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An entropy-driven signal amplifying strategy for real-time monitoring of DNA methylation process and high-throughput screening of methyltransferase inhibitors

Siyi Chen [a], Huan Ma [a], Wang Li [a, b, *], Zhou Nie [a], Shouzhuo Yao [a]

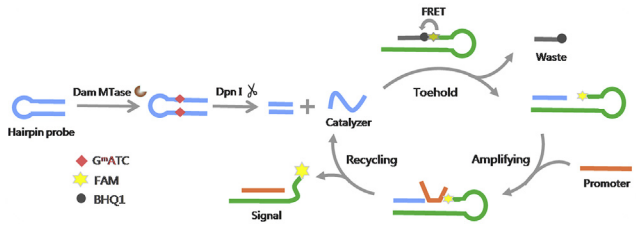
[a] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, PR China

[b] College of Food Science and Engineering, Central South University of Forestry and Technology, Changsha, 410004, PR China

HIGHLIGHTS

- A real-time and one-step fluorescent method for detecting methyltransferase activity is proposed.
- The approach is based on enzyme-free entropy-driven toehold-mediated hairpin displacement.
- High sensitivity with a low detection limit of 0.01 U mL^{-1} is achieved.
- The application of high-throughput screening drugs as methyltransferase inhibitors is demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

Based on the entropy-driven toehold-mediated hairpin displacement (ETHDA), a facile and sensitive one-step fluorescence sensing platform has been proposed for the evaluation DNA methylation process catalyzed by methyltransferase (MTase). In this designed system, a hairpin probe is methylated by DNA adenine methylation (Dam) MTase and cleaved by DpnI endonuclease successively, liberating a catalyzer strand to initiate the signal amplification. The fluorescence intensity is increasing upon the catalyzer strand triggered circulating procedure of the ETHDA process. It is proved that the proposed biosensor is free of intricate procedures and avoids the interference of added amplification enzymes. According to the obtained result, the novel assay is exceedingly sensitive and selective in MTase detection with a low detection limit of 0.01 U mL^{-1} and a wide linear range of 0.01–100 U mL^{-1}, which indicatives of this method a great candidate for monitoring DNA methylation. Moreover, this biosensor can offer practical applications in the high-throughput screening of MTase inhibitors, of which broadens the potential in the clinical diagnostics and related biomedical research.

1. Introduction

DNA methylation, a significant epigenetic modification process, plays an indispensable part in various biological regulations, such as the structure transformation of chromatin and nucleotides, gene silencing and the interaction mode between protein and DNA [1,2]. It has been certified that the level of DNA methylation is various in

* Corresponding author. State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, PR China.
E-mail address: wli@hnu.edu.cn (W. Li).

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different species/organs and undergoes precise regulation. Up to now, numerous researches have reported that uncharacteristic DNA methylation would contribute to tumorigenesis, including cell cycle regulation, inflammatory/stress response and apoptosis [3–5]. Notably, DNA methyltransferases (MTases) play vital role in the process of regulating the level of methylation process [6]. The DNA MTases have been regarded as significant biomarkers of pathologic diagnosis and trend to be a novel family of pharmacological targets for anticancer therapy. Herein, the development of excellent bioassays for the detection of aberrant DNA methylation, especially the activity assay of DNA MTase and screening its inhibitors, is of significance for early clinical diagnosis and cancer classification [7–10].

Conventional approaches for detecting the process of DNA methylation consist of high-performance liquid chromatography (HPLC), radioactive labeling as well as gel electrophoresis [11–14]. However, these aforementioned methods involves in arduous preparation or radioactive substrates, which are complex, high-cost and time-consuming. Lately, considerable progressive studies [15] ranging from colorimetric assays [16–18], electrochemical methods [19–22] to fluorescent sensing platform approaches [23–25] have been developed in DNA MTase activity detection to overcome these drawbacks. Comparatively, fluorescence-based strategy are frequently applied for DNA MTase detection by taking advantage of nucleic acid amplification, such as the rolling circle amplification [8,26] and nicking enzyme signal amplification [27,28]. Noting that these nucleic acid amplification strategies are strictly needing tool enzymes of polymerase or endonuclease, as a result, most of fluorescent bioassays for DNA MTase activity are interferential and discontinuous process. Consequently, the establishment of sensitive, selective, simple and efficient method to simultaneously satisfy the requirements of accurate and precise monitoring the process of DNA methylation and screening inhibitors still remains an urgent challenge. Fortunately, the entropy-driven reactions [29] acted as signal amplification components, which provide a general system for controlling complex nanostructures and programming functional nucleic acids have drawn comprehensive attentions in recent years [30]. Its circulating procedure occurs independently of enzymes, which is superior to other enzyme-involved amplification methods [31]. Meanwhile, entropy-driven hairpin displacement reaction was rarely used for real-time monitoring of enzyme activity.

