Label-Free Colorimetric Assay for Methyltransferase Activity Based on a Novel Methylation-Responsive DNAzyme Strategy

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DNA methylation catalyzed by methyltransferase (MTase) is a significant epigenetic process for modulating gene expression. Traditional methods to study MTase activity require a laborious and costly DNA labeling process. In this article, we report a simple, colorimetric, and label-free methylation-responsive DNAzyme (MR-DNAzyme) strategy for MTase activity analysis. This new strategy relies on horseradish peroxidase (HRP) mimicking DNAzyme and the methylation-responsive sequence (MRS) of DNA which can be methylated and cleaved by the MTase/endonuclease coupling reaction. Methylation-induced scission of MRS would activate the DNAzyme that can catalyze the generation of a color signal for the amplified detection of methylation events. Taking Dam MTase and DpnI endonuclease as examples, we have developed two colorimetric methods based on the MR-DNAzyme strategy. The first method is to utilize an engineered hairpin-DNAzyme hybrid probe for facile turn-on detection of Dam MTase activity, with a wide linear range (6–100 U/mL) and a low detection limit (6 U/mL). Furthermore, this method could be easily expanded to profile the activity and inhibition of restriction endonuclease. The second method involves a methylation-triggered DNAzyme-based DNA machine, which achieves the ultrahigh sensitive detection of Dam MTase activity (detection limit = 0.25 U/mL) by a two-step signal amplification cascade.

DNA methylation is an important epigenetic event that regulates cell function by altering gene expression.1,2 The methylation process refers to the covalent addition of a methyl group, catalyzed by DNA methyltransferase (MTase), to the target cytosine or adenine in the specific DNA sequences.3–5 In recent years, studies on cancer pathology have proved that aberrant DNA methylation is a new generation of cancer biomarkers and DNA MTase is a potential target in anticancer therapy.6–8 Traditional methods for profiling DNA MTase activity require radioactive labeling of DNA substrate, cleavage of DNA by methylation-sensitive nucleases, or separation of methylated fragments by high-performance liquid chromatography (HPLC).9–12 Most of these methods, unfortunately, have the shortcomings of being a time-intensive, DNA-consuming, laborious treatment or the requirement of substrate radiolabeling. Recently, some intriguing techniques have been implemented in the DNA MTase assay for overcoming the above-mentioned drawbacks. For example, a “turn-on” fluorescence method based on the hairpin DNA probe13 has been developed for monitoring MTase-catalyzed DNA methylation. The complexes of DNA and cationic conjugated polymer were also used as potent probes for fluorescent ratiometric detection of the activity of DNA MTase.14 Colorimetric approaches have been reported lately for assaying the MTase using the enzyme-responsive DNA-gold nanoparticles (AuNPs) assembly.15 However, the fabrication of these MTase probes relied on the chemical modification of DNA for the labeling of fluorescence molecules or nanoparticles, which was rather costly, time-consuming, and sophisticated. Therefore, it still remains a challenge to develop facile label-free methods to monitor the DNA MTase activity.

Nowadays, besides the traditional roles as genetic material and the substrates of enzymes for DNA manipulation, the new functions of nucleic acids as recognition elements and enzyme-like biocatalysts have attracted much attention.16–19 DNA enzyme

