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DNAzyme Molecular Beacon Probes for Target-Induced Signal-Amplifying Colorimetric Detection of Nucleic Acids

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A novel DNAzyme molecular beacon (DNAzymeMB) strategy was developed for target-induced signal-amplifying colorimetric detection of target nucleic acids. The DNAzymeMB, which exhibits peroxidase activity in its free hairpin structure, was engineered to form a catalytically inactive hybrid through hybridization with a blocker DNA. The presence of target DNA leads to dissociation of the DNAzymeMB from the inactive hybrid through hybridization with the blocker DNA. This process results in recovery of the catalytically active DNAzymeMB, which can catalyze a colorimetric reaction that signals the presence of the target DNA. In addition, a primer was rationally designed to anneal to the blocker DNA of the blocker/target DNA duplex and displace the bound target DNA during the extension reaction. The released target DNA triggers the next cycle involving hybridization with blocker DNA, DNAzymeMB dissociation, primer extension, and target displacement. This unique amplifying strategy leads to the generation of multiple numbers of active DNAzymeMB molecules from a single target molecule and gives a detection limit down to 1 pM, a value that is nearly 3 or 5 orders of magnitude lower than those of previously reported DNAzyme molecular beacon-based DNA detection methods.

DNA-based biosensors are attractive for target DNA detection owing to straightforward interactions that lead to sequence-specific hybridization.[1−3] One of the most widely used DNA biosensor systems employs a molecular beacon, which undergoes a closed to open conformational change in the presence of target DNA with simultaneous production of a fluorescence signal.[4,5] The target-induced conformational change followed by fluorescence signaling methodology has been utilized to develop various types of molecular beacon probes for the detection of nucleic acids.[6−12] However, generation of the fluorescent signal in molecular beacon probes is a result of a 1:1 stoichiometric hybridization between target and molecular beacon molecules. In other words, one target DNA promotes the fluorescence of only one beacon molecule. Although the requirement for the 1:1 stoichiometry in signal generation leads to accurate target quantification, it limits the sensitivity level of detection. Moreover, conventional molecular beacon probes require that potentially costly processes be used for introduction of fluorescent probes.

DNAzymes (also called deoxyribozyme or catalytic DNAs) are single-stranded DNA molecules of a particular sequence that possess specific catalytic activities and serve as attractive options to achieve signal amplification in DNA detection. This is a consequence of the fact that one DNAzyme molecule is able to catalyze many cycles of a specific chemical reaction. Since the advent of the first Pb^{2+}-dependent RNA cleavage DNAzyme in 1994,[13] hundreds of artificial DNAzymes have been identified by using in vitro selection methodologies. As a result, the range of the reactions that DNAzymes catalyze has significantly increased. Examples are found in DNAzymes that promote RNA,[14,15] DNA,[16] and *N*-glycosidic bonds cleavage,[17] DNA ligation,[18] DNA phosphorylation,[19] DNA adenylation,[20] and peroxidation.[21] DNAzymes have several unique advantages over natural

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proteinogenic enzymes, including higher thermal stability, simple preparation and modification, and very straightforward sequence-specific recognition characteristics. As a consequence of these unique properties, DNAzymes have been widely used as signaling units for the amplified detection of various analytes.[22−26] Many DNAzyme-based detection methods with high sensitivity also have been developed by using enzyme-based amplification strategies, such as nicking enzyme-assisted DNA replication,[27] rolling circle amplification (RCA),[28,29] and polymerase chain reaction (PCR).[30]

In an attempt to improve the sensitivity of target DNA detection, DNAzymes have been recently used in conjunction with molecular beacons to construct novel biosensors.[31,32] In one example, target DNA recognition of the molecular beacon induces a configuration change of the DNAzyme module from an inactive to an active form, the latter of which catalyzes a specific reaction to generate an amplified signal. The most representative example utilizes an RNA cleavage DNAzyme[31] linked with a molecular beacon. The closed form of the beacon prevents hybridization of the DNAzyme with a substrate oligonucleotide containing a fluorophore-quencher label. Target hybridization disrupts the stem portion, revealing a binding site, which then binds the substrate oligonucleotide and catalyzes a cleavage reaction to generate a corresponding fluorescent signal. Another approach employs a single-stranded peroxidase DNAzyme, tethered to a nucleic acid sequence, which forms a loop−stem structure in the absence of target DNA that prevents folding of catalytically active DNAzyme.[32] In the presence of target DNA, the hairpin structure opens to yield a catalytically active DNAzyme that promotes a process that leads to generation of a colorimetric signal. Although intriguing, all of these approaches have relatively low sensitivities (detection limits of 2 and 200 nM for the first[31] and second method,[32] respectively) that are similar to or even lower than those of more conventional molecular beacon-based strategies.

