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ORIGINAL PAPER

A methylation-stimulated DNA machine: an autonomous isothermal route to methyltransferase activity and inhibition analysis

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Abstract The operation of DNA nanomachines is generally triggered by either conformational changes of DNA nanostructure or external environmental stimuli. In the present study, we demonstrate an alternative driving force, DNA methylation, to stimulate DNA machine operation. DNA methylation changes neither DNA sequence and conformation nor external environment, however, blocks its cleavage by corresponding methylation-sensitive restriction endonuclease. We thus designed a strand displacement amplification DNA machine, which could be stimulated upon DNA methylation and then autonomously generates accumulated amounts of peroxidase-mimicking DNAzyme signaling machine products in an isothermal manner. The machine product DNAzyme could catalyze the H_2O_2-mediated oxidation of 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) ($ABTS^{2-}$) to a colored product $ABTS^{-}$. This methylation-stimulated DNA machine was further used as a colorimetric assay for analysis of methyltransferases activities and screening of methylation inhibitors. As compared with classical methylation assay, this facile isothermal DNA machine avoids the introduction of methylation-specific polymerase chain reaction and radioactive labels, which might be employed as an effective tool for DNA methylation analysis.

Keywords DNA machine · Methyltransferase activity · Inhibitor · Isothermal amplification

Introduction

DNA molecular machines are artificially programmed machine-like functional devices that exploit the self-assembly properties of nucleic acids [1–3]. The driving force for DNA machine arises from the increase of internal entropy of DNA nanostructures triggered by either target-induced structural changes [4, 5] or external environmental stimuli, such as pH [6, 7], buffer composition [7], light irradiation [8, 9], and electrochemical actuation [10, 11]. For example, the mechanical motion of DNA was usually carried out by hybridization of one DNA fuel molecule to target sequences, followed by its removal with another DNA sequence that is completely or partially complementary to the first [12, 13]. This strand displacement or chain-exchange activation force has successfully stimulated the mechanical "opening/closing," "extension/contraction" of nucleic acid, which has found applications in nanotransporting and logic gate operations [14].

In addition to the target-induced structural changes and external environmental stimuli, we herein demonstrate an alternative stimulation, DNA methylation modification, as a new activation for DNA machine operation. DNA methylation refers to an enzymatic covalent addition of a methyl

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group from a methyl donor molecule (e.g., *S*-adenosyl-L-methionine (SAM)) to the target cytosine or adenine residue in a site-specific manner [15]. In higher eukaryotes, methylation most prevalently occurs at the C5 position of the cytosine, predominantly within the CpG dinucleotide in a post-replication reaction catalyzed by several cytosine-C5 methyltransferases (C5 MTases, e.g., Hpa II MTase (M.Hpa II)). This heritable post-replication epigenetic event plays an essential role in the interpretation of gene information [16–18]. In the present study, we choose a strand displacement amplification (SDA) DNA machine, as an example to illustrate the possibility of methylation actuation.

Experimental

Materials

The DNA oligonucleotides were synthesized and purified by TaKaRa Biotechnology Co. (Dalian, China) and purified by high-performance liquid chromatography. The following sequences were used: (**1**) upper strand, 5′-AGTCACTGTTGCTCCTCAGCTTCATTCCG GATGGGTAGGGCGGGTTGGG-3′; (**1**) bottom strand, 5′-CCCAACCCGCCCTACCCATCCGGAATGAAGCT GAGGAGCAACAGTGACT-3′; (**2**) 5′-TCAGCTT CATTCCGGATGGGTAGGGCGGGTTGGG-3′; and (**3**) 5′-TCAGCTTCATTCCG-3′.

M.Hpa II, endonuclease Hpa II, Nt.BbVC I, and Klenow fragment exo$^-$ were purchased from New England Biolabs. The deoxynucleotide triphosphate solution mixture (dNTPs) solution was obtained from Takara Corporation. Hemin was purchased from Frontier Scientific (Logan, UT, USA).

Instruments

Absorbance measurements were performed using a Hitachi U-3010 UV–Vis spectrophotometer. Photographs were taken with a Canon A-620 digital camera.

