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Magnetic Nanoparticles-Cooperated Fluorescence Sensor for Sensitive and Accurate Detection of DNA Methyltransferase activity Coupled with Exonuclease III-Assisted Target Recycling

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Abstract

A fluorescence magnetic biosensor for DNA methyltransferase activity was developed based on cooperative amplification of combining with the magnetic nanoparticles synergistic exonuclease III (Exo III)-assisted circular exponential amplification and supramolecular structure ZnPPIX/G-quadruplex. Firstly, a duplex DNA probe, which is constructed by the hybridization of a quadruplex-forming oligomer with a molecular beacon, is assembled on the magnetic nanoparticles (MNPs) as reporter. A hairpin probe (HP) containing sequence of GATC was used as the methylation substrate of DNA adenine methyltransferase (DAM). Once the HP was methylated by DAM, it could be recognized and cleaved by Dpn I, which allows the release of a single-stranded DNA. The DNA (tDNA1) then hybridizes to the MNP probe, which subsequently triggers the exonuclease III-mediated target exponential recycling reaction. Simultaneously, numerous quadruplex forming oligomers are liberated and folded into G-quadruplex-ZnPPIX complexes with the help of zinc(II)-protoporphyrin IX(ZnPPIX) on the MNP surface to give a remarkable fluorescence response. In the developed sensor, a small amount of target DAM can be converted to a large number of stable DNA triggers, leading to a remarkable amplification for the target. Moreover, using MNPs as a vector of the sensor may reduce the interference from the real samples, which increases the anti-interference of the sensing system. Based on this unique amplification strategy, a very low detection limit down to 2.0×10^{-4} U/mL is obtained. Furthermore, the sensor can be commendably used for evaluating DAM activity in different growth stages of *E. coli* cells and screening Dam MTase inhibitors. Therefore, the strategy proposed here provides a promising platform for monitoring activity and inhibition of DNA MTases, and has a great potential to be further applied in early clinical diagnostics and medical research.

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Introduction

DNA methylation, a crucial epigenetic modification of the genome, plays a pivotal role in gene expression, cellular differentiation, and pathogenesis of various human diseases[1-3]. It is closely associated with the activity of DNA methyltransferase (MTase) [4-6]. Alterations in MTase activity may lead to aberrant DNA methylation patterns. Abnormalities in MTase activity usually occur far before other signs of malignancy and could thus be used for early cancer diagnosis. Moreover, recent researches demonstrate that DNA methyltransferases have become predictive biomarkers and potential therapeutic targets in a variety types of cancer[7, 8]. Thus, sensitive and accurate activity assay and inhibitor (anti-methylation drugs) screening for MTases are of great importance for both clinical diagnostics and therapeutics. In addition, effective assay of MTase activity can help in understanding their roles in cancer initiation and progression.

The conventional methods for the DNA MTase assay, including PCR-assisted methods[9], radioactive labeling techniques[10], gel electrophoresis[11], high performance liquid chromatography [12] have been widely used for MTase activity analysis. Except these methods, various new approaches such as colorimetric[13, 14], fluorometric[15, 16], chemiluminescent[17], surface enhanced Raman spectroscopy (SERS)[18], electrochemical[19, 20] have been recently utilized toward the detection of MTase activity. Especially, signal amplification strategies are often combined in the fabrication of gene-specific methylation detection and MTase activity assay. Examples of signal amplification strategies include the use of various nanomaterial-based as amplifying labels[5, 21, 22] and isothermal amplification techniques[14, 17, 23-28]. A variety of DNA MTase activity assay relying on signal amplification strategies have been developed to improve sensitivity. Among them, much attention has been focused on isothermal amplification techniques-based DNA MTase assay in solution, such as strand displacement amplification (SDA)-based assay[14], nicking enzyme signal amplification (NESA)-based assay[17], rolling circle amplification (RCA)-based assay[23, 24] and exonuclease-assisted signal amplification-based assay[25-28]. Especially, exonuclease-assisted signal amplification has become increasingly popular in the

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DNA MTase activity detection due to its simplicity, versatility, and high sensitivity[25-28]. However, in most reported exonuclease-assisted signal amplification-based assay in solution, these methods show moderate sensitivity with detection limits being 0.004 - 0.04 U/mL. In addition, in the implementation of these homogeneous methods for the assay of complex biological samples, there might be some interferences arising from possible endogenous chemicals present in the matrices by perturbing the activity of enzymes, which will affect the accuracy and sensitivity of detection[29]. Although the homogeneous methods are simple in the experimental operation, they show low sensitivity, and are at a disadvantage for the assay of complex biological samples in its practical use. So, new, sensitive and accurate amplification strategies for DNA MTase activity analysis are critical and in urgent need.

Currently, micro- or nanometer sized magnetic particles (beads) has become extremely popular in biological applications due to its unique and excellent physical and chemical stability, minimal background signals, large surface area and size effect[30, 31]. It has been reported that the large surface area of magnetic particles (beads) can significantly improve the detection sensitivity[31]. Experiments also showed that magnetic particles (beads) can expediently implement the separation steps under a magnetic field in the complex matrices, achieving minimal background signals[32, 33]. The extraordinary magnetic separation of magnetic particles, in combination with the large surface area, has been employed to elaborately design and construct universal biosensing platforms in homogeneous solution[34]. In addition, researchers have proven that magnetic particles can protect DNA probes against enzymatic digestion by nuclease due to a steric hindrance effect of magnetic particles that prevents nuclease from binding to the DNA[35]. These attractive characteristics inspired us to constitute new magnetic particles-based analysis platform for DNA MTase activity analysis.