Below, we describe the results of a study probing a new colorimetric DNA detecting system in which a binary peroxidase DNAzyme,[33,34] composed of two separate oligonucleotides that self-assemble into a G-quadruplex structure with hemin, was employed as a catalytic unit to generate amplified signals. Molecular beacons, incorporating the binary peroxidase DNAzymes, termed DNAzyme molecular beacons (DNAzymeMB), were engineered to form inactive hybrids by binding with blocker DNAs and used for target DNA detection. An extremely sensitive colorimetric method was constructed for target DNA detection by coupling the DNAzymeMB/blocker hybrid probes with a unique strand displacement based signal-amplifying strategy. The usefulness of this technique was demonstrated by its application to the 1 pM level detection of a model *Urea* gene. This detection limit is nearly 5 orders of magnitude lower than that of any previously reported colorimetric DNA detection method based on DNAzyme molecular beacons.[32]

EXPERIMENTAL SECTION

Materials. All the chemicals were purchased from Sigma-Aldrich and used as supplied. A hemin stock solution (5 mM) was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at −20 °C. The deoxynucleotide solution (dNTPs), NEB buffer 2 solution, and Klenow fragment exo- were from New England Biolabs, Inc. (Beverly, MA, U.S.A.) and used without further purification. Doubly distilled water was used in all the experiments.

DNAzyme Molecular Beacons and Oligonucleotides. All DNA oligonucleotides were synthesized and HPLC-purified (Genotech Co., South Korea). The binary peroxidase DNAzyme consists of two separate strands: 5′-AGG GAC GGG-3′ and 5′-GTG GAG GGT-3′. A 44-mer segment of ureC gene sequences in *Ureaplasma urealyticum* was used as the target DNA: 5′-GAA GTA ATG GAC TTA GTT ATT ACA AAC GCA TTA ATT CTT GAC TA-3′. Two oligonucleotides were chosen as negative control DNAs, one being a 38-mer segment of *Chlamydia* gene (5′-CTA GGC GTT TGT ACT CCG TCA CAG CGG TTG CTC GAA GC-3′) and the other a single-nucleotide mismatched target *Urea* gene (5′-GAA GTA ATG GAC TTA CTT ATT ACA AAC GCA TTA ATT CTT GAC TA-3′). The sequences of all DNAzymeMBs, blocker DNAs, and primers used in this study are listed in the Supporting Information, Table S1.

Recognition of Target DNA by Using the Inactive DNAzymeMB/Blocker Hybrid. Amounts of 1 μL of 50 μM DNAzymeMB and 1 μL of 50 μM blocker DNA were mixed in buffer (25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100 and 1% DMSO; pH 7.4). The solution was denatured at 94 °C for 4 min, annealed at 50 °C for 5 min, and allowed to cool to room temperature. Then 1 μL of 50 μM target DNA and 1 μL of 25 μM hemin were added to yield a total volume of 50 μL. The resulting solution was incubated at room temperature for 3 h followed by colorimetric measurements. The above procedures were employed to optimize the sequences of DNAzymeMB and blocker DNA.

Signal-Amplified DNA Detection Utilizing Target DNA Displacement Induced by DNA Extension Reaction. The reaction system consists of 1 μL of 50 μM DNAzymeMB/blocker hybrids, 1 μL of 50 μM primer, 2 μL of target DNA solution, 1 μL of 25 μM hemin, 1 μL of Klenow fragment exo- (0.4 units), 2 μL of dNTPs (0.2 mm), 9 μL of distilled water, and 2 μL of NEBuffer 2 (10×) (50 mm NaCl, 10 mm Tris−HCl, 10 mm $MgCl_2$, 1 mm dithiothreitol, pH 7.9). Polymerization reaction was performed at 37 °C for 2 h and blocked by cooling to 4 °C. The product solution was mixed with the detection buffer to make the total volume of 50 μL (25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, and 1% DMSO; pH 7.4), followed by recording absorption intensities. The sensitivity of the strategy was determined by using various concentrations of the target *Urea* gene (0 M and 1, 5, 10, 50, 100, and 500 pM and 1, 5, 10,