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(DNAzyme)\textsuperscript{30–26} is a class of catalytic nucleic acids selected by the systematic evolution of the ligand by the exponential enrichment (SELEX) process. A horseradish peroxidase (HRP) mimicking DNAzyme, PS2.M,\textsuperscript{27,28} contains a special G-quadruplex structure with an intercalated hemin, which can catalyze the oxidation of 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonate di-sodium salt (ABTS\textsuperscript{2−}) by H\textsubscript{2}O\textsubscript{2} to produce the colored radical ion (ABTS\textsuperscript{−•})\textsuperscript{29–34} thereby causing a detectable color change. Recently, several studies have employed this DNA peroxidase-mimic as a signal producer in biosensing events.\textsuperscript{30–39} By rational design of the sequence and structure of DNA, HRP-mimicking DNAzyme was tethered to the various DNA sequences with particular recognition features, fabricating a series of DNAzyme-based sensing platforms which could specifically activate the DNAzyme in response to the target recognition. For example, the hybridization of target-DNA with a molecular beacon\textsuperscript{30} or the recognition of cocaine by its aptamer\textsuperscript{30} led to the activation of DNAzyme for optical detection of DNA or cocaine, respectively. Similarly, Pb\textsuperscript{2+}-specific DNAzyme\textsuperscript{37} or thymine (T)-rich functionalized DNA,\textsuperscript{43,38} coupling with the HRP-mimic DNAzyme, was utilized to develop the bioassays for aqueous Pb\textsuperscript{2+} or Hg\textsuperscript{2+} ion. Furthermore, DNAzyme-based DNA “machines”\textsuperscript{40–43} were engineered by delicate integration of isothermic DNA amplification with target-dependent generation of DNAzyme. After triggered by the target binding-induced DNA conformational change, the DNA “machines” operated along a DNA track and activated the autonomous replication/scission cycles for the synthesis of numerous HRP-mimicking DNAzyme as products, which generated color-change readout and amplified the sensing event. These DNA machines were applied successfully in detecting Hg\textsuperscript{2+} ion\textsuperscript{41} and virus genomic DNA.\textsuperscript{42} However, although many HRP-mimicking DNAzyme-based methods have been reported for sensing of various targets, no such methods are currently available for MTase or nuclease assay. The application of DNAzyme in DNA MTase or nuclease analysis would have several potential advantages, such as generating color change signals observed by the naked eye, facile DNA probe fabrication without any chemical modification of DNA for labeling, and increasing sensitivity by the combination of DNAzyme and nucleic acid amplification.

Herein, we present a novel methylation-responsive DNAzyme (MR-DNAzyme) strategy, which extends the DNAzyme-based technology to sense DNA methylation, to develop simple, colorimetric, and label-free assays for probing MTase activity. Our strategy relies on tethering the methylation-responsive sequence (MRS) of DNA to HRP-mimicking DNAzyme, which enables us to develop an engineered nucleic acid probe susceptible to DNA methylation. The MRS refers to the common DNA-recognition sequence of MTase and the specific methylation-sensitivity restriction endonuclease, which would be methylated and subsequently cleaved by the dual-enzyme (MTase and endonuclease) coupling reaction. The methylation-induced scission of MRS is exploited as a switch to activate DNA peroxidase, thus achieving a turn-on colorimetric bioassay for profiling DNA MTase activity. The feasibility of the present MTase sensing system was examined in the H\textsubscript{2}O\textsubscript{2}/ABTS\textsuperscript{−•} system, and the influence of the MTase inhibitor on the activity of the enzyme was also studied. Besides the application in MTase assay, this approach can be further used to detect the restriction endonuclease by simply changing MRS to the corresponding recognition sequence of endonuclease. Additionally, a machinelike DNA nanodevice that automatically detects the methylation events with high sensitivity has also been fabricated via combining application of in vitro nucleic acid amplification and DNAzyme-catalytic signal-enhancement.

**EXPERIMENTAL SECTION**

**Materials and Measurements.** DNA adenine methylation (Dam) MTase, Nt.BbvC I, Klenow fragment (3′–5′ exo−), DpnI, and 5′-adenosylmethionine (SAM) were purchased from New England Biolabs (NEB) Inc. (Ipswich, MA). EcoRI restriction endonuclease was purchased from Fermentas Inc. (Vilnius, Lithuania). ABTS, DNA oligonucleotides, hemin, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). All other chemicals were of analytical reagent grade and used without further purification. Ultrapure water was obtained from a Millipore filtration system and used throughout. UV–vis absorption spectra were obtained on a Beckman DU-800 spectrophotometer.

