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A cascade signal amplification strategy for ultrasensitive colorimetric detection of BRCA1 gene

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† Electronic Supplementary Information (ESI) available: The sequences of the used oligonucleotides. See DOI:

In this work, we reported a new cascade signal amplification strategy to combine DNAzyme assistant DNA recycling followed with rolling circle amplification (RCA) for highly sensitive colorimetric detection of breast cancer1 (BRCA1) gene. A hairpin probe (HP) was designed, containing the 3'-protruding DNA sequence as target recognition unit and the caged 8–17 DNAzyme fragment in the loop region as a trigger for the next reaction. Once challenged with target DNA, the DNAzyme was liberated from the caged structure. In the presence of Pb^{2+} cofactor, the DNAzyme leaded to the cleavage of a large number of molecular beacons (MBs), accompanied by the releasing of another free DNAzyme fragment for the successive cleavage process and autonomous generation of numerous padlock probes for triggering RCA reaction. The amplified RCA products can form G-quadruplex/hemin DNAzyme to act as a direct signal readout element. The multi-signal amplification of this sening system, provides a sensitive detection of BRCA1 down to the femtomolar level (3.3 fM) with a linear range of 7 orders of magnitude. Moreover, it holds promising potential for single nucleotide polymorphism (SNP) analysis.

Introduction

Breast cancer1 (BRCA1), a tumor suppressor gene, was first identified in 1994.[1] People born with a mutation (abnormality) in this gene are at an increased risk of breast or ovarian cancer. Approximately 40% of inherited breast cancers and more than 80% of inherited breast and ovarian cancers are related to BRCA1 gene mutations.[2] Therefore, sensitive and selective detection of BRCA1 and its mutants in disease-related gene fragments are of great significance for genetic research and clinical diagnosis of breast cancer.

In the past few years, sensitive detection of specific DNA sequences has attracted great research attention for its applications in genetic research, clinical prognosis and clinical treatment. Various methods have been developed for DNA detection including electrochemical,[3-7] colorimetric,[8-11] fluorescent,[12-14] chemiluminescent,[15-18] Raman spectroscopy[19-21] methods and so on. To improve the sensitivity of biosensors, the signal amplification strategies based on enzyme-aided DNA amplification have been developed for DNA detection, such as strand displacement amplification,[22-24] polymerase chain reaction,[25,26] exonuclease,[3,15,27,28] endonuclease[10,23,29-31] and rolling circle amplification (RCA).[22,32,33] Among the reported amplification strategies, RCA has received particular interest due to its excellent property of signal amplification. RCA exploits the

continuous amplification of a circular DNA template with linear kinetics by phi29 DNA polymerase, which generates large amounts of DNA products containing up to 1000 complementary copies of the circular DNA to significantly improve the sensitivity of DNA detection under isothermal conditions.[34] Numerous applications based on RCA have been reported.[35]

Recently, enzyme-free DNA amplification has also been accomplished by an autocatalytic and catabolic DNAzyme-mediated process.[36,37] DNAzymes are a class of functional artificial nucleic acids, which are isolated from combinatorial oligonucleotide libraries by in vitro selection process named systematic evolution of ligands by exponential enrichment (SELEX).[38] The high catalytic activities toward specific substrates and high thermal stability make DNAzymes ideal biocatalysts for developing biosensing platforms.[39,40] G-quadruplex/hemin DNAzyme, as one of DNAzymes, displays the similar catalytic activity of horseradish peroxidase (HRP) enzyme, which has been extensively used as a catalytic label for the amplified detection of DNA.[3,8,9,16,41] In the reported sensing platform, 3,3',5,5'-tetramethylbenzidine (TMB)-H_2O_2 chromogenic reaction has been used as signal readout, and the analytes can be detected by absorbance methods.[8,42,43] 8–17 DNAzyme is a DNA metalloenzyme catalyzing RNA transesterification in the presence of divalent metal ions,[44] and has been employed for amplified detection of various targets.[12,45,46]