Herein, our current work takes a step toward the goal by applying the entropy-driven toehold-mediated hairpin displacement amplification (ETHDA) reaction that, under certain conditions, operates autonomously to detect the DNA methylation in one step. In this system, a hairpin probe was methylated by DNA MTase and cleaved by restriction endonuclease subsequently, releasing a short oligonucleotide named catalyzer. Then, catalyzer initiated the succeeding ETHDA, which eventually separated a fluorophore-labeled strand from a quencher-labeled strand, following the increase of fluorescence intensity. Coupling the addition of promoter strand, catalyzer was liberated and can triggered another catalyzing circulation to amplifying the signals successively and dramatically. Accordingly, we present a substantially novel method for rapidly, sensitively, selectively and facilely monitoring DNA MTase activity, which could be applied for screening inhibitors drugs of DNA MTase for biological therapies [7].

2. Experimental section

2.1. Reagents and materials

DNA adenine methylation (Dam) MTase (*Escherichia coli*), DpnI endonuclease, HhaI methyltransferase (M.HhaI), methyltransferase of DNA cytosine methylation (M.SssI), EcoRI methyltransferase (M.EcoRI), Nb.BbvCI restriction endonuclease, S-adenosylmethionine (SAM) and corresponding buffer solution were supplied by New England Biolabs (NEB) Inc. (Beijing, China). All oligonucleotides listed in Table 1 were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Other chemicals were analytical grade and without further purification.

2.2. Instrumentation

Ultrapure water was provided by a Millipore filtration system (Millipore, Bedford, MA). All fluorescence measurements were carried out on Quanta Master™ 400 Fluorescence Lifetime Spectrometers (Photon Technology International, Spokane, WA) at room temperature.

2.3. General kinetic measurements of Dam MTase activity

The methylation and cleavage processes and the following amplification procedures were accomplished one-step in 100 μL of the reaction mixtures including 200 nM hairpin probe, 80 μM SAM, 10 units DpnI and various amounts of Dam MTase, 1 × Dam MTase reaction buffer (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 5 mM dithiothreitol), 100 nM FAM-DNA, 150 nM BHQ1-DNA, 100 nM promoter DNA in Mg^{2+} contained Tris-HCl buffer. The fluorescence intensity was monitored at a time interval of 1 s. The emission wavelength was 485 nm and the excitation wavelength was 515 nm. Slit widths for the excitation and emission were both 5 nm.

2.4. Analysis of DNA MTase activity by gel electrophoresis

The gel electrophoresis assay was carried out under the conditions as aforementioned except the changes below: 2 μM hairpin probe, 10 units DpnI and different amounts Dam MTase. 20% non-

Table 1
Sequences of DNA used in the experiment.

Name	Sequence (5′-3′)
Hairpin probe	CGGTCAGCCTTAAGTTGGGATCGAGATGAAGCACTGACTCGATCCCAACTTAAGGCTGACCG
FAM-DNA	TCGAGTCAGTGCTTCATCTCGATAGGGCCGTAAGTTATTTGGTTAACTTACGG-FAM
BHQ1-DNA	BHQ1-CCCTATCGAGATGAAGCAC
Catalyzer	TCGAGATGAAGCACTGACTCGA
Promoter-24 (P24)	TAACTTACGGCCCTATCGAGATGA
Promoter-26 (P26)	TAACTTACGGCCCTATCGAGATGAAG
Promoter-28 (P28)	TAACTTACGGCCCTATCGAGATGAAGCA
Promoter-30 (P30)	TAACTTACGGCCCTATCGAGATGAAGCACT
Promoter-23 (P23)	ACGGCCCTATCGAGATGAAGCAC
Promoter-25 (P25)	TTACGGCCCTATCGAGATGAAGCAC
Promoter-27 (P27)	ACTTACGGCCCTATCGAGATGAAGCAC

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denaturing polyacrylamide (PAGE) gel (19:1 acrylamide/bisacrylamide) was utilized in 1 × TBE (90 mM Tris-HCl, 90 mM Boric acid, 20 mM EDTA, pH 7.5) and 10 mM $MgCl_2$ at 40 V for 10 min and then 120 V 2 h. SYBR Green II was used for fluorescent indicator.

