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Real Time in Vitro Regulation of DNA Methylation Using a 5-Fluorouracil Conjugated DNA-Based Stimuli-Responsive Platform

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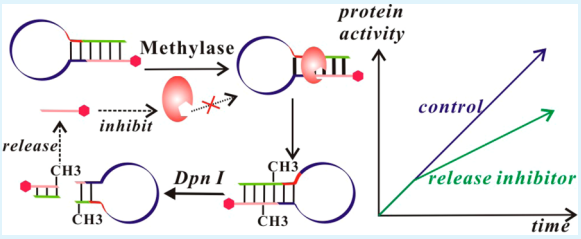
ABSTRACT: DNA methylation, catalyzed by methylases, plays a critical role in many biological processes, and many methylases have been regarded as promising targets for antimicrobial drugs. In this work, we report a stimulus responsive, self-regulating anticancer drug release platform, comprising a multifunctional DNA that upon methylation by methyltransferase (MTase) releases 5-fluorouracil (5-Fu) and in turn inhibits subsequent expression of MTase. The multifunctional DNA with anticancer drug are first methylated by DNA adenine methylation (DAM) methyltransferase (MTase) and then cut by the methylation-sensitive restriction endonuclease Dpn I. Removal of duplex from the functional DNA by the methylation/cleavage process will release the anticancer drug, resulting in inhibition of the activity of DAM in turn. Consequently, the enzyme activity of DAM MTase can be self-regulated. Furthermore, we found that the inhibition efficiency of 5-Fu significantly increase as it is functionalized with DNA.



KEYWORDS: *signal amplification, functional DNA, self-regulation, anticancer drug, nicking endonuclease*

INTRODUCTION

DNA methylation, an epigenetic modification crucial for interpretation of genes, is the most common enzymatic base modification in both prokaryotic and eukaryotic genomes.[1−4] This biochemical process occurs by site-specific, enzymatic covalent addition of a methyl group from a donor molecule (e.g., S-adenosyl-L-methionine (SAM)) to the target cytosine or adenine residue, by the ubiquitous enzyme methyltransferase (MTase).[5−11] It has been well-known that DNA methylation plays an important role in normal methylation patterns and the corresponding alteration in gene expression, such as genomic imprinting, suppression of repetitive elements.[12−17] Moreover, aberrant DNA methylation patterns are closely associated with genetic instability and the repression of tumor suppressor genes, which may eventually lead to carcinomas.[18−23] Therefore, detection of MTase activity and identification of its inhibitors are crucial to biomedical research and early phase cancer diagnosis.

5-fluorouracil (5-Fu) is a well-known inhibitor of MTase that has been popularly used for cancer therapy for about 40 years.[24−26] It not only reactivates the expression of genes that have been epigenetically silenced,[27,28] but also interrupts DNA replication in the nucleus and kills cancer cells.[29−31] However, 5-Fu is poor in selectivity that causes high incidence of toxicity.[32−34] Therefore, a "smart" system that instantaneously, autonomously senses abnormal increases in MTase activity, and in response releases this inhibitor to inhibit and down-regulate MTase activity, is of great therapeutic potential in cancer therpay.[35]

In addition to engineering down-regulating system, a platform that enables real-time monitoring of MTase activity is also required to sense abnormal increase in MTase activity. A variety of methylation assays, such as methylation-specific polymerase chain reaction, radioactive labeling, gel electrophoresis and immune reaction, have been developed to monitor MTase activity.[36−38] However, these assays are usually time-consuming with laborious operations. Various nanoprobes have been employed to monitor the activity of MTase.[39−58] Particularly, molecular beacon (MB)-based DNA probe has proven to sensitively to monitor the activity of MTase in real time and high throughput.[45,59−62]

In this study, we propose an in vitro stimuli-responsive methylation regulation platform, which integrates a real-time monitoring assay and a methylation-dependent inhibitor release system. A hairpin DNA with 5-Fu modification is designed as a platform to monitor the MTase activity. This platform contains MTase recognition site of DNA adenine Methylation (DAM) MTase, as a model target, in the middle of the stem of the

