Analytical Biochemistry 590 (2020) 113532

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Detection of DNA methyltransferase activity using template-free DNA polymerization amplification based on aggregation-induced emission

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ARTICLE INFO

Keywords:
Quaternized tetraphenylethene salt
Fluorescence
Terminal deoxynucleotidyl transferase
Hairpin probe
DNA adenine methyltransferase

ABSTRACT

A sensitive and selective fluorescence assay for DNA methyltransferase (MTase) activity detection was designed based on aggregation-induced emission (AIE) and target initiated template-free DNA polymerization. Quaternized tetraphenylethene salt was synthesized as the AIE probe, which binds to single-stranded DNA by electrostatic interaction. A hairpin probe was designed with a specific sequence for DNA MTase. In the presence of DNA MTase, the methylation reaction initiated DNA polymerization with terminal deoxynucleotidyl transferase (TdT), which activated the fluorescence intensity through AIE. The designed DNA sensor displayed a linear response to concentrations of DNA adenine methyltransferase (Dam) MTase from 0.5 U·mL^{-1} to 100 U mL^{-1}, with a limit of detection of 0.16 U mL^{-1}. The assay was also effective for detection of DNA MTase activity in human serum and for showing the inhibitory effect of 5-fluorouracil on Dam MTase.

1. Introduction

Fluorescent chemosensors play an important role in clinical diagnostics, environmental monitoring and analytical basic research with prominent sensitivity and specificity. With the advantage of inexpensive instrument and rapid detection, several analytes including nucleic acid, protein, small biological molecules, enzyme and metal ions could be quantitatively analyzed by fluorescence method with high accuracy [1,2]. Many biological samples have been analyzed by fluorescence in situ, obtaining much more spatial information [3,4]. In the presence of target, fluorescent probes were employed to turn on fluorescence based on changing the distance between fluorophore and quenched probe. Since then, several strategies have been designed for targets analysis by using signal on or signal off model of the fluorescence. To date, several fluorescence activation and quench mechanisms were employed for sensitive detection. However, many of these fluorescence probes were limited in biosensing applications with low turn-on ratios and poor photobleaching thresholds.

To address this problem, a novel category of fluorogens with aggregation-induced emission (AIE) phenomenon were discovered by Tang's group [5]. This unique performance makes the fluorogens nonemissive in dissolved condition but become distinctly emissive by aggregation [6,7]. This phenomenon was due to the mechanism of the restriction of intramolecular rotations (RIR) [8]. Benefiting from advanced features, such as the novel light-up probe, AIE fluorogens were widely used in chemo- and biosensor analysis, such as nucleic acid (DNA [9], RNA [10]) detection, protein(Acetylcholinesterase [11], Caspase-3/7 [12]) detection, ion detection [13,14] and cancer cell track [15]. Specifically, tetraphenylethylene (TPE), a typical AIE chromophore, is easy for chemical modification. TPE can be conjugated with single strand DNA or RNA by click-reaction, enhancing the biocompatibility and bioactivity. Other TPE salt was synthesized with charge. In general, TPE and its derivatives have been applied in various chemo-/biosensors [11,16–19].

Recently, a template-free DNA extension reaction has received much attention. Terminal deoxynucleotidyl transferase (TdT) is a template-independent DNA polymerase which could catalyze the addition of deoxyribonucleoside triphosphate (dNTP) at the 3′-OH group of DNA to produce a nonspecific and long sequence directly [20]. With the advantage of template-free, this amplification reaction was easily operated and applied in different sensing analysis. The single strand DNA with the end of 3′-OH could be used as the primer for TdT reaction. Therefore, the TdT assisted isothermal amplification has become an essential strategy in nucleic acid sensor detection [21,22].

We designed a facile and untrasensitive biosensor for detection of DNA methyltransferases (MTase) activity. Quaternized

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https://doi.org/10.1016/j.ab.2019.113532
Received 23 October 2019; Received in revised form 4 December 2019; Accepted 4 December 2019
Available online 07 December 2019
0003-2697/

tetraphenylethene salt (TPE-Z) was synthesized and used as the AIE fluorescence probe in this strategy. In the presence of DNA MTase, the quencher group labeled DNA probe could be cleaved by methyl-sensitive endonuclease and then extended with TdT. Through the electrostatic force, the positively charged TPE-Z could bind to the negatively charged extension DNA strand, inducing strong fluorescence with AIE effect. Taking advantage of TdT enzyme amplification and AIE phenomenon, fluorescence turn-on biosensor was established for precise analysis of MTase activity.