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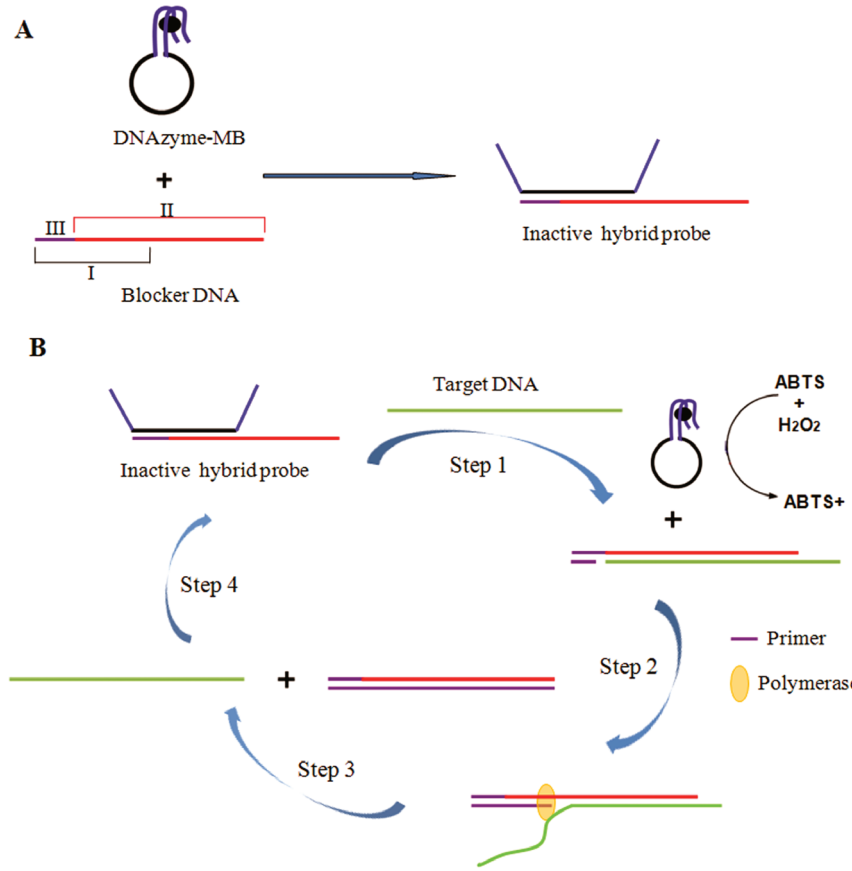
Scheme 1. Schematics Describing the Principles Behind the Strategies[a]



[a] (A) Formation of the catalytically inactive hybrid probes through hybridization of DNAzymeMB and blocker DNA. The blocker DNA consists of a DNAzymeMB complementary region I, a target binding region II and a primer binding site III. (B) Colorimetric target DNA detection based on target-induced amplification cycle. Target DNA hybridizes with the blocker DNA of the inactive hybrid probe and releases the catalytically active DNAzymeMB for generation of colorimetric signals (step 1). Following this, the primer anneals to the blocker DNA and is extended to synthesize the cDNA of blocker DNA (step 2). During the polymerization reaction, the target DNA is dissociated (step 3) and triggers amplification cycle by hybridizing with another inactive hybrid probe (step 4).

50, and 100 nM), and the specificity was assessed by using 100 nM of the *Chlamydia* gene and single-nucleotide mismatched target *Urea* gene as negative controls.

Colorimetric Measurement. For the colorimetric measurement, the ABTS/H_2O_2 substrates were added to the product solution to yield a total volume of 100 μL (ABTS (7.2×10^{-4} M) and H_2O_2 (4.4×10^{-5} M)). Absorption intensities were recorded at $\lambda = 415$ nm by using a Cary 100 UV−vis spectrophotometer (Varian, Palo Alto, CA).

Gel Electrophoresis. The product solution mixed with 6× loading buffer (Bioneer, South Korea) was subjected to electrophoresis analysis on a 15% nondenaturing PAGE. The analysis was carried out in 1× TBE (pH 8.3) at a 100 V constant voltage for about 90 min. After ethidium bromide staining, gels were scanned using a UV transilluminator.