2.5. Evaluation of inhibitors of DNA MTase

To further study the inhibitors screening ability of the presented assay, the inhibition effects of gentamycin, benzylpenicillin, ampicillin, carbenicillin, kanamycin (broad-spectrum antibiotic), 5-fluorouracil (anti-cancer drugs), as two sorts inhibitors, on the Dam MTase activity were carried out with various concentrations. The inhibitor of Dam MTase was incubated at varying concentrations with DNA-Dam for 15 min before other ingredients were added. Other procedures were the same as shown in the aforementioned methylation experiment.

3. Results and discussion

3.1. Principle of real-time monitoring of DNA methylation based on ETHDA

The mechanism of the proposed method is based on the selective methylation and cleavage of Dam MTase and DpnI, and then amplify the DNA methylation process by ETHDA strategy. ETHDA is a design approach that allows a certain input oligonucleotide to release a specific output oligonucleotide, which is driven forward by the configurational entropy of the released molecule and the energy of base-pair formation, therefore, the above process provides an amplifying circuit element. As illustrated in Scheme 1, the developed assay contains two stages: (1) the hairpin probe substrate is methylated by Dam MTase and cleaved by DpnI consecutively, (2) ETHDA magnifies fluorescent signal and detects the methylation of hairpin probe. In the first stage, Dam MTase recognizes the specific sequence (5′-GATC-3′) in the stem of hairpin probe, and then transfers a methyl group from SAM to adenine residues as well as change the adenine into N-6 methyl adenine simultaneously [6,32]. DpnI subsequently cleaves the methylated recognition sites between N-6 methyladenine and thymine, which generates a 22-base catalyzer oligonucleotide and stimulates the next ETHDA. In the second amplification procedure, the whole ETHDA is designed on the foundation of thermodynamically driven entropy gain process (Fig. S1). The 22-base catalyzer strand serves as the primer and reacts with the short overhang toehold 8-base region of two-stranded hairpin probe complex (FAM-DNA and BHQ1-DNA). Consequently, the binding between FAM-DNA and BHQ1-DNA is too weak to keep attached, so BHQ1-DNA spontaneously dissociated from FAM-DNA and the catalyzer takes the place of BHQ1-DNA. And then the 5-base gap is newly exposed, which facilitates the binding of promoter strand. After the promoter strand substituting catalyzer, the released catalyzer strand could activate the next cycle. The reaction mechanism driven forward thermodynamically by the entropic gain of the liberated DNA strands, requires no enzymes and alters no covalent bonds within the circulating system.

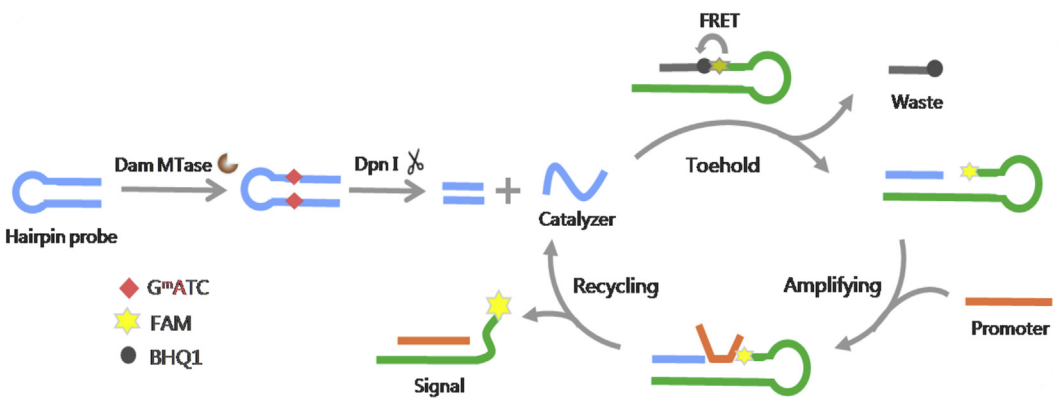
In spite of the two procedures, this approach can examine the DNA methylation in one-step, which means the enzyme reaction and toehold-hairpin displacement reaction could happen sequentially without operating step by step. Therefore, an extraordinarily simple and distinguished real-time monitoring method of DNA methylation is introduced. The innovative part of our designed method is applying hairpin probe in the amplification procedure, which elucidates less strands involved in the ETHDA [33]. In comparison with the multi-strands methods, our proposed toehold-mediated hairpin displacement reaction achieves high amplification and has obvious advantages, including preventing interferences from excessive strands, reducing the difficulties of strand design and easier controlling of the proportion of various strands.