In this work, we take advantage of the topological effect of DNAzyme, combining DNAzyme assistant DNA recycling and RCA strategy to develop a colorimetric DNA sensing in which multi-step cascade amplification were successfully achieved. In our strategy, the sequence of 8–17 DNAzyme was caged in the loop region and its activity was suppressed. Upon sensing of target DNA, the DNAzyme can be released from this hairpin DNA probe and activated for the cyclic cleavage toward the MB substrate which contained anothor DNAzyme and template for RCA. The catalytic action of DNAzyme resulted numerous padlock probes to trigger the RCA for forming G-quadruplex/hemin DNAzyme for catalyzing colorimetric reaction of TMB–H_2O_2, which provided amplified optical signals. The DNAzyme assistant DNA recycling, RCA strategy and the G-quadruplex/hemin mimic-peroxidase induced signal amplification, provided highly amplified efficiency and sensitive quantification in single nucleotide polymorphism (SNP) discrimination.

Experimental

Materials and chemicals

All HPLC-purified oligonucleotides (listed in Table S1) were synthesized by Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). The oligonucleotide stock solutions (100 μM) were prepared in 20 mM Tris−HCl buffer (200 mM NaCl, 2 mM $MgCl_2$, 20 mM KCl, pH 7.4). The Phi29 DNA polymerase, T4 DNA ligase and deoxynucleotide solution mixture (dNTPs) were obtained from NEW ENGLAND Biolabs (NEB). Hemin, H_2O_2 and TMB (3.3′,5.5′-tetramethylbenzidinesulfate) were obtained from Aladin Chemistry Co. Ltd. (Shanghai, China). A stock solution of hemin was prepared in DMSO and stored in the dark at -20 ^oC as 10 mM stock solution. Sequences of the oligonucleotides were designed with the help of online software (from the Website of Integrated DNA Technologies (IDT)).

Procedures for DNA detection

Before DNA detection, hairpin probes (HPs) and molecular beacons (MBs) were pretreated with the following procedure: heated to 90 °C and incubated for 5 min, and then slowly cooled down to

room temperature. The obtained DNA solutions were stored at 4 °C for further use. To detect target DNA, the hybridization between HP (5 μL, 0.1 μM) and different concentrations of target DNA (5 μL) was performed for 30 min. The 8–17 DNAzyme aided DNA cycles was carried out in 25 μL of reaction mixtures containing 10.0 μM MB, 1.0 mM Pb^{2+}, 20 mM Tris−HCl buffer (200 mM NaCl, 2 mM $MgCl_2$, 20 mM KCl, pH 7.4) and the above hybridization solution. The reaction mixture was incubated for 80 min. Afterward, a volume of 25 μL of solution containing 1 × ligase buffer, 20.0 μM primer, 20 U T4 DNA ligase was hybridized with the padlock probes from the cleavage of MB substrates at 37 °C for 1 h. Then the formed circular templates can initiate the RCA reaction by addition of 40 U of phi29 DNA polymerase and 10.0 mM dNTP in 25 μL of RCA buffer and incubated for 2 h at 37 °C. After the amplification reaction, 105 μL of Tris–HCl buffer solutions (20 mM, pH 6.0) were added into the above solution. Finally, 10 μL of TMB (100.0 mM) and 10 μL of H_2O_2 (20.0 mM) were added to the mixture to start the chromogenic reaction. H_2O_2-mediated oxidation of TMB was catalyzed by G-quadruplex/hemin DNAzyme for 25 min at room temperature (avoiding direct light exposure). The resulting solutions were observed by the naked eye or quantized by UV-vis absorption spectroscopy (Perkin Elmer Instruments, USA).

Results and discussion

Sensing strategy

Herein, a simple and isothermal 8-17 DNAzyme recycling strategy coupled with RCA was developed for the achievement of ultrasensitive detection of DNA, which was schematically illustrated in Scheme 1. The binding of target DNA with the recognition region on the hairpin probe initiates the release of the DNAzyme from the caged structure, acting as triggers in following reaction. Upon the addition of the cofactor Pb^{2+}, the DNAzyme is activated to catalyze the cleavage of MB substrate into two pieces at the rA position, causing the release of another free DNAzyme. The released DNAzyme can then bind another MB to retrigger a new cycle of cleavage. Eventually, each released 8–17 DNAzyme can undergo a number of cycles to trigger the cleavage of abundant MB substrates to produce a large number of padlock probe which can be catalyzed to form a ‘‘circular’’ DNA template for the initiation of RCA reaction. Therein, many complementary copies with G-rich oligomer can be generated. This G-rich oligomer can self-assemble G-quadruplex structure into catalytic HRP-mimicking DNAzyme in the presence of hemin. Subsequently, it catalyzes colorimetric reaction of TMB–H_2O_2, which provides amplified optical signals.

