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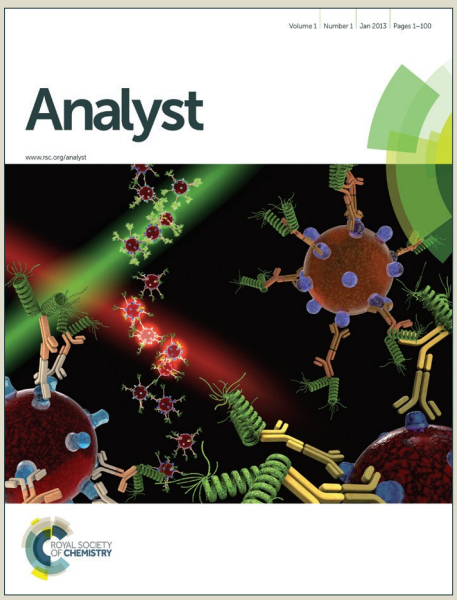

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ARTICLE

Detection of DNA Methyltransferase Activity using Allosteric Molecular Beacons

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Abnormal DNA methylation patterns caused by altered DNA methyltransferase (MTase) activity are closely associated with cancer. Herein, using DNA adenine methylation methyltransferase (Dam MTase) as a model analyte, we designed an allosteric molecular beacon (aMB) for sensitive detection of Dam MTase activity. When the specific site in an aMB is methylated by Dam MTase, the probe can be cut by restriction nuclease DpnI to release a fluorophore labeled aptamer specific for streptavidin (SA) which will bind to SA beads to generate highly fluorescent beads for easy signal readout by a microscope or flow cytometer. However, aMBs maintain a hairpin structure without binding ability to SA beads in the absence of Dam MTase, leading to weakly fluorescent SA beads. Unlike existing signal amplified assays, our method is simpler and more convenient. The high performance of the aptamer and the easy bead separation process make this probe superior to other methods for the detection of MTase in complex biological systems. Overall, the proposed method with a detection limit of 0.57 U/mL for Dam MTase shows great potential for further applications in the detection of other MTases, screening of MTase inhibitors, and early diagnosis of cancer.

Introduction

DNA methylation, one of the earliest discovered DNA modifications, plays an important role in many biological processes, such as gene transcription, cell growth and proliferation, in both prokaryotes and eukaryotes[1]. In bacteria, methylation is related to self-protection mechanisms, while in mammals methylation closely relates to embryogenesis, epigenetic and genomic imprinting. Recent research has found that the degree of abnormal methylation is a vital epigenetic marker for related human diseases and is closely associated with cancer[2], such as gastric cancer[3] and lung cancer[4]. Because DNA MTase has become a potential target in anticancer therapy, detection of DNA methylation and DNA MTase activity has great significance in disease diagnosis and drug discovery.

Traditional analysis methods for DNA methylation and DNA MTase activity mainly rely on PCR[5], radio-labeling[6], HPLC[7] and gel electrophoresis[8]. All of these methods require cumbersome procedures, making them time-consuming and labor-intensive. Currently，a number of methods have been developed for DNA MTase activity, such as the use of Au nanorods for colorimetric assay[9] and FRET assay[10], MoS_2 nanosheets[11] and graphene oxide[12] as quenchers for fluorescence assay, electrochemical assay[13-16] combined with a specific enzyme or quantum dots, chemiluminescence[15, 17, 18] and bioluminescence assay[19] based on hybridization chain reaction (HCR) or rolling circle amplification (RCA). In addition, multiple molecular beacons (MBs)[20] have been designed for DNA methylase detection with the help of enzymatic recycling amplification[21-23] and DNAzyme recycling amplification[24, 25]. However, the requirement of several other enzymes for signal amplification increases the complexity of the reaction system, leading to susceptibility to matrix effects especially in biological solutions. Therefore, development of a simple strategy for detection of DNA MTase without sophisticated probe design, multiple enzymatic amplification or system interferences still needs to be addressed.