RESULTS AND DISCUSSION

Principle Behind the Target-Induced Signal-Amplifying DNA Detection Assay. The new colorimetric strategy for ultrasensitive nucleic acid detection consists primarily of an inactive DNAzymeMB/blocker hybrid probe and target-induced signal-amplifying module, composed of a primer and polymerase (Scheme 1). The inactive hybrid probe was first prepared by hybridizing a peroxidase DNAzymeMB with a blocker DNA (Scheme 1A). The DNAzymeMB possesses a loop sequence that is the same as part of the target DNA and stem sequences at both ends, which self-assemble into the binary peroxidase DNAzyme in the presence of hemin. It is noteworthy that the stem is formed by self-assembly of binary peroxidase sequences but not by self-complementary sequences, as is the case in traditional molecular beacons. The blocker DNA possesses a region I that is complementary to part of the sequence of DNAzymeMB, a target binding region II that is complementary to part of the sequence of the target DNA, and a primer binding site III buried in region I. Through hybridization of region I within blocker DNA, the DNAzymeMB is prevented from forming a hemin-containing active conformation. Importantly, the target DNA binding region II was designed to be longer than region I. As a result, the target DNA can dissociate the DNAzymeMB by forming more favorable hybrid with the blocker DNA.

Hybridization of the target DNA with the blocker DNA, which displaces the DNAzymeMB from the catalytically inactive hybrid, leads to the formation of catalytically active DNAzymeMB in the presence of hemin. The released free active DNAzymeMB catalyzes the oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid) generating a blue-green colorimetric signal (step 1, Scheme 1B). At the same time, the uncovered primer binding site III within the blocker DNA becomes available for primer annealing followed by the extension reaction by polymerase (step 2). During primer extension, the target DNA is displaced owing to the strand displacement activity of polymerase,

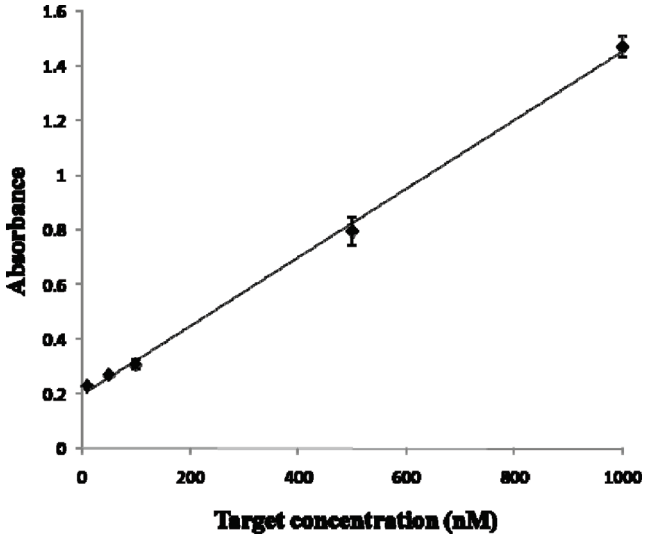


Figure 1. Absorption intensity at 415 nm from the ABTS oxidation for the analysis of target *Urea* gene at different concentrations after a fixed time interval of 5 min. An amount of 1 μM DNAzymeMB 1/blocker 1-1 hybrid probe was employed. Data are averages of three independent experiments.

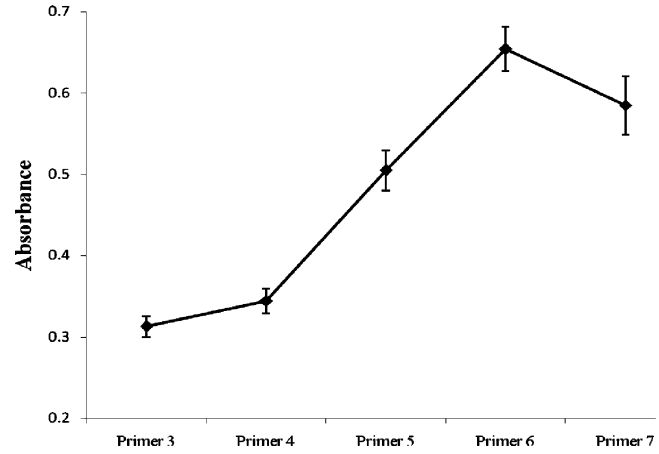


Figure 2. Effect of primer length on the absorption intensity at 415 nm for the analysis of 100 nM target *Urea* gene in the presence of polymerase/dNTP after a fixed time interval of 5 min. The reaction systems contain 500 nM primers with different lengths and 500 nM corresponding DNAzyme/MB/blocker hybrids as described in Supporting Information Table S1. Data are averages of three independent experiments.