Herein, we developed a highly sensitive and accurate detection method of DNA MTase (using Dam MTase as an example) on the basis of cooperative amplification of combining with the magnetic nanoparticles synergistic exonuclease III (Exo III)-assisted circular exponential amplification and supramolecular structure ZnPPIX/G-quadruplex. Although the use of nanomaterial as amplifying labels or

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isothermal amplification techniques for MTase activity detection has been reported previously, these previous studies did not involve coupling of the isothermal amplification techniques to nanomaterial (magnetic nanoparticles) to achieve cooperative amplification and perform fluorescence detection. In the proposed method, first, a DNA strand designed as quadruplex-forming oligomer was engineered to form inactive hybrids by binding with a molecular beacon and assembled on the surface of MNP as detection probe for the DNA MTase assay. The hybrids can be protected from enzymatic digestion effectively after binding onto MNP surface. A hairpin probe (HP) with the sequence of 5'-G-A-T-C-3' in the stem part, which functions as the reaction substrate, is catalyzed to a methylated hairpin probe (5'-G-mA-T-C-3') by Dam MTase in the presence of SAM. After that, the methylation-sensitive restriction endonuclease Dpn I cleaves the methylation-responsive sequence of hairpin probe, producing a short DNA product (tDNA1). The tDNA1 can then hybridize to the 3' protruding terminus of hybrids on the surface of MNP, which subsequently triggers the next Exo III-mediated target recycling reaction on the surface of MNP. Finally, a circular exponential amplification for the target Dam can be achieved. Moreover, the incorporation of ZnPPIX into G-quadruplex structures leads to similar enhancement of the fluorophore fluorescence. In the proposed strategy, a small amount of target Dam MTase can be converted to a large number of stable DNA triggers, leading to a remarkable amplification and improving the detection sensitivity. Due to the cooperative amplification and the low fluorescence background by MNPs, the magnetic nanoparticles synergistic exonuclease III (Exo III)-assisted circular exponential amplification allows sensitive and accurate quantification of Dam MTase with a sensitivity of 2.0×10^{-4} U/mL. Furthermore, Dam MTase activity in different growth stages of E. coli cells and the application of screening Dam MTase inhibitors was also performed with satisfactory results, indicating that this strategy will become a reliable and sensitive Dam MTase quantification method in complex biological matrices.

Scheme 1

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Experimental Section

Chemicals

All the HPLC-purified oligonucleotide sequences were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) and listed below: quadruplex-forming oligomer (oligo 1): 5'-CAC TGG GTT GGG CGG GAT GGG TTT TTT-$(CH_2)_6$-biotin-3'; unlabeled molecular beacon(MB): 5'-TCT CCA ACT ATG TTA TCC CGC CCA ACC CAG TGA GTT GGA GA-3'; hairpin probe(HP): 5'-GAG ATC AAG GTC TGA CTT TTT GTC AGA CCT TGA TCT CCA ACT GAA CAC-3'; Protoporphyrin IX zinc (II) (ZnPPIX) was purchased from Sigma-Aldrich and used without further purification. Streptavidin-MNBs (350 nm diameter) were from Bangs Laboratories Inc (Fishers, IN). Dam MTase and S-adenosylmethionine (SAM) were obtained from New England Biolabs Inc. Exonuclease III (Exo III), gentamycin, 5-fluorouracil and benzylpenicillin were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Other chemicals (analytical grade) were obtained from standard reagent suppliers. Water (≥ 18.2 MΩ) was used and sterilized throughout the experiments.

Apparatus

All the fluorescence measurements were performed on a Hitachi F-7000 spectrofluorimeter (Hitachi, Japan). The excitation wavelength was 418 nm, and the spectra are recorded between 580 and 655 nm. The fluorescence emission intensity was measured at 592 nm.

Magnetic nanoparticle probe preparation

The DNA duplex probe was first prepared by mixing oligo 1 and MB in molar ratio of 1：1.5 in 10 mM PB solution (pH 7.4, 0.1 M NaCl). The DNA sequences were first heated to 90 °C for 5 min and then allowed to cool to room temperature before use. The cleaned streptavidin-magnetic nanoparticle was incubated into the above DNA duplex probe solution and gently shaking for 2 h at room temperature and then thoroughly rinsed with PBS. The nonspecific DNA duplex probe was removed with

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0.15 mol/L NaOH solution. Then, the functionalized magnetic nanoparticle was washed three with 100 μL of PBS buffer. All washing steps in this work were performed under a magnetic field.

Methylation and Cleavage of Hairpin Probe

The methylation of hairpin probe was carried out in 20 μL of reaction mixtures containing various amounts of Dam MTase, 400 nM HP, 1× Dam buffer (50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM EDTA, 5 mM 2-mercaptoethanol), and 160 μM SAM at 37 °C for 1 h firstly. After methylation, 20 μL of methylation products was added to 20 μL of buffer solution containing 1× NEB buffer 4 (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 1 mM DTT (pH 7.9)) and 10 U Dpn I for the cleavage reaction. The mixture was incubated at 37 °C for 1 h, followed by inactivation at 80 °C for 20 min.

Exo III-Assisted Cycling Amplification and Fluorescence Dam MTase detection

The DNA duplex probe functionalized magnetic nanoparticle was then mixed with the aforementioned methylated mixture, followed by the addition of 30 μL 10 mM Tris-HCl solution (pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$) containing 60 unit Exo III to incubate for 2 h at 37 °C. Then, 10 μL 20 μM ZnPPIX solution were added and incubated at 37 °C for 30 min to induce the liberated oligo 1 on the magnetic nanoparticle to fold into a ZnPPIX/G-quadruple supramolecular structure complex. The resulting magnetic nanoparticle was thoroughly rinsed three times with PBS buffer under a magnetic field. Finally, the appropriate buffer (20 mM HEPES) were added and made the mixture last volume 50 μL. The fluorescence intensity of the mixture solution was measured in a 100 μL quartz cuvette at room temperature. The fluorescent spectra were measured using a spectrofluorophotometer. The excitation wavelength was 418 nm, and the spectra are recorded between 570 and 650 nm. The fluorescence emission intensity was measured at 592 nm. The control experiments were carried out under the same condition without adding Dam MTase.