We have previously reported the design and development of a new type of molecular probe named allosteric molecular (aMB) for highly sensitive detection of nucleic acids, proteins and small molecules in complex biological samples[26, 27]. In brief, an aMB is a single-fluorophore-labeled single-stranded DNA probe consisting of an aptamer specific for streptavidin (SA)[28], a specific sequence for target recognition, a short sequence that is complementary to part of the SA aptamer, and a fluorophore for signal readout. In the absence of target, a stable hairpin structure is formed by intramolecular hybridization between the SA aptamer sequence and the complementary sequence, temporarily disabling the probe's ability to bind with SA-beads. When the target is present, the hairpin structure can be opened and subsequently the probe regains the binding ability to SA-beads making the beads highly

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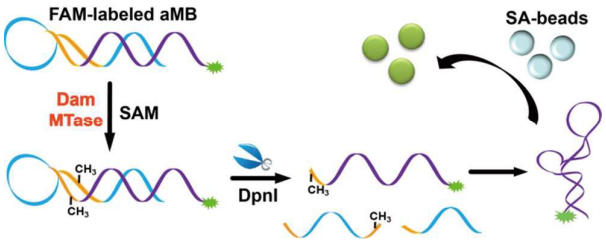

Scheme 1 Schematic illustration of allosteric molecular beacons (aMBs) for the detection of Dam MTase activity.

fluorescent. The target can be quantitatively detected by reading the fluorescence intensity of SA-beads via fluorescence microscopy or flow cytometry. The structural features of our aMBs enable sensitive detection of targets with high signal-to-background (S/B) ratio even in biological medium, because unbound probes and interferences can be easily separated from the SA-Beads.

In this study, we proposed to apply aMB strategy for DNA MTase activity assay, using DNA adenine methylation methyltransferase (Dam MTase) as the model analyte. This enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to the 6-positon of adenine in recognition sequence GATC[29]. In the presence of Dam MTase, the palindromic sequence designed in the stem of an aMB is methylated and incised by DpnI, an endonuclease from *diplococcus pneumoniae* able to recognize and cleave methylated sites GA^{m}TC, thereby activating the probe's binding affinity to SA-beads. As a consequence, Dam MTase activity can be quantified by a microscope or flow cytometer (**Scheme 1**). We have achieved sensitive and selective detection of Dam MTase both in buffer and complex solution via a single-labeled aMB. The inhibitory effect of a model drug 5-fluorouracil on Dam MTase activity was further studied. The design offers new opportunities to study DNA MTase activity and inhibition for disease diagnosis and cancer research. Because the signal can be simply photographed by a microscope, it could be further applied in the area of high content screening and high throughput screening for drug discovery.

Experimental section

Materials and chemicals

Dam MTase, S-adenosylmethionine (SAM), DpnI, MboI from *moraxella bovis*, and 10x Cutsmart buffer were obtained from New England Biolabs Inc. (Beijing, China). Streptavidin beads were obtained from GE Healthcare. All other chemicals were analytical reagent grade and used without further purification. All oligonucleotides used in this study were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and sequences were as follows:

Dam5 FAM-*ATTGACCGCTGTGTGACGCAACACTCAAT***GA*TC**G
GTGAGCG**GA*TC**ATTGA

Dam8 FAM-*ATTGACCGCTGTGTGACGCAACACTCAAT***GA*TC**G
GTGAGCG**GA*TC**ATTGAGTG

Dam10 FAM-*ATTGACCGCTGTGTGACGCAACACTCAAT***GA*TC**G
GTGAGCG**GA*TC**ATTGAGTGTT

Dam12 FAM-*ATTGACCGCTGTGTGACGCAACACTCAAT***GA*TC**G
GTGAGCG**GA*TC**ATTGAGTGTTGC

The bolded letters in all aMBs represent the recognition sequence for Dam MTase and DpnI. The stem of the aMBs is underlined, and the SA aptamer sequence is italicized.

Optimization of aMBs

To achieve the best S/B ratio of the probe, we optimized the length of the sequence that is complementary to part of the SA aptamer sequence to ensure that the hairpin structure forms for initial inhibition of the binding affinity to SA. The concentrations of aMBs and Dam are 50 nM and 20 U/mL, respectively, and the DNA methylation time is 2.5 hr.

Assay of Dam MTase activity

To prepare the hairpin structure, 50 nM aMBs in 100μL buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH=7.5) was heated to 95° C for 8 min and then cooled slowly to 37° C for another 20 min. The buffer above was used for both the DNA methylation reaction and the DpnI incising reaction. In a typical experiment, 100 μL buffer containing 50 nM aMBs, 160 μM SAM, 4U DpnI and different concentrations of Dam MTase was first incubated at 37°C for 2.5 hr. After enzyme reaction, 1.5 μL SA-beads (about 2×10^4 beads) were added and the mixture was allowed to rotate slowly for 30 min at 37° C in the dark. After washing with Tris-HCl buffer, the SA-beads were filtered to eliminate unbound aMBs and resuspended in buffer before flow cytometry analysis or fluorescence microscopy imaging.

