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A novel label-free fluorescence strategy for methyltransferase activity assay based on dsDNA-templated copper nanoparticles coupled with an endonuclease-assisted signal transduction system†

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Evaluating DNA methyltransferase (MTase) activity has received considerable attention due to its significance in the fields of early cancer clinical diagnostics and drug discovery. Herein, we proposed a novel label-free fluorescence method for MTase activity assay by coupling double-stranded DNA (dsDNA)-templated copper nanoparticles (CuNPs) with an endonuclease-assisted signal transduction system. In this strategy, dsDNA molecules were first methylated by DNA adenine methylation (Dam) MTase and then cleaved by the methylation-sensitive restriction endonuclease DpnI. The cleaved DNA fragments could not act as efficient templates for the formation of fluorescent CuNPs and thus no fluorescence signal was produced. Under optimized experimental conditions, the developed strategy exhibited a sensitive fluorescence response to Dam MTase activity. This strategy was also demonstrated to provide an excellent platform to the inhibitor screening for Dam MTase. These results demonstrated the great potential for the practical applications of the proposed strategy for Dam MTase activity assay.

Introduction

DNA methyltransferase (MTase) is an important DNA-modified enzyme that can transfer a methyl group from *S*-adenosyl-L-methionine (SAM) to adenine or cytosine residues.[1–3] In recent years, studies on cancer pathology have revealed that the aberrant expression of DNA MTase has profound implications in tumorigenesis and genetic diseases.[4,5] Thus, the development of efficient tools for monitoring DNA MTase activity is of considerable significance in the fields of early cancer clinical diagnostics and drug discovery.

Routine methods for DNA MTase activity assay required radioactive labelling of the DNA substrate or separation of methylated fragments by HPLC and gel electrophoresis.[6–8] However, most of these methods encountered the problems of complicated procedures, and time-consuming steps with the involvement of radioactive materials. The desire to avoid these drawbacks has motivated the development of diverse alternative technologies including electrochemistry, fluorescence, and colorimetric assays.[9–11] In these strategies, the MTase activity was generally evaluated from the signal changes of indicators labelled on DNA fragments before and after treatment with DNA MTase and a restriction enzyme. Such indicators generally include electroactive species, enzymes, fluorescent dyes, and nanoparticles. Despite the considerable advances, the labelling technologies of these indicators on DNA substrates still carry significant drawbacks including time-consuming sample preparation, and need for sophisticated operators. Moreover, these labels may interfere with the interaction between MTase and DNA substrates, and thus decrease the performance of DNA MTase activity assay. Some groups resorted to a label-free bioassay strategy based on direct electrochemical determination of 5-methyl cytosine using its oxidation signal, or methylation-responsive DNAzyme colorimetric assay.[12,13] However, these methods exhibited limited sensitivity. Therefore, it still remains a challenge to develop facile label-free strategies to monitor the DNA MTase activity with high sensitivity and high specificity.

Recently, a type of newly emerged functional fluorescent probe, DNA-templated metal nanoparticles (NPs), has widely been used for label-free detection of biomarkers due to their outstanding optical properties, low toxicity, facile surface modification and good biocompatibility.[14–16] More impor-

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tantly, such novel fluorescent probes exhibit facile integration with nucleic acid-based target-recognition abilities and DNA signal amplification mechanisms.[14–18] Hence, applying these excellent properties of DNA-templated NPs may be highly desired to produce a highly efficient signal transduction strategy for label-free DNA MTase activity detection. Recently, cytosine-rich DNA-templated silver nanoparticles have been widely applied for the detection of various targets including metal ions, proteins, DNA, and cells.[19–22] However, their sequences are complicatedly designed, which limits their practical applications. In order to further broaden the biochemical applications of such novel functional fluorescent probes, double-stranded DNA (dsDNA) with random sequences can be exploited as alternative templates for synthesizing fluorescent copper nanoparticles (CuNPs).[23] It is supposed that CuNP formation on a DNA scaffold might be attributed to the high-affinity clustering of Cu^0, which is formed through chemical reactions between Cu^{2+} and the reducing agent in solution. The formed CuNPs can exhibit excellent fluorescence properties. The specificity of CuNPs towards the dsDNA templates as well as their excellent fluorescence properties makes them suitable for biomarker detection. Very recently, several groups have extended dsDNA-templated CuNPs as fluorescent probes for biomarker assays such as small molecules, DNA, microRNA, and proteins,[17,18,24–31] which have demonstrated that dsDNA-templated CuNPs can be used as ideal fluorescent probes in biochemical applications. Hence, applying these excellent properties of dsDNA-templated CuNPs in Dam MTase activity may be highly desired to produce a highly efficient and simple strategy. To the best of our knowledge, research into dsDNA-templated CuNPs for DNA MTase activity assay has been still rare so far.