Based on the autonomous cleavage in Cycles I and II, the DNA fragment containing the base sequence of 8–17 DNAzyme could be exponentially released for the generation of a large amount of padlock templates. Combined with DNAzyme assistant DNA recycling, RCA strategy and G-quadruplex/hemin DNAzyme amplification, the newly designed colorimetric biosensor actually achieves tandem multi-step cascade amplification which is hopeful to offer an ultrahigh sensitivity for the assay of nucleic acid.

Feasibility of the fabricated DNA biosensor for target DNA detection

To verify the feasibility of the designed cascade amplification strategy for target DNA detection, the absorption spectra under different conditions were investigated. As shown in Fig. 1, in the absence of target DNA or HP/MB, the absorption intensity of the solution is relatively low

because the cleavage activity of the caged 8–17 DNAzyme in hairpin probe toward MB substrate was suppressed (curves a, b and c). While in the presence of target DNA (10.0 pM), an obvious increase in absorption intensity can be observed after RCA (curve d). This signal increase could be attributed to the hybridization of target DNA with hairpin probe and the cleavage of released 8–17 DNAzyme toward MB substrate. Then, the sequences of padlock probe can be liberated and the RCA reaction can be proceeded. Through the RCA, the complementary copies can self-assemble G-quadruplex structure into peroxidase-mimicking DNAzymes. These DNAzymes catalyze the conversion of colorless TMB to blue-colored TMB with the presence of H_2O_2, causing an increase in absorption intensity. These results strongly indicate that the feasibility of currently designed cascade amplification strategy for target DNA detection.

Optimization of signal amplification time of the sensing strategy

Amplification time is important factor to determine the performance of this assay. The amount of MB fragments produced by Pb^{2+} assistant DNA recycling and the number of long RCA products produced by RCA, were depended on the reaction time. So the response signals for target DNA (10.0 nM) are investigated with different incubation time of the cascade signal amplification strategy. Firstly, the 8–17 DNAzyme aided DNA cycles was carried out in reaction mixtures for 0 to 140 min before being subjected to the RCA process for 2 h. As shown in Fig. 2 (a), the absorption intensity increased rapidly with the augment of the reaction duration of Pb^{2+} assistant DNA recycling and trended to a constant value at the time of 80 min because almost all the MB substrates were cleavaged. The action time of 80 min was chosen for the optimal condition. Secondly, RCA reaction mixture was also incubated for 0 to 140 min to estimate the effect on the amplication process. In theory, a long RCA reaction time was expected to generate more repeated sequences. In Fig. 2 (b), the peak value was attained at 120 min, which corresponded to the saturated state of RCA products. The optimal reaction time of RCA was chosen as 2 h.

Comparison on the effect of the signal amplification of different MBs

For improving the preformance of the sensing system, we designed four different oligonucleotides of MB. After the signal amplification of DNAzyme assistant DNA recycling and RCA, many RCA products with G-rich oligomer can be generated. We chose four common G-quadruplex structures and the base sequences are as follows,

5′ -ggTTggTgTggTTgg-3′ (TBA aptamer)

5′ -ggggTTggggTTggggTTgggg-3′ (T2)

5′ -gggTTAgggTTAgggTTAggg-3′ (telomeric repeat unit)

5′ -gggTAgggCgggTTggg-3′ (AG4)