3.2. Feasibility analysis of DNA methylation

To confirm that the catalyzer is obtained after substrate hairpin probe treating with Dam MTase and DpnI, a 20% non-denaturing PAGE is achieved. As depicted in Fig. 1, Lane 3 and 4 show only one band due to the existence of either Dam MTase or DpnI, which means no methylation/cleavage reaction occurs. When lane 5 represents the coexistence of Dam MTase and DpnI, two new bands are appearing, which is in accord with lane 2 of catalyzer band, indicating that methylation and cleavage of the hairpin probe take place successively and release catalyzer and double-stranded DNA at the same time. A denaturing PAGE is also presented to demonstrate the occurrence of methylation/cleave reactions (Fig. S2). The result further confirms that catalyzer strand is attained.

3.3. Real-time monitoring of DNA methylation process

As displayed in Fig. 2A, the fluorescence intensity enhanced in response to the existence of both Dam MTase and DpnI (red line). Nevertheless, in the absence of Dam MTase (blue line) or DpnI (green line), these samples show almost no improve of fluorescence intensity comparing to the background control (black line). To



Scheme 1. Schematic illustration of Dam MTase assay using thermodynamically real-time entropy-driven toehold-mediated hairpin displacement amplification reaction.

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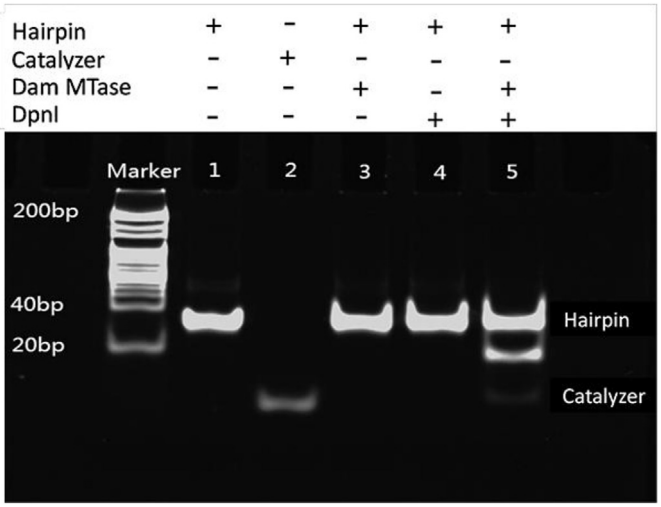

Fig. 1. Non-denaturing PAGE analysis of digestion of Dam MTase and DpnI. The products are separated by 20% PAGE and stained by SYBR Green II. Lane Marker is from 20bp to 200bp.