2. Experimental section

2.1. Materials and instrumentation

N-bromosuccinimide (NBS), 4-methylbenzophenone, zinc powder and benzophenone were got from Aladdin. Titanium tetrachloride ($TiCl_4$), dichloromethane (DCM), triethylamine (Et_3N), 5-fluorouracil, tetrahydrofuran (THF) and benzoyl peroxide (BPO) were purchased from J&K Scientific Ltd. DNA adenine methylation (Dam) MTase, M.SssI MTase, S-adenosylmethionine (SAM), Dpn I, *Hpa*II and Terminal deoxynucleotidyl transferase (TdT) were received from the New England Biolabs (NEB). Purified oligonucleotides (5′-GTTGGGATCGA GAAGTTTTCTTCTCGATCCCAA C-Dabcyl -3’; 5′-GTTGGGATCGAGAA GTTTTCTTCTCGATCCCAAC-NH_2-3′) were got from Sangon Biological Engineering Technology & Services Co., Ltd. Human serum sample was got from Lablead Biotech. Co., Ltd. (Beijing, China). Deionized water was used throughout the experiments.

Fluorescence spectra were registered on a Hitachi F-4600 fluorescence spectrometer (Hitachi Ltd., Japan). Excitation and emission slits were set for 5.0 nm and 10.0 nm band-pass. In the experiment, the excitation wavelength for the TPE-Z was set at 303 nm, and the emission spectra were recorded between 400 nm and 600 nm. The fluorescence emission wavelength at 460 nm was used for investigating the performance of the designed assay. ^{1}H NMR and IR spectra were measured with Bruker ARX 500 and Nicolet 510P FT-IR Spectrometer respectively.

2.2. Synthesis of TPE-Z

TPE-Z, as the AIE probe, was synthesized according to the previous reported methods [23,24]. 1, 2-Bis[4-(bromomethyl)-phenyl] −1,2-diphenylethene (2) was synthesized at first, the detail of the process showing in the supporting materials. 104 mg (0.2 mmol) compound (2) and 30 mL trimethylamine were added into a 250 mL round-bottom flask. This mixture was refluxed with 12h and cooled to room temperature. After being rinsed and filtrated, the white solid (TPE-Z) was gained in 34.23% yield (38.4 mg). ^{1}H NMR (500 MHz, D_2O), δ (TMS, ppm): 7.13 (m, 18H), 4.21 (s, 4H), 3.06(m, 12H), 1.24(t, 18H).

2.3. Methylation and fluorescence detection with TPE-Z

The methylation was carried out in 50 μL of reaction mixture containing hairpin probe (50 nM, chemical modified quencher Dabcyl at 3’ end of the nucleic acid), different concentration of Dam MTase, 10 × Dam MTase buffer, SAM (80 U mL^{-1}), D*pn*I (4 U) and buffer at 37 ˚C for 2 h. After digestion, 10 μL of reaction mixture containing TdT enzyme (5 U), 0.5 μL dNTPs (1 mM), 5 × TdT buffer (4 μL) and 5.3 μL H_2O was introduced to the above pretreated solution. This mixture was incubated at 37 ˚C for 3 h for DNA extended. After that, 80 μL TPE-Z (7.4 μM) was added the reaction products for fluorescence measurements.

2.4. Influence of 5-fluorouracil on dam MTase activity

5-fluorouracil as the drug model was used for investigating the influence on Dam MTase activity. Different concentration of 5-fluorouracil was added into the methylation reaction solution, which included DNA MTase, SAM, D*pn*I and the hairpin probe. The subsequent procedures were the same as the above description.

2.5. Detection of MTase from bacterial cells

The cell culture and the preparation of cells extracts were performed according to the previous report [25]. GW5100 (Dam-positive) and JM110 (Dam-negative) *E. coli* cells were cultivated in culture-medium (5 g/L yeast extract, 10 g/L Trypton, 10 g/L NaCl) at 37 ˚C for 12 h in a shaker (250 rpm). Then, the cell suspension was diluted by 20 times for 2.5 h of cultivation. Subsequently, the *E. coli* cells were collected by centrifugation and rinsing with PB solution. The resulting cells were lysed using RIPA lysis buffer. The total extracted protein concentrations were determined by the Bradford Protein Assay Kit, and the contained MTase was determined according to the proposed method as above mentioned.