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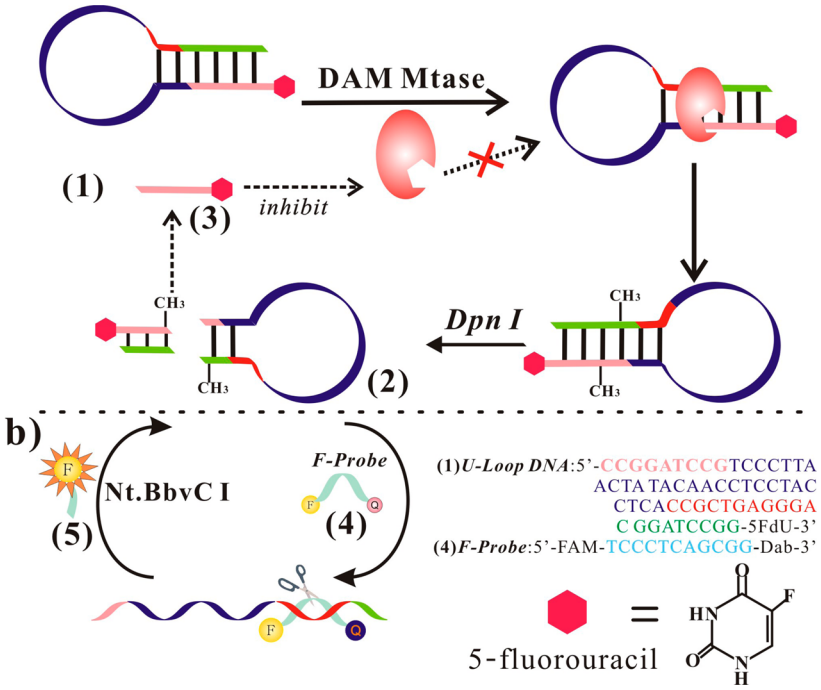

Figure 1. Schematic illustration of the principle of the stimuli-responsive platform for regulation of DNA methylation.

probe. As the probe is methylated by the MTase, the hairpin probe responsively releases the modified 5-Fu and inhibits the MTase activity in turn, which is tightly associated with cancer therapy.[63−65]

MATERIAL AND METHODS

Materials. All DNA were synthesized and purified by Takara Corporation (Dalian, China). The Dam MTase (*Escherichia coli*), Dpn I endonuclease, SAM, Nt.BbvC I endonuclease, and the corresponding buffer solution were purchased from New England Biolabs Inc. 5-fluorouracil was obtained from Bio Basic Inc. Other chemicals were of analytical grade and were used without further purification. All solutions were prepared with Milli-Q water (18 MΩ cm resistivity) from a Millipore system.

Methylation of Probe. The reaction mixtures (20 μL) consisted of 250 nM Probe, 1 μM F-Probe, 1 × NEB buffer 2 (10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT), 1 × SAM(80 μL),20 unit of Dpn I endonuclease, 8 unit of Dam MTase, and 10 unit of Nt.BbvC I. After adding the buffer, Probe (**1**), F-Probe (**4**), SAM, Nt.BbvC I and Dpn I into the mixture in order, the Dam MTase was added to initiate methylation reaction for 1 h.

Fluorescence Measurements. The fluorescence measurements were immediately performed after adding the product mixture (20 μL) to buffer (200 μL). All the fluorescence spectra were recorded on a Hitachi F-4500 spectrophotometer equipped with a Xenon lamp excitation source. The excitation wavelength was λ = 494 nm, and the spectra were recorded between = 505 and 700 nm. The fluorescence measurements were carried out at 25 °C. Meanwhile, the time scan of fluorescence started after Dam MTase was present.