The sequences of four MBs contain the 8–17 DNAzyme fragments and the complementary sequences of the above four G-rich oligomers. Fig. 3 showed the UV–vis absorption spectra and the photographs of TMB-H_2O_2 system in the presence of 10.0 pM target and blank, MB1, MB2, MB3, MB4. In comparison with blank (curve a and photograph a), weak change in the solution color and absorption spectrum was observed in the case of b, indicating that the sequence of TBA aptamer in the presence of Pb^{2+} can't improve the peroxidase activity of hemin immensely. Fig 3 indicated that in the presence of Pb^{2+} and hemin, the peroxidase-mimicking DNAzymes formed by the G-rich oligomers of T2 (curve c), telomeric repeat unit (curve d) and AG4 (curve e) show obvious activity for catalyzing colorimetric reaction of TMB–H_2O_2. Especially, the largest change

in solution color and increase of absorbance at 367 nm and 650 nm were observed in photograph e and curve e. In the whole experimental process, we chose the sequence MB4 for the detection of the target DNA.

Detection performance of the designed sensing system

The quantification performance of cascade signal amplification strategy for target DNA detection was investigated under the above optimal conditions. The absorption values A300–A750 after termination of the TMB–H_2O_2 coloration were recorded in the presence of a series of target DNA solution with various concentrations. As shown in Fig. 4A, the colorimetric responses increased gradually with the increase of the concentration of target DNA, which implied that more and more RCA products were obtained. The absorbance at 650 nm showed a good linear relationship between the absorption intensity and the logarithm of the target DNA concentration in the range from 10.0 fM to 10.0 nM, as shown in Fig. 4B. The regression equation was A = 3.06133 + 0.20092 $\log_{10}$C and the regression coefficient (r) was 0.9968. And we achieved a calculated detection limit of 3.3 fM for target DNA in terms of the 3σ rule. The RSD for 11 repetitive measurements of 1.0×10^{-14} M target DNA was 5.47%, indicating a good reproducibility of the assay. The comparison of our strategy with other reported methods was illustrated in Table S2. The advantages can be summarized as follows, (1) satisfactory sensitivity which can be ascribed to cascade signal amplification coupled DNAzyme assistant DNA recycling, RCA with G-quadruplex/hemin DNAzyme amplification. (2) extremely simple and cost-effective which can be ascribed to the homogeneous and label-free detection in solution phase. The above results verified that the cascade signal amplification strategy was efficient for ultrasensitive detection of BRCA1 gene.

In order to further evaluate the performance of the developed DNA sensing system, SNP analysis was performed which can discriminate completely complementary target and the mismatched targets. The specificity of the proposed method was investigated by exposing HPs to different kinds of DNA sequences (10.0 nM), including completely complementary target DNA (TD), single-base mismatched DNA (1 MT), two-base mismatchted DNA (2 MT), non-complementary DNA (NC) and blank. As shown in Fig. 5, the absorption intensity of 1 MT and 2 MT was only about 45.3% and 30.6% of that of completely complementary DNA, respectively. In addition, the noncomplementary DNA shows almost the same absorption intensity with the blank solution. Thus, our strategy exhibited good performance in the discrimination of one-base mismatched sequences.

Conclusions

In summary, a label-free DNA colorimetry biosensor has been developed for highly sensitive detection of BRCA1 gene. This new cascade signal amplification strategy coupled DNAzyme assistant DNA recycling, RCA with G-quadruplex/hemin DNAzyme amplification. The cofactor Pb^{2+} assistant DNA recycling can produce a large number of cleaved MB fragments-padlock template probe with the challenge of target DNA, which then initiated the reaction of the RCA for realizing the cascade signal amplification. This strategy can detect as low as 3.3 fM target DNA with excellent differentiation ability for even single mismatches. Notably, it also exhibited the distinct advantages of ultra-high sensitivity and specificity, simplicity in probe design and biosensor fabrication and short analysis time. Moreover, the whole reaction is performed in the

homogeneous solution, making the method extremely simple and cost-effective. The sensing system could prove its potential application in the detection of cancer biomarker of the early stages of cancer.

Acknowledgements

We gratefully acknowledge the financial and facility supports from central laboratory of Henan Provincial People's Hospital.