3. Results and discussion

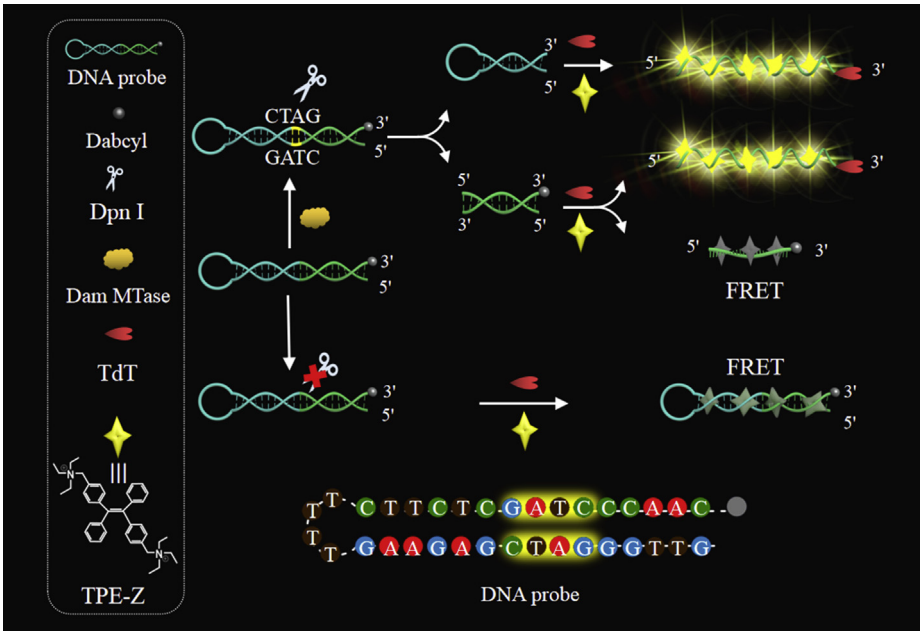
3.1. Design strategy for DNA methylation assay

The MTase assay is based on the same recognition site for restriction endonucleases and methylated reaction. A hairpin structure DNA probe is designed for the dual enzyme-linkage (Dam MTase and Dpn I endonuclease) reaction, which includes the palindromic sequence of 5′-G-A-T-C-3′ in the stem and labeled with a quencher group on 3′ end. Before the methylation and polymerization reaction, the fluorescence resonance energy transfer (FRET) was occurred between the TPE-Z and quencher. The design of fluorescence assay for Dam MTase activity detection is schematically shown in Scheme 1. This strategy involves three principal processes: (1) methylation and restriction endonuclease enzyme-linkage reaction; (2) TdT assisted DNA polymerization and (3) fluorescence detection with AIE effect. In the first step, the hairpin probe was employed as the reaction substrate. After being methylated by Dam MTase and SAM, the hairpin probe was digested by Dpn I, resulting in three pieces of ssDNA. One of these ssDNA was labeled with a quencher group on 3′ end, which could not be polymerized. The other two ssDNA with 3′-OH end performed as primers to initiate DNA polymerization with TdT and dNTPs, which produced long strand of ssDNA. Then, the long ssDNA is obtained with more negative charge and binds to more TPE-Z molecules through electrostatic force. After that, the AIE effect is occurred and fluorescence intensity light up as the quencher far away from TPE-Z molecules.

3.2. Feasibility

In order to confirm the quench effect, 3′-NH_2 end of the hairpin probe was used in the process of methylation assay. Under the same experiments condition, the fluorescence intensity was observed with different end of hairpin substrate. According to the electrostatic interaction, the longer ssDNA will bind to more TPE-Z with significant fluorescence increase. As shown in Fig. 1A, the fluorescence signal was increased after bound to the positive charged TPE-Z. Compared with the control experiment (curve a), the fluorescence signal was 612% increased when the quencher group labeled hairpin (curve c). By contrast, only 303% of signal increase was obtained when the hairpin was labeled with NH_2 (comparison of curve b and d). These data suggested that the use of quencher group could decrease the background signal and increase the signal-to-noise (S/N) ratio.