MboI reaction

A 25 nM solution of annealed aMBs in 50 μL 1X Cutsmart buffer (20 mM Tris–Ac, 10 mM $Mg(Ac)_2$, 50 mM KAc, 100μg/mL BSA, pH 7.9) was prepared. Then, 5U MboI was added and the mixture was incubated at 37°C for 2 hr. Then 1.5 μL SA-beads was added and the mixture was incubated at 37°C in the dark. After washing and filtering, the resuspended SA-beads were analysed by flow cytometry.

Selectivity study

The selectivity of the Dam activity was evaluated by using M.SssI, another kind of MTase, as negative control. The procedures were the same as that of Dam activity detection with replacement of 20 U/mL M.SssI.

Evaluation of DNA MTase inhibitor

The influence of drugs on Dam MTase activity was investigated by using 5-fluorouracil (5-FU) as a model inhibitor. Different concentrations of 5-FU were first mixed with Dam MTase (20 U/mL). The mixture was added to 100 μL buffer containing 50 nM aMBs, 160 μM SAM and 4U DpnI and incubated at 37°C for 2.5 hr. Then 1.5 μL SA-beads were added and incubated at 37°C in the dark. After washing and filtering, the resuspended SA-beads were subjected to flow cytometry analysis or fluorescence microscopy imaging.

Results and Discussion

Design and Optimization of aMB for DNA MTase Assay

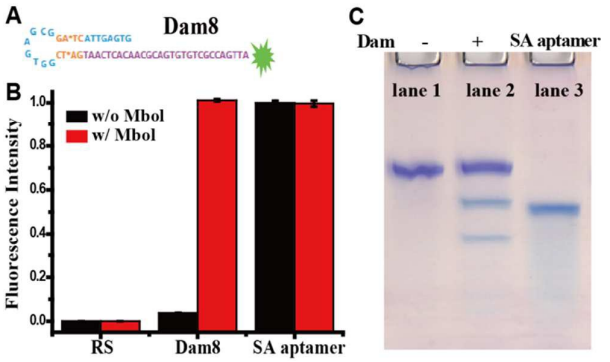

Figure 1 (A) Structure and sequence of Dam8; (B) Fluorescence intensity of SA-beads after incubating with Dam8 probe with or without MboI treatment. (C) Denatured PAGE gel stained by nucleic acid dye (Stains-All), lane1: Dam8 without Dam MTase; lane2: Dam8 with Dam MTase, DpnI and SAM; lane 3: SA aptamer with equal quantity as Dam8.

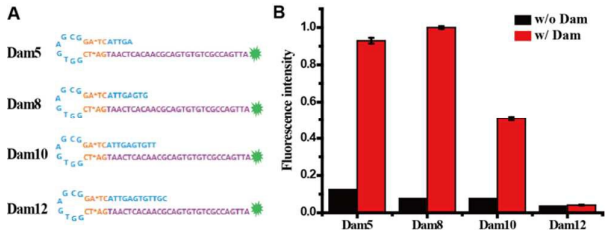

Figure 2 Sequence optimization of aMB probe. (A) Structures and sequences of four designed aMBs. (B) Fluorescence intensity of SA-beads after incubation in aMB probe solution with or without Dam MTase.

To test the feasibility of aMB for DMTase activity analysis, an aMB with an 8-base sequence complementary to SA aptamer sequence, named Dam8, was first designed and synthesized. As shown in **Figure 1A**, the purple part with a fluorophore represents the 5'-FAM-labeled SA aptamer sequence, the orange palindrome represents the specific recognition site, GATC, the two blue parts represent the flexible linker and the short sequence complementary to SA aptamer. The probe can stably fold into a hairpin structure with an 12bp stem. We first verified the formation of the hairpin structure with correct palindrome area by treating the probe with restriction endonuclease MboI, which can recognize the same palindromic sequence GATC as DpnI but cleave it when no methylation is present. As shown in **Figure 1B**, without MboI treatment, SA-beads with Dam8 exhibited fluorescence as low as that with random sequence (RS). However, when treated with MboI, SA-beads with Dam8 were strongly fluorescent, with intensity similar to that of SA-beads with SA aptamer. This experiment suggested that Dam8 was able to form hairpin structure and be recognized by restriction the enzyme. More importantly, after cutting by MboI, the SA aptamer sequence was released from Dam8 to bind strongly to SA-Beads, leading to enhanced fluorescence of beads for signal readout.

