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Dual amplified and ultrasensitive electrochemical detection of mutant DNA Biomarkers based on nuclease-assisted target recycling and rolling circle amplifications

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ABSTRACT

Based on nicking endonuclease (NEase)-assisted target recycling and rolling circle amplification (RCA) for *in situ* generation of numerous G-quadruplex/hemin complexes, we developed a new, dual amplified and ultrasensitive electrochemical biosensor for mutant human p53 gene. The target mutant DNA hybridizes with the loop portion of a dithiol-modified hairpin probe (HP) self-assembled on a gold sensing electrode and forms nicking site for the NEase, which cleaves the HP and releases the target DNA. The released target DNA again hybridizes with the intact HP and initiates the DNA recycling process with the assistance of the NEase, leading to the cleavage of a large number of the HPs and the generation of numerous primers for RCA. With rationally designed, G-quadruplex complementary sequence-encoded RCA circular template, subsequent RCA results in the formation of long DNA sequences with massive tandem-repeat G-quadruplex sequences, which further associate with hemin and generate significantly amplified current response for highly sensitive DNA detection down to 0.25 fM. The developed method also exhibits high specificity for the target DNA against single-base mismatched sequence. With the ultrahigh sensitivity feature induced by the dual signal amplification, the proposed method can thus offer new opportunities for the detection of trace amounts of DNA.

1. Introduction

Sensitive and selective detection of sequence-specific DNA has become increasingly important in modern life sciences owing to its potential applicability ranging from genetic research of diseases to clinical diagnosis and therapy (Wang, 2000; Rosi and Mirkin, 2005; Li et al., 2010a). Over the past decades, various analytical protocols, including fluorescent (Zuo et al., 2010; Ryoo et al., 2012), electrochemical (EC) (Zhang et al., 2009; Wang et al., 2013), chemiluminescent (Liu et al., 2011) and colorimetric (Ji et al., 2011; Liu and Lu, 2006) approaches, have been implemented to achieve sensitive detection of DNA. Among these techniques, the EC approach as a promising tool for DNA detection has attracted particular attention in different fields owing to its inherent advantages such as simplicity, rapidness and low cost (Lei and Ju, 2012; Paleĉek and Bartoŝík, 2012). Despite the attractive progresses, the development of EC DNA sensors with ultrahigh sensitivity, selectivity and simplicity and the discrimination of single-base mismatch still remain major challenges.

In order to push down the detection limit, the development of effective signal amplification strategies for DNA detection continues to attract tremendous research interest (Comstock et al., 2011; Sorgenfrei et al., 2011). Although traditional polymerase chain reaction (PCR) (Heid et al., 1996) has been the most widely used method for DNA detection, it suffers the drawbacks of high cost, easy contamination and expert operation (Nallur et al., 2001; Giljohann and Mirkin, 2009). In recent years, various signal amplification strategies have been suggested to overcome the PCR-related shortcomings by using amplification labels such as enzymes (Patolsky et al., 2001; Gao et al., 2011), nanomaterials (Wang et al., 2003; Xu et al., 2009) and DNA biobarcode (Li et al., 2010b) or using the nuclease-assisted target recycling strategies. The target DNA recycling amplification methods via nuclease, such as polymerase-mediated isothermal strand displacement amplification (ISDA) (Guo et al., 2009; He et al., 2010), exonuclease III-aided signal amplification (Wu et al., 2011; Liu et al., 2013; Su et al., 2011) and nicking endonuclease signal amplification (NESA) (Xu et al., 2009; Chen et al., 2010; Ji et al., 2012), wherein a single target can be amplified to cyclically produce multiple hybridization events, have increasingly become attractive alternatives for the detection of trace levels of target DNA. NESA has received particular interest due to the distinct specificity for single-base

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mismatch detection capability and high sensitivity (Li et al., 2011; Xue et al., 2012a). In a typical NESA design, the target DNA hybridizes with a probe to create a nicking endonuclease (NEase) restriction site, which is recognized by sequence-specific NEase. After the NEase cleaves the probe in a DNA duplex into two pieces, the target DNA is released and then initiates next hybridization, cleavage, and dissociation process, which results in target recycling and signal amplification (Yin et al., 2012). Owing to the specific recognition of sequences, these NEase-assisted target recycling strategies obviate extra probe modification or conjugation steps and the addition of many exogenous reagents. Additionally, by taking the advantage of the NEase, reactions can be performed under an isothermal condition without using specialized instrumentation, which offers the NEase-assisted target recycling method with the potential to be widespread for routine analysis.

The discovery of the enzyme-assisted DNA recycling strategy can offer new amplification routes for highly sensitive DNA monitoring by integrating other signal enhancement approach to achieve dual or multiple amplifications within one assay protocol. Here, by coupling NEase-assisted target DNA recycling with rolling circle amplification (RCA), we developed a new hybrid signal amplification strategy for highly sensitive EC detection of the mutant human p53 gene cancer biomarker DNA. RCA, as an advanced DNA amplification technique alternative to PCR, can achieve significant signal amplification via the production of thousands of repeated sequences under mild reaction conditions and with speediness, high efficiency, and specificity (Liu et al., 1996; Ji et al., 2012). Due to the drastic signal amplification power, RCA has been widely employed in various sensing schemes for the analyses of proteins and nucleic acids (Hu and Zhang, 2010; Yan et al., 2012; Xue et al., 2012b). In our approach, the target mutant p53 genes hybridize with the hairpin DNA probes on the senor surface and create nicking sites for NEase to initiate the target DNA recycling amplification process to cleave numerous probes. The cleaved probes on the sensor surface are subject to *in situ* RCA to generate massive long DNA sequences with repeat G-quadruplex units, which associate with hemin to form G-quadruplex/hemin complexes. Direct electron transfer between hemin and the sensing electrode during the potential scan thus can offer amplified current response for highly sensitive detection of the mutant p53 gene.