The DNA sequences were as follows: DNA-Dam, 5′-GGG TAG GGC GGG TTG GGA TCG AGA ATG TTT TCA TTC TCG ATC CCA AC-3′; DNA-Eco, 5′-TAC CGG AAT TCA GCA GTT TTC TGC TGA ATT CGG GTA GGG GTT GGG-3′; DNA-Mac1, 5′-CAT CAC GTA CGT AAG CAT GCA CGT TTA TTA TTA GTC AGA CCT TGA TCT CCG TGT TTT T-3′; DNA-Mac2, 5′-CCC AAG CCG CCC TAC CGG CTG AGG TCT GTG CAG GTA CGT GAG TAT G-3′. The methylation experiment was performed in 10 μL of methylase buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl\textsubscript{2},
pH 7.5) containing 2 µM DNA-Dam, 80 µM SAM, 4 units DpnI, and a varying amount of Dam MTase at 37 °C for 2 h. After the methylation reaction, 2 µL of 10 mM hemin (prepared in DMSO), 20 µL of 10× HEPES solution (final concentration was 25 mM HEPES, 200 mM NaCl, 10 mM KCl, and 0.05% Triton X-100, pH 5.2), and 128 µL of H2O were added to the sample. Half an hour later, 20 µL of 20 mM ABTS2− and 20 µL of 20 mM H2O2 were added to initiate the biocatalyzed oxidation of ABTS2−. UV−vis absorption spectra were obtained at 415 nm in 7.5 min at 27 ± 2 °C.

The inhibitor of Dam MTase was incubated at varying concentrations with 2 µM DNA-Dam in 10 µL of methylase buffer for 15 min. Then, 80 µM SAM, 4 units Dam MTase, and 4 units DpnI were added and the resulting solution was incubated at 37 °C for 2 h. The detection procedure was the same as shown in the aforementioned methylation experiment. The calculation of relative activity of Dam MTase in the presence of inhibitors was referenced from the literature.

**Assay of EcoRI Nuclease Activity and Inhibition by DNA-Eco Probe.** This assay followed the similar protocol as the aforementioned MTase assay with some modification. The detailed procedure of the assay is described in the Supporting Information.

**Assay of Dam MTase Activity by DNA-Based Machine.** For the assay using a DNA-based machine, 1 µM DNA-Mac1, 1 µM DNA-Mac2, 0.5 mM dNTPs, 80 µM SAM, 4 units of DpnI, 4 units of Nt.BbvC I, 2 units of Klenow fragment (3′→5′ exo−), and a varying amount of Dam MTase were incubated in 10 µL of methylase buffer (50 mM NaCl, 10 mM Tris-HCl, and 10 mM MgCl2, pH 7.5) at 37 °C for 2 h. Then, 2 µL of 10 mM hemin, 20 µL of 10× HEPES solution, and 128 µL of H2O were added, and the resulting solution was incubated for 0.5 h. Absorption spectra were obtained at 415 nm in 2.5 min after the addition of 20 µL of 20 mM ABTS2− and 20 µL of 20 mM H2O2.

**Enzyme Activity Assays by Gel Electrophoresis.** In the gel electrophoresis assay, all the contents in the sample were the same as the aforementioned. After incubation at 37 °C, the sample was applied to a polyacrylamide (PAGE) gel (20% acrylamide, 19:1 acrylamide/bisacrylamide) to separate the cleaved products from the substrate. The electrophoresis was carried in 1× tris-borate-EDTA (TBE) buffer (90 mM Tris, 90 mM boric acid, and 10 mM EDTA, pH 8.0) at 180 V for 1.5 h. The gels were silver-stained.

**RESULTS AND DISCUSSION**

**Design Strategy of Hairpin-DNAzyme Hybrid Probe.** The recognition principle of our MR-DNAzyme strategy is based on the specificity of some restriction endonucleases for methylated sites. Hence, the rational design of DNA probe sequence is crucial for achieving an efficient dual-enzyme (MTase and endonuclease) coupling reaction. Dam MTase and DpnI restriction endonuclease are chosen as the model MTase and endonuclease, respectively. Dam MTase catalyzes the transfer of a methyl group from SAM to the N6 position of the adenine residues in the palindromic sequence 5′-G-A-T-C-3′. DpnI endonuclease can specifically recognize and cleave the fully methylated (adenine methylation) GATC sites (5′-G-mA-T-C-3′). Because these enzymes share the same recognition site, the tetranucleotide sequence GATC is employed as MRS in our probe design. Scheme 1 shows the design strategy of the hairpin-DNAzyme hybrid probe for the MTase assay. The 16 base pairs containing MRS (region II) are used in the stem of the hairpin. As several adjacent bases are necessary for accelerating the enzyme reaction,13 five base pairs (region III) and seven base pairs (regions IV) are added to the two ends of the recognition site. Four T bases (region V) are exploited as the loop of the hairpin, and the HRP-mimicking DNAzyme segment (region I) is placed at the terminus (5′ end) of the hairpin probe.

