Sensors and Actuators B 244 (2017) 450–457

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Label-free, non-enzymatic and ultrasensitive electrochemical nucleic acid biosensing by tandem DNA-fueled target recycling and hybridization chain reaction

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ARTICLE INFO

Article history:
Received 17 September 2016
Received in revised form
29 November 2016
Accepted 3 January 2017
Available online 4 January 2017

Keywords:
DNA biosensor
Electrochemistry
DNA-fueled target recycling
Hybridization chain reaction
Signal amplification

ABSTRACT

Herein, the DNA-fueled target recycling was effectively married with the hybridization chain reaction (HCR) for the autonomous dual signal amplification, by which an isothermal, non-enzymatic, label-free and ultrasensitive electrochemical DNA biosensor was developed for the first time. The DNA-fueled target recycling was operated onto the three-strand duplex DNA probe immobilized electrode according to the principle of the target DNA-triggered two cascaded toehold-mediated strand displacement reactions, accompanied with the association of plenty of DNA fuel strands on the electrode surface. The introduced DNA fuel strand possesses an emerging DNA fragment, which could be used to further propagate the HCR between two DNA hairpins, inducing the formation of a typical one-dimensional linear DNA concatamer on the electrode surface. The formed DNA concatamers were then bound with Hexaammineruthenium (III) chloride (RuHex) through electrostatic interaction for the significantly enhanced electrochemical response toward target DNA. A very low detection limit of 20 aM with an excellent selectivity toward target DNA could be achieved. The developed biosensor creates an opportunity for the simple, flexible and enzyme-free coupling of the target recycling and post-amplification strategies and thus opens a promising avenue for the electrochemical detection of nucleic acid with low abundance in bioanalysis and clinical biomedicine.

1. Introduction

The development of DNA biosensors capable for the detection of trace amount of target analytes is being greatly motivated by its potential applications ranging from virus detection to the diagnosis of genetic diseases [1–4]. Currently, the polymerase chain reaction (PCR) could be considered as the most commonly used method for the DNA amplification [5,6]. But it involves the complex thermal replication cycles, which may easily induce the false-positive signal. Compared with it, the alternative isothermal signal amplification strategies are more attractive for biosensor fabrication owing to their substantial characteristics including the easier to operate, constant temperature and reduced false positive rate [7,8]. The developed isothermal amplification method further includes nuclease-based or enzyme-free strategy based on its dependent or independent upon the use of protein enzyme [9–11]. The nuclease-based isothermal amplification is usually involved in the individual or combined use of various nucleases including exonuclease, endonuclease and polymerase, etc to achieve the recycling of target itself and/or the concomitant generation of large amounts of target-associated nucleic acid sequences for signal amplification [12–18]. Although sometimes the superior detection performance toward targets could be acquired especially in the buffer solution, the employment of nucleases increases the complexity of the experimental system, the assay cost, and even the risk of false-positive signal output, which limits its wide applications especially in complex matrix. Thus, the development of enzyme-free isothermal amplification strategies that allow targets to be profiled with high confidence is highly advocated for the diagnostic purposes.

The enzyme-free amplification strategy is usually designed based on the dynamic DNA assembly principle and exhibits the advantages such as lost cost, easy construction and uncompromised biocompatibility [19–22]. Toehold-mediated strand displacement reaction has been well known as a programmable form of dynamic DNA assembly to develop enzyme-free amplification strategy for

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http://dx.doi.org/10.1016/j.snb.2017.01.017
0925-4005/

Table 1
DNA sequences used in the experiments[a].

Name	Sequence (5′–3′)
Immobilization strand (IP)	SH-$(CH_2)_6$-TTTTTTGAAATGGTGGAAAGGAGGCGAGTCTTCC AGTGTGATGA
Protector strand-1 (PS-1)	CACTGGAAGACTCGCCTATATCCATAAATT
Protector strand-2 (PS-2)	CCTTTCCACCATTTC
Fuel strand (FS-a)	GCACGTCCACGGTGTCGCTTGAATCACTGGAAGACTCGCCT CCTTTCCACCATTTC
Fuel strand (FS-b)	CACTGGAAGACTCGCCTCCTTTCCACCATTTC
Ferrocene-labeled Fuel strand	GCACGTCCACGGTGTCGCTTGAATCACTGGAAGACTCGCCT CCTTTCCACCATTTC-ferrocene
Hairpin DNA-1 (HP-1)	ATTCAAGCGACACCGTGGACGTGCACCCACGCACGTCCAC GGTGTCGC
Hairpin DNA-2 (HP-2)	GCACGTCCACGGTGTCGCTTGAATGCGACACCGTGGACGT GCGTGGGT
Target DNA (TD)	TCATCACACTGGAAGACTC
One-base mismatch target (1MT)	TCATCAAACTGGAAGACTC
Two-base mismatch target (2MT)	TCATCAAGCTGGAAGACTC
Non-complementary sequence	ACGTTGCATATCGACTAGC
Target DNA2 (TD2)	GCACGTCCACGGTGTCGCTTGAATTCA TCACACTGGAAGAC TCGCCTCCTTTCCACCATTTC

