Versatile DNAzyme-Based Amplified Biosensing Platforms for Nucleic Acid, Protein, and Enzyme Activity Detection

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Supporting Information

ABSTRACT: DNAzymes have been widely applied as signal amplifiers for enzyme-free and highly sensitive detection of DNA. A few of them have also been employed for amplified detection of other biomolecules via a target-triggered assembly of split or mutated DNAzyme strategy. However, most of these designs adopt Mg2+-dependent DNAzyme as the catalytic unit, which suffered from low catalytic cleavage activity. Meanwhile, some DNAzymes with high catalytic activity are not suitable for these designs because the slight modification of the catalytic core might result in remarkably decreased or even no catalytic activity of these DNAzymes. On the basis of DNAzyme topological effect or the terminal protection of small-molecule-linked DNA, we developed two versatile sensing platforms for amplified detection of different biotargets. Since no modification is necessary for the catalytic core of the DNAzyme in these designs, they can employ any DNAzyme with high catalytic activity as an amplified unit, which affords a high amplified efficiency for the sensing platform. A catalytic and molecular beacon design was further employed to realize the true enzymatic multiple turnover of DNAzyme. These designs together allow a high sensitivity for the biotargets, resulting in a detection limit of 20 pM, 0.2 U/mL, and 1 ng/mL for target DNA, DNA adenine methylation methyltransferase (Dam MTase), and streptavidin, respectively, much lower than previously reported biosensors. In addition, the proposed sensing strategy is versatile. By conjugating with various recognition units, it can be employed to detect a wide range of biotargets, varying from nucleic acids to proteins with high sensitivity.

The presence and concentrations of certain biomolecules (e.g., nucleic acids and proteins) inside a human body can reflect the person’s health.1 For example, variations in nucleic acid sequences are found to be the sources of several genetic diseases and individual differences in drug metabolism,2−4 whereas some proteins have been verified to be associated with the occurrence and development of certain cancers.5−7 In the early stages of the above-mentioned diseases, the concentrations of their biomarkers in human body are usually very low. Therefore, the development of highly sensitive assay methods for accurate detection of trace amounts of such molecules is of great importance for the early diagnosis and individual therapy of serious diseases. Signal amplification is an efficient way to construct biosensing systems with high sensitivity. Various amplification strategies are employed to address this issue. One of the widely used strategies is the well-known “biobarcode assay”,8−12 which has been applied in amplified detection of a variety of biotargets including nucleic acids,8,9 proteins,10,11 and telomerase.12 A key to its high sensitivity is the use of gold nanoparticle-based amplifying probes, which were modified with hundreds of thiolated single-stranded barcode oligonucleotides. A target molecule can theoretically trigger the release of hundreds of barcode strands, and the detection of the barcode strands can then afford signal amplification for the target. In addition, the combination of the catalytic cycle of biocatalysts with a recognition event to afford an amplified signal is also an efficient strategy to improve the sensitivity of a biosensor. In the past five years, quite a few amplified sensing platforms have been proposed for DNA13−15 and other biomolecules16−19 detection by employing protein enzymes as biocatalysts, and nucleic acid probes or aptamers as recognition components. Despite their high sensitivities, the irreversible denaturation of enzymes under harsh conditions, such as heat or heavy metal ions, will greatly limit the practical applications of these sensing systems.

DNAzymes are nucleic acids which are isolated from combinatorial oligonucleotide libraries by in vitro selection.20 Similar to protein enzymes, DNAzymes show high catalytic hydrolytic cleavage activities toward specific substrates, while they are more stable than enzymes, and can be denatured and renatured many times without losing their catalytic activities toward substrates. This unique advantage makes DNAzymes ideal biocatalysts for amplified sensing applications. As a result, recent years have seen increasing interest in conjugation of
DNAzymes to recognition events to design enzyme-free amplified assay platforms for detection of DNA and other biomolecules. For most of these sensing platforms, their designs involved split DNAzyme sequences with no catalytic cleavage activity, which will be assembled to form activated structures upon the stimuli of targets. Unfortunately, these designs generally adopt Mg\(^{2+}\)-dependent DNAzyme as catalytic cleavage units, which suffered from low catalytic activity with a maximum catalytic rate of 0.08 min\(^{-1}\). Meanwhile, some DNAzyme with high catalytic cleavage activity (such as Zn\(^{2+}\)-dependent DNAzyme with a catalytic rate of 1.35 min\(^{-1}\)) are not suitable for these designs, because the slight modification of the catalytic core might result in remarkably decreased or even no catalytic activity of these DNAzymes (see the Supporting Information, Figure S1). Moreover, these designs are limited to these targets that can trigger the assembly of split or mutated DNAzyme molecules. Therefore, a sensitive and general strategy is desired for DNAzyme-based amplified sensing platform.