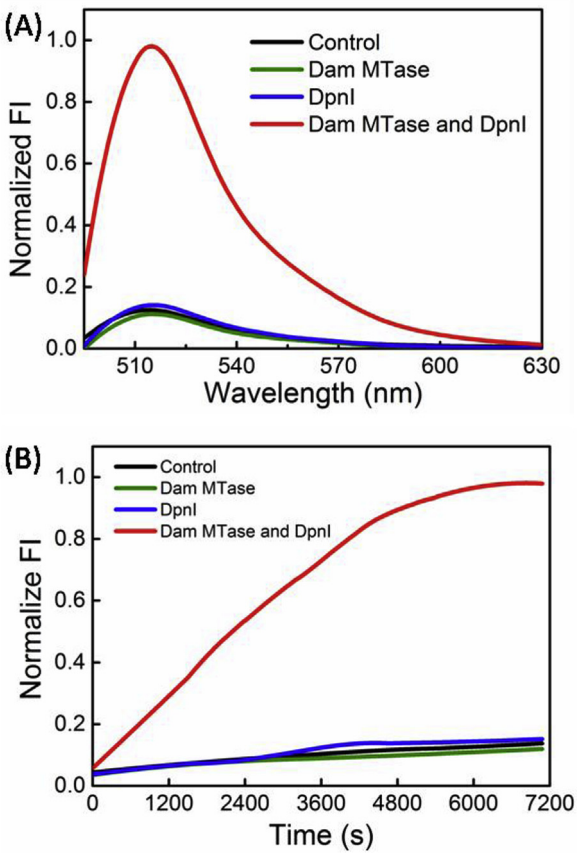

Fig. 2. (A) Fluorescence analysis of amplification products of entropy-driven toehold-mediated hairpin displacement reaction after 100 min incubation in the presence of Dam MTase and DpnI (red line), Dam MTase only (green line), DpnI only (blue line) and control (without Dam MTase and DpnI) (black line). (B) Corresponding of Real-time fluorescence monitoring of DNA methylation with 100 U mL^{-1} of Dam MTase and DpnI, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substantiate the DNA methylation and Dam MTase activity, a real-time fluorescence measurement of ETHDA is carried out with Dam MTase and DpnI. The fluorescence signal increases when the reaction time prolonged, and reaches a plateau around 100 min (Fig. 2B). Finally, we select 100 min as the optimized reaction time, which denotes the exceptionally time-saving of this approach.

3.4. Optimization of assay conditions

To obtain the optimum analytical performance, the effects of the length of promoter strand and the concentration of Mg^{2+} are investigated, respectively. The length of promoter DNA strand (Table 1) can determine the effectiveness of ETHDA. Above all, the catalyzer would not be liberated from FAM-DNA if the length of promoter strand was too short, so it definitely influences the circulation of catalyzer and the augment of fluorescence signal. Moreover, the long promoter strand could directly displace hairpin region of BHQ1-DNA and the fluorescence background increases without the existence of catalyzer so that false output signals are produced. Therefore, the length of promoter strand needs to be considered cautiously and precisely. As depicted in Fig. 3A, promoter 23 (P23) to 30 (P30) is fixed in 3′ terminal and extended in 5′ terminal, the fluorescence intensity is enhancing with the prolongation of strand length. Fig. 3B exhibits similarly result as promoter 24 (P24) to 30 (P30) is fixed in 5′ terminal and elongated in 3′ terminal. The results show that the fluorescence signal increased and the signal to background ratio is also enhanced with the extension of promoters. It could be seen that P30 is the option for next experiments as the fluorescence intensity increased remarkable. Due to the bivalent cation Mg^{2+} could stabilize the double strand DNA, the efficiency of replacement reaction is related to the concentration of Mg^{2+}. As illustrated in Fig. 3C, even though the fluorescence intensity enhances gradually with the increase of Mg^{2+} amount, the optimal signal to background ratio is exhibited when the concentration of Mg^{2+} is 10 mM. Therefore, 10 mM was chosen as the experimental concentration of Mg^{2+}.

3.5. ETHDA reaction for the amplification of Dam MTase activity

The strengths of ETHDA reaction is that not only the low amounts of Dam MTase can be detected but also no enzyme is involved in the next amplification steps, indicating that the fluorescence signal is magnified as well as the interference of various enzymes has been avoided. To substantiate the amplification of Dam MTase activity via ETHDA reaction, the contrast experiments, including the comparison of fluorescence intensity with or without promoter strand added, are designed. The consequences apparently prove that the ETHDA dramatically elevates the detection sensitivity in contrast to the control group without the addition of promoter strand (Fig. 4A and B). The linear range of the assay for Dam MTase activity is from 0.01 to 100 U mL^{-1} (Fig. 4C). The fluorescence signal FS ($FS = F_x$-F_0, F_x is the fluorescence signal when Dam MTase concentration is x U mL^{-1} and F_0 is the fluorescence signal of the sample without Dam MTase) of 25 U mL^{-1} Dam MTase by ETHDA (red bar, inset of Fig. 4C) is 14.7 times of that detection without promoter strand-aided detection (black bar), and FS of 100 U mL^{-1} Dam MTase by ETHDA is 7.1 times higher than that of without promoter, revealing a responsive detection efficacy using ETHDA. Therefore, the method reflects that promoter dramatically enhances the sensitivity of this method with the existence of ultra amount catalyzer only. Generally, the procedures of DNA methylation and further amplification can be simplified in one facile and straightforward step without any interference of other enzymes, which is superior to most of complicated and intricate existing methods (Table S1). Additionally, the detection limit of our approach is relatively lower than other approaches [15,19].

