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Collapse of DNA Tetrahedron Nanostructure for "Off–On" Fluorescence Detection of DNA Methyltransferase Activity

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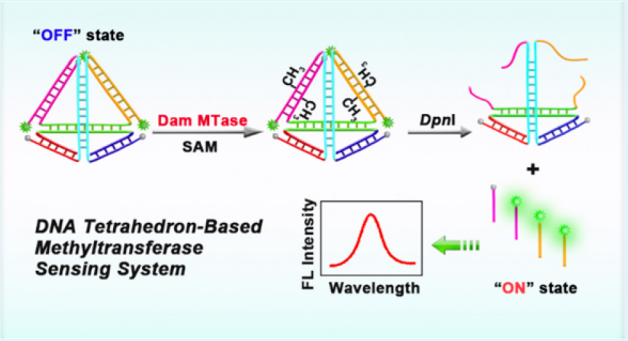
Supporting Information

ABSTRACT: As a potential detection technique, highly rigid and versatile functionality of DNA tetrahedron nanostructures is often used in biosensing systems. In this work, a novel multifunctional nanostructure has been developed as an "off–on" fluorescent probe for detection of target methyltransferase by integrating the elements of DNA tetrahedron, target recognition, and dual-labeled reporter. This sensing system is initially in an "OFF" state owing to the close proximity of fluorophores and quenchers. After the substrate is recognized by target methyltransferase, the DNA tetrahedron can be methylated to produce methylated DNA sites. These sites can be recognized and cut by the restriction endonuclease *Dpn*I to bring about the collapse of the DNA tetrahedron, which leads to the separation of the dual-labeled reporters from the quenchers, and thus the recovery of fluorescence signal to produce an "ON" state. The proposed DNA tetrahedron-based sensing method can detect Dam methyltransferase in the range of 0.1–90 U mL^{-1} with a detection limit of 0.045 U mL^{-1} and shows good specificity and reproducibility for detection of Dam methyltransferase in a real sample. It has been successfully applied for screening various methylation inhibitors. Thus, this work possesses a promising prospect for detection of DNA methyltransfrase in the field of clinical diagnostics.



KEYWORDS: *DNA nanotechnology, DNA tetrahedron, DNA methyltransferases, fluorescence biosensor, DNA methyltransferase inhibitors*

INTRODUCTION

DNA methylation, an epigenetic event, plays a pivotal role in gene expression, cell growth and proliferation, and chromatin organization.[1,2] It has been well confirmed that the DNA methylation process is catalyzed by DNA methyltransferase (MTase), which can transfer a methyl group from *S*-adenosyl methionine (SAM) to target adenine or cytosine residues in the recognition sequences.[3] Recent studies have proved that the aberrant DNA methylation can influence the interaction between DNA and protein and alter genes expression which might result in tumorigenesis[4,5] and tumor metastasis.[6,7] Notably, DNA MTase has been treated as a potential drug target for anticancer therapy.[8] Therefore, it is imperative to develop a sensitive and accurate method for activity assay and inhibitor (antimethylation drugs) screening of MTase in both clinical diagnostics and therapeutics.

Conventional techniques, including enzyme-linked immunosorbent assay (ELISA),[9] high performance liquid chromatography (HPLC),[10] and radioactive [methyl-3H]-SAM labeling methylation techniques,[11] have been extensively adopted for detection of MTase activity. However, these approaches encounter several drawbacks, including the involvement of radioactive reagents, complicated sample preparations, and bulky and expensive instrumentations, which limit their wide applications in practice. Besides the above traditional methods, various biosensing-based approaches, such as colorimetric,[12] fluorometric,[13,14] surface-enhanced Raman scattering (SERS),[15] and electrochemical methods,[16] have recently been utilized toward the detection of MTase activity by coupling with