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Gel electrophoresis

Gel electrophoresis was used to confirm the feasibility of the methylation process of Dam MTase. Samples for gel electrophoresis assays were prepared as follows: (1) hairpin probe and Dam MTase; (2) hairpin probe and Dpn I; (3) hairpin probe, Dam MTase, and Dpn I. The samples were put on a 12 % nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1×TBE (9 mM Tris-HCl (pH 7.9), 9 mM boric acid, 0.2 mM EDTA) with ethidum bromide (EB) staining. The imaging of the gel was performed using Bio-Rad Gel Doc XR+ imaging systems

Dam MTase inhibition evaluation

To further extend the potential application of this assay in the inhibition assay, the influence of drugs on Dam MTase activity was investigated by using gentamycin, 5-fluorouracil, and benzylpenicillin. First, the influence of inhibitor, these drugs, on the activity of Dpn I and Exo III was investigated by mixing Dam MTase, SAM, and the HP for 1 h to ensure the absolute methylation. Then, 10 units Dpn I, 60 unit Exo III and 2 μM different inhibitors were added. The following procedures were the same as those describe above. Subsequently, the influence of drugs on the activity of Dam MTase was evaluated. All the inhibition experiments were carried out in conditions similar to those of Dam MTase activity assay except for 1 μM concentrations of different inhibitors in the samples. Briefly, the influence of different inhibitors on the activity of Dam MTase was investigated by mixing Dpn I, SAM, different inhibitors, and the HP. Then, 1.0 unit of Dam MTase was added. The following procedures were similar as above. In addition, to investigate the relationship between the concentration of 5-fluorouracil and the inhibition ratio, different 5-fluorouracil concentrations were added into these samples. The following procedures were the same as those described above.

Culture of Bacterial Cells

The DH5a (Dam positive) and JM110 (Dam negative) *E. coli cells* were cultured according to the procedure from the literature with slight modifications. Briefly, a

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colony was inoculated into 3 mL of liquid medium (5 g/L yeast extract, 10 g/L Trypton, 10 g/L NaCl) and incubated at 37°C in a shaker (250 rpm) for 12 h. Then, 500 μL of the cell suspension was subsequently added into 50 mL of medium and incubated for 2.5 h or 12 h. Subsequently, 3 mL of the cell suspension was centrifuged at 13000 rpm for 30 s to obtain a cell pellet followed by washing twice with Milli-Q water. The resulting *E. coli cells* were lysed using lysis buffer. The protein contents were determined using the Bradford Protein Assay Kit according to the manufacturer's recommended protocol.

Results and discussion

Principle of the Magnetic Nanoparticles Synergistic Exonuclease III (Exo III)-assisted Circular Exponential Amplification Assay for Dam MTase

The mechanism of our sensing system is depicted in **Scheme 1**. As can be seen, the Dam MTase assay involves three principal processes: (1) magnetic nanoparticle probe preparation, (2) a Dam MTase and Dpn I endonuclease coupling reaction, and (3) exonuclease III-assisted target recycling amplification and fluorescence detection. In the first step, an inactive duplex DNA hybrid probe was first prepared in the solution by hybridizing a DNA strand designed as a quadruplex-forming oligomer with a traditional unlabeled molecular beacon (MB). The molecular beacon contains three domains, a loop region that is complementary to the quadruplex-forming oligomer, a "target" binding stem region I that is complementary to part of the sequence of the trigger DNA, and a stem region II in which part of the sequence is complementary to the sequence of region I. The duplex DNA hybrids probe was immobilized on the magnetic nanoparticle by a strong biotin/streptavidin interaction as detection probe for the DNA MTase assay. In the second step, a hairpin probe (HP) containing sequence of 5'-G-A-T-C-3' was used as the methylation substrate of DAM. Once the hairpin substrate was methylated by DAM, it could be recognized and cleaved by Dpn I, which allows the release of a single-stranded oligodeoxynucleotide (ssODN). The ssODN then as "target" DNA (tDNA1) hybridizes to the duplex DNA probe immobilized on the magnetic nanoparticle and initiates exo III-assisted "target"

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recycling amplification. This duplex DNA probe is ingeniously designed with the protruding 3'-end and cannot be digested by Exo III which specifically cleaves duplex DNA from blunt or recessed 3'-termini. When the probe is challenged with tDNA1, the hybridization of tDNA1 with the dangling ssDNA at the 3'-end of molecular beacon leads the probe to have a blunt 3'-terminus. Exo III then can catalyze the stepwise removal of mononucleotides from this terminus, releasing tDNA1 and ultimately liberating the quadruplex-forming oligomer. Thus, the fluorescent supramolecular structure, ZnPPIX/G-quadruplex, can be formed on the magnetic nanoparticle surface with the help of ZnPPIX to give a fluorescence response. Furthermore, the released tDNA1 is free to bind to another 3'-protruding terminus of duplex probe to trigger a new cleavage process, which constitutes the cycle I in Scheme 1. At the same time, the DNA fragment II (the region II of MB) is also released during each cleavage process, which can be used as a secondary target DNA (tDNA2) to activate the successive cleavage process. The tDNA2 has a longer base sequence than that of region I. After its recognition with duplex DNA probe, the Exo III can also only catalyze the cleavage of duplex DNA probe, releasing again the DNA fragment II as secondary trigger DNA (tDNA2) for the next cleavage cycle and liberating the quadruplex-forming oligomer for amplified fluorescence response. This constituted the cycle II in Scheme 1. On the basis of the autonomous cleavage in cycle I and II, a small amount of target Dam can be converted to a large number of stable DNA triggers, leading to a remarkable amplification for the target. Therefore, the current strategy by a functionalized magnetic nanoparticles synergistic exonuclease III (Exo III)-assisted circular exponential amplification is hopeful for sensitively monitoring activity of Dam MTase.