Herein, we developed a novel label-free fluorescence strategy to monitor DNA MTase activity based on dsDNA-templated CuNPs coupled with an endonuclease-assisted signal transduction system. In this strategy, dsDNA refers to the DNA-recognition sequence of MTase and restriction endonuclease, which could be methylated and then cleaved by the dual-enzyme couple reaction. The cleaved DNA fragments were used as dsDNA template switches for fluorescent CuNP formation, thus producing a turn-off fluorescence bioassay strategy for monitoring MTase activity. Compared to the reported literature, this developed label-free fluorescence strategy for MTase activity and inhibitor screening seems to be more convenient, cost-effective, and easily executable because there is no requirement of complicated design and labelling technologies. We demonstrated that this label-free strategy can be applied for DNA MTase activity assay with high sensitivity and specificity.

Experimental

Reagents and materials

The DNA oligonucleotides used in this work were purchased from Takara Biotech. Co. Ltd (Dalian, China). The base sequences of oligonucleotides were as follows: P1: 5′-GTATTCTGATCTCTCTAC-3′; P2: 5′-GTAGAGAGATCAGAATAC-3′. The base sequences of other oligonucleotides are listed in Table S1.† Dam MTase and DpnI endonuclease were supplied by New England Biolabs (Beijing, China). 3-(*N*-Morpholino)-propane sulfonic acid (MOPS), 5-fluorouracil, copper sulfate ($CuSO_4$), and sodium ascorbate were from Shanghai Chemical Reagent Company (Shanghai, China). All other reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA) with an electric resistance >18.3 MΩ cm.

Assay of Dam MTase activity and fluorescence measurements

To prepare duplex DNA substrates of Dam MTase, DNA probes P1 and P2 were preliminarily mixed in 300 mM MOPS buffer containing 150 mM NaCl. Subsequently, the obtained mixture was annealed by heating to 90 °C for 10 min followed by slowly cooling down to room temperature for 1 h.

The assay of Dam MTase activity was performed in 200 μL methylase buffer (50 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.4) containing 1 μM annealed dsDNA, 5 units of DpnI endonuclease, and a varying amount of Dam MTase at 37 °C. 30 min later, 20 μL 28 mM sodium ascorbate was added. After incubation for 5 min at room temperature, 60 μL 467 μM $CuSO_4$ was added into the solution and the fluorescence spectra of the formed CuNPs were recorded 10 min later. In order to prevent the effect of ascorbate on enzyme activity, ascorbate was added into the solution after enzyme treatment and then incubated with nucleic acids.

All fluorescence measurements were performed on a Fluoro-Max-4 spectrometer (HORIBA Jobin Yvon, Inc., New Jersey) at room temperature. The fluorescence emission spectra of CuNPs were obtained from 520 nm to 660 nm with a 340 nm excitation wavelength.

Inhibition of Dam MTase activity

The inhibitor of Dam MTase was incubated at various concentrations from 0 to 5 μM with 1 μM dsDNA (P1/P2) in 200 μL of methylase buffer. Then, SAM, 10 U mL^{-1} Dam MTase, and 5 units of DpnI endonuclease were added and the resulting solution was incubated at 37 °C for 30 min. Other experimental procedures were the same as aforementioned.

Results and discussion

Analytical principle of this strategy

In this strategy, Dam MTase and DpnI restriction endonuclease were chosen as the model MTase and endonuclease, respectively. Fig. 1 depicts the principle diagram of the developed label-free fluorescence strategy for Dam MTase activity assay. In this strategy, dsDNA containing the tetranucleotide sequence 5′-GATC-3′ was designed to act as both Dam MTase's and DpnI endonuclease's substrate.[32–34] Moreover, such dsDNA with more than 9 base pairs (bp) was also exploited as