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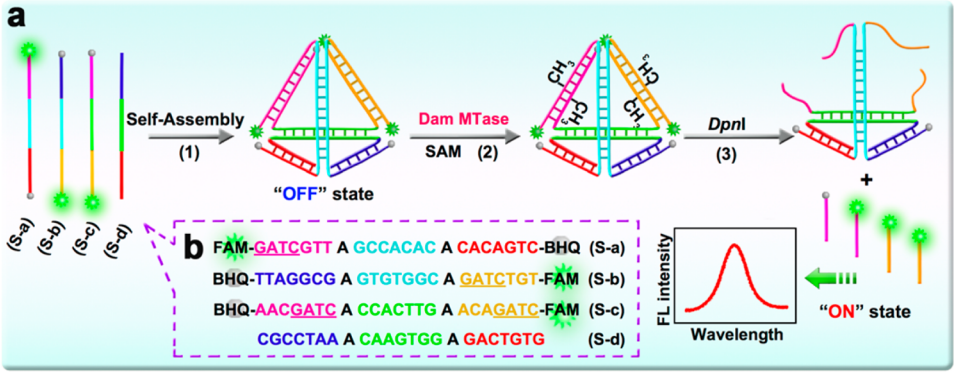
Scheme 1. Construction of "Off−On" Fluorescence Sensing System with DNA Tetrahedron: (a) Schematic Representation of (1) Assembly of DNA Tetrahedron, (2) Methylation by Dam MTase with the Help of SAM, and (3) Cleavage of Methylated Sites by *Dpn*I To Collapse the DNA Tetrahedron for Recovering the Fluorescence Signal; (b) Complementary Sequences in Four Oligos (S-a, S-b, S-c, and S-d) for Construction of Tetrahedron



different enzymes or nanomaterial amplification strategies. For instance, Bi et al.[17] reported a hybridization chain reaction-based branched rolling circle amplification strategy, while Jing et al.[16] designed an AuNPs-loaded probe for MTase sensing. Another report also utilized Cu(I)-based click chemistry of functionalized AuNPs for colorimetric sensing of MTase activity.[18] Among these sensing platforms, fluorescence biosensing is considered to be the most promising platform for point-of-care testing (POCT) because of its advantages of ease to use, high sensitivity, rapid response, and low cost. For example, the fluorescent monitoring methods combined with the molecular beacon-based quadratic isothermal exponential amplification[19] and hybridization chain reaction coupling with DNAzyme recycling[20,21] have been developed for the rapid and sensitive determination of MTase activity, and a majority of these strategies have been designed in multiple-step and indirect signal read-out. Developing a rapid and sensitive fluorescence sensing strategy for detection of MTase activity is a continuous demand.

Currently, DNA-based nanotechnology endows exciting opportunities to explore the powerful biosensing strategies.[22−24] A three-dimensional (3D) DNA nanostructure has been constructed by one-step four strands annealing to form a complex interlocked DNA tetrahedral nanostructure.[25,26] Some functional molecules can be modified on the vertexes, embedded between the double-stranded DNA (dsDNA) of the tetrahedron edges, hanged on the edges, and encapsulated in the cagelike structure of the tetrahedron, which further enriches the vast application of transmolecular-level detection. Benefiting from these properties, Fan and colleagues[23] constructed a series of DNA tetrahedron-based structure for biosensing assay of microRNA, prostate-specific antigen (PSA),[23,27] cocaine,[28] and cancer cells.[29] Additionally, several research groups have used DNA tetrahedral nanostructure to design the sensing strategies for cellular imaging.[30−36] For instance, a dual-color encoded DNAzyme tetrahedron was developed for multiple toxic metal ions imaging,[31] and a molecular beacon was embedded in DNA tetrahedron for imaging low-copy mRNAs in cell.[35] More recently, David et al.[36] adopted the structure of tetrahedron to build a nano-SNEL for mRNA fluorescent detection. Besides, some researches showed a meaningful application of DNA tetrahedron as an effective tool to deliver siRNA[37] or drugs[38−40] into cells. These works demonstrate that DNA tetrahedron holds excellent mechanical properties and rich molecular modification sites, which inspires us to explore DNA nanostructure-based methodology for DNA MTase activity analysis.

This work developed a DNA tetrahedron-based fluorescence biosensing system for sensitive detection of MTase activity using DNA adenine methylation (Dam) MTase as a model analyte. The DNA tetrahedron was assembled with three rationally designed strands dual-labeled with fluorescein (FAM) and black hole quencher (BHQ)[41] and one assistant strand at its "OFF" state owing to the close proximity of fluorophores and quenchers (Scheme 1). The two edges of DNA tetrahedron contained the recognition sites of Dam MTase, which catalyzed the methylation of adenosine residues within symmetric region in the presence of SAM. The methylated sites could be cleaved by DpnI to collapse the DNA tetrahedron, which led to the fluorescence recovery, as "ON" state. The proposed method showed good performance for detection of Dam MTase in real sample and screening of various methylation inhibitors, indicating promising application in clinical diagnostics.