Assay of M.Hpa II activity

The methylation of DNA (**1**) was performed by incubating DNA (**1**) (200 nM) with a varying amount of M.Hpa II in 50 μL MTase buffer (50 mM Tris–HCl, 5 mM 2-mercaptoethanol, 10 mM EDTA, pH 7.5) containing SAM (80 μM) at 37 °C for 2 h and then at 65 °C for 20 min to inactivate M.Hpa II. Then, 5 μl of the resulting solution was further incubated with Hpa II (20 units) in NEBuffer 4 (20 mM Tris–acetate, 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9) at 37 °C for 1 h and then at 65 °C for 20 min to inactivate Hpa II (total volume, 50 μL). The machine was operated by adding Klenow fragment (5 units), Nt.BbVC I (10 units), and dNTPs (0.2 mM) to the reaction mixture, which was incubated at 37 °C for 1 h and then 80 °C for 20 min (total volume, 100 μL). Then, 5 μL of hemin (20 μM) was added to the reaction mixture. Absorption spectra were obtained at 416 nm in 180 s after the addition of 10 μL of 40 mM H_2O_2 and 100 μL of 2 mM $ABTS^{2-}$.

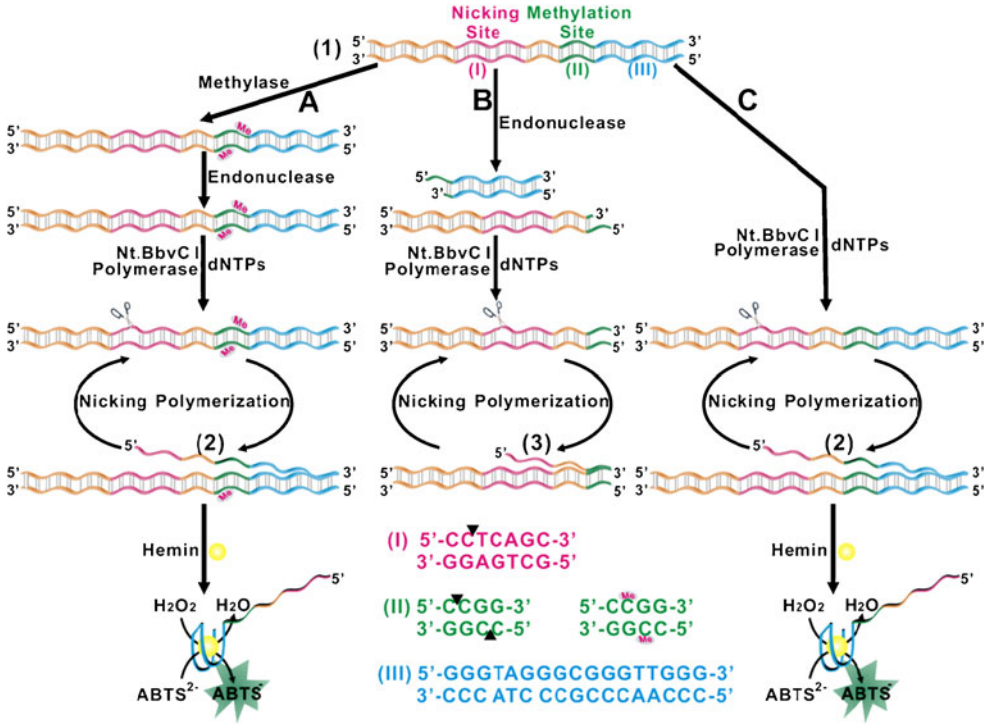
Inhibition assay of M.Hpa II activity

Different drugs were pre-incubated with DNA (**1**) and M.Hpa II in the MTase buffer (50 mM Tris–HCl, 5 mM 2-mercaptoethanol, 10 mM EDTA, pH 7.5) at 37 °C for 15 min. Then, SAM was added, and the obtained mixture, which contained 200 nM DNA (**1**), 100 U/ml M.Hpa II, and 80 μM SAM, demanding concentration drugs (total volume, 50 μL), was incubated for another 2 h and then heated to 65 °C for 20 min to inactivate the M.Hpa II. The experimental conditions for further endonuclease Hpa II treatment, SDA DNA machine operation, and colorimetric measurement were the same as the MTase activity assay. It should be noted that, eliminating the possible inhibition of RG108 on the other three enzymes involved in our machine system besides M.Hpa II, control experiments were performed as follows: DNA (**1**) was first treated with 100 U/mL M.Hpa II, and then 50 μM RG108 was added to the system when (**1**) was treated with Hpa II. The further operation was the same as other assays.