Gel Electrophoreses. The samples were put on a polyacrylamide gel (20% acrylamide, 29:1, acrylamide/bisacrylamide) to separate the cleaved products from the substrate. The electrophoresis was carried in 1xTBE (pH 8.0) at 150 V constant voltages for 5 h.

MTT Assays. Cytotoxicity was estimated using an MTT assay. Briefly, cells were seeded in 24-well plates and cultured overnight to reach ∼80% confluence. Fresh media containing cy3 labeled ss-DNA (100 nM) were incubated with cells for 72 h. 20 μL 5 mg/mL thiazolyl blue tetrazoliul bromide (MTT, Sigma-Aldrich, USA) solution was then added to each well, followed by 4 h incubation at 37 °C. Next, cells were lysed with 10% acid SDS solution (pH 2−3). After centrifugation, the absorbance of supernatant was measured at 570 nm using a microplate reader (Bio-Rad 680, USA).

Confocal Microscopic Imaging. Cell images were taken with a Leica confocal microscope setup. HeLa cells were seeded on glass covers lips in 24-well culture plates at a density of 5 × 10^5 cells/mL and incubated at 37 °C for 24 h. They were then washed twice with phosphate buffer (PBS) and incubated with fluorescently labeled ss-DNA (for investigation of cellular uptake, Cy3 was labeled to ss-DNA in fresh RPMI 1640 medium for 2 h at 37 °C. Cells were then washed twice with PBS, fixed with 3%paraformaldehyde/sucrose and the nuclei were stained using 3 μg/mL Hoechst 33258. The coverslips were mounted on a glass slide. All images were obtained using a Laser confocal microscope (Leica TCS SP5).Wavelength sets was 561 nm Ex/565−600 nm Em for Cy3.

RESULTS AND DISCUSSION

We employed an adenine methylation (Dam) MTase as model platform for our stimulus-response system.[66] The Dam MTase specifically recognizes the symmetric tetranucleotide $\begin{matrix}5'....GATC....3'\\3'....CTAG....5'\end{matrix}$ region in double stranded (ds-) DNA and methylates the adenosine residues.[67−69] Upon methylation by Dam MTase, a corresponding methylation-sensitive restriction endonuclease Dpn I recognizes the methylated region and cleaves the methylated ds-DNA. We harnessed this methylation-specific cleavage property and designed our stimuli-response "smart" regulation platform (Scheme 1a). A 57 base hairpin DNA, included a Dam MTase recognition $\left(\begin{matrix}5'....GATC....3'\\3'....CTAG....5'\end{matrix}\right)$ site (green) near the end of the stem region, was designed as a probe (**1**) to monitor the Dam MTase activity. An antitumor drug, 5-fluorouracil (5-Fu), was appended at the 3′ terminus.[70,71] In the presence of both Dam MTase and Dpn I, probe (**1**) is methylated and then cleaved (Figure 1a) into two fragments, one consisting of the loop region (blue) and the majority of the stem, and the other consisting of the four base pairs at the terminus of the stem region, including the 5-Fu modification. This short terminal