EXPERIMENTAL SECTION

Reagents and Materials. All DNA strands were synthesized, labeled, and purified with HPLC by Sangon Biotechnology Co., Ltd. (Shanghai, China). Their sequences are listed in Table S1 (Supporting Information). Dam MTase, M.SssI, M.CviPI, Alu I, *Dpn*I endonuclease, DNase I, Exonuclease III, Nb.BvCI, SAM, and the corresponding buffers were purchased from New England Biolabs Inc. (Beijing, China). Azacitidine, decitabine, and 5-fluorouracil were purchased from Sigma-Aldrich (St. Louis, MO). Other reagents were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). All solutions were prepared with ultrapure water from Millipore Milli-Q water purification system (Millipore, Bedford, MA). Serum samples were obtained from the First Affiliated Hospital of Chongqing Medical University.

Apparatus. The concentrations of DNA solutions were quantified by UV−vis spectroscopy (Shimadzu, Kyoto, Japan) at 260 nm. A Bio-Rad T100 thermal cycler (Bio-Rad, USA) was used by temperature gradients for the formation of DNA tetrahedron. Fluorescence spectra were measured with a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Palo Alto, CA) at room temperature in all experiments. The polyacrylamide gel electrophoresis (PAGE) analysis was carried out on a Bio-Rad electrophoresis analyzer (Bio-Rad, USA) and imaged on Bio-Rad ChemDoc XRS (Bio-Rad, USA).

Formation of DNA Tetrahedron. DNA tetrahedrons were prepared with different DNA strands (Supporting Information, Table S1) according to a previous protocol.[42] Briefly, equimolar

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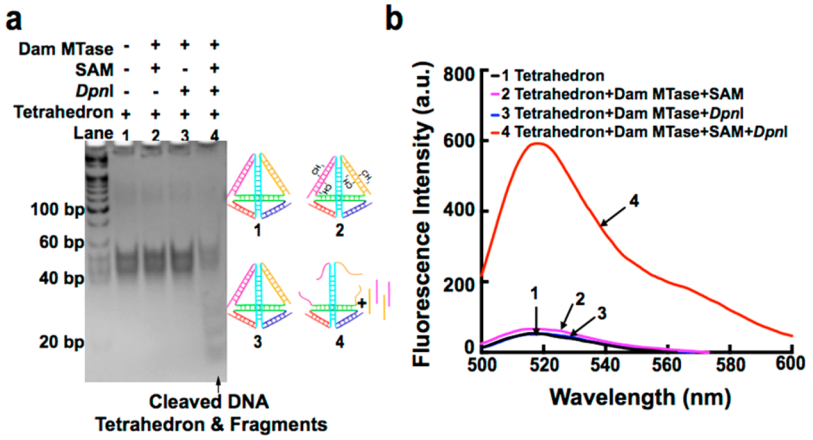

Figure 1. Feasibility of Dam MTase detection using the designed DNA tetrahedron. (a) Native-PAGE (12%) analysis of 500 nM DNA tetrahedron (lane 1), methylated DNA tetrahedron (lane 2), the mixture of DNA tetrahedron, Dam MTase, and *Dpn*I (lane 3), and the mixture of cleaved DNA tetrahedron and fragments (lane 4). (b) Fluorescence spectra of (1) 500 nM DNA tetrahedron and (2–4) DNA tetrahedron mixed with Dam MTase (40 U mL^{-1}) and (2) SAM (180 μM), (3) *Dpn*I (200 U mL^{-1}), and (4) SAM (180 μM) and *Dpn*I (200 U mL^{-1}) after being incubated at 37 °C for 60 min.

quantities of four strands with a final concentration of 1 μM were dissolved in TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0). The resulting mixture was heated to 95 °C for 10 min and cooled to 4 °C in 30 s using a Bio-Rad T100 thermal cycler (Bio-Rad, USA). The assembled DNA tetrahedron was allowed to store at 4 °C for at least 1 week.