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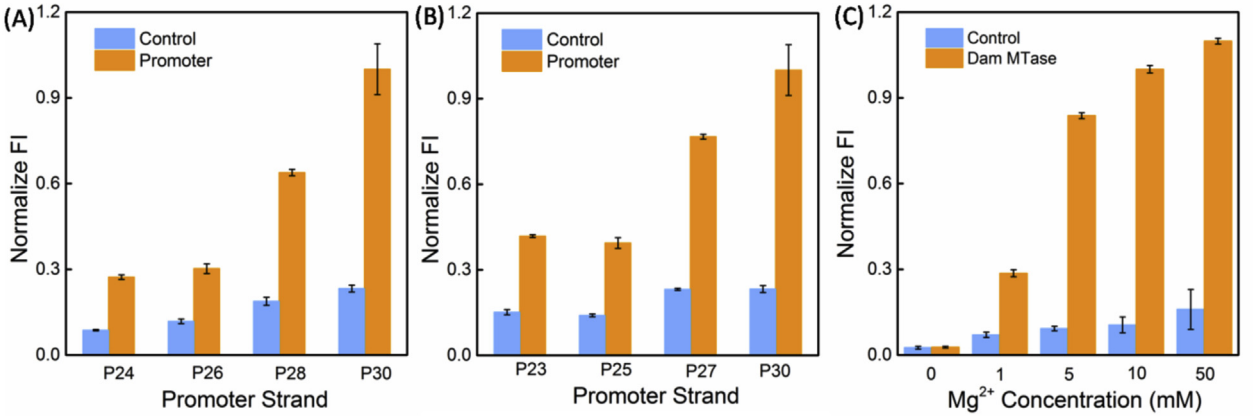

Fig. 3. Optimization of the experimental conditions. Control represents the reaction without promoter strand. (A) The increase of fluorescence intensity with the elongation of promoter 5′ terminal and (B) the influence of fluorescence signal with the extension of promoter 3′ terminal in the process of ETHDA. (C) The effect of Mg^{2+} concentration on the sensing performance of the proposed method.

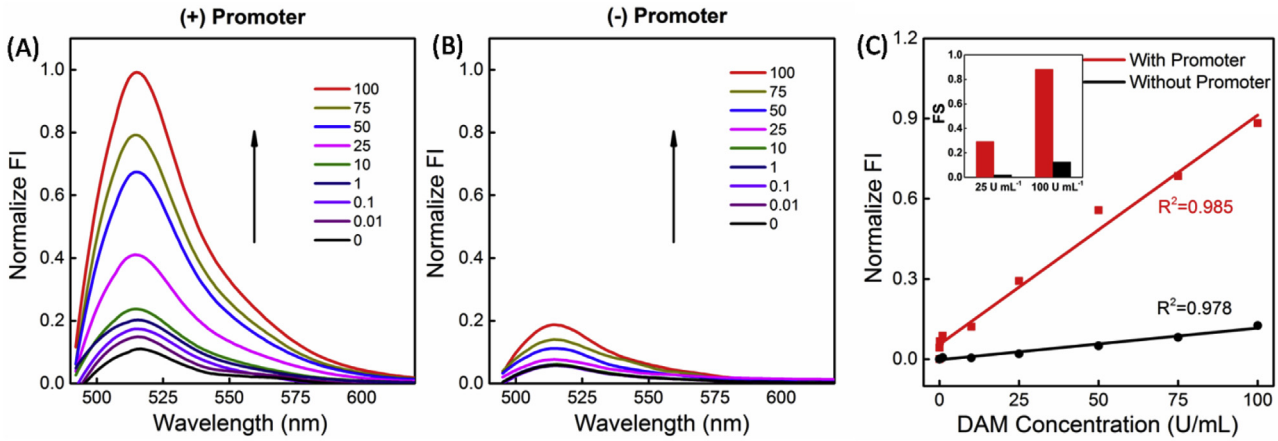

Fig. 4. Fluorescent signal in the presence of different concentration of Dam MTase from 0 to 100 UmL^{-1} with (A) or without (B) promoter strand. (C) Linear curve of the assay for Dam MTase activity with or without promoter based on ETHDA. The inset of part C shows the comparison of the method based on ETHDA (red line) and that based on the reaction without promoter adding (black line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

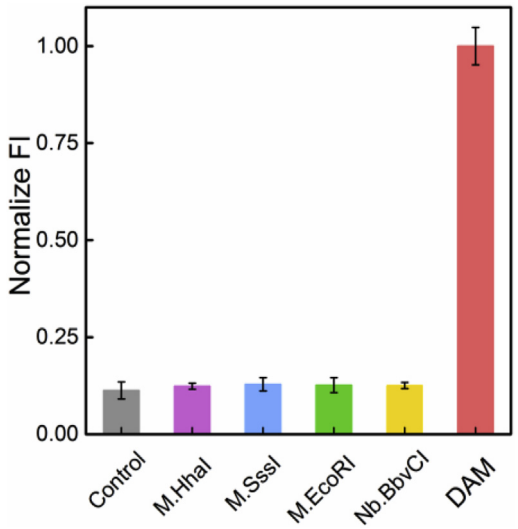

Fig. 5. The selectivity of the sensing system. All concentrations of Dam MTase, DpnI, M.HhaI, M.SssI, M.EcoRI and Nb.BbvCI are 100 U mL^{-1}.