and a complementary strand of the blocker DNA is synthesized generating a blocker−cDNA duplex (step 3). To prevent its undesired extension by the polymerase in step 2, the blocker DNA was modified to contain a phosphate group at the 3′ end. The released free target DNA hybridizes with another DNAzymeMB/blocker hybrid probe (step 4), triggering another cycle of DNAzymeMB dissociation, primer extension, and target displacement.

Utilizing the unique target DNA recycling strategy, a single target molecule can activate multiple numbers of DNAzymeMB molecules from the inactive hybrids so that they can catalyze the colorimetric reaction. Consequently, by simply monitoring the color change or absorption intensity resulting from the colorimetric reaction, the target DNA can be conveniently detected with high sensitivity.

Construction of Catalytically Inactive DNAzymeMB/Blocker Hybrid Probes. In contrast to conventional molecular beacons that generate fluorescent signals in their open configuration, the DNAzymeMB employed in the new procedure generates colorimetric signals in its closed configuration, where the stem portion self-assembles with hemin into the peroxidase DNAzyme unit that catalyzes the H_2O_2-mediated oxidation of ABTS. The DNAzymeMB 1 (Supporting Information Table S1), whose stem portion is composed of only basic binary peroxidase DNAzyme sequences, exhibits the highest catalytic activity in comparison to those from the free binary peroxidase DNAzyme and the DNAzymeMB 2, which contains five additional self-complementary sequences in its stem portion (see the Supporting Information for details, Figure S1). Therefore, DNAzymeMB 1 not containing an additional self-complementary stem sequence was employed to construct the catalytically inactive hybrid probe through hybridization with a blocker DNA. The catalytic activity of DNAzymeMB 1 was almost completely inhibited by blocker 1-1 (Supporting Information Table S1), which is partially complementary to the stem portion at the 5′ end of DNAzymeMB 1. More importantly, nearly all of the original activity of DNAzymeMB 1

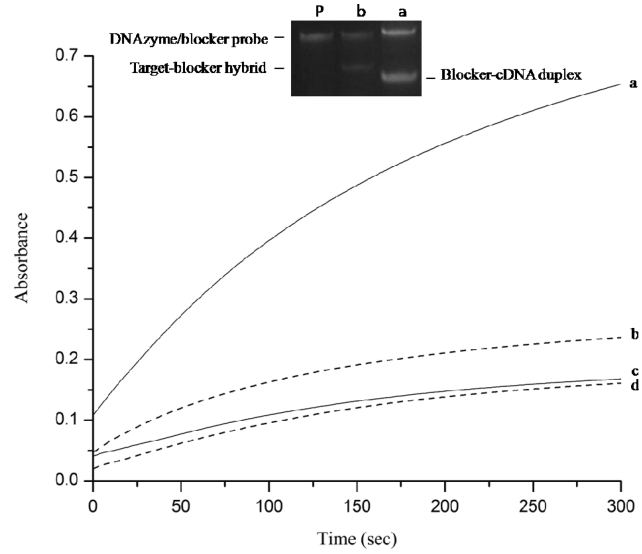


Figure 3. Time-dependent absorption intensities at 415 nm associated with oxidation of ABTS in the analysis of 100 nM (curves a and b) and 0 nM target *Urea* gene (curves c and d) in the presence (solid line) or absence (dashed line) of polymerase/dNTP. Amounts of 500 nM DNAzymeMB 6/blocker 6 hybrid probes and 500 nM primer 6 were employed. Inset: polyacrylamide gel electrophoresis image obtained from the sample corresponding to curves a and b (lanes a and b). Lane P is obtained from the sample only containing DNAzymeMB/blocker hybrid probes.

was recovered upon addition of the target DNA (see the Supporting Information for details, Figure S2).