2. Experimental

2.1. Materials and reagents

Hemin, dimethyl sulfoxide (DMSO), 6-mercaptohexanol (MCH) and tris(2-carboxyethy)phosphine hydrochloride (TCEP) were purchased from Sigma (St. Louis, MO, USA). A hemin stock solution (10 mM) was prepared in DMSO and stored in dark at −20 °C. Tris–HCl, 4-(2-hydroxyethyl) piperazine-1 ethanesulfonic acid sodium salt (HEPES), and dNTPs were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The NEase, N.BstNB I (an endonuclease that recognizes the specific nucleotide sequence of 5′-GAGTC-3′in a double-stranded DNA) and 10 × NE Buffer 3 (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (DTT), pH 7.9) were purchased from New England Biolabs (Ipswich, MA, USA). Phi29 DNA polymerase, T4 DNA ligase and 10 × T4 DNA ligase buffer were obtained from Fermentas (Lithuania).

All synthetic oligonucleotides with sequences listed below were ordered from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

Dithiol-modified hairpin capture probe (HP): 5′-SH–$(CH_2)_6$-TTTTTT*ACACAGTGTA***CTCACACGAATTCATCTGAC*GAGTC*TTC-C↓AGTGTGATGA*TACACTGTGT*TTTTTT-$(CH_2)_6$–SH-3′. The HP contains four functional regions: (1) the italic parts at the two ends represent the complementary sequences of the stem arm; (2) the bold part is complementary to the bold part of the padlock probe; (3) the underlined part is the complementary sequence to the mutant target DNA and (4) ***GAGTC*** is the recognition sequence of N.BstNB I, and the arrow indicates the nicking position.

Padlock probe: 5′-p **ATTCGTGTGAG**AAAACCCAACCCGCCCTACC-CAAAA **GGAAGACTCGTCAGATGA**-3′ (the bold portion matches the bold sequence of the hairpin probe; the underlined letters is completely complementary with the G-quadruplex sequence, and p in the padlock probe represents phosphate at the 5′ end).

Mutant human p53 gene target: 5′-TCATCACACTGGAAGACTC-3′;

Single-base mismatched sequence (sDNA): 5′-TCATCACACTG-GAAGAATC-3′;

Non-complementary sequence (nDNA):5′-GACGTCTGACTTCCTG-CGA-3′.

All other reagents were of analytical grade and used as received. Aqueous solutions were prepared using ultrapure water (specific resistance of 18 MΩ-cm).

2.2. Preparation of the HP-modified sensing electrode

The gold electrode (AuE, 3 mm in diameter) was cleaned by immersion in a freshly prepared piranha solution (a 3:1 v/v mixture of concentrated H_2SO_4 and 30% H_2O_2) for 30 min. After rinsing thoroughly with water, the electrode was polished with 0.3 and 0.05 μm aluminum slurry, followed by sequential sonication in distilled water, ethanol and distilled water for 5 min each to remove residual alumina powder. The well-polished electrode was then subjected to electrochemical pretreatment in 0.5 M H_2SO_4 with potential scanning from 0.2 to 1.6 V until remarkable voltammetric peaks were obtained. After being dried with nitrogen, the electrode should be immediately used for HP immobilization.

The above gold electrode was then incubated with 0.25 μM pretreated HP in a immobilization buffer solution (10 mM Tris–HCl, 10 mM TCEP, 0.1 M EDTA, 10 mM $MgCl_2$, pH 7.4) for 12 h at room temperature. $MgCl_2$ was added into the electrolyte to induce the formation of the hairpin structure of the HP, as reported by previous literatures (Gao et al., 1999; Chen et al., 2010). After that, the electrode was immersed in 1 mM MCH solution for 1 h to reduce nonspecific DNA adsorption and to optimize the orientation of the capture probes, followed by rinsing with ultrapure water.

2.3. Target hybridization and nicking enzyme-assisted target recycling

For target hybridization, the MCH/HP/AuE was incubated with 10 μL of 10 mM Tris–HCl solution (1 mM EDTA, 100 mM NaCl, pH 7.4) containing different concentrations of target DNA for 1 h at room temperature. After washing with Tris–HCl buffer, 10 μL of N. BstNB I enzyme (3 units μL^{-1}) in NEBuffer 3 was added and incubated at 55 °C. After 60 min of nicking, the electrodes were rinsed with NEBuffer 3 and dried under a stream of nitrogen gas.

2.4. RCA generation of G-quadruplex/hemin complexes

For subsequent RCA process, 10 μL of solution containing 1 × ligase buffer (40 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8), 50 nM padlock probe, 0.2 U T4 ligase was added onto the electrode surface for hybridization with the HP fragments produced in nicking enzyme-assisted target recycling and incubated at 37 °C for 1 h. The introduction of the T4 ligase links the 5′ and 3′ termini of the padlock probe together to form