiments were carried out on an Autolab PGSTAT-30 potentiostat/galvanostat (Eco Chemie BV, Utrecht, The Netherlands) with FRA software. UV–vis absorption spectra were carried out on a Shimadzu UV-3600 UV–vis–NIR photospectrometer (Shimadzu Co.) Transmission electron microscopy (TEM) images were taken with a JEOL model 2000 instrument operating at 200 kV accelerating voltage. Atomic force microscopy (AFM) images were performed in ambient conditions using a molecular imaging Pico SPM in tap mode with a 10 μm scanner.

2.3. Preparation of Au NPs and Fe_3O_4 NPs

Au NPs were prepared according to the literature [26]. Briefly, 0.6 ml of 0.1 M $NaBH_4$ was added to 20 mL aqueous solution containing 2.5×10^{-4} M $HAuCl_4$ under stirring. The solution turned to orange–red color immediately, indicating the formation of Au NPs. These particles were stirred at 25 °C for 3 h and then kept in a refrigerator at 4 °C for further use.

The Fe_3O_4 NPs were synthesized by controlled co-precipitation of Fe (II) and Fe (III) ions with 0.5 M NaOH as the reductant according to previous report [27]. Typically, 1 M $FeCl_3{\cdot}6H_2O$, 0.5 M $FeCl_2{\cdot}4H_2O$ was dissolved in 25 mL of 0.4 M HCl solution degassed with nitrogen under vigorous stirring. The co-precipitation of Fe_3O_4 NPs was carried out in a three-neck round-bottom flask. Before the co-precipitation reaction, the above mixture solution was added to 250 mL of 0.5 M NaOH, which was preheated to 80 °C. This reaction was protected under N_2 atmosphere and was vigorously stirred. Black powder was collected by sedimentation with the help of an external magnetic field and washed several times with water until stable ferro fluid was obtained. Finally, the particles were redispersed in water and stored at 4 °C.

2.4. Preparation of DAHF bioconjugates

The preparation procedure of the DAHF bioconjugates was showed in Scheme 1A. The layer-by-layer assembly of oppositely