The DNAzymeMB 1/blocker 1-1 hybrid probes were employed to detect different concentrations of target DNA (Figure 1). The results show that, as the concentration of target DNA increases from 10 nM to 1 μM, the absorption intensity increases in a highly linear ($R^2 = 0.998$) fashion. According to the definition that the detection limit is the lowest analyte concentration required to produce a signal greater than 3 times the standard deviation of the noise level, the limit of detection for this system is 10 nM, a value which is about 20-fold lower than that of previously

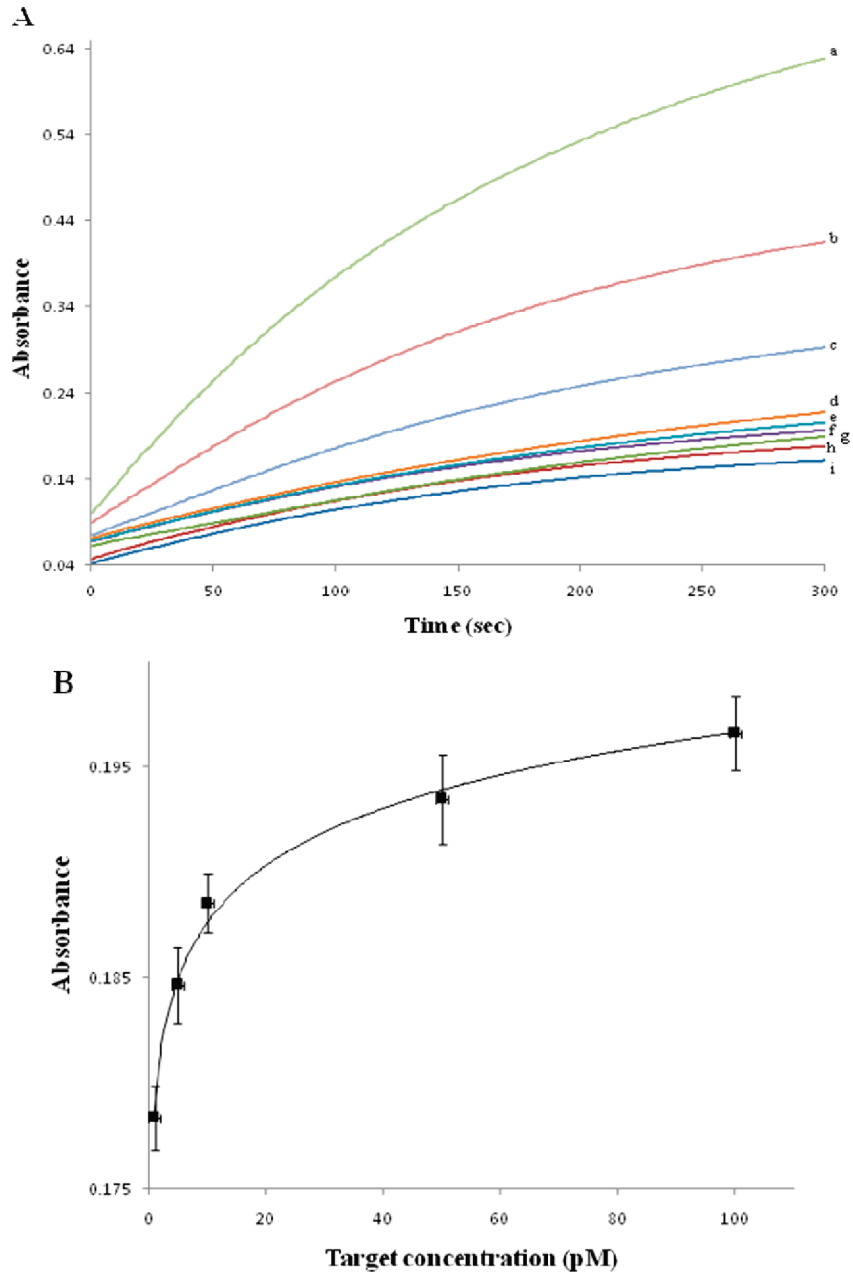


Figure 4. (A) Time-dependent absorption intensity at 415 nm from the oxidation of ABTS for the analysis of target *Urea* gene at different concentrations: (a) 50, (b) 10, (c) 5, and (d) 1 nM, (e) 500, (f) 100, (g) 10, and (h) 1 pM, and (i) 0 M. All the reaction systems contain 500 nM DNAzymeMB 6/blocker 6 hybrid probes and 500 nM primer 6. (B) Calibration curve of absorbance after a fixed time interval of 5 min upon analyzing various concentrations of target *Urea* gene from 1 to 100 pM. Data are averages of three independent experiments.