### EXPERIMENTAL SECTION

#### Materials and Reagents.
Dam MTase (*Escherichia coli*), Dpn I endonuclease, S-adenosyl methionine (SAM), Exonuclease I (Exo I), and Exonuclease I buffer were purchased from New England Biolabs Inc. Streptavidin (SA), bovine serum albumin (BSA), and immunoglobulins G (IgG) were purchased from Dingguo Biotech. Co. (Beijing, China). All other chemicals were obtained from Shanghai Chemical Reagents (Shanghai) and used without further purification. Milli-Q water (resistance >18 M\(\Omega\) cm) was used in all experiments. DNA oligonucleotides used in this work were synthesized and...
purified by Takara Biotechnology Co., Ltd. (Dalian, China), and their sequences are shown in Table 1. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter.

**Preparation of Cellular Homogenate.** The CCRF-CEM cells (1.3 x 10⁸ cells) were first centrifuged for 5 min at 25 °C (3000 rpm) with the supernatant being removed, and the cells precipitate was dispersed in 1 mL of buffer solution (25 mM HEPES, 100 mM NaCl, 2.0 mM MgCl₂, pH 7.0). Then, the dispersed cells were strong sonicated for 30 min in an ice-water bath. Finally, the resulting cellular homogenate was stored in 4 °C.

**Procedures for Nucleic Acid Analysis.** To detect target DNA, the hybridization between probe 1 (5 μL, 1 μM) and different concentrations of target DNA (5 μL) was performed in buffer solution of 70 μL volume (25 mM HEPES, 100 mM NaCl, 2.0 mM MgCl₂, pH 7.0) for 30 min. Then, 10 μL of 1 μM MB and 10 μL of 1 mM Zn²⁺ were added into the above solution. After thoroughly mixing the components, the rate of the fluorescence increase was monitored every 20 s using a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ). The instrument settings were chosen as follows: λ_ex = 494 nm (slit 5 nm) and λ_em = 518 nm (slit 5 nm).

**Procedures for Dam MTase Activity Detection.** The methylation experiment was carried out in 40 μL of methylase buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, pH 7.5) containing 50 nM DNA probe 2, 80 μM SAM, 4 units DpnI, and a varying amount of Dam MTase at 37 °C for 2 h. After the methylation reaction, 10 μL of 1 μM MB and 40 μL of buffer solution (25 mM HEPES, 100 mM NaCl, pH 7.0) were added into the sample solution and incubated for 20 min. Then 10 μL of 1 mM Zn²⁺ were then added to the mixture to initiate the cleavage. When the catalytic reaction was allowed to proceed for 25 min, the FluoroMax-4 spectrofluorometer was then used to record the fluorescence intensity change at 518 nm upon excitation at 494 nm.

**Procedures for Streptavidin Analysis.** To detect SA, probe 5 (5 μL, 1 μM) and SA with different concentrations (5 μL) were added into the 1 × Exonuclease I buffer of 25 μL volume (67 mM Glycine-KOH, 6.7 mM MgCl₂, 10 mM thioglycol). Then, the mixture was incubated at room temperature for 10 min to allow complete interaction between SA and biotin. Third, Exo I (5 μL, 2 U/μL) was added in the mixture and incubated at 37 °C for 1 h to perform the digestion reaction. After digestion, the Exo III was inactivated by heating the system to 85 °C for 20 min, and subsequently, 10 μL of 1 μM MB, 10 μL of 1 mM Zn²⁺, and 40 μL of buffer solution (25 mM HEPES, 100 mM NaCl, pH 7.0) were added into the above mixture. Lastly, the rate of the fluorescence increase was monitored every 20 s using a FluoroMax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ). The instrument settings were chosen as follows: λ_ex = 494 nm (slit 5 nm) and λ_em = 518 nm (slit 5 nm).