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region dissociates above 10 °C, liberating the ss-DNA modified 5-Fu (**3**), and allowing it to bind to and inhibit MTase activity (Figue 1a). To enable real-time monitoring of the activity of this platform, we employed a second cleavage step involving the duplex DNA formed by the loop fragment and an additional fluorescent probe (F-probe (**4**)). This F-probe (**4**) consists of an 11 base ss-DNA sequence complementary to the loop fragment (**2**), modified with a fluorophore (FAM) and a quencher (Dabcyl) at the 5′ and 3′ termini, respectively. Unhybridized, FAM and Dabcyl are spatially separated by 11 bases, approximately 3.3 nm; this distance is less than the Förster radius and thus the fluorescence is quenched because of FRET. Upon methylation and cleavage of probe (**1**), the loop fragment (**2**) hybridizes with F-probe (**4**), forming a duplex containing the recognition site of the nicking endonuclease Nt.BbvC I, $\begin{pmatrix} 5'....CC\blacktriangledown TCAGC....3' \\ 3'....GGAGTCG....5' \end{pmatrix}$.[72,73] The Nt.BbvC I specifically recognizes and cleaves the F-probe strand via a nicking endonuclease signal amplification (NESA) strategy (Figure 1b), separating FAM and Dabcyl, and thus enhancing fluorescent signal. After this cleavage, the loop fragment (**2**) dissociates from the duplex DNA and is free to hybridize with more F-probe (**4**), generating a hybridization-cleavage cycle, accumulating (**5**) and amplifying fluorescent readout. This readout step allows us to detect in real time the methylation activity and performance of our inhibitor. The solidity of the proposed stimulus responsive, self-regulating system lies in two factors: the real-time responsibility of NESA and the inhibition efficiency of ss-DNA-modified 5-Fu (**3**). We, thus, first examined the fluorescent variety of F-probe (**4**) upon incubation with probe (**1**) (without 5-Fu modification). A slight fluorescent signal increase was observed exact after Dam MTase and Dpn I were added to the mixture, suggesting a conformational change of F-probe (**4**) from random coil to rigid duplex (**2**/**4**) that spatially separated FAM and Dabcyl (Figure 2). This fast response indicates that the methylation and cleavage reaction was quick. Meanwhile, the further addition of nicking enzyme Nt. BbvC I to the mixture resulted in a dramatic fluorescence recovery. However, the fluorescent signal continued to increase in the next hours rather than quickly plateaued, indicating the effective and successive cleavage of (**2**/**4**) duplex by nicking enzyme that generated the hybridization-cleavage cycle and accumulated amounts of released FAM (**5**).

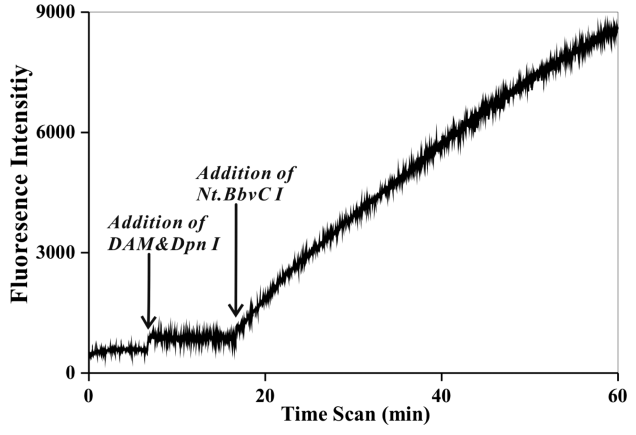

Figure 2. Time course of the fluorescent variety of F-probe (**4**) (1 μM) upon incubation with unmodified probe (**1**) (25 nM). The arrows indicate the addition of different enzymes to the mixture. Dam MTase (8 units), Dpn I (20 units), Nt. BbvC I (10 units), respectively.

5-fluorouracil (5-Fu) is a pyrimidine analogue that has been a part of the therapeutic armamentarium for a variety of solid tumors for over forty years.[74−76] However, poor bioavailability is the major disadvantage of 5-Fu due to low solubility in aqueous solutions.[77] Therefore, we hypotheses that modifying DNA to 5-Fu could increase its solubility and the inhibit efficiency. To evaluate inhibition efficiency of 5-Fu and ss-DNA modified 5-Fu (**3**) toward Dam MTase in vitro, we compared their median effect dose (IC_{50}), concentration of drug needed for a 50% reduction, with our monitoring system (Figure 3).The median effect dose of ss-DNA modified 5-Fu (**3**), IC_{50} = 0.30 ± 0.02 μM, is lower than that 5-Fu, IC_{50} =6.0 ± 0.2 μM. This result indicated that the ss-DNA-modified 5-Fu (**3**) show higher inhibition efficiency than 5-Fu.