Gel Electrophoreses. The assembly of DNA tetrahedron was identified by 12% native PAGE in 1 × TBE running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 100 V constant voltages for 50 min. The effects of SAM (180 μM), *Dpn*I (200 U mL^{-1}), and Dam MTase (40 U mL^{-1}) on the structure of DNA tetrahedron (500 nM) were also identified by 12% PAGE in 1 × TBE buffer at 100 V for 50 min. After gold view (GV) staining, the gels were visualized under UV light and finally photographed with a gel imaging analysis system (Bio-Rad, USA).

Fluorescence Measurements. After mixing 1 × Dam MTase buffer (10 mM EDTA, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.5), 1 × CutSmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 100 μg mL^{-1} BSA, pH 7.9), DNA tetrahedron (500 nM), or other MTase-probe, SAM (180 μM) and *Dpn*I (100 U mL^{-1}), a variety of concentrations of Dam MTase were added with a final volume of 100 μL to incubate at 37 °C for 1 h. The fluorescence spectra were recorded from 500 to 600 nm in a quartz cuvette at an excitation wavelength of 488 nm and slit size of 5 nm at room temperature. The fluorescence intensity was measured at 518 nm.

To investigate the specificity of the proposed method, M.SssI (or other methyltransferases) was selected as the potential interfering enzyme. The specificity experiments were implemented with different methyltransferases (30 U mL^{-1}) in place of Dam MTase at the same experimental procedures.

Evaluation of Dam MTase Inhibition. The inhibition experiment was carried out in a mixture of 1 × Dam MTase buffer and 1 × CutSmart buffer containing 500 nM DNA tetrahedron, 40 U mL^{-1} Dam MTase, and 180 μM SAM, which was incubated at 37 °C for 60 min to ensure the absolute methylation process. After 100 U mL^{-1} *Dpn*I and various concentrations of inhibitors (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 μM) were added to the mixture and incubated at 37 °C for 30 min, the effect of inhibitor on Dam MTase activity was evaluated by the fluorescence measurement.

RESULTS AND DISCUSSION

Feasibility of DNA Tetrahedron Nanostructure for Dam MTase Detection. The assembly of DNA tetrahedron was first confirmed with gel electrophoresis. The product showed the lowest mobility compared with those formed by any three strands and double-stranded DNA (dsDNA) (Supporting Information, Figure S1). The DNA tetrahedron, the mixture of DNA tetrahedron, Dam MTase, and SAM, and the mixture of DNA tetrahedron, Dam MTase, and *Dpn*I all displayed a clear and noninteracted band (lanes 1–3, Figure 1a), while the mixture of DNA tetrahedron, SAM, *Dpn*I, and target Dam MTase showed various cleavage fragments (lane 4, Figure 1a), indicating that *Dpn*I realized digestion of DNA tetrahedron by cleaving the methylation regions, and the Dam MTase catalyzed methylation of DNA tetrahedron was a key step for the digestion.

The fluorescence measurement further confirmed the feasibility of the "off–on" method. The fluorophore-labeled DNA tetrahedron showed a weak fluorescence signal due to the presence of adjacent quenchers (curve 1, Figure 1b). The weak signals did not change when SAM or *Dpn*I was introduced into the fluorophore-labeled DNA tetrahedron solution (curves 2 and 3, Figure 1b), suggesting that DNA tetrahedron still kept at the "OFF" state. However, after SAM and Dam MTase were added together into fluorophore-labeled DNA tetrahedron, the presence of *Dpn*I led to a strong fluorescence signal, indicating the "ON" state (curve 4, Figure 1b). These results suggested that SAM was necessary for Dam MTase to catalyze the methylation of tetrahedron, and the methylated DNA tetrahedron was further digested by *Dpn*I to recover the fluorescence of FAM labeled on DNA strand, which leading to an "off–on" method for fluorescence measurement of Dam MTase activity.

Sensitivity of DNA Tetrahedron-Based Method for Dam MTase Measurement. The performances using different DNA probes including hairpin, dsDNA, single-labeled tetrahedron, and multilabeled tetrahedron for detection of Dam MTase were examined. These probes were first mixed with 180 μM SAM and 200 U mL^{-1} *Dpn*I; they showed very low fluorescence signal, though little difference (Figure 2). Upon addition of 40 U mL^{-1} Dam MTase, these probes were first methylated by transferring the methyl group from SAM to adenine or cytosine residues in the recognition sequences; the methylated sites were then recognized by *Dpn*I to cleave the hairpin, dsDNA, or tetrahedron structure, which led to greater enhance of the fluorescence signal. Both the hairpin and dsDNA probes increased the fluorescence signal by 6.2–6.5