The feasibility of the designed sensing system

To demonstrate the methylation process of Dam MTase, a nondenaturating PAGE experiment with ethidum bromide (EB) as the fluorescent indicator was carried ou. As shown in **Fig. 1A**, there is only one band of the original probe when either Dpn I or Dam MTase is absent (**Fig. 1A, lanes 1 and 2**), indicating that no

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methylation/cleavage event occurs. As a comparison, when both Dam MTase and Dpn I are present, a new band can be observed (**Fig. 1A, lane 3**), suggesting that a methylation reaction happens, and the methylated DNA is then cut into cleavage segments.

Fig. 1

To investigate the feasibility of the proposed strategy for Dam MTase activity assay, the progress of the reaction was monitored via the fluorescence measurements. As shown in **Fig. 1B**, in the presence of only MNPs/oligo 1/MB and ZnPPIX, a negligible fluorescence signal was observed (curve a) due to the "caged" inactive G-quadruplex sequence in MNPs/oligo 1/MB probe. With the addition of HP, Dpn I and Exo III, the fluorescence signal barely changed (curve d), because in the absence of the target (Dam MTase), the methylation of HP did not occur, resulting in no active G-quadruplex sequence and thus no fluorescence signal amplification. With the further addition of the target (Dam MTase) to the reaction system, a significantly increased signal was observed (curve e), indicating the occurrence of the methylation of HP, the cleavage of the methylated HP, as well as the two subsequent cycling cleavage processes illustrated in Scheme 1. However, in the absence of Dpn I (curve b) or Exo III (curve c), small fluorescence signals were observed, which clearly demonstrated that both the cleavage of the methylated HP by Dpn I and the subsequent Exo III assisted target recycling were necessary to achieve significantly amplified signals.. Therefore, these results clearly demonstrated the feasibility of functionalized magnetic nanoparticles synergistic exonuclease III (Exo III)-assisted circular exponential amplification strategy for MTase activity assay.

To further verify the feasibility of the proposed strategy for Dam MTase activity assay, the fluorescence image of MNPs obtained in the presence of Dam MTase and in a control experiment are depicted in **Fig. 2**. As shown in **Fig. 2**, a well-defined fluorescent image of MNPs can be observed in the presence of Dam MTase. This fluorescent image can be contributed to the trigger DNA (tDNA1) initiating Exo III-aided recycling amplification in the presence of Dam MTase, generating the fluorescent ZnPPIX/G-quadruplex supramolecular structure functionalized magnetic

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nanoparticle. In the presence of Dam MTase, hairpin probe is catalyzed to a methylated hairpin probe (5'-G-mA-T-C-3') by Dam MTase. Then, the methylated hairpin probe is cleaved to generate trigger DNA (tDNA1) by the methylation-sensitive restriction endonuclease Dpn I. The tDNA1 initiates Exo III-aided DNA recycling amplification in the surface of MNPs, liberating the activy G-quadruplex structure in the surface of MNPs and further generating the fluorescent ZnPPIX/G-quadruplex supramolecular structure functionalized magnetic nanoparticle in the help of ZnPPIX. In the absence of Dam MTase, it was noted that no bright spots can be observed. A possible reason was that no methylation event occurs. Thus, the hairpin probe can not be cleaved to generate trigger DNA (tDNA1) by the methylation-sensitive restriction endonuclease Dpn I. Exo III-aided DNA recycling amplification was inhibited. The G-quadruplex sequence is "caged" by the hybridization with a traditional molecular beacon. The caged structure of the G-quadruplex prohibits the formation of the fluorescent ZnPPIX/G-quadruplex supramolecular structure in the surface of MNPs. These results indicated that the magnetic nanosensor has been successfully achieved.

Fig. 2

Optimization of assay conditions

Effect of DpnI and SAM concentration

In the the methylation and cleavage reation of hairpin probe, the effects of the concentrations of DpnI and SAM were investigated, respectively. Dpn I endonuclease can only cut the sequence of 5'-G-Am-T-C-3' when the internal adenine is methylated. Therefore, it is important to investigate the effect of Dpn I concentration on the assay. As can be seen from **Fig. S1A(see supporting information in ESI†)**, with the increasing concentrations of DpnI, the fluorescence intensity increased and tended to a maximum at 8 unit. Thus, 10 unit of DpnI was chosen for the following experiments.

As the donor of methyl group, SAM is the critical factors in DNA methylation process catalyzed by Dam MTase. Therefore, it was necessary to optimize the

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concentration of SAM. **Fig. S1B(see supporting information in ESI†)** shows the effects of the concentration of SAM on the fluorescence signal. It could be seen that the fluorescence signal increased gradually with an increase in the concentration of SAM concentration, and then reached an equilibrium value at the concentration of 80 mM. However, considering that SAM is unstable in vitro experiments, a higher concentration of 160 mM was employed for the sensing system.