Methylation of Dam8 by Dam MTase was further confirmed by polyacrylamide gel electrophoresis analysis (**Figure 1C**). When there was no Dam MTase present, only one DNA band was observed (lane 1). In contrast, two additional bands appeared when Dam MTase was added to the probe solution containing Dam 8, DpnI and SAM (lane 2), suggesting the occurrence of methylation and the cleavage reaction. The size of the larger newly generated fragment was found to be similar to that of SA aptamer (lane 3). These experiments demonstrated that the expected hairpin structure of aMB was formed and can be methylated and cleaved by respective enzymes to generate free SA aptamer for further binding to SA-bead for signal readout.

In the aMB probe, the binding affinity of the aptamer sequence is inhibited by the short complementary sequence. The longer the sequence, the higher the inhibition efficiency, and the weaker the binding of free probe to SA Beads, resulting in lower background. However, after cutting by theDpnI, the complementary sequence should dissociate from the aptamer sequence to allow binding of the aptamer to SA beads to yield highly fluorescent beads. Since the length of the sequence complementary to part of the SA aptamer would affect the performance of an aMB, we designed and synthesized four aMBs with various complementary lengths to optimize the probe sequence, Dam5, Dam8, Dam10, Dam12 (**Figure 2A**). The numbers in the names stand for the length of the stem part excluding the enzyme recognition site. As the results in **Figure 2B** show, with increasing stem length, the background was gradually decreased, due to the increasing stability of aMB hairpin structure and decreasing binding of the probe to SA beads. However, the signal with Dam MTase treatment was slightly increased then sharply decreased. The shorter stem probe such as Dam5 results in unstable stem structure, affecting enzyme reaction efficiency. With the longer stem probes, such as Dam10 and Dam12, the stability of the duplex structure may be too high, so that even after the enzyme cleavage it may still inhibit the binding of SA aptamer to SA-beads. The results showed that eight bases (Dam8) had the best S/B ratio, which was chosen for subsequent experiments.

Detection of Dam MTase in buffer and complex system

The quantitative detection of Dam MTase by the proposed assay was performed by using the optimized aMB probe Dam8 with a series of concentrations of Dam MTase reading by flow cytometry. **Figure 3A** shows that the fluorescence intensity gradually increased with increasing concentration of Dam MTase from 0-200 U/mL. In the presence of higher concentrations of Dam MTase, more aMBs were methylated and cleaved by DpnI, resulting in an increase in the release of SA aptamer and fluorescence enhancement of the SA-beads. From the fitting curve of **Figure 3B**, the limit of detection was determined to be 0.57 U/mL in buffer and the linear range was from 0.7 to 30 U/mL (R^2=0.9328). This result is comparable with other reported methods,[30-35] which have to rely on signal amplification assisted by nanomaterials or enzymes.

In addition to flow cytometry, analysis can also be performed by using fluorescent microscopy to read out the signal intensities of the microbeads, thus allowing flexible

detection schemes. As shown in **Figure 4**, the enhancement of the fluorescence intensity corresponded to the increased concentration of Dam MTase.

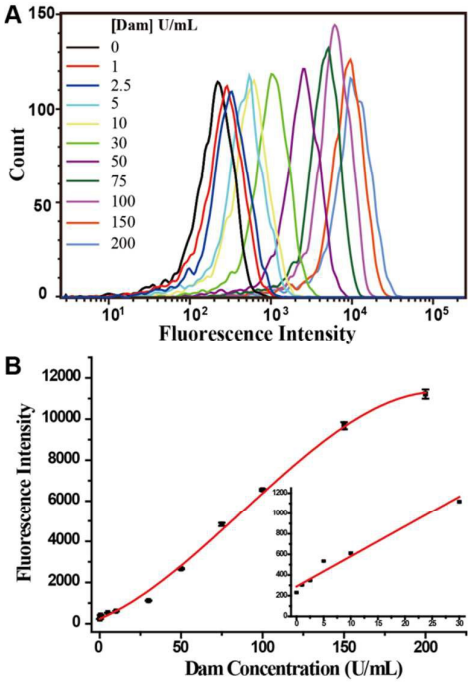

Figure 3 Response of Dam 8 to different concentrations of Dam MTase in buffer solution monitored with a flow cytometer (A) and the response curve of corresponding fluorescence peak intensities versus Dam MTase concentrations (B) and linear fitting from 0.7-30 U/mL (inset)