Results and discussion

SDA DNA machine is an isothermal nucleic acid amplification protocol that mimics in-vivo DNA replication [19]. An all-purpose SDA-based DNA machine involves the primer-directed nicking activity of a restriction enzyme and an exonuclease-deficient polymerase, which are capable of initiating DNA replication at a nicking site and subsequently, the downstream displacement of the strand [20–23].

One interesting feature of DNA methylation is that methylated DNA could block its cleavage by corresponding methylation-sensitive restriction endonuclease (e.g., M.Hpa II and Hpa II endonuclease (Hpa II)). Based on this knowledge, the principle of our designed methylation-stimulated DNA machine is outlined in Scheme 1. A rational tailored double-stranded DNA "track" (**1**), which contains a recognition site for nicking enzyme Nt.BbvC I (Domain I, purple) and another recognition site for M.Hpa II and its cognate restriction endonuclease Hpa II (Domain II, green), is employed as the DNA machine operation template. "Track" (**1**) also contains a Domain III (blue), which consists of a sequence of G-quadruplex (upper



Scheme 1 Schematic for the operation principle of the methylation-activated DNA machine

strand) and its complementary strand. Hemin could stack on G-quadruplex to form a horseradish peroxidase-mimicking DNAzyme [24], which catalysis the oxidation of 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt ($ABTS^{2-}$) by H_2O_2 to a colored product $ABTS^{\cdot-}$, acting as an optical readout. The operation of this methylation-stimulated machine is initiated by MTase M.Hpa II as a stimulus (route A). When domain II is methylated by M.Hpa II, the cleavage of domain II by Hpa II is inhibited, and the DNA "track" (**1**) is intact. Then, Nt. BbvC I and exonuclease-deficient polymerase (Klenow fragment) and deoxynucleotide triphosphate (dNTPs) fuels are added to this methylated system to trigger the SDA operation. In detail, Nt.BbvC I results the scission of domain I by Nt.BbvC I and yields a replication site for Klenow. The following replication results in a new nicking site for Nt.BbvC I. The further cleavage at this site results in the initiation of a secondary polymerization cycle while displacing sequence (**2**) that self-assembles into the G-quadruplex/hemin DNAzyme structure. This repeated synthesis of DNAzyme (**2**) is regarded as operation products of this methylation-stimulated DNA machine. However, in the absence of MTase (route B), the original unmethylated "track" (**1**) is digested by Hpa II, and the duplex DNA (**1**) is cut into two parts at domain II. The further operation of the SDA machine could only produce short, useless waste product (**3**). We also designed a control route to verify the solidity of the SDA machine operation (route C). In the absence of both M.Hpa II and Hpa II, scission of domain I by Nt.BbvC I results a new replication site for Klenow, and the methylation-triggered DNA machine is transformed to a conventional structural change actuated SDA machine.

The methylation-stimulated DNA machine is verified by comparison of the absorbance changes of $ABTS^{\cdot-}$ oxidized by DNAzyme (**2**) generated in the three operation routes (Fig. 1). Clearly, as compared with the unmethylation-treated process (curve b, route B), the methylation-triggered process (curve a, route A) produced increased amounts of product DNAzyme (**2**), which confirmed the improved operation efficiency. In addition, the control route (curve c, route C) produced slightly higher amount of (**2**) than in route A, which in another way verified our design. Further support that confirms the methylation-stimulated DNA machine was obtained from gel electrophoresis experiments (Fig. S1 in Electronic Supplementary Material).

We also performed another control experiment to explore the efficiency of our DNA machine. Figure S2 in Electronic Supplementary Material shows the calibration curve corresponding to the absorbance values of $ABTS^{\cdot-}$ generated by different concentrations of artificial synthesized machine product (**2**). According to this calibration curve, 930 nM of machine product (**2**) would induce similar color change of $ABTS^{2-}$ as shown in Fig. 1 (curve a, route A). Thus, we concluded that the DNA machine would generate 930 nM of product (**2**) upon full methylation. Given the concentration of DNA machine template (**1**) is 10 nM, one strand of machine template may produce 93 strands of machine product when the template is fully methylated.