To further confirm the ss-DNA-modified 5-Fu (**3**) shows higher activity than 5-Fu, we generally compared the activity of ss-modified 5-Fu (**3**) and free 5-Fu in vivo against HeLa with MTT assay.[78] Treatment of HeLa 72 h of incubation, the cell viability of HeLa for ss-DNA-modified 5-Fu (**3**) is lower than that of free 5-Fu with each concentration, indicating that the ss-DNA-modified 5-Fu (**3**) shows higher activity than 5-Fu (Figure 4) . Meanwhile, the cell viability of HeLa for ss-DNA and control are identical within the experimental error, suggesting that ss-DNA has little effect on the HeLa cells viability. This result, in turn, implies that 5-Fu is the effector that reduced the viability of HeLa cells. To confirm the cellular uptake ability of ss-DNA-modified 5-Fu (**3**), we incubated cy3-labeled ss-DNA with HeLa cells for 72 h (Figure 5). The cy3-labeled ss-DNA is predominantly localized in the cytoplasm, suggesting that this short ss-DNA could immigrate into cells. These results demonstrated that ss-DNA-modified5-Fu (**3**) show higher inhibition efficiency than free 5-Fu in vivo and vitro and could be uptake by cell.

To test our stimuli-responsive regulating system, we monitor the methylation process of probe (**1**), in which 5-Fu was modified at the end of the hairpin stem (curve a, Figure 6). We also employed unmodified probe as control to reveal the inhibition efficiency of the self-regulate system (curve b, Figure 6). When both Dpn I and Dam MTase are absent in the probe solution, probe (**1**) (curve c, Figure 6) has the same fluorescent signal with unmodified hairpin probe (curve d, Figure 6), indicating that the 5-Fu modification has little effect on the structure of probe (**1**) and the probe (**1**) could be employed in our new NESA monitoring strategy. As methylated and cleaved by Dam MTase and Dpn I, the fluorescent signal of probe (**1**) (curve a, Figure 6) and unmodified probe (curve b, Figure 6) significantly increased because of producing cleaved loop fragment (**2**) during the DNA methylation process. However, the probe (**1**) produced a less-cleaved loop fragment (**2**) because of the lower activity of Dam MTase, suggesting that the released ss-DNA-modified 5-Fu (**3**) inhibited the activity of DAM MTase in return.

To further evaluate the inhibition efficiency of the self-regulation system, we also monitored the methylation process of unmodified probe with 25 nM inhibitor (5-Fu, curve e in Figure6; ss-DNA modified 5-Fu (**3**), curve f in Figure 6), in which the concentration of 5-Fu is the same to the probe (**1**). Addition of these inhibitors significantly reduced the fluorescent signal, especially ss-DNA modified 5-Fu (**3**) (curve f, Figure 6). This result is in agreement with that ss-DNA modified 5-Fu (**3**) exhibited higher inhibition efficiency