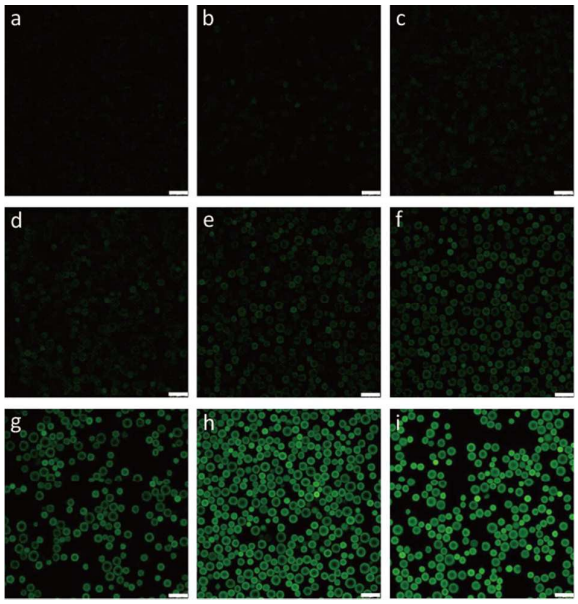

Figure 4 Confocal imaging of SA-beads treated with Dam 8 probe and different concentrations of Dam MTase ranging from 0, 0.25, 0.5, 2.5, 10, 20, 50, 100, 200 U/mL (a-i). Scale length was 75 μm.

To demonstrate the potential application of the probe for detection of Dam MTase in complex biological samples, the assay was performed in 20% LB medium samples and the resulting beads were analysed by flow cytometry (**Figure 5**). Fluorescence intensity of the SA microbeads was found to increase proportionally with the increase of Dam MTase concentration in LB medium media. After methylation by Dam MTase and cutting by DpnI, the probe releases SA aptamer, which binds to the SA beads and enriches the fluorescence effect. Such a combination of probe enrichment and separation from interfering species in solution leads to excellent detection sensitivity. The limit of detection was found to be 1.03 U/mL and the linear range was from 1.1 to 10 U/mL (R^2=0.9832).

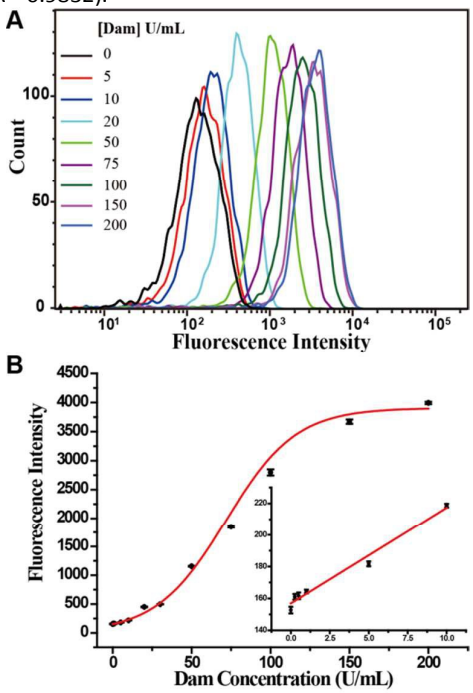

Figure 5 Response of Dam 8 to different concentrations of Dam MTase in 20%LB solution monitored by flow cytometry (A); response curve of corresponding fluorescence peak intensities versus Dam MTase concentrations (B); and linear fitting from 1.2-10 U/mL (inset).

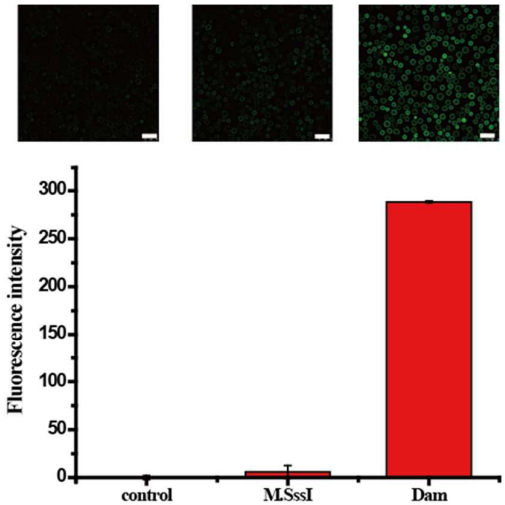

Figure 6 Response of Dam 8 probe to Dam MTase and M.SssI MTase. MTase concentration: 20 U/mL. Control: no MTase. Scale length was 75 μm.

