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Ultrasensitive detection of DNA based on target-triggered hairpin assembly and exonuclease-assisted recycling amplification

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ABSTRACT

An ultrasensitive electrochemical detection of target DNA was developed based on target-triggered hairpin assembly and exonuclease III (Exo III)-assisted recycling quadratic amplification strategy. The detection employed a gold nanoparticles (AuNPs) modified Au electrode and two specially designed hairpin probes P1 and P2. P1 probe contained G-quadruplex-forming sequence and target DNA recognition region, and was immobilized on the electrode. P2 probe was used as a secondary complementary sequence which can displace target DNA and hybridize with P1 probe. In the absence of target DNA, these hairpin structures of P1 and P2 can coexist. While in the presence of target DNA, it can trigger the self-assembly process of P1 and P2 and initiate the Exo III-assisted two recycling process, resulting in the formation of G-quadruplex structure on electrode surface. Finally, with the addition of hemin, numerous G-quadruplex-hemin complexes formed on the electrode surface and gave a pronounced electrochemical response in differential pulse voltammogram (DPV). Taking K-ras proto oncogene as an example, the proposed DNA biosensor exhibited a wide detection range from 10 fM to 20 nM, and an extremely low detection limit of 2.86 fM. Moreover, it can clearly discriminate one-base difference in DNA sequence, thus can identify the mutation of the target gene. The proposed DNA biosensor has potential applications in the fields of clinic diagnosis, biomedicine, food and environment microbial monitoring.

1. Introduction

Detection of trace disease-related sequence-specific DNA has significant applications in clinic diagnosis [1], gene therapy [2], sickness prevention and forensic investigations [3,4] and thus has attracted substantial research efforts. So far, various analytical techniques have been employed for DNA detection. Compared with those colorimetric [5], chemiluminescent [6], or fluorescent [7] assays, the electrochemical DNA biosensors have attracted particular attention owing to its excellent characteristics, such as high sensitivity, low cost, simplicity, high specificity and easy to miniaturization [8,9]. With the development of synthesis technique of nucleic acid probe, the electrochemical DNA biosensors have been widely applied in the detection of target genes [10,11], environmental monitoring [12], and food quality control [13], *etc*.

Because of the small concentration of sequence-specific target DNAs in biological samples, the development of signal amplification strategies is essential for DNA biosensors to obtain sufficient sensitivity [14]. Generally, several amplification strategies were involved in the fabrication of DNA biosensors. The traditionally used signal amplification was mostly based on DNA-amplification techniques, such as hybridization chain reaction (HCR) [15], strand displacement amplification (SDA) [16], and rolling circle amplification (RCA) [17]. With the development in nanotechnology, a variety of nanomaterials, including gold nanoparticles (AuNPs) [18], graphene [19], carbon nanotubes [20], and nanocomposites [21,22] were employed in the fabrication of DNA biosensors as a carrier to increase the loading of the immobilized biomolecules or active signal-labels.

The target-triggered hairpin assembly with target DNA recycling strategy consists of two parts. One is the hybridization reaction triggered by the target. The other is strand displacement reaction. These reactions take place without the use of enzymes and complicated processes [7,23,24]. However, extremely high sensitivity and satisfactory detection range are not easy to be achieved by using a single amplification strategy. Thus two or more amplification strategies were sometimes introduced into the fabrication of DNA biosensors [25,26]. G-quadruplex-hemin complex has been widely used in various biosensor configurations as signal amplification labeling [5,27,28]. Owing to its characteristic redox signal and peroxidase-like activity, the formation of G-quadruplex-hemin

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can provide intensive electrochemical signal in the fabrication of biosensors.

K-ras proto oncogene is one of the members of ras family which can regulate cell growth as a molecular switch. The codon 12 mutation will disorder cell growth and cause cancer, such as carcinoma of the pancreas [29]. According to statistics, the frequency of K-ras mutations is around 80-100 80–100% in pancreatic cancers [30]. Therefore, the detection of K-ras is of great significance since it can be utilized as a biomarker in the early diagnosis of pancreas cancer [31,32]. Herein, an ultrasensitive, label-free DNA biosensor for K-ras gene sequence was developed by coupling exonuclease III (Exo III)-assisted target-triggered hairpin assembly with target DNA recycling strategy and employing G-quadruplex-hemin as a signal label.

2. Experimental

2.1. Materials and reagents

6-mer6-mercaptohexanol (MCH), hemin, $HAuCl_4$, 1, 6-hexanedithiol (HDT) were purchased from Sigma-Aldrich. Exonuclease III (Exo III) was obtained from Takara Biotechnology Co., Ltd. 4- (2-hydroxyethyl)-1-piperazine ethanesulfonic acid sodium salt (HEPES) and tris (2-carboxyethyl) phosphine (TCEP) were purchased from Sangon Biotech Co., Ltd. Other chemicals were of analytical grade. All solutions were prepared with ultrapure water (18.2 MΩ cm) obtained from a Millipore water purification system.

All oligonucleotides were synthesized and HPLC-purified by Sangon Biotech Co., Ltd. And all oligonucleotides (100 μM) were stocked in 20 mM Tris-HCl buffer (200 mM NaCl, 2 mM $MgCl_2$ and 20 mM KCl, pH 7.4), and diluted with 10 mM Tris-HCl buffer (1 mM EDTA, 0.1 M NaCl, 10 mM TCEP, pH 7.4). These oligonucleotides were heated to 95 °C for 5 min, and then slowly cooled down to room temperature. The sequences of the oligonucleotides were as below.