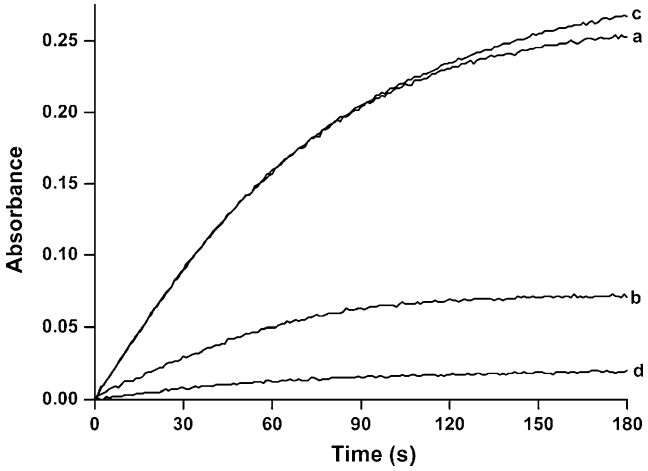


Fig. 1 Time-dependent absorbance changes of the methylation-stimulated DNA machine upon operation in routes A (curve *a*), B (curve *b*), and C (curve *c*). Curve *d* is the absorbance change induced only by hemin. In all experiments, the methylation of DNA (**1**), (curve *a*) was performed by incubating DNA (**1**) (200 nM) with different amounts of M.Hpa II (e.g., 200 U/mL in route A) in 50 μL MTase buffer (50 mM Tris–HCl, 5 mM 2-mercaptoethanol, 10 mM EDTA, pH 7.5) containing SAM (80 μM) at 37 °C for 2 h and then at 65 °C for 20 min to inactivate M.Hpa II. Then, 5 μL of the resulting solution was further incubated with Hpa II (20 units) in NEBuffer 4 (20 mM Tris–acetate, 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9) at 37 °C for 1 h and then at 65 °C for 20 min to inactivate Hpa II (total volume, 50 μL). The machine was operated by adding Klenow fragment (5 units), Nt.BbVC I (10 units), and dNTPs (0.2 mM) to the reaction mixture, which was incubated at 37 °C for 1 h and then 80 °C for 20 min (total volume, 100 μL). Then, 5 μL of hemin (20 μM) was added to the reaction mixture. Absorption spectra were obtained at 416 nm in 180 s after the addition of 10 μL of 40 mM H2O2 and 100 μL of 2 mM $ABTS^{2-}$