described colorimetric DNA detection strategy based on DNAzyme molecular beacons.[32]

To induce the strand displacement polymerization reaction, a primer binding site needs to be inserted into the blocker DNAs in a manner so that both the blocker DNA and the DNAzymeMB sequences vary according to the primer sequence. As a result, the primer sequence should influence not only signal amplification but also the catalytic activity of the corresponding DNAzymeMB. To assess the effect of the primer length on the colorimetric signal, absorption intensities from ABTS oxidation induced by 100 nM target *Urea* gene were compared in reaction systems (Figure 2) employing different lengths (12 nt primer 3 to 8 nt primer 7) of primers and their corresponding DNAzyme/blocker hybrids (Supporting Information Table S1). The absorption intensities were observed to first increase with decreasing primer length (from primer 3 to primer 6) due to the higher catalytic activity of DNAzymeMBs designed for the primers with shorter length (Figure S3, Supporting Information). However, the absorption intensities then decrease with further decreases of the primer length (from primer 6 to primer 7), indicating that the 8 nt primer 7 is too short to promote a highly efficient extension reaction. As a result, the primer 6 reaction system which gives the most intense signal was selected for use in further studies.

Detection Performance of the Assay. In order to confirm that target-induced signal amplification by the strand displacement activity of polymerases takes place during the extension reaction,

the absorbance caused by ABTS oxidation in the assay system containing polymerase/dNTP was determined as a function of time upon the analysis of 100 nM target *Urea* gene. The change was compared with that arising from the same assay system but not containing the components for DNA extension (Figure 3). Absorption intensities resulting from both systems increased with time owing to the peroxidase mimicking activity of DNAzymeMBs activated by the target DNA. Importantly, the system containing polymerase/dNTP produced a much higher absorbance than the one without polymerase/dNTP, indicating that more catalytically active DNAzymeMBs were released from the inactive DNAzymeMB/blocker hybrid probes. The results clearly confirm that the bound target DNA was displaced from the blocker DNA by the strand displacement activity of polymerases during the formation of blocker DNA−cDNA duplex and that recycling occurred to generate more free DNAzymeMB molecules. This result was further confirmed by using electrophoretic analysis of the DNA molecules involved in this method. As shown in the inset in Figure 3, the DNAzyme/blocker DNA hybrid probe was observed on the most upper position and the band for target−blocker DNA hybrids appeared in the lower position upon the application of target DNA. The band for the blocker DNA−cDNA duplex, a byproduct of target recycling process, was also observed at the expected position only in the assay system containing polymerase/dNTP.

The target DNA recycling process involving target hybridization, DNAzymeMB dissociation, primer extension, and target displacement enabled the achievement of a much higher sensitivity compared to those of previously reported methods based on simple target hybridization.[31,32] To determine the sensitivity of this system, time-dependent absorbance intensities arising from different concentrations of target *Urea* gene were measured (Figure 4A). As the concentration of target DNA increased, the absorbance increased. Figure 4B is shown the calibration curve obtained from the analysis of various concentrations of target DNA in the range of 1−100 pM. The detection limit was found to be 1 pM, a value that is much lower than those from other methods using DNAzyme molecular beacons.[31,32]

The specificity of the new technique was demonstrated by analyzing a sample containing 100 nM of the *Chlamydia* gene, another pathogen causing a sexual transmitted disease (Figure 5A). No significant absorption increase is observed to take place when the *Chlamydia* gene is employed. Only a weak background signal is detected, which is close to that of a negative control not containing the target *Urea* gene. This new technique also displays the ability to distinguish a single-nucleotide mismatch by adjusting the overhang length of blocker DNA within the DNAzymeMB/blocker hybrid probe. The system containing a DNAzymeMB/blocker hybrid probe whose blocker DNA overhang length is 6 nt (blocker 8, Supporting Information Table S1) was employed for analyzing a perfectly complementary target DNA and a single-nucleotide mismatched target DNA. As shown in Figure 5B, perfectly matched target *Urea* gene gave rise to about 3-fold higher absorbance signal than the single-nucleotide mismatched target *Urea* gene, which induced only a slightly increased absorbance as compared to the background (target free) signal. Thus, this DNA detection method displays a high degree of specificity for target DNA detection.