3.6. Selectivity of the proposed method

Besides the sensitivity, selectivity is another significant factor to assess the performance of a biosensor platform. To examine the selectivity of the sensor, M.HhaI, M.SssI, M.EcoRI and Nb.BbvCI are selected to function as interference enzymes to challenge the sensor (see Fig. 5). M.HhaI, an enzyme methylates all cytosine within the double-stranded dinucleotide recognition sequence 5′-GCGC-3’. M.SssI can methylate all cytosine residues in the double-stranded dinucleotide recognition sequence of 5′-CG-3′. M.EcoRI has the capacity for recognizing the sequence 5′-GAATTC-3′ of DNA. Nb.BbvCI is a restriction endonuclease that only cleaves one strand of DNA substrate of 5’-GCTGAGG-3’. As expected, the fluorescence enhancement upon the addition of M.HhaI, M.SssI, M.EcoRI and Nb.BbvCI rarely occurs. However, only existed Dam MTase can induce a noteworthy fluorescence enhancement. The results demonstrate that the amplification strategies for Dam MTase activity exhibited a great selectivity originating from the high specific sequences recognition between Dam MTase and the hairpin probe.

3.7. Dam MTase activity inhibition evaluation

Since Dam MTase is associated with the level of bacterial

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virulence and diseases [34–38], the inhibition of Dam MTase activity may contribute to principal applications in cancer therapy and antibiotic development. High-throughput screening of inhibitors of DNA MTase receives more and more interests. Therefore, our proposed straightforward biosensing platform is further investigated in high-throughput screening of Dam MTase inhibitors. If transferring of the methyl group to the DNA residue is blocked in the presence of inhibitor, the specific site of the DNA sequence will not be recognized and cleaved by DpnI. Experimental results present that the inhibitory effect of the inhibitors can be indeed quantitatively analyzed using fluorescence intensity. Considering that DpnI is involved in this sensing system, it is essential to ensure whether these inhibitors have impact on it. It has been found that all employed inhibitors (ampicillin, 5-fluorouracil, gentamycin, benzylpenicillin, carbenicillin and kanamycin) exhibit negligible influence on the activity of DpnI when the concentration of each inhibitor is 1.0 μM (Fig. S3). As a result, 1.0 μM of various inhibitors are used to estimate the influence on Dam MTase activity. As presented in Fig. 6A, these drugs inhibit the activity of Dam MTase to various degrees. In comparison with other inhibitors, the gentamycin, a well-known broad spectrum anticancer drug, shows the strongest inhibitory effect at the same concentration which causes about 52% reduction of Dam MTase activity. 5-fluorouracil and benzylpenicillin also present effective inhibitory toward Dam MTase, the inhibition efficiency is 47% and 32%, respectively.

Moreover, we make further efforts to investigate the concentration-dependent inhibitory effect of gentamycin, 5-fluorouracil and benzylpenicillin on the activity of Dam MTase. As shown in Fig. 6B, C and 6D, the inhibitory effect gradually enhances with the increase of inhibitor concentration. Finally, the IC50 value, represented the inhibitor concentration that is required to reduce enzyme activity by 50%, for gentamycin, 5-fluorouracil and benzylpenicillin are calculated as 0.86 μM, 1.28 μM and 9.11 μM, respectively. These findings demonstrate that the developed fluorescent sensor could be used as a promising platform to screen the inhibitor of Dam MTase and to discover efficient antimicrobial drugs.