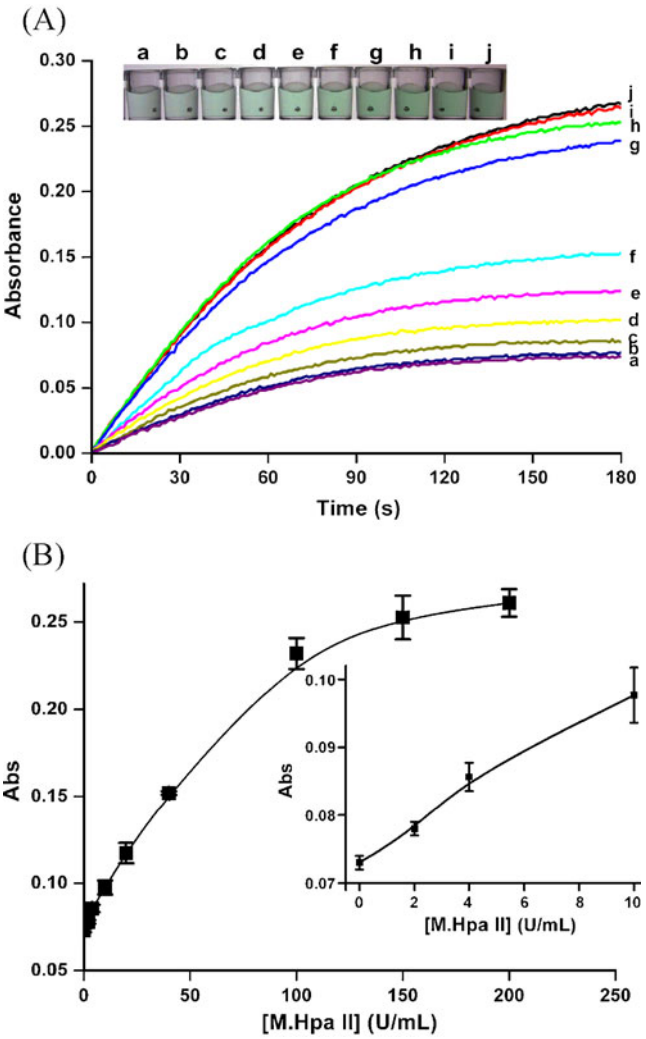


Fig. 2 **a** Time-dependent absorbance changes of the methylation-stimulated DNA machine upon challenging with different amounts of M.Hpa II: (*a*) 0 U/mL, (*b*) 2 U/mL, (*c*) 4 U/mL, (*d*) 10 U/mL, (*e*) 20 U/mL, (*f*) 40 U/mL, (*g*) 100 U/mL, (*h*) 150 U/mL, (*i*) 200 U/mL, and (*j*) 0 U/mL of M.Hpa II and 0 U/mL of Hpa II. *Inset* is the visual color changes of $ABTS^{\cdot-}$ oxidized by the machine product DNAzyme (**2**) upon treatment with different amounts of MTase M.Hpa II. **b** The derived calibration curve for M.Hpa II activities. *Inset* is the amplified zone of the low concentration range of calibration curve. The experimental conditions were detailed in the caption of Fig. 1. *Error bars* are the standard deviations of measurements taken from three independent experiments

The signal amplification ability of this methylation-stimulated DNA machine enables it as a colorimetric assay to monitor MTases activities. The machine "track" (**1**) was treated with M.Hpa II for 120 min and Hpa II (20 unit) for 60 min, followed by 60 min of the SDA machine operation. Figure 2a depicts the time-dependent absorbance changes of $ABTS^{\cdot-}$ oxidized by DNAzyme (**2**) that produced upon challenging the DNA machine with different concentrations of M.Hpa II. As the concentration of M.Hpa II increased, the color generated by the machine was intensified; implying more DNA (**1**) was methylated and intact, enhanced amounts of DNAzyme (**2**) were generated as a result. Curve j of Fig. 2a reflects the absorbance changes of $ABTS^{\cdot-}$ observed in route C. Since the absorbance change of the system upon treating with 200 U/mL of M.Hpa II (curve i) was similar to curve J, we thus concluded that almost 100% of (**1**) was methylated by 200 U/mL of M.Hpa II. The derived calibration curve (Fig. 2b) and the limit of detection (LOD) for M.Hpa II were calculated as 1.2 U/mL (3σ). Since DNAzyme could also catalyze the oxidation of luminal in the presence of H_2O_2 and generate chemiluminescence signal [24], LOD of this DNA machine-based MTase assay could be further improved by using luminol as substrate.

The methylation-stimulated DNA machine could also be employed for screening MTase inhibitors. Since MTase widely exists in pathogens, the pharmacological inhibition of DNA MTase has broad potential application in cancer therapy and antimicrobial infection [25]. Thus, a facile method for screening MTase inhibitors is highly desirable [26–31]. We systematically challenged the DNA machine with four representative methyltransferases inhibitors:

5-aza-2′-deoxycytidine (5-Aza-CdR), 5-fluorouracil (5-FU), procaine, and RG108 (Fig. 3a). Suitable inhibitor will inhibit the operation of this DNA machine (route A); as a result, less machine product (**2**) is generated. These drugs inhibited the activities of M.Hpa II differently; only RG108 reveals strong direct inhibition toward M.Hpa II, while