In the hairpin probe, the DNAzyme sequence (region I) is partially caged in the duplex structure of the stem by hybridization with region III. As a result, the G-riched DNAzyme segment is prohibited to combine with the hemin molecule and fold into the active G-quadruplex/hemin HRP-mimicking DNAzyme structure. When both Dam MTase and DpnI restriction endonuclease are present in solution, the enzyme-linkage reaction will occur according to the mechanism shown in Scheme 1. First, Dam MTase methylates the recognition sequence to generate the methylation duplex DNA (5′-G-mA-T-C-3′), acting as the substrate for DpnI (path A). Then, DpnI cleaves the methylated probe into two parts (path B). One part is a new ssDNA possessing the loop and the blunt terminus. The other one is a new hybrid containing an 18-base DNAzyme segment and a 7-base ssDNA. Since its Tm value is estimated to be 22 °C (Tm = (4 °C)(G/C pairs) + (2 °C)(A/T pairs)), the newly formed 7 base pairs hybrid is unstable and will be separated into two short ssDNA fragments at the reaction temperature (37 °C). The released DNAzyme assembles with hemin to form the G-quadruplex that exhibits peroxidase-like activity which can oxidize ABTS2− to the colored product ABTS3− (λmax = 415 nm, ε = 3.6 × 104 m−1 cm−1) by H2O2 (path C). Therefore, taking advantage of the present strategy, we can easily monitor the DNA methylation catalyzed by MTase by the naked-eye or with UV−vis spectroscopy.

DNAzyme-Based Detection of Dam MTase Activity. Figure 1A shows the UV–vis absorption spectra of the DNA-Dam samples treated without (I) and with (II) Dam MTase in the presence of DpnI. Figure 1B shows the corresponding colorimetric detection of MTase activity. Addition of Dam MTase and DpnI induced a significant increase of absorbance (Figure 1A) and the color changed from colorless to green (4, Figure 1B), indicating that the methylation-induced cleavage of DNA-Dam occurred and activated HRP mimicking DNAzyme which catalyzed the generation of colored ABTS$^\cdot$$^-$. The probe solution treated by Dam MTase alone remained colorless (3, Figure 1B), implying that DpnI is essential to methylation recognition and DNAzyme activation. Moreover, the specificity of DNA-Dam probe for Dam MTase was demonstrated by the unchanged color of sample 5 treated by EcoRI MTase (5, Figure 1B). In addition, for further investigation of the mechanism of our method, the methylation events of DNA-Dam were probed by electrophoresis analysis with a 20% nondenaturing PAGE gel (Figure 1C). The single band of DNA-Dam in lanes 2 and 3 suggests that no probe cleavage occurs in the presence of Dam MTase or DpnI, respectively. After treatment of DNA MTase and DpnI, a new band appeared in lane 4 and was identified as the cleft DNAzyme sequence by comparison with the control band of 18-base DNAzyme in lane 0, which is direct evidence for cleavage of DNA-Dam and the successful releasing of DNAzyme sequences after DNA-Dam methylation. The single band of DNA-Dam in lane 5 indicates that the EcoRI MTases cannot methylate the specific MRS for Dam MTase. The results of electrophoresis are in agreement with those of colorimetric detection. Therefore, all the aforementioned results demonstrate that it is rational to probe DNA methylation by DNA-Dam, providing a solid foundation for the following experiments.

This DNAzyme-based method can also be used to monitor the process of DNA methylation catalyzed by MTase. In order to study the methylation process, the absorbance changes (ΔAbs) at 415 nm versus different methylation times were recorded (shown in Figure S1 in the Supporting Information). With the increase of methylation time, the absorbance change gradually increases. This is consistent with the fact that the longer the treatment time for MTase is, the more the amount of active DNAzyme is produced. However, when the methylation time is prolonged, the increase rate of absorbance change is gradually decreased, suggesting that the methylation catalyzed by Dam MTase is slowed. This phenomenon is probably due to the consumption of the substrates, which is similar to the phenomenon previously reported by Li et al. For achievement of the balance between short detection time and high signal intensity related to methylation time, 2 h is selected for the methylation reaction in this work unless otherwise specified.