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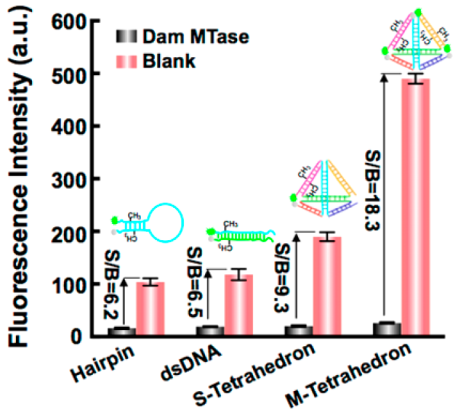

Figure 2. Fluorescence signals of different probes (500 nM) in the mixture of 1 × Dam MTase buffer and 1 × CutSmart buffer containing 180 μM SAM and 200 U mL^{-1} *Dpn*I before and after addition of Dam MTase (40 U mL^{-1}). S/B indicates the signal change or signal-to-background ratio. Error bars indicate the standard deviations of three independent experiments.

times, while the single-labeled tetrahedron showed an increase of 9.3 times (Figure 2), indicating that the stable and rigid DNA tetrahedral structure[42−45] as a nanomolecule was a more suitable candidate for sensitive detection of Dam MTase compared with the flexible dsDNA and hairpin structures. This can be attributed to the fact that the DNA tetrahedral nanostructure can provide a good dispersion and a well-defined spacing in the reaction system. Moreover, the change of fluorescence signal upon addition of Dam MTase in multi-labeled tetrahedron system was about 2 times that in single-labeled tetrahedron system, which would further improve the performance of the DNA tetrahedron for target MTase sensing.

Analytical Performance of Multilabeled DNA Tetrahedron Nanostructure for Biosensing of Dam MTase. DNA tetrahedron with an edge length of 7 bp showed the best fluorescence resonance energy transfer (FRET) efficiency.[42,43] This work used such a length to construct the multilabeled DNA tetrahedron nanostructure for obtaining highly efficient FRET. The highest S/B ratio was obtained from the DNA tetrahedron assembled with group 3 (Figure S2a), with which the optimal tetrahedron concentration was 500 nM; the concentrations of SAM and *Dpn*I were optimized to be 180 μM and 100 U mL^{-1}, respectively (Figure S2b−d), and the optimal incubation time was 60 min (Figure S2e).

Under the optimal conditions, the fluorescence signal of the biosensing system increased with the increasing Dam MTase concentration from 0.1 to 90 U mL^{-1} (Figure 3a) due to the fact that more DNA tetrahedron was methylated and then cleaved to release more FAM-labeled DNA. The plot of $(F - F_0)/F_0$ vs Dam MTase concentration in the range of 4−90 U mL^{-1} showed a good linear relationship (R^2 = 0.995) (Figure 3b). A good linear response was also obtained in the concentration range of Dam MTase from 0.1 to 4.0 U mL^{-1} with the regression equation of Y = 1.729X + 0.1364 (R^2 = 0.996) (inset in Figure 3b). The limit of detection (LOD, defined as 3σ of the signal from the blank) was estimated to be 0.045 U mL^{-1}, which was much lower than those in the reported fluorescence detections and slightly lower than that based on nicking enzyme-assisted signal amplification (Supporting Information, Table S2). Additionally, no primer or extra decorator probe was required in this MTase sensing system, obviously reducing nonspecific amplification and background signal. Moreover, with multisite modification, the DNA tetrahedron sensing process generated more cleaved fragments, which remarkably enhanced the fluorescence signal of the MTase sensing.

The specificity of the proposed method for detection of methyltransferase was assessed on a panel of Dam MTase, CpG Methyltransferase (M.SssI),[46] GpC Methyltransferase (M.CviPI),[47] and Alu I MTase.[48] M.SssI and M.CviPI can methylate all cytosine residues with the base moiety of $\binom{5'\text{-CG-}3'}{3'\text{-GC-}5'}$ and $\binom{5'\text{-GC-}3'}{3'\text{-CG-}5'}$ in dsDNA, respectively, and Alu I MTase methylates the cytosine residues in the moiety of $\binom{5'\text{-AGCT-}3'}{3'\text{-TCGA-}5'}$. In the biosensing system containing 500 nM multilabeled DNA tetrahedron nanostructure, 180 μM SAM and 100 U mL^{-1} *Dpn*I in 1 × Dam MTase buffer and 1 × CutSmart buffer, an obvious fluorescence signal enhancement was observed upon addition of Dam MTase, while the addition of M.SssI or other methyltransferases did not show any change of fluorescence signal (Figure 3c), indicating high specificity of the sensing method, which might be originated from the fact that the interactions of MTase and *Dpn*I with their substrates are sequence-specific.[49−51]