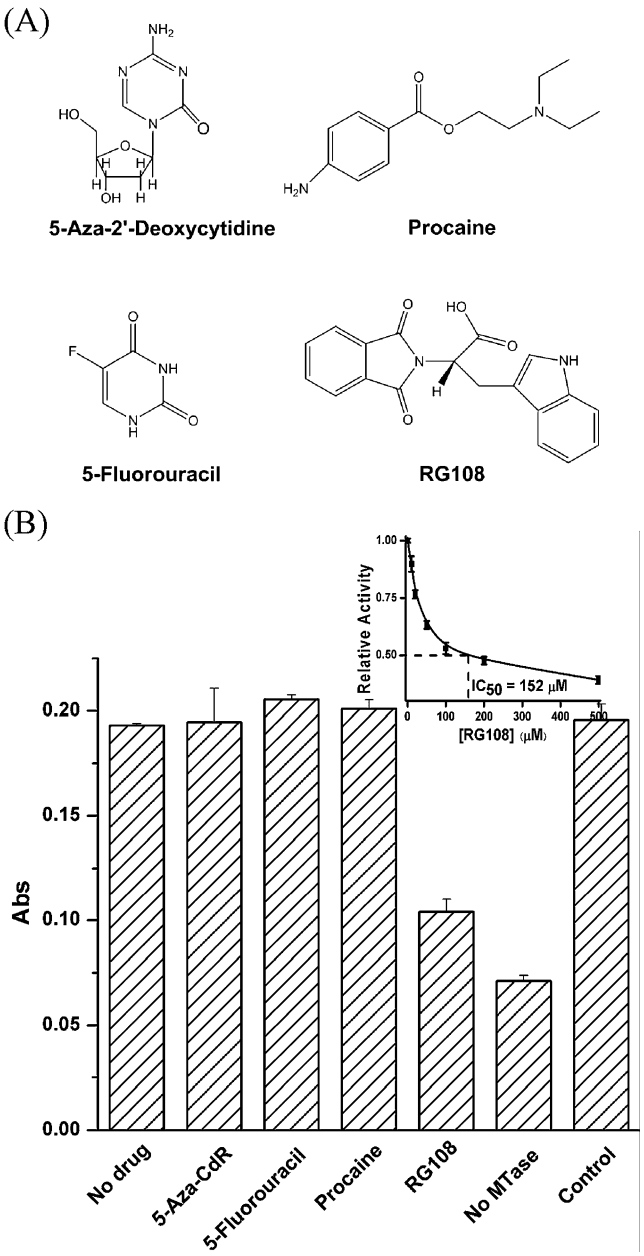


Fig. 3 **a** The chemical structures of the four candidate inhibitors of M.Hpa II. **b** Influence of different inhibitor candidates (all of 500 μM) on the absorbance intensity of the machine operation product DNAzyme (**2**), upon complex with hemin, and further reacting with $ABTS^{2-}$ and H_2O_2. The concentration of M.Hpa II used for inhibition assay was 100 U/mL. *Inset* is the inhibition ratio at different concentrations of RG108. All other experimental conditions were detailed in the caption of Fig. 1. *Error bars* are the standard deviations of measurements taken from three independent experiments

5-Aza-CdR, 5-FU, and procaine do not inhibit (Fig. 3b), which also coincide with our gel electrophoresis results (Fig. S3 in Electronic Supplementary Material). Considering that multiple enzymes work simultaneously in this machine, we also performed a control experiment to exclude the possible inhibition effect of RG108 on other enzymes involved in the nuclease cleavage and SDA operation steps. In this control experiment, track (**1**) was first treated with M.Hpa II, then RG108 was involved in the further endonuclease cleavage and SDA machine operation steps, and the obtained result suggested that RG108 had no influence on the activity of other three enzymes (Fig. 3b, control). The inhibition mechanism of RG108 was explained as that RG108 blocks the active site of DNA MTase [32]. We also investigated the concentration-dependant inhibitory effect of RG108 on the activity of M.Hpa II. The IC50 value, half-maximal inhibitory concentration, was obtained from the plot of relative activity versus RG108 concentration (Fig. 3b, inset) and calculated as 152 μM, which is also in accordance with the previous study (115 nM, normalized to 1 nM of enzyme) [33]. Very recently, Yao et al. reported a methylation-stimulated DNA machine that could be used as a label-free colorimetric assay for methyltransferase activity [28]. The DNA adenine methylation (Dam) MTase employed in their work mainly exists in bacteria. In contrast, the cytosine-C5 MTase employed in our work widely exists in tumor-related pathology, which might be of broader interest to methylation studies.

Conclusion

In summary, we have demonstrated DNA methylation as a new stimulus to trigger the autonomous operation of DNA machine, which amplifies the methylation event and produces colorimetric readout in an isothermal manner. The signal amplification ability of this methylation-stimulated DNA machine enables it as a label-free colorimetric assay for analysis of MTase activity and screening of MTase inhibitors, which avoids the introduction of methylation-specific polymerase chain reaction and radioactive labels. In addition, since this DNA machine-based colorimetric assay does not require multiple steps and laborious experimental operation and could work autonomously, it could be easily scalable and amenable to automation; thus, autonomous methylation analysis is highly expected. This facile assay could be employed as an effective tool for DNA methylation analysis.

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