**RESULTS AND DISCUSSION**

**Application of the DNAzyme-Based Sensing System to DNA Detection.** The highly sensitive and selective detection of nucleic acid is a current focus of research in the field of clinical diagnostics and gene therapy. Many design strategies employing nucleic acid enzymes as the amplifying biocatalyst have been developed for DNA detection. The design principles are shown in Scheme 2. Sando and co-workers designed target-assisted self-cleavage probes as a new type of enzyme- and reagent-free nucleic acid sensors (Scheme 2A). This sensing system is simple and sensitive with wide applicability, although it needs label fluorophores in the middle section of the oligonucleotide, with a long detection time of 2 h. Burke and co-workers described a new mode of allosteric regulation of ribozyme for nucleic acids detection (Scheme 2B), whereas Kossen and co-workers used a target-activated ribozyme system to detect viral nucleic acids (Scheme 2C). Both of them also show high sensitivity to target nucleic acid. However, these probes employ ribozymes as the amplifying biocatalyst which are unstable, and their structures are very complex. In order to overcome these limitations, the topological effect of DNAzyme was employed to design an amplified sensing system for DNA detection using the 8–17 DNAzyme as an amplifying biocatalyst. In comparison with ribozyme, the structure of 8–17 DNAzyme is more simple and stable. Moreover, the MB substrate can provide a low background for the sensing system.

**Application of the DNAzyme-Based Sensing System to DNA Detection.** The topological effect of DNAzyme was first employed to design an amplified sensing system for DNA detection using the 8–17 DNAzyme as an amplifying biocatalyst. The design strategy of the sensing system is depicted in Scheme 1. The hairpin-structured probe 1 includes two domains: domain I is complementary to the sequence of the target DNA, and domain II corresponds to the nucleic acid sequence of 8–17 DNAzyme. In the absence of the target DNA, the 8–17 DNAzyme (domain II) is caged by partially hybridizing with domain I to form a hairpin structure, and is inactive to MB substrate, which provides a low background for the sensing system. In the presence of the target DNA, the hairpin structure is opened, and the 8–17 DNAzyme is liberated from the caged structure with its catalytic activity being restored. The activated 8–17 DNAzyme can hybridize with the MB substrate to form the CAMB system and catalyze the cleavage of the MB substrate in the presence of cofactor Zn²⁺. After cleavage, the quenched MB fluorophore/quencher pair was separated from each other, resulting in an obvious increase of fluorescent signal and a free DNAzyme strand. Eventually, each target-induced activated 8–17 DNAzyme can
undergo many cycles to trigger the cleavage of many MB substrates, achieving an amplified detection signal for the target.

In order to achieve the system’s best sensing performance, the effect of the molar ratio of MB to probe 1 on the fluorescence response of the sensing system is investigated. As can be seen from Figure S2 (see the Supporting Information), an increasing molar ratio of MB to probe 1 result in the increase of the signal-to-background ratio (SBR) of the fluorescence signal until it reached 2:1. However, when the ratio exceeded 2:1, the SBR of the fluorescence signal decreased with an increasing molar ratio of MB to probe 1. A molar ratio of MB to probe 1 at 2:1 was therefore chosen for further experiments.

Time-dependent fluorescence enhancements arising from different concentrations of target DNA were first measured. As shown in Figure 1a, in the absence of target DNA, the background fluorescence of the sensing system is very low, because in such a case the 8–17 DNAzyme is caged and inactive to MB substrate. However, in the presence of target DNA, the 8–17 DNAzyme is liberated from the caged structure and its catalytic activity is restored. Each activated DNAzyme can catalyze the cleavage of many MB substrates and induce a fluorescence enhancement. When the target DNA concentration reached 200 nM, an approximately 16-fold fluorescence enhancement was observed. The large fluorescence enhancement together with the multiple turnover capability of the activated 8–17 DNAzyme could improve the sensitivity of the sensing system. The calibration curve for this sensing system was shown in Figure 1b. A dynamic range from 100 pM to 200 nM for target DNA was achieved with a linear relationship range until 10 nM. A detection limit of 20 pM (3δ/slope) was estimated, which is much lower than the previously reported Exonuclease III-aided amplification fluorescence sensing system for DNA (with a LOD of 200 pM).33

In order to evaluate the selectivity of the sensing system, we compared the fluorescence response induced by DNA strands containing single-base, two-base-mismatched, and three-base-mismatched oligonucleotides with that of target DNA. All results are shown in Figure 2. The completely matched DNA target triggered obviously larger fluorescence enhancement than that of mismatched DNA sequences. This result might be ascribed to the relatively long stem of the probe 1, which makes the hairpin structure thermodynamically stable, and being unfavorable for the hybridization between mismatched sequences and the probe 1. This sensing system seems to be able to discriminate perfectly matched DNA target from mismatched DNA stands.