Effect of molar ratio of oligo 1 to MB, the concentration of oligo 1 and the amounts of Exo III

In the sensing system, the assembly density of duplex DNA probe on the MNPs surface would have an important effect on the performance of DNA methyltransferase activity assay. The full hybridization equilibrium between oligo 1 and MB is a key factor for ensuring efficient assembly density of duplex DNA probe on the MNPs surface. Therefore, the effect of molar ratio of oligo 1 and MB was evaluated using a fixed concentration of oligo 1, namely 2 μM. As shown in **Fig. S2A(see supporting information in ESI†)**, the fluorescence intensity increased with the increase in molar ratio. When the ratio value reached 1 : 1.5, the maximum net signal (F-F0) was achieved, where F and F0 are the fluorescence intensities of the sensor in the presence and absence of Dam MTase, respectively. Thereafter, the fluorescence response exhibited a gradual decrease with a further increase in the molar ratio. This was probably because a large excess of MB disturbed their hybridization with oligo 1 and the assembly of duplex DNA probe on the MNPs surface. As a result, the molar ratio of 1 : 1.5 was selected for further investigation. In addition, oligo 1 also serves as a signal probe for the formation of the fluorescent ZnPPIX/G-quadruplex supramolecular structure after the assembly of duplex DNA probe on the MNPs surface. Therefore, the concentration of oligo 1 was optimized. **Fig. S2B (see supporting information in ESI†)** shows the variance of fluorescence intensity with the concentration of oligo 1. As a result, 2 μM was selected as the optimal concentration due to its maximum net signal (F-F0).

In addition, Exo III-aided DNA recycling amplification was a crucial step. To achieve the best sensing performance of the system, the amount of Exo III was

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optimized in the presence of 50 U/mL Dam methyltransferase. As shown in **Fig. S2C (see supporting information in ESI†)**, the fluorescence response to Dam MTase increased quickly with increasing Exo III concentration (the black line). However, in the absence of Dam MTase, the control group also showed a slight increase in background fluorescence intensity (the red one). As a result, the signal to background ratio (S/N) reached the maximum value at the Exo III amount of 60 U. Thus, 60 U of Exo III was chosen as the optimal experimental conditions, and used in the subsequent experiments.

Effect of the time for DNA methylation and the reaction time of Exo III

Performance of the proposed sensing system is still strongly influenced by other assay conditions such as the reaction time of DNA methylation and Exo III reaction. The time for DNA methylation is an important factor for the sensing systems. The trigger primer for Exo III-assisted recycling amplification reaction derived from the methylation and cleavage reaction. Therefore, the different methylation reaction time were evaluated. It is clear from **Fig. S3A(see supporting information in ESI†)** that the response increases substantially when reaction time changes from 0 to 60min. No significant increase in the sensor response occurred from 60 to 100 min. As a result, the optimal reaction time for the methylation was selected as 60 min in subsequent studies due to its maximum net signal (F-F0). To achieve the best sensing performance, a long Exo III reaction time are expected to generate large numbers of "uncaged" G-quadruplex. Therefore, we further investigated the influence of Exo III-assisted recycling amplification reaction time upon the fluorescence signal. As shown in **Fig. S3B (see supporting information in ESI†)**, in the presence of 60 U of Exo III, the net signal (F-F0) reached maximum at the reaction time of 120 min. Therefore, the Eox III reaction time of 120 min is used in the subsequent research.

In the fluorescence sensor, the fluorescence signal generated by ZnPPIX/G-quadruplex is dependent on the amount of the ZnPPIX molecule bound to G-quadruplex. Therefore, the concentration of ZnPPIX was also optimized. As shown in **Fig. S3C (see supporting information in ESI†)**. The experimental results indicated that a concentration of 20 μM ZnPPIX could provide maximum S/N ratio

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for the sensing system.

Fluorescence Detection of Dam MTase Activity.

To investigate the analytical performance of the proposed method, we measured the Dam MTase at various concentrations under the optimal conditions. **Fig. 3A** demonstrates the fluorescence emission spectra of the sensing system upon the different Dam MTase concentrations. As can be seen, as the concentration of Dam MTase increases, large numbers of trigger DNA (tDNA1) are produced for Exo III-aided DNA recycling amplification to synthesize the ZnPPIX/G-quadruplex, resulting in the increase of fluorescence intensity correspondingly. As shown in the inset of **Fig. 3B,** a good linear relationship between the fluorescence intensity change, F-F0 (in which F0 and F are the fluorescence intensity detected in the absence and presence of Dam MTase, respectively) and the logarithm of Dam MTase concentration ranging from 0.0005 to 50 U/mL was obtained, with a regression equation of (F-F0) = 1195 + 326.5·log C and a correlation coefficient of 0.992. The limit of detection of Dam Mtase was approximately 2.0×10^{-4} U/mL in terms of the rule of 3 times standard deviation over the blank response, which was lower than previously reported assays **(Table S1 ESI†)**. Such extremely high sensitivity of the proposed method can be attributed to following two factors: (i) the high amplification efficiency of Exo III-aided exponential amplification and enhancement of the fluorescence ZnPPIX/G-quadruplex, (ii) lowered background signal interference due to using MNPs as the separation. In addition, the relative standard deviation (RSD) for five replicate determinations of Dam MTase from the same batch at the 0.5 U/mL level was 3.5 % and the RSD for interday was 4.3 %, indicating an acceptable repeatability of the proposed method. These results suggest that the proposed method can be used for quantitatively analyzing MTase activity conveniently and efficiently.

Fig. 3

We further evaluated the selectivity of this assay for Dam activity. To investigate the selectivity of the proposed method, M.Sss I and HhaI that function as

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methyltranferases with the recognition sequence of 5'-CG-3'and 5'-GC-3', respectively, were selected as interference methyltranferases to assess the selectivity of this method. Due to the specific site recognition of Dam MTase toward its substrate, the proposed method can easily discriminate Dam MTase from M.Sss I and HhaI MTase. As shown in **Fig. 4A**, significant fluorescence enhancement is observed in the presence of Dam MTase. In contrast, no distinct fluorescence signal is observed in the presence of M.SssI and HhaI. These results demonstrated that the amplified strategy for Dam MTase activity exhibited a good selectivity originating from the high specific sequence recognition between Dam Mtase and the hairpin probe.