To further validate the enzymatic reaction, PAGE experiment was carried out to reveal the products of each step. As seen from Fig. 1C, the bands of lane 1–6 exhibited the same length. When the hairpin probe existed alone, only one band was shown in lane 1. After being treated with TdT, Dam and TdT, Dpn I and TdT, lane 2–4 were similar as lane a, which suggested that the methylated reaction and polymerization were



Scheme 1. Illustration of Dam MTase activity assay based on Aggregation-Induced Emission and template-free DNA polymerization.

not occurred. When the Dam and Dpn I were added, a new short band was exhibited in lane 5 due to the methylation reaction occurred. After mixed with Dam, Dpn I, and TdT, a new long trailing band was appeared in lane 6. This further conformed that 3′-OH of hairpin was formed after methylation cleavage reaction and polymerization was occurred by TdT. These results clearly demonstrated that the hairpin probe was modified with Dam and digested by Dpn I, releasing the cleavage fragments for polymerization with TdT. After the whole

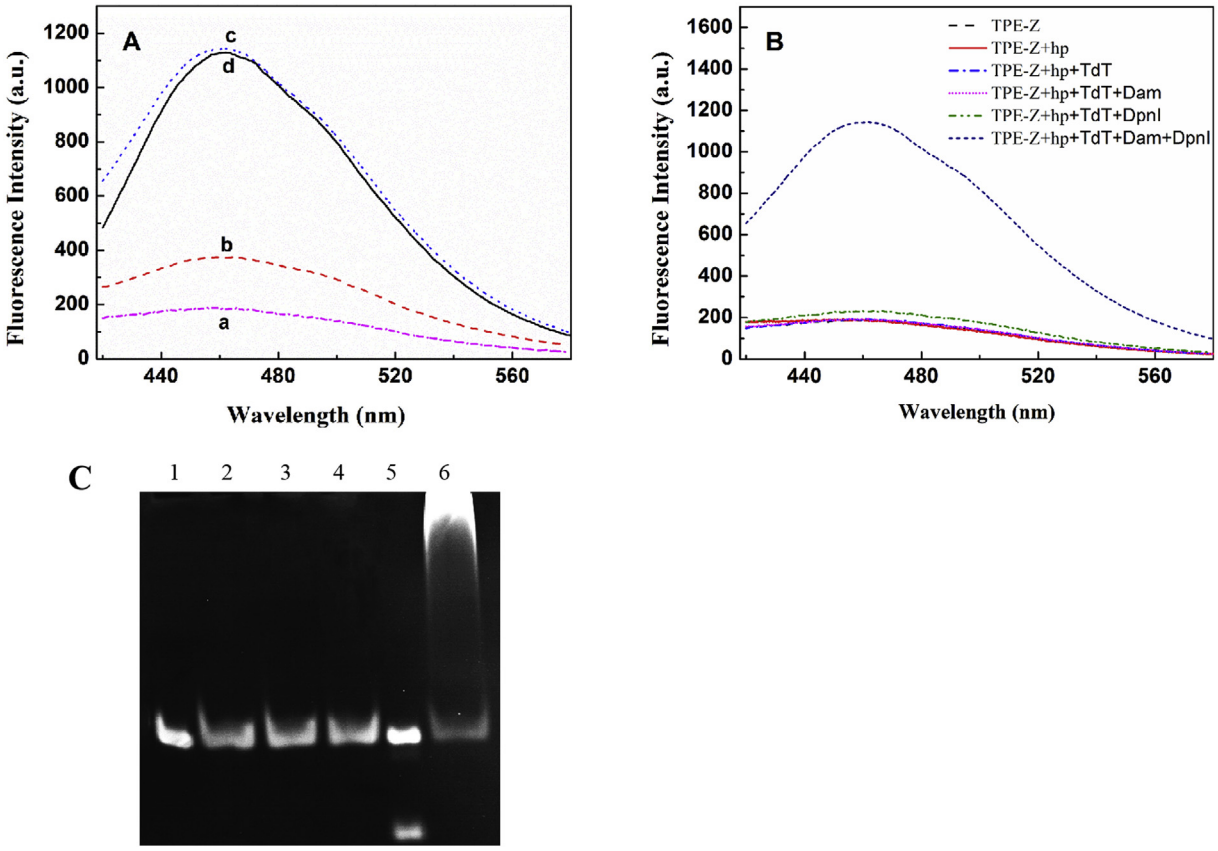


Fig. 1. (A) Fluorescence emission spectra of TPE-Z bonds hairpin DNA labeled with (a) or without quencher (b), and in the presence of DNA MTase by polymerization with (c) or without quencher (d). (B) Fluorescent intensity of TPE-Z in different conditions. (C) Polyacrylamide gel electrophoresis (PAGE) analysis of Dam MTase by hairpin probe: (1) control; (2) treated with TdT; (3) treated with TdT and Dam; (4) treated with TdT and DpnI; (5) treated with Dam and DpnI; (6) treated with Dam, DpnI and TdT.

experiment, the long DNA strand was produced for binding to TPE-Z molecules. Therefore, the Dam MTase activity could be detected by the fluorescence intensity.

In addition, the fluorescence intensity was detected to verify the feasibility of the designed sensor for Dam MTase activity assay. As expected, the fluorescence intensity in Fig. 1B was in a good agreement with those obtained from PAGE measurements. As shown in the gel image, lane 1–6 exhibited short band which suggested the DNA strands were quite short. The fluorescence intensities of TPE-Z were weak after binding with short strand DNA. After the reaction completed, the fluorescence intensity was strong as the binding between the TPE-Z and long strand DNA. The fluorescence signals were varied with the processes based on enzymes reactions. All these experimental results clearly proved that the essential role of Dam MTase, Dpn I and TdT for the amplification strategy.