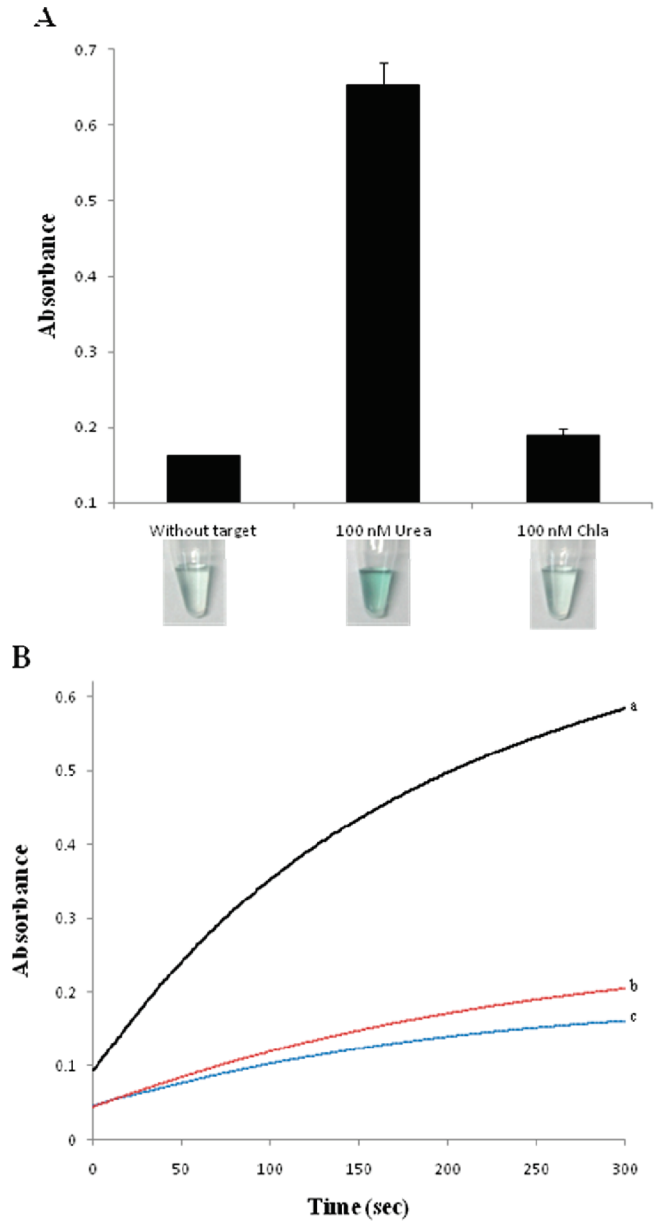


Figure 5. (A) Absorption intensity at 415 nm obtained from the oxidation of ABTS for the analysis of negative control without target *Urea* gene, 100 μM target *Urea* gene, and 100 nM nontarget *Chlamydia* gene after a fixed time interval of 5 min. Data are averages of three independent experiments. (B) Time-dependent absorption intensity at 415 nm associated with the oxidation of ABTS in the analysis of 100 nM perfectly complementary (curve a), 100 nM single-nucleotide mismatched (curve b) target *Urea* gene, and control sample without target *Urea* gene (curve c).

CONCLUSIONS

In the investigation described above, we have developed a highly sensitive DNA colorimetric detection assay system that employs inactive DNAzymeMB/blocker hybrids linked with the isothermal strand displacement polymerization reaction. In the strategy, the presence of target DNA promotes the release of catalytically active DNAzymeMB from the inactive hybrid probe and simultaneously triggers an amplification cycle consisting of target hybridization, DNAzymeMB dissociation, primer extension, and target displacement. By using this method, an extremely high sensitivity for the detection of nucleic acids is achieved. For example, the detection limit of a model *Urea* gene was determined

to be 1 pM, a value that is nearly 5 orders of magnitude lower than that of other previously described colorimetric DNA detection strategy based on DNAzyme molecular beacons.[32] Moreover, the procedure used to achieve amplified DNA detection by the strand displacement polymerization can be performed under isothermal condition, thus eliminating the requirement for thermal cycling. The results of this study should substantially broaden the perspective for future development of DNA-based biosensors for the detection of other analytes, such as proteins and small molecules.

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SUPPORTING INFORMATION AVAILABLE

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