4. Conclusions

In summary, we have developed an ETHDA strategy for accurate sensing the activity and inhibition of Dam MTase. The extraordinary merit of our method is that we could real-time monitor the

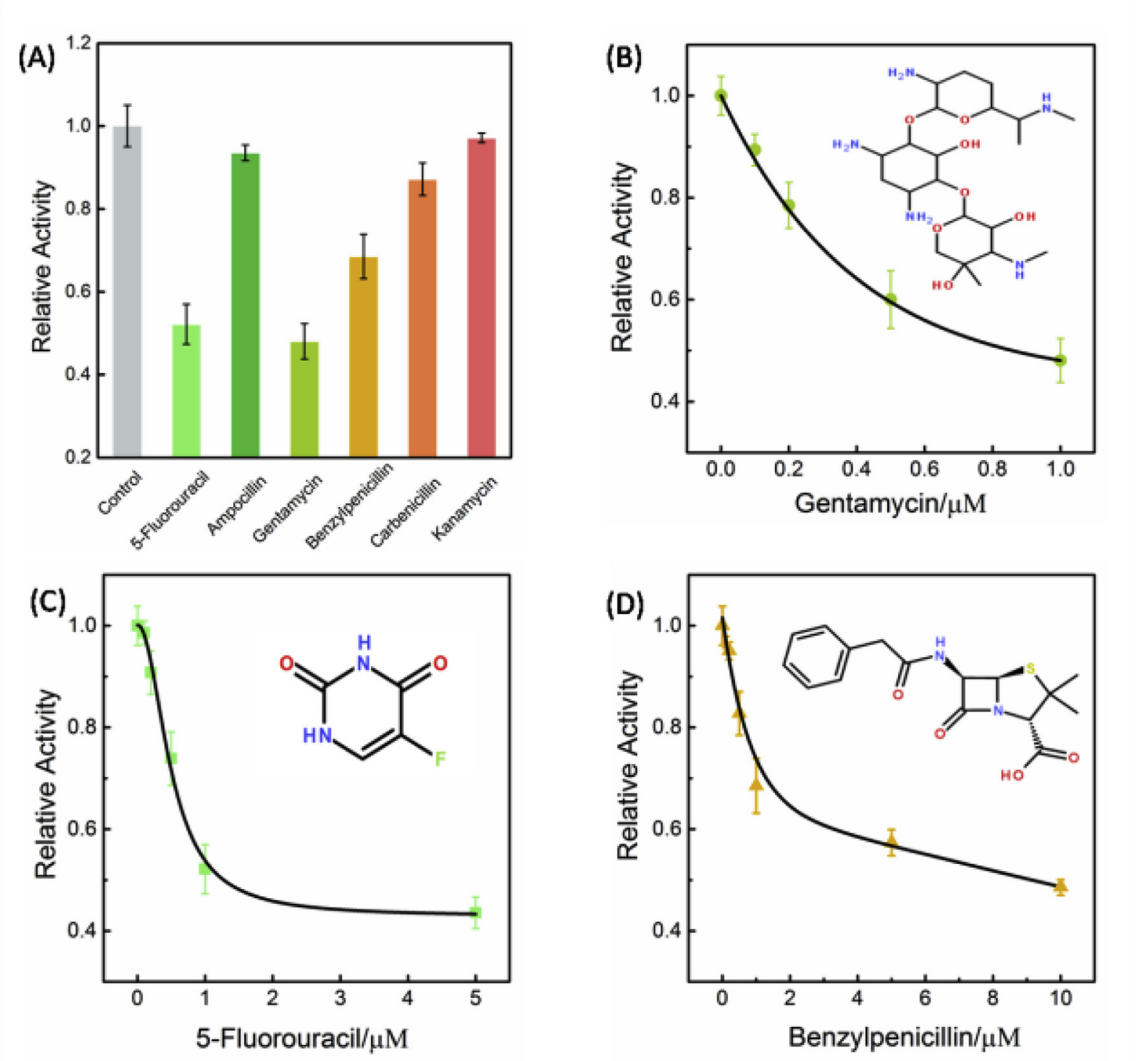

Fig. 6. (A) The influence of different inhibitors on the activity of Dam MTase. The concentrations of all these drugs are 1 μM. The inhibitory influence of various concentrations of gentamycin (B), 5-fluorouracil (C) and benzylpenicillin (D) on Dam MTase activity.

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fluorescence signal and the whole process could be performed in simple and facile one-step. Furthermore, this approach presents a high sensitivity through accelerating and circulating ETHDA to amplify fluorescent intensity with a detection limit of 0.01 U mL^{-1}, the wide linear range is 0.01–100 U mL^{-1}. In addition, the amplification procedure does not involve in enzymes which avoid interfering of other enzymes and the whole reaction can be performed under isothermal condition. It is convenient to achieve the high-throughput screening of target MTase inhibitors. We anticipate that this method will open up new opportunities for screening antibiotics drugs of Dam MTase, which provides a valuable approach for further applying in biomedical research and early clinical diagnosis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.03.017.

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