In order to evaluate the applicability of the sensing system, we conducted analyses of target DNA in cellular homogenate, a realistically complex media containing a variety of proteins and other contaminants. To avoid the interference of background fluorescence signal of cellular homogenate around 520 nm, varying concentrations of target DNA were added into the diluted cellular homogenate samples (2.6 × 10^5 cells/mL). As can be seen from Figure S3 (see the Supporting Information), the DNA calibration curve in the cellular homogenate was similar to that in the buffer solution, which suggested that the

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**Figure 1.** Sensitivity of the sensing system for target DNA detection. (a) Time-dependent fluorescence response over background fluorescence with varying concentrations of target DNA. The concentrations for probe 1 and MB are 50 and 100 nM, respectively. The buffer contained 25 mM HEPES, 100 mM NaCl, and 2.0 mM MgCl2, pH 7.0. Inset: Responses at low DNA concentrations. (b) Calibration curve of the sensing system for target DNA. The curve was plotted with the initial rate of fluorescence enhancement vs DNA concentration. The inset shows the linear responses at low DNA concentrations. Error bars show the standard deviations of measurements taken from three independent experiments.

**Figure 2.** Fluorescence response of the sensing system toward perfectly matched and mismatched DNA targets. The concentrations for probe 1 and MB are 50 nM and 100 nM, respectively. The concentrations of perfectly matched and mismatched DNA targets were fixed at 10 nM.
proposed sensing system can be applied to detect target DNA in real samples.

**Application of the Sensing System to Dam MTase Activity Analysis.** To further demonstrate the applicability of our DNAzyme topological effect-based sensing platform for various targets, another sensing system was designed for the detection of the Dam MTase activity. As shown in Scheme 1B, a hairpin-structured oligonucleotide (probe 2) was designed as both recognition and amplified units. The loop of the hairpin (region I) corresponds to the sequence of 8−17 DNAzyme. Its stem is formed by a 13 base pairs double stranded DNA, which contains the recognition site sequence of the Dam MTase (region II). In such case, the 8−17 DNAzyme activity is suppressed because it cannot form the active conformation to hybridize with the MB substrate. Nevertheless, when both the Dam MTase and DpnI restriction endonuclease are present in the test solution, the Dam MTase will catalyze the methylation reaction on the recognition sequence of the hairpin stem to yield the methylation dsDNA. Subsequently, the methylated dsDNA are specially recognized and cleaved by DpnI restriction endonuclease. As a result, an activated 8−17 DNAzyme with blunt terminus was liberated, which can catalyze the cleavage of the MB substrate to induce a fluorescence enhancement. The fluorescence of the sensing system increased with increasing concentration of the Dam MTase.

To obtain the best performance of the sensing system, the sequence of the hairpin-structured probe was first optimized. We synthesized three different hairpin probes (probe 2, probe 3 and probe 4) that included 1, 3, and 4 base pairs in the region III, respectively (Table 1). An increase of the base pairs in the region III from 1 (probe 3) to 3 (probe 2) resulted in a larger SBR of fluorescence signal (see Figure S4 in the Supporting Information), suggesting that the more base pairs in the region III could facilitate the hybridization of the recognition site base pairs of the Dam MTase to promote a highly efficient enzyme reaction. However, further increase of the base pairs in the region III from 3 (probe 2) to 4 (probe 4) might result in a high melting temperature of the DpnI-catalyzed cleaved hairpin-structured product, which shows a weak ability to hybridize with and cleave the MB substrates and, therefore, a lower SBR than that of probe 2. Probe 2 was then chosen in further experiments. The molar ratio of MB to probe 3 and the incubation time for the methylation reaction were also optimized. Experimental results indicated that a molar ratio of 2:1 for MB to probe 2 with an incubation time of 2 h for the methylation reaction could provide maximum SBR for the sensing system (see the Supporting Information, Figure S5 and Figure S6).

The activity of Dam MTase was then investigated under the optimized conditions. As can be seen from Figure 3a, the fluorescence intensity gradually increases with increasing Dam MTase concentration. This is in accordance with the fact that under higher concentrations of Dam MTase, more activated 8−17 DNAzymes are released, and the activated 8−17 DNAzymes can catalyze the cleavage of more MB substrates to induce a gradually increase of fluorescence intensity. Figure 3b depicts the calibration curve of the sensing system, and a linear relationship from 0.5 to 40 U/mL was observed. A detection limit of 0.2 U/mL is estimated for Dam MTase (3σ/slope), and is lower than the previously reported method which coupled hairpin fluorescent probes with enzyme-linkage reactions.  