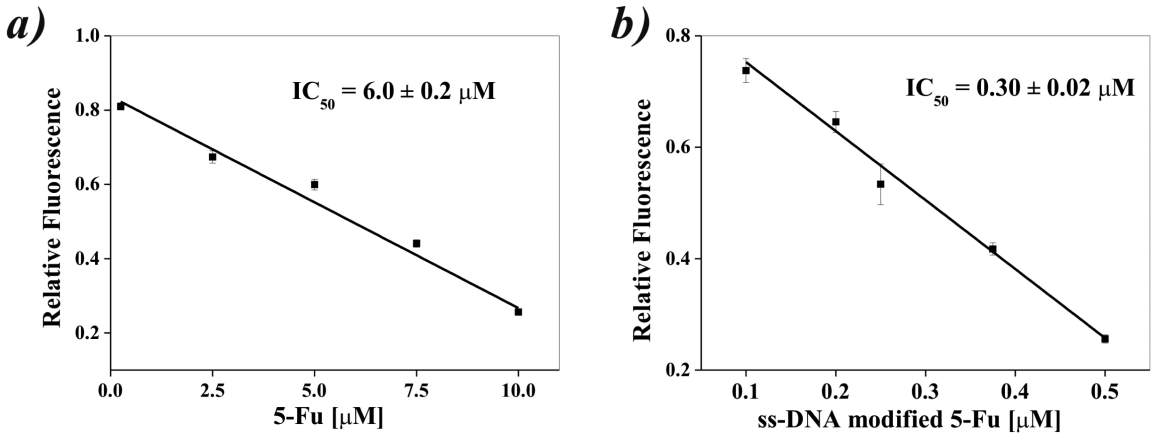

Figure 3. Median effect dose (IC_{50}) of 5-Fu (a) and ss-DNA modified 5-Fu (b) separately. The experiments were carried out in 1 × NEB buffer 2, with 250 nM Probe, 1 μM F-Probe, 1 × SAM(80 μL), 20 unit of Dpn I endonuclease, 8 unit of Dam MTase, and 10 unit of Nt.BbvC I.

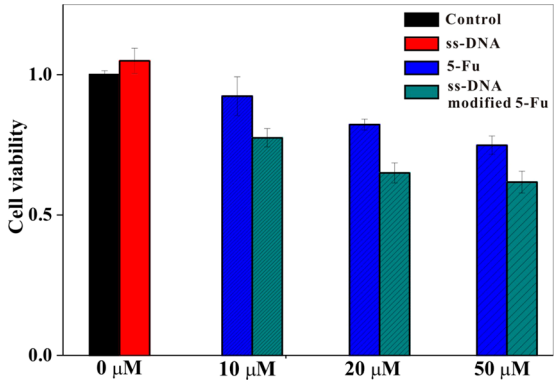

Figure 4. Effect of 5-Fu and ss-DNA-modified 5-Fu (**3**) on cell viability in vivo with MTT assay. Cells were seeded into 96-well plates and treated with various doses of 5-Fu and SAHA for 72 h, and then incubated with MTT reagent for 4 h. Cell viabilities were determined by measuring absorbance at 560 nm.

than 5-Fu. Meanwhile, the fluorescent signal of probe (**1**) (curve a, Figure 4) was higher than unmodified probe with ss-DNA-modified 5-Fu (**3**) (curve f, Figure 6), indicating that the released ss-DNA modified 5-Fu (**3**) from probe (**1**) inhibited the activity of DAM MTase and down-regulated MTase in turn.

Furthermore, native gel-electrophoresis provides additional evidence of the DNA methylation process of Probe (1) (Figure 7). When Dpn I is absent, there is only one band of the original Probe (**1**), indicating that no cleavage reaction occurred and no loop fragment (**2**) produced (lane e, Figure 7). The new band of loop fragment (**2**) appeared in lane f and g when Dpn I was added. These results suggest that the methylation reaction of probe (**1**) produced loop fragment (**2**) upon the addition of DAM and Dpn I.

CONCLUSION

In the present study, we have developed an in vitro stimuli-responsive methylation regulation platform, which integrates a real-time monitoring assay and a methylation-dependent