3.3. Optimization

A series of experiments were carried out to find the optimal conditions for MTase assay. The following factors were optimized: the concentration of TPE-Z(a), SAM(b), TdT(c) and dNTPs (d), and the TdT incubation time (e). Respective data and Figures were provided in the supplementary material. The following experimental parameters were investigated to give best results: Optimal concentration of TPE-Z (a), SAM(b), TdT(c) and dNTPs (d) were 7.4 μM, 80 U/mL, 5.0 U/mL and 5 μM respectively; Optimal reaction time of TdT: 180 min (e).

3.4. Dam MTase detection

The fluorescence signal was directly related to the quantity of TPE-Z, which was bind to the long DNA strand through the electrostatic interaction. The long DNA strand was produced by methylation and polymerization. So, the fluorescence intensity was dependent on the concentration of Dam MTase. The designed assay for Dam MTase activity detection was carried out under the optimized condition. Fig. 2A shows the fluorescence intensity changes with the different concentration of Dam MTase. A linear plot was obtained in the range of 0.5–100 U mL^{-1} (Fig. 2B), and the detection limit of this sensor is estimated to be 0.16 U mL^{-1} (3σ, n = 11). Notably, the sensitivity of this optical method for Dam MTase detection was superior to other reported assay in Table S1 (see supplementary material).

3.5. Selectivity

The specificity of this assay was examined with other methyltransferases, including M. SssI and HhaI. M. SssI recognized the 5′-CG-3′ sequence (CpG) and HhaI methylated the cytosine residues in 5′-GGCC-3’. The fluorescence intensity of Dam MTase was about five-fold higher than M. SssI and HhaI, even the concentration of other methyltransferases was the same or two-fold (Fig. 2C). This indicated that the sequence 5′-GATC-3′ can be only methylated by Dam MTase. Consequently, the single DNA was prolonged with polymerase and bound the AIE probe. These results demonstrated that the designed biosensor exhibited good selectivity.

In addition, the practical applicability of this designed biosensor was investigated in 20% diluted human serum samples (Table S2, supplementary material). Different concentrations of Dam MTase at 1, 10, 50 U mL^{-1} were separately spiked into the diluted human serum samples. The recoveries were obtained in the range of 98.34–100.83% and the RSD was 3.3%, 4.2% and 4.7%, respectively. Therefore, the designed biosensor has the potential to quantify of Dam MTase in a complex system.