Fig. 4

Assay of Dam Activity in *E. coli* cells

A big challenge for an excellent enzyme activity assay is its ability to be applied in real biological samples. To determine whether the as-proposed method could be applied in real samples, we investigated the endogenous DAM activity of *E. coli* cells in different growth stage using using our developed method. The exponential growth stage of *E. coli* occurred from 1.5 to 3 h, and then the growth rate slowed down significantly and subsequently entered into the stationary growth stage after 12 h. The result showed that the activity of Dam is slightly higher in the exponential growth stage than in the stationary growth stage (**Fig. 4B**) with the Dam activity being 0.59 ± 0.05 U/mg and 0.45 ± 0.04 U/mg, respectively. This phenomenon may be attributed to the higher level of DAM at the exponential growth stage, which is required for the fast growth of *E. coli* cells[36]. In addition, we also evaluated Dam activity was observed in DAM-negative *E. coli* of JM110, no obvious Dam activity was observed (**Fig. 4B**). These results suggested that the obtained signal is derived from Dam activity, not from the nonspecific cleavage of HP probe. Taken together, the developed assay can be readily applied to evaluate Dam activity in complex biological samples.

Dam MTase activity inhibition evaluation

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The pharmacological inhibition of DNA MTase may contribute to important applications in antibiotic development. We choose two antibiotics (benzylpenicillin and gentamycin) and one anticancer drug (5-fluorouracil) as model to investigate the influence of inhibitors on DNA methylation. Given Dpn I and Exo III enzymes involved in the DNA methylation process, it was essential to ensure whether these drugs have influence on them. Control experiments were carried out as described in experimental procedures. The result indicated that all the inhibitors (gentamycin, 5-fluorouracil, and benzylpenicillin) exhibited negligible influence on the activity of both Dpn I and Exo III when the concentration of each inhibitor was 1.0 μM **(Fig. S4 see supporting information in ESI†)**. As a result, 1.0 μM of different inhibitors was used to estimate the influence on Dam MTase activity. As presented in **Fig. 5A**, all drugs could inhibit the activity of Dam MTase. In comparison with other inhibitors, the 5-fluorouracil, a well-known broad spectrum anticancer drug, exhibited the strongest inhibitory effect at the same concentration.

In addtion, we further investigated the concentration dependant inhibitory effect of 5-fluorouracil on the activity of Dam MTase. It is observed in **Fig. 5B** that the inhibitory effect gradually enhanced with the increase of inhibitor concentration. Finally, the 5-fluorouracil were found to inhibit 50 % Dam MTase activity (The inhibitor concentration required to reduce Dam MTase activity by 50 %, was obtained from the plot of relative activity versus inhibitor concentration.) with 0.58 ± 0.03 μM, These findings demonstrated that the proposed sensor can be used to study the MTase inhibitor and applied for screening of MTase inhibitors.

Conclusion

In summary, we have successfully developed a versatile, highly sensitive and selective fluorescence sensing strategy for sensitive and accurate detection of the activity and inhibition of MTase based on cooperative amplification of the magnetic nanoparticles synergistic Exo III-assisted circular exponential amplification and supramolecular structure ZnPPIX/G-quadruplex. Such a strategy using MNPs shows a low background, and exhibits stable and sensitive sensing results, guaranteeing the

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reliable quantitative detection in complex biological samples. In the sensor, Exo III-assisted target exponential amplification reaction can be performed in an isothermal condition without the requirement for high-precision thermal cycling, making the proposed method more simple and cost-effective. By taking advantage of cooperative amplification, the proposed method exhibits excellent sensitivity with a detection limit of 2.0×10^{-4} U/mL. Owing to the specific site recognition of MTases, this proposed approach could discriminate Dam from other methyltranferases with high selectivity. The application in *E. coli* cell demonstrated that the assay has a great potential for sensitive detection of Dam activity in complex biological samples due to using MNPs as the separation and amplification elements. Importantly, the proposed method can be applied to screen inhibitors of Dam and might be further applied for early clinical diagnosis. In addtion, with minor modification of the hairpin probe and the change of corresponding restriction endonuclease, the proposed sensing strategy might be used as a universal method for the detection of other DNA MTases such as cancer-related Dnmt1. Given attractive analytical characteristics, the sensing strategy might find many important applications in clinical diagnosis and biomedical research.