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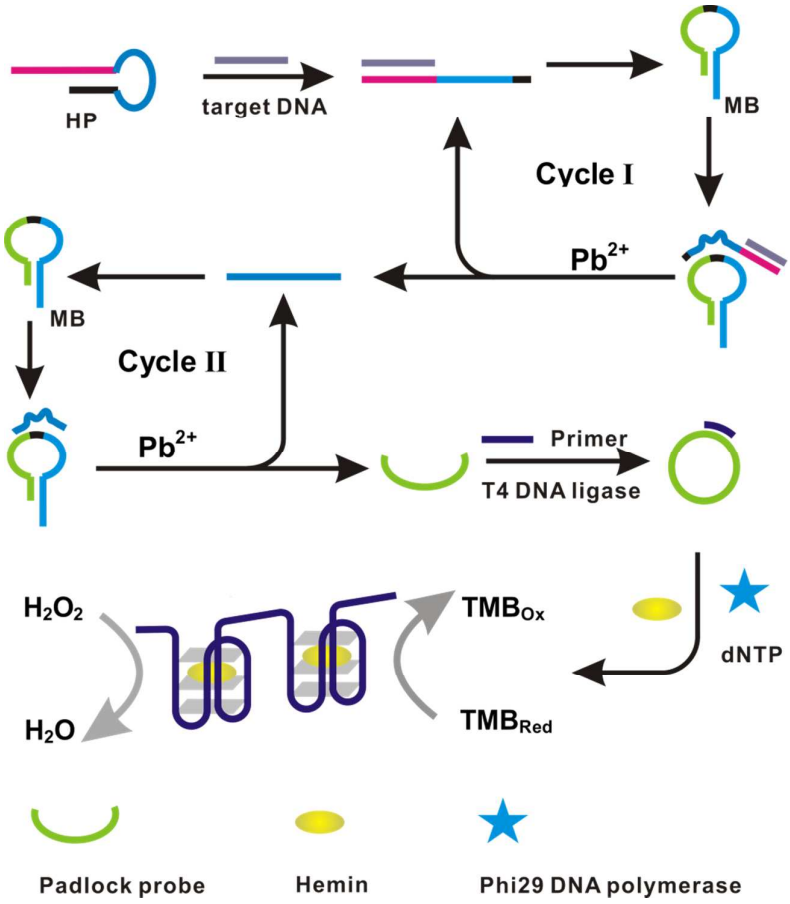

Scheme 1 Schematic illustration of colorimetric biosensor for breast cancer1 gene detection based on DNAzyme assistant DNA recycling and rolling circle amplification.

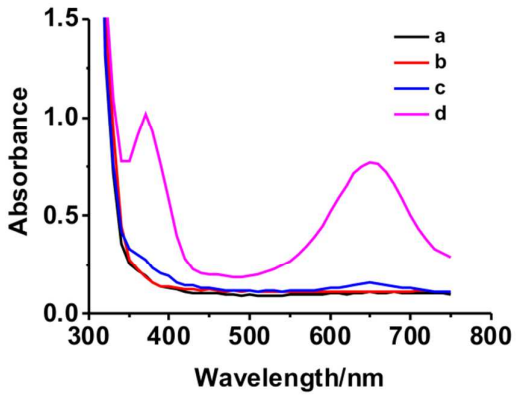

Fig. 1 Absorption spectra of sample solutions under different conditions: (a) no HP, MB and target DNA, (b) no HP and MB, 10.0 pM target, (c) no target DNA, 0.1 μM HP and 10.0 μM MB, (d) 10.0 pM target DNA, 0.1 μM HP and 10.0 μM MB.

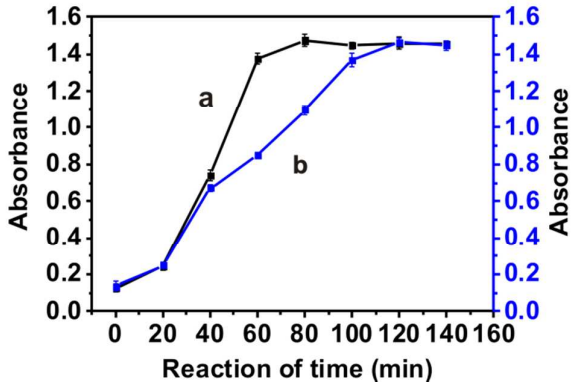

Fig. 2 Dependence of absorption intensity for 10.0 nM target DNA on the reaction time of (a) DNAzyme assistant DNArecycling and (b) RCA. When one parameter changes, the others are under their optimal conditions. The data depicted the averages of three experiments and the error bars represented the standard deviation of three trials.