![Figure 3](image-url)  
**Figure 3.** (a) Fluorescence emission spectra of the sensing system on exposure to different concentrations of Dam MTase: (1) 0, (2) 0.5, (3) 1, (4) 4, (5) 8, (6) 10, (7) 20, (8) 30, and (9) 40 U/mL. (b) Calibration curve of the sensing system for the Dam MTase activity. Inset shows the linear responses at low DNA concentrations. Error bars show the standard deviations of measurements taken from three independent experiments.

In order to evaluate the specificity of this assay, the fluorescence response of the sensing system to other DNA MTase such as AluI MTase, CpG MTase and GpC MTase were also recorded. All results are displayed in Figure S7 (see the Supporting Information). It was found that 40 U/mL Dam MTase could induce a significant fluorescence increase, whereas all other DNA MTase at a concentration of 40 U/mL did not induce an obvious fluorescence enhancement, demonstrating that our sensing system has a high specificity for DNA MTase.

**Terminal Protection Strategy for Amplified Detection of Protein.** To employ DNAzyme as amplifier to detect protein, a terminal protection strategy was then employed with its analytical principle illustrated in Scheme 1C. A biotin-labeled DNA (probe 5, Table 1) was designed as both recognition and amplified unit. It contains an 8−17 DNAzyme sequence (domain I) and a T-rich sequence (domain II) which was design to decrease the steric hindrance effect of the SA molecule on the hybridization of 8−17 DNAzyme with the MB substrate. In the absence of SA, the DNA probe 5 was hydrolyzed successively into mononucleotides by Exo I and cannot catalyze the cleavage of the MB substrate, therefore, providing a zero-background for the sensing system. In the
presence of SA, the probe 5 is protected from the degradation by Exo I, because the bound SA molecules may convey significant steric hindrance, preventing Exo I from approaching and cleaving the phosphodiester bond adjacent to the 3’ end. Consequently, the probe 5 containing the 8-17 DNAzyme sequence can catalyze the cleavage of the hairpin-structured MB substrate to induce a significant fluorescence enhancement.

Similarly, in order to achieve the best sensing performance, the molar ratio of MB to probe 5 and the Exo I concentrations were optimized. Experimental results showed that a molar ratio of 2:1 for MB to probe 5 and 10 U of Exo I could provide maximum SBR for the SA sensing system (see the Supporting Information, Figure S8 and Figure S9). Under the optimization conditions, time-dependent fluorescence enhancements arising from different concentrations of SA were recorded. As shown in Figure 4a, in the absence of SA, no fluorescence enhancement was observed, indicating that the DNA probe 5 was hydrolyzed successively into mononucleotides by Exo I and cannot catalyze the cleavage of the MB substrate. In the presence of SA, however, a large fluorescence increase was observed. Figure 4b depicts the calibration curve of the sensing system, and a detection limit of 1 ng/mL was estimated for SA according to the 3σ rule, which is much lower than the reported fluorescence staining-based system.35

To evaluate the specificity of the assay, the fluorescence response of the sensing system to other proteins such as IgG and BSA were also recorded. As shown in Figure 5, 100 ng/mL of SA could induce a significant fluorescence enhancement of the sensing system, whereas all other proteins at a concentration of 100 μg/mL did not give an obvious fluorescence increase, indicating that our sensing system shows good selectivity. Such a high specificity might be originated from the fact that terminal protection required relatively stable interaction between small molecules and target proteins.

**CONCLUSIONS**

In summary, we have developed versatile sensing platforms for amplified detection of nucleic acids, DNA MTase activity, and protein based on topological effect of 8-17 DNAzyme and terminal protection of small-molecule-linked DNA, respectively. Since no modification is necessary for the catalytic core of the DNAzyme in these designs, they can adopt any DNAzyme with high catalytic cleavage activity as an amplified unit, which affords a high sensitivity for the sensing platform. A CAMB strategy was further employed to amplify the detection signal by cycling and regenerating the DNAzyme to realize true enzymatic multiple turnover of catalytic beacons. These designs together allow a high sensitivity for the biotargets. In addition, because various recognition units might be fused in the sensing system, the new platform can be employed to detect various targets with high sensitivity and may find wide applications in biomedical fields.

**ASSOCIATED CONTENT**

*Supporting Information*

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure 4. (a) Time-dependent fluorescence response over background fluorescence with varying concentrations of SA. The concentrations for probe 5 and MB are 50 and 100 nM, respectively. (b) Calibration curve of the sensing system for SA. The curve was plotted with the initial rate of fluorescence enhancement vs SA concentration. Error bars show the standard deviations of measurements taken from three independent experiments.
Notes
The authors declare no competing financial interest.

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■ REFERENCES