Furthermore, the reproducibility of the method was investigated with intra-assay and inter-assay. The intra-assay variability was evaluated by parallel detection of the spiked Dam MTase level of two prepared human serum by the same batch of TPE-Z. The variation coefficients (CVs) of the intraassay for this method were 9.7 and 8.6% at Dam MTase concentrations of 10 and 50 U mL^{-1}, respectively. The inter-assay variability was evaluated by determining, in duplicate, the spiked Dam MTase in one serum sample with different batches of TPE-Z. The CV of the inter-assay was 14.3% for the fluorescence determined at 10 U mL^{-1}. These results were indicated an acceptable reproducibility for this method.

The endogenous DAM activity of *E. coli* cells were further investigated by using the designed system. A certain number of *E. coli* cells lysate were added to the reaction mixture and the fluorescence intensity were recorded with the total protein of *E. coli* cells. The total protein concentration was measured using the Bradford Protein Assay. JM110 (Dam-negative) and GW5100 (Dam-positive) were employed in this experiment. As shown in Fig. 3A, the signals were obtained nearly the same between the Lysis and JM110. Obviously, the signal for GW5100 was much higher than others, owing to the endogenous methyltransferases not for other interfering proteins. Furthermore, the inhibition for DAM from *E. coli* cells was also examined by gentamycin. The signal was obtained much lower than GW5100 only, suggesting the efficient inhibitory effect. In addition, a linear relationship was obtained between the fluorescence ratio and the total protein concentration in the range of 25.4–198.6 ng (Fig. 3B). These results suggest that the developed assay can be applied to DAM activity assay in the practical complex samples.

3.6. Universality of the designed strategy

The proposed MTase assay is a universal biosensing system and is able to investigate more sequence-specific biotransformations through redesigning the hairpin probe. The present Dam/DpnI pair can be substituted with M.SssI/*Hpa*II pair. The recognition site 5′-GATC-3′ was replaced to 5′-CCGG-3′ for evaluating M.SssI MTase (Fig. S3). Different from the Dam/DpnI system, the *Hpa*II endonuclease cannot cleave the methylated hairpin substrate, resulting in the low fluorescence intensity. Without M.SssI MTase, the hairpin can be cleaved to three parts, which triggered the TdT assisted polymerization. As shown in Fig. 4, a good linear relationship was obtained for M.SssI MTase ranging from 0 to 40 U mL^{-1} with a detection limit of 0.03 U mL^{-1} (3σ, n = 11). Therefore, the universal applicability of the designed system can analyze other MTase through changing the sequence of hairpin probe.

3.7. Influence of drug on dam MTase activity

The inhibition of this assay was investigated to provide further the application of antibacterial therapy [26]. 5-fluorouracil as the anticancer drug is chosen for a representative inhibitor in this assay. There are three enzymes involved in this reaction system, including DpnI, TdT and Dam MTase. So, the effect of 5-fluorouracil on DpnI and TdT was evaluated before Dam MTase. The activity of the DpnI and TdT had been relatively unaffected with 5-fluorouracil in the range of 0–10 μM (Fig. S2). Subsequently, the influence of 5-fluorouracil on Dam MTase activity was investigated. After the addition of 5-fluorouracil, the relative activity of Dam decreased with the increasing concentration of 5-fluorouracil (Fig. 2D), showing visible dose-dependent inhibition of Dam. In the presence of 5-fluorouracil, it was difficult to transfer the methyl group to the DNA sequence, which resulted in fluorescence intensity decrease. The half maximal inhibition values (IC_{50}) of 5-fluorouracil are calculated to be about 2 μM, which are approximate to those in previous report [27,28]. In addition, the lower and higher values of IC_{50} for inhibition are reported with 0.6 μM [29] and 100 μM [30] respectively.