[a] The underlined letters in 1MT and 2MT indicate the mismatched bases.

the biosensor fabrication [23–26]. The currently proposed enzyme-free amplification strategy can be further simply classified as the target recycling and post-amplification modes with the catalytic hairpin assembly (CHA) circuit and hybridization chain reaction (HCR) as the typical examples, respectively. The CHA circuit was executed with the catalytic assembly of two DNA hairpins into duplex DNA, accompanied with the target recycling to contribute for the indirect amplification toward target amounts [27–32]. But the CHA strategy is often confronted with the relatively rigorous design principle for the adopted hairpin DNA. Also, the two hairpin substrates in a CHA circuit may potentially react nonspecifically, even in the absence of target, which leads to relatively high background signals. Thus, the development of new enzyme-free target recycling strategy with the flexibility in sequence designs and the stability in assembly products should be explored. As for the HCR, it is operated with the alternate opening and assembly of two DNA hairpins for the generation of DNA concatamer in the presence of DNA triggers [33–37]. It now has been widely employed as the excellent enzyme-free post-amplification strategy for the fabrication of various biosensors toward the analysis of nucleic acid, protein and small molecules [38–41]. Although great advances have been made toward the target detection by using the enzyme-free amplification strategies, the designing of new enzyme-free DNA amplification-based biosensing system to upgrade the detection performance including reliability and sensitivity is still highly desirable to satisfy the requirement for the profiling trace amounts of biomarkers, and then serve for the clinical early diagnosis and treatment of some major diseases. It is tentatively conceived that the effective combination of enzyme-free target recycling and post-amplification strategies would be beneficial for the biosensor performance improvement. Unfortunately, it has been rarely reported for the biosensor fabrication via the grafting or marriage of these two strategies [42–44].

Herein, an isothermal, enzyme-free, label-free and ultrasensitive electrochemical DNA biosensor platform was proposed by coupling DNA-fueled target recycling and HCR-based post-amplification. The DNA-fueled target recycling strategy is autonomously operated by two cascaded toehold-mediated strand displacement reactions [23,45,46], which features with the simplicity in sequence design and operational flexibility in biosensor fabrication, and also the minimized background noise. Accompanied with the DNA-fueled target recycling process, the plenty of DNA fuel strands would be successfully associated on the electrode surface, which contains the exposed DNA fragment at 5′-terminus for the further propagation of HCR between two DNA hairpins. The formed linear DNA concatamer could then bind with Hexaaminеruthenium (III) chloride (RuHex) for the label-free and amplified electrochemical detection of target DNA. The currently proposed tandem DNA-fueled target recycling and HCR strategy may hold great potential for the ultrasensitive nucleic acid biosensor fabrication served for the applications in bioanalysis and clinical diagnosis.

2. Experimental

2.1. Materials and chemicals

Hexaaminеruthenium (III) chloride (RuHex), 6-Mercaptohexanol (MCH) and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acrylamide/bisacrylamide 39:1 40% gel stock solution, *N,N,N′,N′*tetramethylethylenediamine (TEMED), ammonium persulfate (APS) and ethidium bromide (EB) were purchased from Yantai Science and Biotechnology Co., Ltd. (Yantai, China). Fetal bovine serum was obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). The HPLC-purified oligonucleotide sequences were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China) and listed in Table 1. The designed target DNA in current system is a disease-related sequence of the p53 gene fragment. All other reagents were of analytical grade without further purification.

2.2. Immobilization of three-strand DNA duplex probe

The gold electrode (2 mm diameter) was firstly polished with 0.05 μm alumina powder to obtain a mirror surface, followed by sonication in acetone and water for 5 min, respectively. The gold electrode was then electrochemically cleaned in a 0.5 M H_2SO_4 solution within a potential window between −0.2 and +1.5 V at a scan rate of 100 mV/s to remove any remaining impurities. The mixture of thiolated immobilization strand (SH-IP, 0.5 μM), protector strand-1 (PS-1, 0.6 μM) and protector strand-2 (PS-2, 0.6 μM) in 10 mM Tris-HCl (0.1 M NaCl, 1 mM EDTA, pH 7.4) was heated to 85 °C for 5 min and then allowed to cool down to room temperature for at least 60 min to form the three-strand DNA duplex probes. The above mixture was incubated with TCEP (10 mM) for 30 min to reduce the disulfide bonds of the SH-IP. Then, droplets of 10 μL of the above duplex probe were pipetted on the electrode surface