Selectivity of aMB

The selectivity of the aMB was further investigated using M.SssI MTase as the interference enzyme. M.SssI MTase belongs to the DNA cytosine methyltransferase family which modifies the cytosine residue (C5) within the double-stranded dinucleotide recognition sequence 5'-CG-3'. As flow cytometry and fluorescence microscopy results in **Figure 6** show, the fluorescence response of the M.SssI MTase treated aMBs was practically the same as the control response. In contrast, Dam MTase led to very high fluorescence intensity. Therefore, the proposed assay exhibits extraordinary selectivity and can easily discriminate Dam MTase from other interference enzymes.

Inhibition study

Dam MTase is essential for bacterial virulence, and its inhibitors are likely to have a broad antimicrobial action. Therefore, we applied the Dam MTase assay for an inhibition study with 5-fluorouracil (5-FU) as the model drug. The activity of Dam MTase (20 U/mL) was detected for a series of different concentrations of 5-FU. **Figure 7A-B** shows that the relative activity of Dam MTase decreased with increasing concentration of 5-FU. The IC_{50} (half maximal inhibitory concentration) value was determined to be 0.91 μM, which is agreed well with other reports.[31, 36, 37] Fluorescence images of SA-beads (**Figure 7C**) show the decrease of fluorescence intensity with increasing of inhibitor concentration. Hence, microscopy is a good detection method for inhibitor screening. DNA methylation occurs in mammals as well as bacteria, and abnormal methylation in humans is indicative of cancer. This method has the potential to investigate DNA MTase inhibition, possibly leading of new inhibitors for both antimicrobial drug development and cancer therapy.

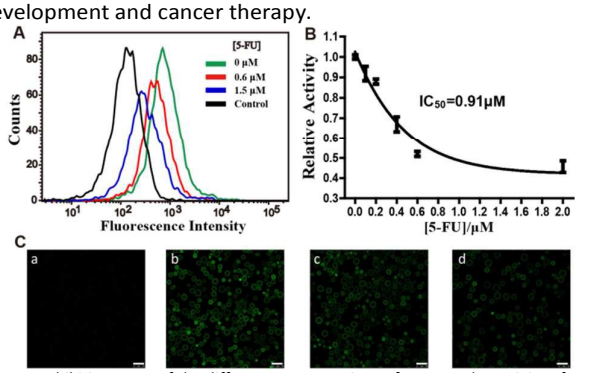

Figure 7 Inhibition assay of the different concentrations of 5-FU on the activity of Dam MTase. (A) The flow cytometry data of inhibition assay; (B) The Fitting curve to determine the IC_{50}; (C) confocal imaging of SA-beads; a, no Dam MTase; b-d, Dam MTase (20 U/mL) with 5-FU concentration of 0, 0.2, 0.6 μM, respectively. Scale bar was 75 μm.

Conclusions

In summary, we have developed a facile and simple strategy for Dam MTase activity and inhibition studies based on allosteric molecular beacons (aMBs). In this assay, only one singly labeled probe and a necessary assistant endonuclease are needed without any other assisting sequences or enzymes, thereby simplifying the reaction system. In addition, the easy bead separation process makes this probe superior to others for suitability in complex biological system. Furthermore, the high affinity and specificity of SA aptamer towards SA and the microbead enrichment process enable a detection limit of 0.57 U/mL without any other additional amplification steps. Hence, our method is much simpler and less expensive compared to other methods.

Inhibitors of human DNA MTase have close correlation with cancer-related diseases. Using this method, the suitable inhibitor of target MTase can be readily screened and its performance can be evaluated via flow cytometry for possible use in drug discovery. It is worth noting that the developed strategy can be used as a universal tool for the detection of other DNA MTases in both prokaryotes and eukaryotes by simply changing the palindromic methylation sequence and the corresponding endonuclease. Therefore, multiple aMBs aiming at multiple targeting MTase with different fluorescence labels can be applied to rapid screening of multiple possible inhibitors. Moreover, considering the flexible detection schemes of flow cytometry and fluorescent microscopy, the proposed strategy holds great promise for DNA MTase quantitative analysis and high throughput drug screening.

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