4. Conclusions

In summary, a positively charged fluorescence probe (TPE-Z) was

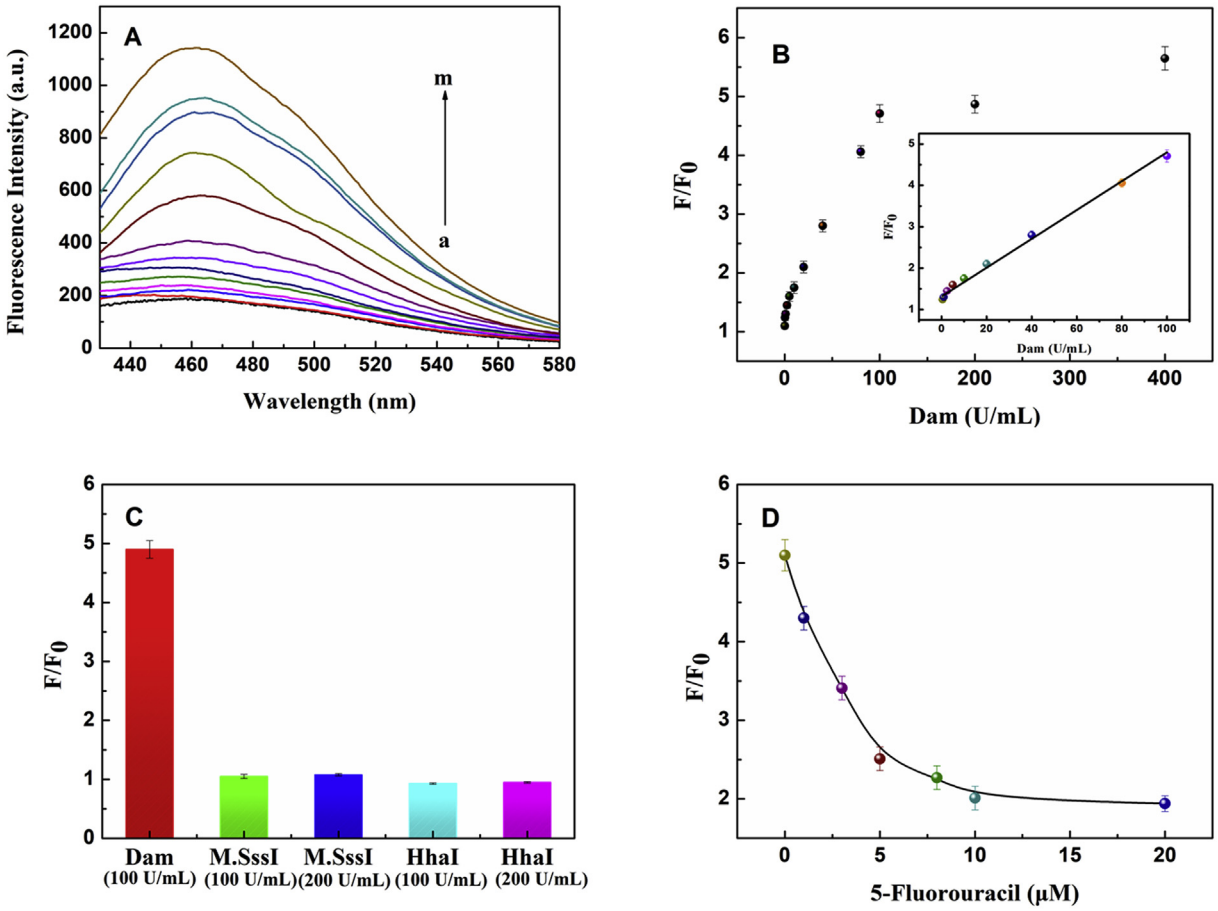


Fig. 2. (A) Fluorescence spectra of TPE-Z in response to Dam MTase in different concentrations (0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 40, 80, 100, 200, 400 U/mL). (B) Corresponding fluorescence ratio at 462 nm. Inset represent the linear relationship between the signal and Dam MTase concentrations from 0.5 U/mL to 100 U/mL. (C) Selectivity study. (D) Inhibition assay for Dam MTase with different concentration of 5-Fluorouracil. The error bars were based on three repetitive experiments performed.