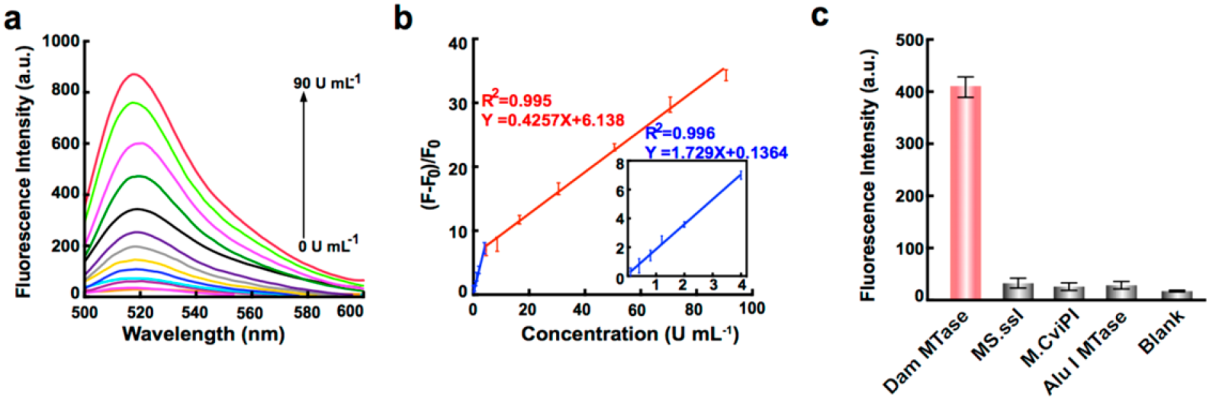

Figure 3. Fluorescence spectral response of the biosensing system to Dam MTase: (a) fluorescence spectra at 0, 0.1, 0.4, 0.8, 1.2, 2, 4, 8, 16, 30, 50, 70, and 90 U mL^{-1} Dam MTase, (b) calibration curve for Dam MTase concentration, and (c) fluorescence spectra with different methyltransferases (30 U mL^{-1}). Error bars show the standard deviation of three experiments.

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The stability of the DNA tetrahedron-based MTase-probe was investigated by incubating 500 nM DNA tetrahedron with or without DNase I, exonuclease III and Nb.BbvCI, respectively. As shown in Figure S3a, no significant fluorescence enhancement was observed with or without the treatment of DNase I, exonuclease III and Nb.BbvCI, respectively. PAGE assays also confirmed the minimal degration effect of nuclease treatment on DNA tetrahedron (Figure S3b), suggesting the stability of DNA tetrahedron-based MTase probe.

The reproducibility of the biosensing system was examined by repeatedly detecting its fluorescence signals at 1.0 and 30 U mL^{-1} Dam MTase for 8 consecutive days. The fluorescence signal showed a statistically insignificant change (Figure S4). The relative standard deviation (RSD) at 1.0 and 30 U mL^{-1} Dam MTase was 6.6% and 5.4%, respectively, suggesting that the developed method possessed a satisfactory reproducibility to accurately measure Dam MTase activity. Furthermore, recovery experiments were carried out by adding various concentrations of Dam MTase in *E. coli* culture medium, which showed the recoveries ranging from 98% to 107.5% (Table S3). Moreover, this method showed the recovery of 91.2%−107.8% for triplicate detection of Dam MTase spiked in human serum samples (Table S4), indicating its practical application in Dam MTase analysis of complex biological medium. Thus, this method had a potential prospect in the field of laboratory medicine.