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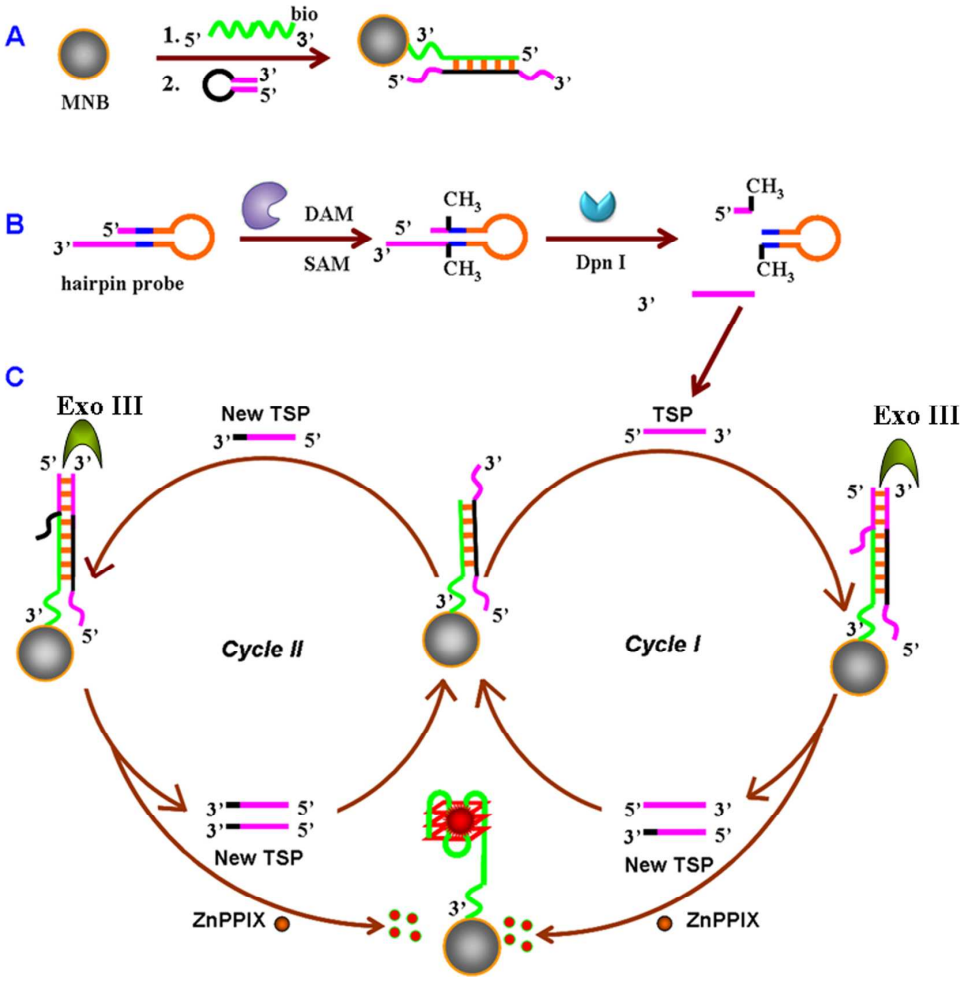

Scheme 1. Schematic Illustration of the magnetic nanoparticles synergistic exonuclease III (Exo III)-assisted circular exponential amplification for Dam MTase activity.

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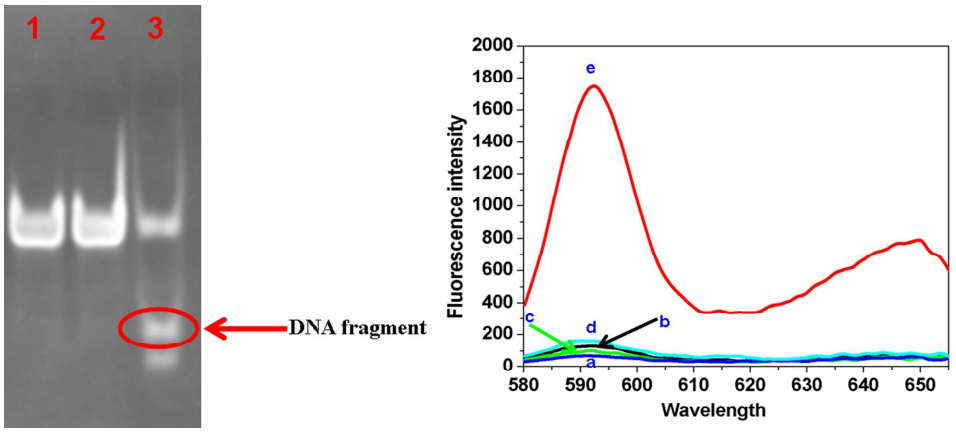
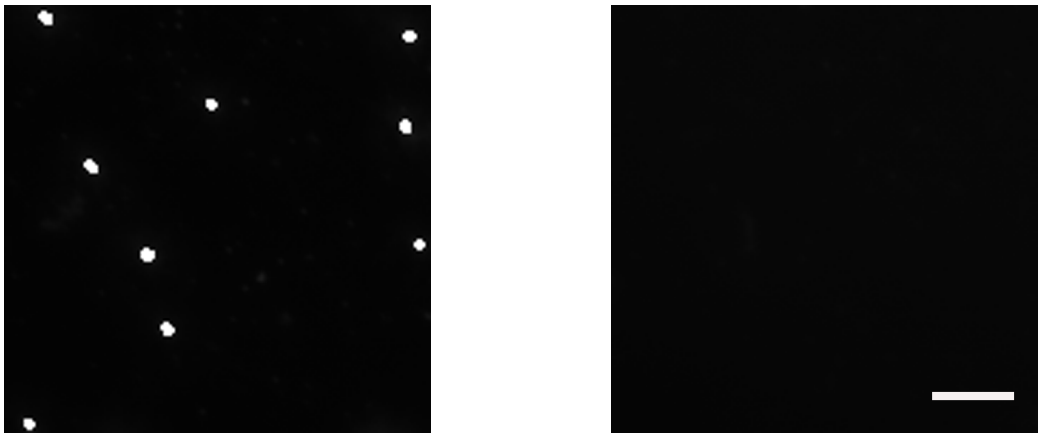


(A) **(B)**

Fig. 1. (A) Gel electrophoresis analysis of Dam MTase. The samples are treated (lane 1) HP + Dam MTase (5 U/mL); (lane2) HP + Dpn I (10 U); (lane 3) HP + Dam MTase (5 U/mL) + Dpn I (10 U). The methylation time for gel electrophoresis is 1.5 h. **(B)** Fluorescence emission spectra for the biosensing platform containing: (a) MNPs/oligo 1/MB + ZnPPIX, (b) HP + Dam MTase (50 U/mL) + MNPs/oligo 1/MB + Exo III (60 U) + ZnPPIX, (c) HP + Dam MTase (50 U/mL) + Dpn I (10 U)+ MNPs/oligo 1/MB + ZnPPIX, (d) HP + Dpn I (10 U) + MNPs/oligo 1/MB + Exo III (60 U) + ZnPPIX, and (e) HP + Dam MTase (50 U/mL) + Dpn I (10 U)+ MNPs/oligo 1/MB + Exo III (60 U) + ZnPPIX.