The activity of Dam MTase was investigated by the hairpin-DNAzyme probe under the same conditions with different concentrations of Dam MTase (0–400 U/mL). As shown in Figure 2A, the approximately linear increase of the time-dependent
absorbance at 415 nm is observed within 7.5 min, resulting from the continuous generation of ABTS$^{+}$ catalyzed by DNAzyme. The absorbance value gradually increases with the increase of the concentrations of Dam MTase. This is in accordance with the fact that at higher concentrations of Dam MTase, more DNAzymes are released. The calibration curve for analyzing the activity of Dam MTase (Figure 2A, inset) indicates that the linear range of this DNAzyme-based Dam MTase assay is from 6 to 100 U/mL with a detection limit of 6 U/mL. The detection range of this method is wider than that of previously reported MTase assays, and the detection limit is close to that of the method based on cross-linking AuNP aggregation. Hence, as observed in the case of Dam MTase, the present method based on the hairpin-DNAzyme probe can be used to detect the activity of MTase conveniently and efficiently.

**Assay of the Inhibition of Dam MTase.** Since DNA methylation plays an important role in both prokaryotes and eukaryotes, the pharmacological inhibition of DNA MTases provides a broad spectrum of therapeutic applications, such as antibiotics and anticancer therapeutics. The validity of our method in assaying the inhibition of Dam MTase was tested by using 5-fluorouracil, an anticancer drug, as a model inhibitor. Because there are two enzymes involved in the assay system, it is necessary to evaluate the influence of 5-fluorouracil on DpnI before studying the effect of 5-fluorouracil on Dam MTase. The control experimental results indicate that 5-fluorouracil has no influence on the activity of DpnI when the concentration of 5-fluorouracil is not more than 10 µM (Figure S2 in the Supporting Information). Figure 2B depicts the effect of the 5-fluorouracil on the activity of Dam MTase. The increase of the inhibitor concentration lowers the content of methylated DNA; therefore, the events of digestion by DpnI decrease and the lower content of the DNAzyme is produced. The IC$_{50}$ value, the inhibitor concentration required to reduce enzyme activity by 50%, is acquired from the plot of relative activity (RA) of Dam MTase versus 5-fluorouracil concentration and is found to be 2.74 µM. These facts indicate that our method can be used to study the MTase inhibitor and employed for MTase inhibitors screening.

**Colorimetric Detection of EcoRI Endonuclease Activity and Its Inhibition.** Since this MTase assay relies on the recognition and cleavage of the methylation-specific endonuclease, it is reasonable to expect that the present detection system can be used to assay restriction endonuclease activity. To demonstrate the versatility of the DNAzyme-based method in the restriction enzyme detection, we have developed another hairpin-DNAzyme probe, DNA-Eco, for sensing the activity of restriction endonuclease EcoRI. The sequence of DNA-Eco is similar to that of DNA-Dam, except that the recognition site of Dam MTase is replaced by EcoRI recognition site 5′-GAATTCC-3′. The detailed mechanism of the EcoRI assay is shown in the Supporting Information and Scheme S1. As depicted in Figure S3A in the Supporting Information, the cleavage of DNA-Eco by EcoRI was observed by the color change of the solution from colorless to green (sample 2). In addition, no color change occurred after treatment of BamHI or HindIII (samples 3 and 4), indicating the good specificity of this method for EcoRI. Figure S3B in the Supporting Information shows the effect of EcoRI concentrations on the time-dependent absorbance changes. The increase in EcoRI concentration induces a monotonous increase in absorbance values. The resulting calibration curve, Figure S3C in the Supporting Information, indicates that the linear range of this DNAzyme-based method is from 5 to 600 U/mL with a detection limit of 5 U/mL. The inhibition of EcoRI by pyrophosphate (PP) was observed, and the IC$_{50}$ value for PP on EcoRI activity was found to be 1.49 mM (Figure S4 in the Supporting Information). Thus, our enzyme activity assay based on DNAzyme can be facilely expanded for colorimetric detection of restriction endonuclease activity and inhibition.