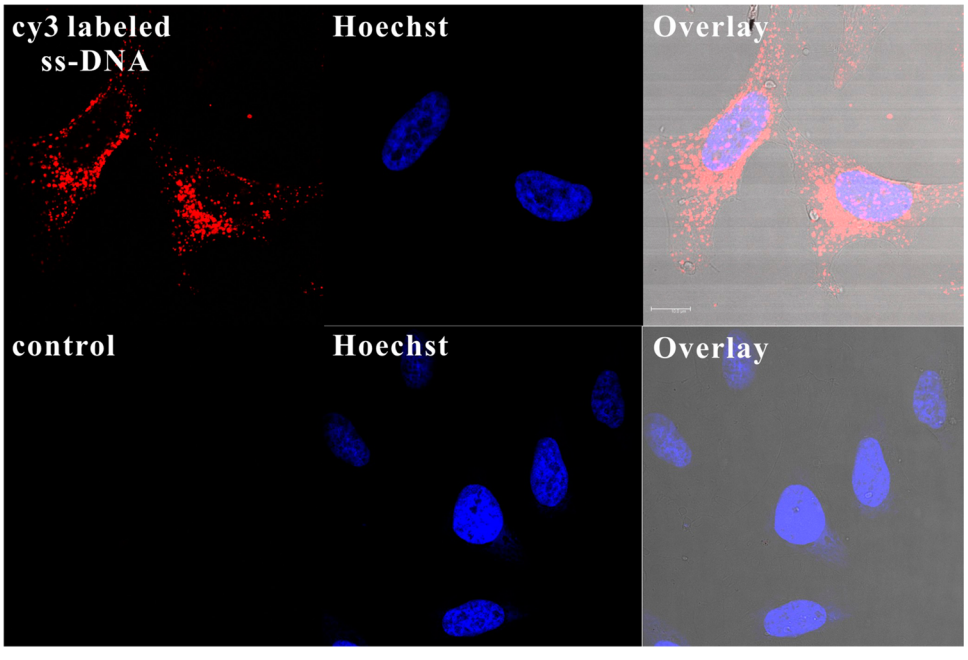

Figure 5. Confocal microscopic pictures for intracellular localization of cy3-labeled ss-DNA and control. Scale bars: 10 μm.

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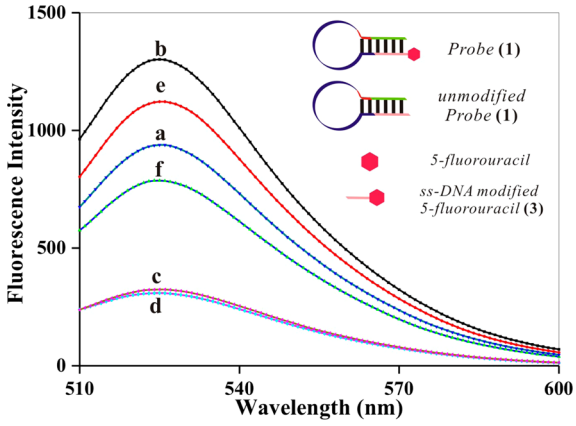

Figure 6. Fluorescence spectra of DNA Methylation toward variety 25 nM probes upon incubation with MTase and endonuclease. (a) Probe (**1**), (b) unmodified Probe (**1**), (c) probe (**1**) without Dam MTase, (d) unmodified probe (**1**) without MTase, (e) unmodified probe (**1**) and 25 nM 5-Fu, (f) unmodified probe (**1**) and 25 nM ss-DNA modified 5-Fu (**3**).

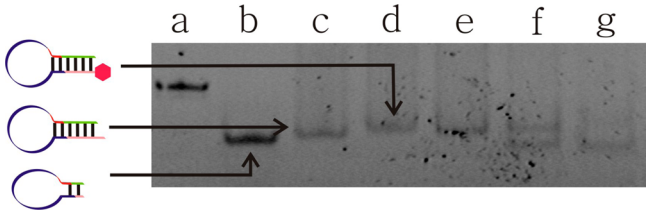

Figure 7. Native polyacrylamide gel electrophoresis (PAGE) demonstrates the product of DNA methylation: (a) 100 bp marker, (b) loop fragment (**2**), (c) unmodified probe (**1**), (d) probe (**1**), (e) methylated probe (**1**), (f) methylated and cleaved unmodified probe (**1**), (g) methylated and cleaved probe (**1**).

inhibitor release platform. Modified with 5-Fu, this platform responds to DNA Methylation and releases ss-DNA-modified 5-Fu (**3**) to inhibit the activity of DAM MTase in return. Meanwhile, we found that the inhibition efficiency of 5-Fu significantly increase as it is functionalized with DNA. Given that 5-fluorouracil is a widely used drug for cancers, we expect that this method may eventually find application in cancer therapy.

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Notes

The authors declare no competing financial interest.

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