Hairpin probe 1 (P1): 5′-HS-$(CH_2)_6$-GGGTGGGCG*GGATGGGT*TACGCCACCAGC TCCA*ACCCATCC*TT-3′

Hairpin probe 2 (P2): 5′-AA*GGATGGGTGGAG*CTGGTGGCGTAGGCA *CTCCAC CCATCC*AGAC-3′

Target DNA (TD): 5′-TGGAGCTGGTGGCGTAGGCA-3′

Single-base mismatched sequence (M1): 5′-TGGAGCTG<u>A</u>TGGCGTAGGCA-3′

Three-base mismatched sequence (M3): 5′-TGGAGCT<u>CCA</u>GGCGTAGGCA-3′

Non-target DNA 1: 5′-GGCAGCAATTTCACCAGTACTA-3′

Non-target DNA 2: 5′-GATTTTCTTCCTTTTGTTC-3′

The italic letters are the sequence of the stem arms, and the underlined letters are the mismatched bases.

2.2. Preparation and modification of Au electrode

The bare Au electrode (3 mm in diameter) was carefully polished with wet alumina slurry (0.3 and 0.05 μm) and then sonicated in ultrapure water and ethanol for 3 min, respectively. Then the electrode was electrochemically activated by immersing into 0.5 M H_2SO_4 and cyclic scanning within the potential range from −0.2 to +1.5 V. The electrode was dried at nitrogen atmosphere.

The surface of Au electrode was modified with AuNPs through HDT, which possesses free −SH group at both ends and acts as a connection bridge to immobilize AuNPs on gold electrode [33,34]. Firstly, the cleaned Au electrode was immersed in 10 mM HDT ethanol solution for 3 h to ensure HDT to be self-assembled on the electrode. The obtained HDT-Au electrode was then rinsed by ethanol and ultrapure water to remove the loosely bound HDT. AuNPs were prepared through the reported procedure [35] and characterized by TEM and UV–vis spectrophotometer. The average diameter of AuNPs is about 12 nm and the UV–vis spectrum shows a typical absorption peak at 520 nm. The HDT-Au electrode was subsequently dipped in AuNPs solution overnight under ambient condition. Finally, the prepared AuNPs-HDT-Au electrode was washed with water and dried with nitrogen.

2.3. Fabrication of the electrochemical DNA biosensor

The AuNPs-HDT-Au electrode was first incubated in 100 μL of 10 mM Tris-HCl working solution with 350 nM P1 probe for 12 h at room temperature. And then the electrode was immersed in 2 mM MCH for 2 h, followed by rinsing with water and drying with nitrogen.

To detect the concentration of target DNA, the P1 probe modified AuNPs-HDT-Au electrode was dipped into 100 μL 10 mM Tris-HCl working solution (100 mM NaCl, 1 mM $MgCl_2$ and 10 mM KCl, pH 7.4), containing 500 nM P2 probe, 50 U Exo III, and different concentration of TD, for 2 h at 37 °C (the optimal reaction temperature of Exo III). Subsequently, 2 μL of 20 mM hemin and 98 μL of 20 mM HEPES buffer (50 mM KCl, 1% DMSO, pH 7.4) were added into the hybridization system mentioned above. The electrode was incubated for another 1 h at room temperature to induce the liberated part I to fold into a G-quadruplex-hemin complex. After rinsing for 30 s, the resultant electrode was used for the electrochemical measurement.

2.4. Electrochemical measurements

The electrochemical measurements were conducted on a CHI 660e electrochemical workstation. Differential pulse voltammetry (DPV) was performed in 20 mM HEPES testing buffer (20 mM KCl, pH 7.4) in the potential range from −0.1 to −0.6 V. Cyclic voltammetry (CV) and electrochemical impedence spectroscopy (EIS) were carried out in 0.1 mM $[Fe(CN)_6]^{3-/4-}$ (containing 0.1 M KCl) to characterize the different interfacial processes during the modification of the electrode. For CV measurements, the potential range was from −0.2 to −0.6 V at a scan rate of 50 mV/s. For EIS measurements, the frequency range was from 0.1 Hz to 10 kHz.

3. Results and discussion

3.1. The principle of the electrochemical DNA biosensor

The principle of the electrochemical DNA biosensor is depicted in Scheme 1. The DNA biosensor is primarily constructed with two hairpin structures and Exo III, where the target DNA can induce hairpin assembly leading to Exo III-assisted autocatalytic recycling reaction. Two special hairpin probes termed P1 and P2 were rationally designed, which can stably coexist in the solution in the absence of target DNA. Both hairpin probes contain three major parts (part I, II and III locate in hairpin probe P1, and part IV, V and VI locate in hairpin probe P2). Part I is a G-quadruplex-forming sequence which is blocked via hybridization with part III; and part II is the target DNA recognition region. Part IV is complementary to part III, and is caged via hybridization with part VI. Part V has the same sequence with target DNA, which can serve as a secondary target to displace target DNA and trigger the cleavage of probes by Exo III. Thus, P1 probe can firstly be immobilized on AuNPs-HDT-Au electrode through Au-S interaction. It can coexist with P2 probe in the detection solution containing Exo III. However, the existence of the target DNA can trigger two independent cycles of reactions: (1) The target DNA first interacts with part II and opens the hairpin structure of P1. Through a branch migration process, part III can hybridizes with part IV, leading to the displacement of target DNA