**Dam MTase Activity Assay Using Methylation-Responsive DNA-Based Machine.** The method employing the hairpin-DNAzyme probe for the MTase assay has the merit of being label-free, simple, and inexpensive. Also the detection limit of this method is comparable to that of the method based on cross-linking AuNP aggregation, but it is still higher than that of MTase assays based on a fluorescent probe or destabilization-induced AuNP aggregation. In order to further improve the sensitivity, we have developed a novel methylation-responsive DNA-based machine for the highly-sensitive detection of MTase activity via the amplification cascade comprising strand displacement amplification (SDA) and DNAzyme catalysis. The principle of the DNA machine is depicted in Scheme 2. This machine is a hybrid including two ssDNA: one is used as the methylation-responsive element named DNA-Mac1 and the other is the template for amplification named DNA-Mac2. DNA-Mac2 consists of three regions. Region I is complementary to part of DNA-Mac1. Region II has a nicking site for nicking endonuclease Nt.BbvC I when it forms a double strand. Region III is a complement sequence to the DNAzyme that is synthesized by the DNA-based machine as product. DNA-Mac1 is a hairpin DNA containing three segments, which can be methylated and cleaved by the Dam MTase/DpnI coupling reaction. One (segment A) is an 18 base-pair duplex stem with a MRS (red) in the middle, tethering a 5 base loop. Another (segment B) can hybridize with a 14-base part of region I of DNA-Mac1, yielding an entire form of the DNA machine. The third one (segment C) is an unpaired four “T” bases sequence at the elongation of the 3′ terminus, which serves as a block to prohibit the undesired replication initiated at the 3′ end of DNA-Mac1. When segment A is not methylated, DNA-Mac2 is blocked with DNA-Mac1 to prevent it from forming an active machine. In the presence of the Dam MTase, the segment A is methylated and cleaved by DpnI sequentially, resulting in the separation of DNA-Mac1 into three parts. Two parts, a new hairpin and a small ssDNA fragment, are released. The ssDNA containing segment C as the third part, which is fully complementary to region I of DNA-Mac2, hybridizes with the rest of region I, which switches on the DNA machine. Formation of the duplex initiates the replication of the track (regions II and III of DNA-Mac2) in the presence of polymerase and dNTPs mixture. The replicated strand in region II includes the nicking site for Nt.BbvC I. The cleavage of region II by nicking enzyme restarts the replication by polymerase to produce a secondary DNAzyme replacing the original one. Subsequently, with the coordination of hemin, the DNAzyme catalyzes the oxidation of ABTS$^{−}$ by H$_2$O$_2$ to generate a color-change read-out. Therefore, an enzyme-induced methylation recognition event activates the DNA machine to automatically and repeat-

edly synthesize the DNAzyme, which will significantly amplify the sensing process.

The feasibility of the methylation-responsive DNA-based machine in the MTase assay was investigated by the colorimetric analysis in the presence of H$_2$O$_2$/ABTS$^2^-$ (Figure 3A). In comparison with the colorless sample without Dam MTase treatment (sample 2), the obvious color change from colorless to green was observed when the DNA-Mac1/2 was incubated with Dam MTase (sample 3). This result suggests that the methylation of DNA-Mac1 induces its cleavage by DpnI and activates the DNA machine to synthesize the DNAzyme. The produced DNAzyme self-assembles to the G-quadruplex-hemin structure and catalyzes the generation of green oxidation product, providing a “read out” of the operation of machine and initial methylation events. There was no color change in sample 4 treated by EcoRI MTase, which implies that this DNA machine has good specificity for Dam MTase. Additionally, the autonomous synthesis of DNAzyme by the DNA machine was further confirmed by gel electrophoresis experiments (shown in Figure 3B). A 22 base ssDNA containing HRP mimicking the DNAzyme sequence was used as a control for the product of the DNA machine (lane D). Without the presence of Dam MTase, no machine product band was observed (lane IV). However, after the treatment of Dam MTase, a clear band of the DNAzyme, the product of DNA machine, appears (lanes II or III), indicating that the machine is activated by MTase. As the operation of the machine is prolonged, the band of the machine product becomes increasingly clear, which demonstrates the continuous generation of DNAzyme product after activation of the DNA machine (lanes II and III). The absence of machine product band in lane V reveals that EcoRI MTase cannot methylate DNA-Mac1 and activate the DNA machine.