Application for Evaluation of Inhibitors to Dam MTase Activity. Since Dam MTase is associated with the level of methylation in various diseases, the inhibition of Dam MTase activity may contribute to important applications in demethylation drugs development. 5-Fluorouracil,[52,53] azacitidine,[54,55] and decitabin,[55,56] approved by FDA for the treatment of cancer, are epigenetically targeted inhibitors. Considering that *Dpn*I involves in the methylation process, the influence of the inhibitors on the activity of *Dpn*I was first evaluated by methylating the DNA tetrahedron with Dam MTase and SAM and then adding *Dpn*I and different concentrations of inhibitors in the mixture. The fluorescence intensity gradually decreased with the increase of the inhibitor concentration (Figure 4a). 5-Fluoruracial showed negligible influence on the activity of *Dpn*I when its concentration was less than 1.2 μM. In contrast, azacitidine and decitabine down to 1.0 μM could obviously influence the activity of *Dpn*I. Thus, the effects of these inhibitors on the activity of Dam MTase were examined at the concentrations less than 1.0 μM, at which they hardly affect the activity of *Dpn*I. As shown in Figure 4b,c, all these inhibitors greatly decreased the activity of Dam MTase, which led to less methylated DNA tetrahedron and thus lower fluorescence intensity. With the increasing concentrations, the inhibitory effect of azacitidine and decitabine gradually enhanced compared with 5-fluorouracil. The half-maximal inhibitory concentration (50% relative activity) for 5-fluorouracil, azacitidine, and decitabine was 0.95, 0.73, and 0.86 μM, respectively. Azacitidine and decitabine are the well-known broad methyltransferase inhibitors,[56] so they inhibit Dam MTase more effectively than 5-fluorouracil. These results demonstrated that the proposed biosensing system has a practical application for screening of the MTase inhibitor.

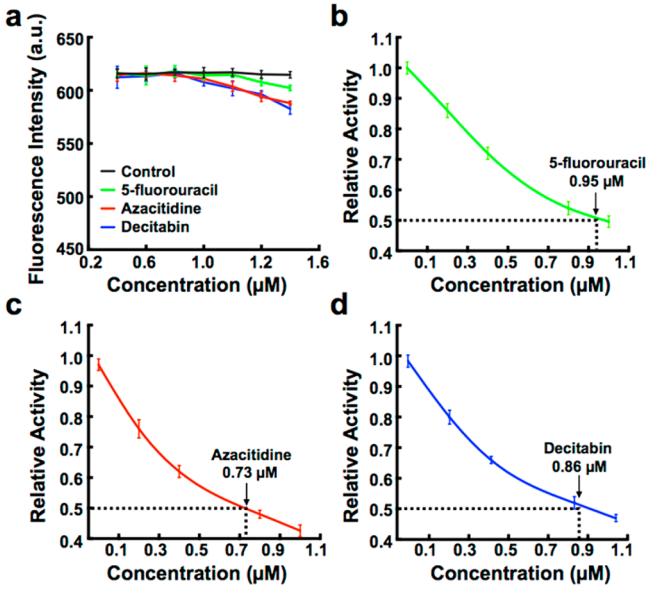

Figure 4. (a) Effect of inhibitors at different concentrations on *Dpn*I activity at 100 U mL^{-1}. (b−d) Relative activity of Dam MTase (40 U mL^{-1}) against (b) 5-fluorouracil, (c) azacitidine, and (d) decitabine at different concentrations. The relative activity denotes the ratio of fluorescence intensity in the presence to absence of inhibitor. Error bars indicate the standard deviations of three independent experiments.

CONCLUSIONS

This work uses DNA tetrahedron nanostructure to develop a fluorescence "off−on" approach for highly sensitive detection of Dam MTase activity. The multifunctional DNA tetrahedron nanostructure exhibits many outstanding merits, such as facile synthesis, stable and rigid structure, and rich modification sites. More importantly, the DNA tetrahedron nanostructure demonstrates highly efficient biosensing performance for sensing Dam MTase activity in real samples. The proposed biosensing system has been utilized for selectively and sensitively measuring Dam MTase with a lower detection limit than those reported previously, even with the help of nicking enzyme-assisted signal amplification. It has successfully been applied for screening inhibitors of methyltransferase. We anticipate that this strategy based on DNA nanostructures will contribute significantly for biological detection, clinical diagnostics, and drug discovery.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b13551.

Figures for PAGE analysis, condition optimization and reproducibility of sensing method, stability of DNA tetrahedron to nuclease treatment, and tables for oligonucleotids sequences, recovery results, and comparison among analytical performances (PDF)

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Notes

The authors declare no competing financial interest.

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