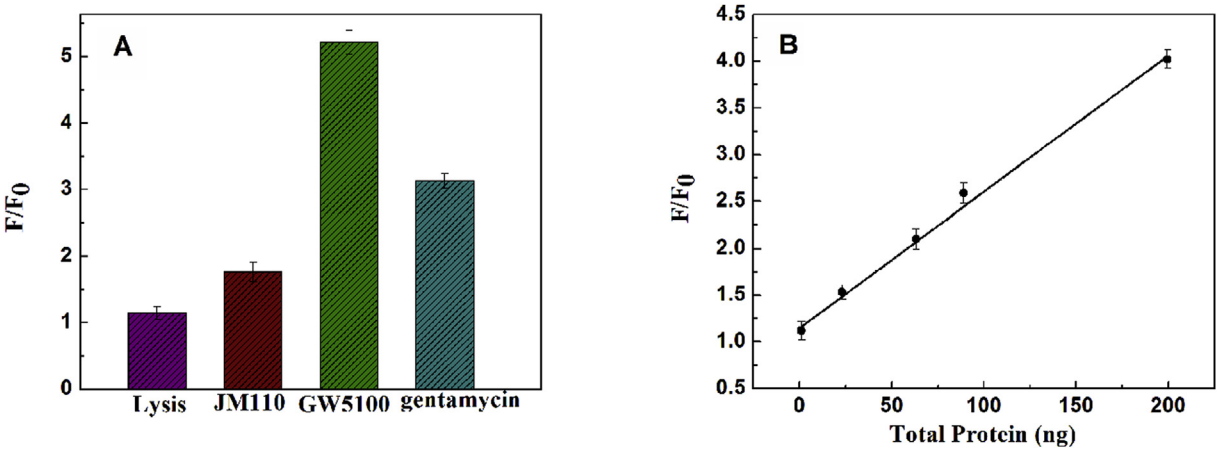


Fig. 3. (A) Evaluation of the activity of Dam in *E. coli* cells, GW5100 cells with 198.6 ng total proteins and the inhibition of gentamycin. (B) The linear calibration between the fluorescence ratio and total protein contents.

synthesized for sensitive detection of DNA methyltransferase activity. Through electrostatic attraction, TPE-Z was aggregated on the DNA strand with fluorescence intensity enhanced. Integrating the template-free DNA polymerization, methyl-sensitive endonuclease and aggregation-induced emission (AIE) phenomenon, the detection limit of this method was 0.16 U mL^{-1} with a linear range from 0.5 to 100 U·mL^{-1} Compared with other fluorescent detection methods, this assay is facile, quite simple and possibly rapid. In addition, the inhibitor of MTase was measured, which was possibly used for screening suitable anticancer drug. Furthermore, the real sample investigation was also implemented with the diluted human serum samples and cells. Therefore, this strategy holds great promise application for early diagnosis of methylation related diseases.

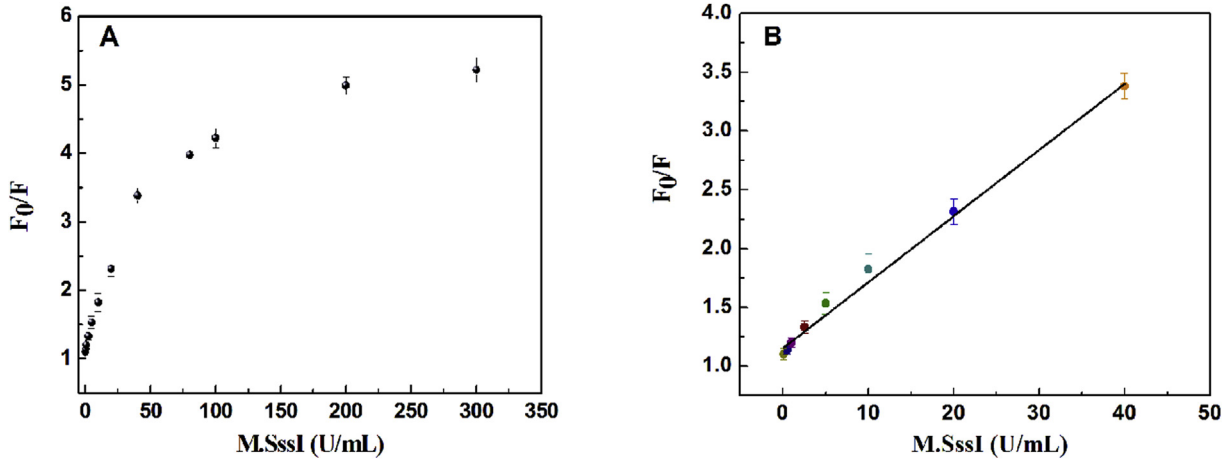


Fig. 4. (A) Fluorescence ratio in the presence of increasing amounts of M.SssI MTase. (B) The corresponding linear calibration curve.

CRediT authorship contribution statement

Shuyan Niu: Conceptualization, Methodology. **Cheng Bi:** Data curation, Investigation, Validation. **Weiling Song:** Writing - review & editing.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (21705086)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2019.113532.

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