(A) **(B)**

Fig. 2. Fluorescence microscopy images of a glass slide drop-coated with MNPs solution using (A) 0.005 U/mL Dam methyltransferase and (B) a control experiment. Scale bar, 30 pixel.

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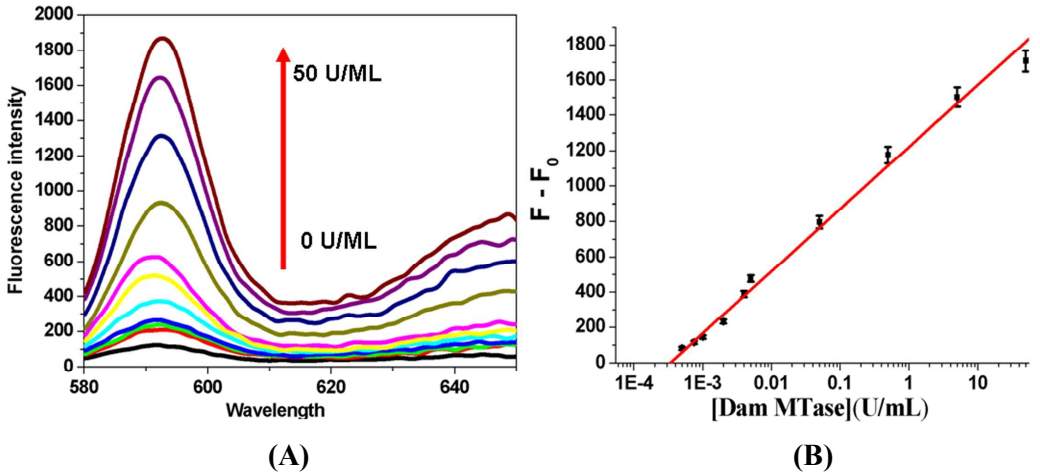

Fig. 3. (A) Fluorescence emission spectra in the presence of different concentrations of Dam MTase ranging from 0 to 50 U/mL, (B) Relationship between thefluorescent response and the logarithm of Dam MTase concentrationthe from 0.0005 to 50 U/mL.

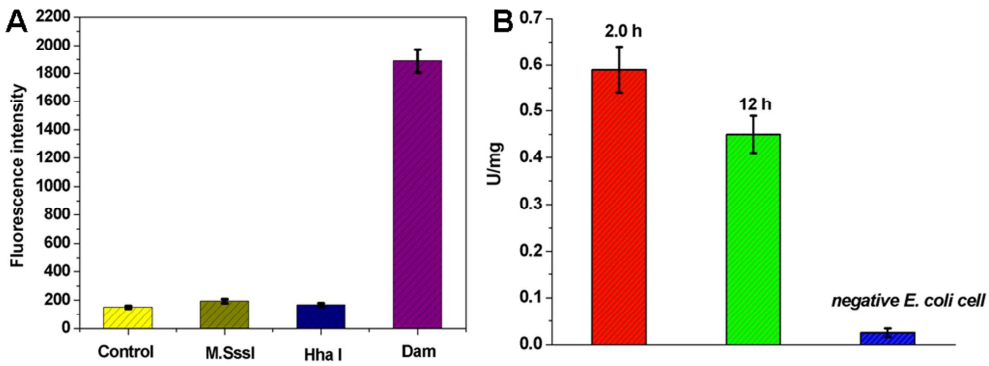

Fig. 4. (A) Selectivity of the sensing system. Both the concentrations of Dam Mtase, M.Sss I and HhaI are 50 U/mL.(B) The activity of DAM at different growth stages of E. Coli cells DH5a and in DAM negative E. Coli cells JM110.

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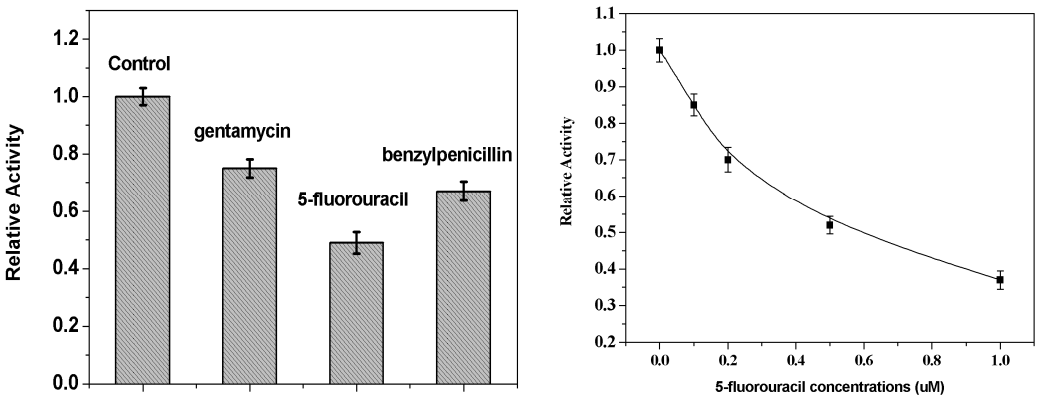

（A） （B）

Fig. 5 (A) Effect of different inhibitors on the activity of Dam MTase. (B) The inhibitory effect of different concentrations of 5-fluorouracil on Dam MTase activity. The concentration of Dam MTase is 50 U/mL.