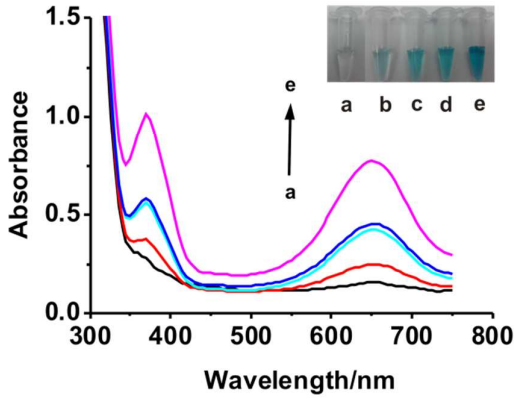

Fig. 3 The UV–vis absorption spectra of 1 mM TMB-5 mM H_2O_2 (in 20 mM Tris–HCl buffer solution, pH 6.0) after it reacted with 10.0 pM target, (a) blank, (b) MB1, (c) MB2, (d) MB3, (e) MB4. The inset shows the corresponding color changes.

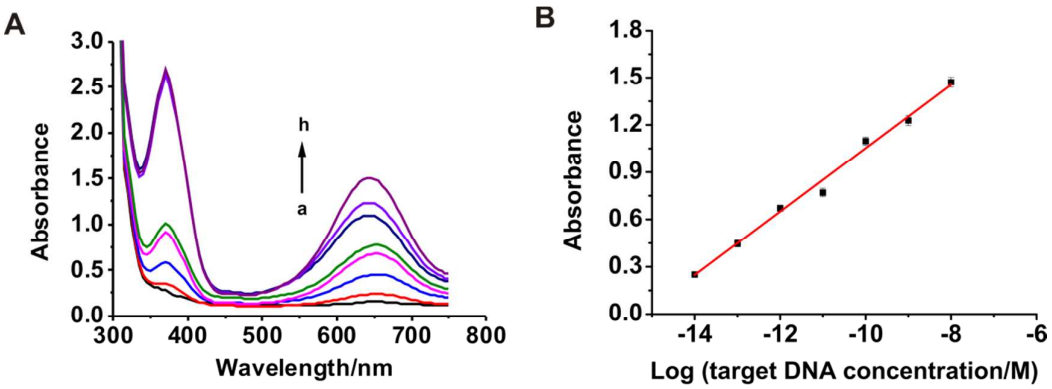

Fig. 4 (A) Absorption intensity of amplified biosensor for detection of target DNA at different concentrations, as follows: (a) 0 M, (b) 1×10^{-14} M, (c) 1×10^{-13} M, (d) 1×10^{-12} M, (e) 1×10^{-11} M, (f) 1×10^{-10} M, (g) 1×10^{-9} M, (h) 1×10^{-8} M target. (B) Calibration curve of signal response (A300–A750) vs. target DNA concentrations. Signal responses were obtained via monitoring dual wavelengths after a fixed time interval of 25 min. The error bars represent the standard deviation of three measurements under optimal conditions.

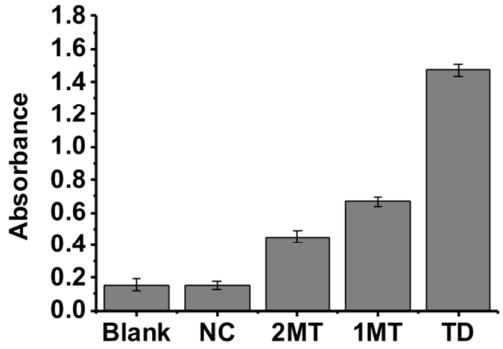

Fig. 5 Histograms of the absorption intensity toward the blank and the four various DNA sequences including non-complementary (NC), two-base mismatched (2 MT), single-base mismatched (1 MT), and complementary target DNA (TD). The concentration of each DNA sequence was 10.0 nM. The error bars represent the standard deviations of three parallel tests.