Figure 4A shows the time-dependent absorbance changes observed upon analyzing different concentrations of Dam MTase (from 0 to 400 U/mL) by the DNA machine. As the concentration of Dam MTase increases, more DNA machines are activated for the synthesis of the DNAzyme, causing that the generation of colored ABTS$^-$ is accelerated and the rate of absorbance changes increases correspondingly. It is noteworthy that the DNA machine method can remarkably amplify the methylation events in comparison with the aforementioned method based on the hairpin-DNAzyme probe. As shown in the inset plot of Figure 4A, the UV–vis signal intensity (SI, SI = ∆Abs$_x$ – ∆Abs$_0$, ∆Abs$_x$ is the absorbance change in 2.5 min when Dam MTase concentration is $x$ U/mL and ∆Abs$_0$ is the absorbance change in 2.5 min when the sample is treated without Dam MTase) of 25 U/mL Dam MTase by DNA machine was 5.9 times of that by the hairpin-DNAzyme method, and the SI of 100 U/mL Dam MTase by DNA machine was 2.8 times higher than that by the hairpin-DNAzyme method, which indicates that the strand displacement amplification used in the DNA machine can efficiently further improve the sensitivity of the DNAzyme-based assay, especially at a low concentration of Dam MTase. The calibration curve (Figure 4B) indicates that the detection limit of this DNA machine-based method is 0.25 U/mL, which is much lower than that of the method using the hairpin-DNAzyme probe (6 U/mL) and more sensitive than that of previous reported MTase assay. In addition, similar to other enzyme-catalyzed
reactions, this method can be expected to reach a lower detection limit by further optimizing the methylation, cleavage, or replication conditions.

CONCLUSIONS

We propose a simple and label-free methylation-responsive DNAzyme (MR-DNAzyme) strategy for the colorimetric detection of MTase activity. This novel strategy is based on the specific recognition of the MTase and methyl-sensitive endonuclease and the unique catalytic property of HRP-mimicking DNAzyme. The feasibility of this MR-DNAzyme strategy is demonstrated in two cases: DNAzyme-hairpin hybrid probe and methylation-responsive DNA-based machine. In comparison with the traditional method to detect DNA methylation, these DNAzyme-based methods have several advantages: first of all, the assay of MTase activity can be simply visualized by the naked eye; second, the DNA probes used do not require the labor-intensive and expensive modification of DNA; third, the preparation of the hairpin-DNAzyme probe is facile, low-cost, and accessible to numerous laboratories; fourth, the combination of DNAzyme-mediated signal transduction and the DNA amplification method allow a significant improvement of sensitivity for the MTase assay. Furthermore, this strategy can be easily extended to assay endonuclease or screen its suitable inhibitor. The present work, as the first example of the DNAzyme-based MTase or nuclease assay, also demonstrates a new application to the analytical function of HRP-mimicking DNAzyme. Therefore, the MR-DNAzyme strategy developed here provides a promising platform for the high-throughput screening of novel MTase or the drugs with potential methylation-inhibition ability. These new methods may also contribute to the future application of DNAzyme-based technologies to the enzyme activity analysis for therapeutic purposes.

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SUPPORTING INFORMATION AVAILABLE

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. Assay of Dam MTase activity by DNA-based machines. (A) The time-dependent absorbance changes upon analyzing Dam MTase. The curves from bottom to top were obtained with different concentrations of Dam MTase: 0, 0.25, 1, 5, 25, 100, and 400 U/mL, respectively. The inset of part A shows the comparison of the method based on DNA-based machines (black) and that based on the hairpin-DNAzyme probe (gray). (B) The absorbance change (ΔAbs) in 2.5 min was plotted as a function of the Dam MTase concentration.