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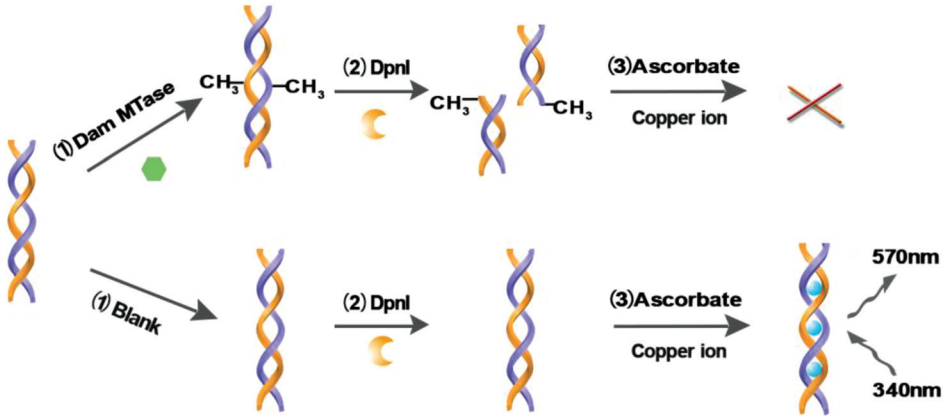

Fig. 1 Schematic diagram of the developed label-free fluorescence strategy for Dam MTase activity assay based on dsDNA-templated CuNPs coupled with an endonuclease-assisted signal transduction system.

templates for fluorescent CuNP formation.[21] Under the treatment of Dam MTase, dsDNA was methylated and further cleaved by DpnI endonuclease. The obtained dsDNA residues with less than 9 bp did not act as templates for fluorescent CuNP formation, and thus no fluorescence signal was observed in the reaction solution containing copper ions and ascorbate. However, in the absence of Dam MTase, dsDNA probes were not methylated, thus prohibiting the degradation by DpnI endonuclease and keeping dsDNA intact. On addition of copper ions and ascorbate to the reaction solution, the dsDNA-templated CuNPs were formed and exhibited a strong fluorescence response at 570 nm.

Assay of Dam MTase activity

The key to signal transduction in the developed strategy is its dependence on dsDNA templates with more than 9 base pairs for fluorescent CuNP formation. Hence, the fluorescence properties were preliminarily investigated based on exploiting ssDNA and dsDNA with 9 bp and 18 bp as templates for CuNP formation, respectively. As shown in Fig. S1 (ESI†), when dsDNA with 18 bp (P1/P2) was used as a template, an obvious fluorescence was obtained in the reaction solution containing copper ions and ascorbate. However, the fluorescence was negligible in the presence of dsDNA with 9 bp (P3/P4) or ssDNA (P1 or P2) as templates. These results indicated that the preferential dsDNA template with 18 bp over ssDNA and short dsDNA with 9 bp promoted the formation of fluorescent CuNPs in the reaction solution containing copper ions and ascorbate, originating from the presence of a scaffold in the longer dsDNA template to support fluorescent CuNPs.[23]

Typical fluorescent characteristic curves of the developed strategy are presented in Fig. 2. As can be seen in Fig. 2, when the dsDNA substrates were treated by Dam MTase and DpnI endonuclease, no fluorescence response was observed, originating from the methylation/cleavage reaction that occurred and the obtained short dsDNA fragments did not act as an efficient template for CuNP formation. However, when dsDNA

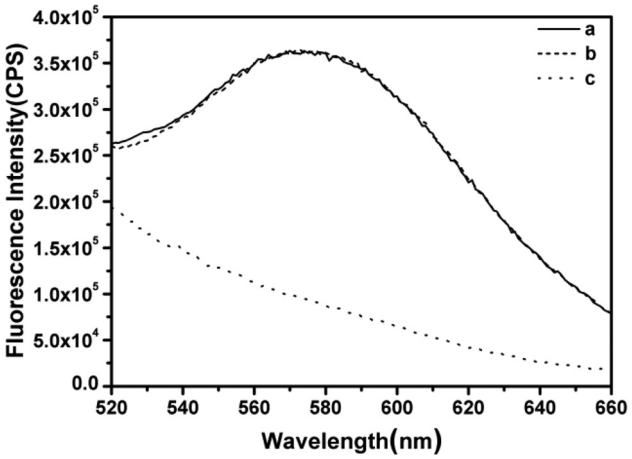

Fig. 2 Fluorescence spectra of the obtained dsDNA-templated CuNPs in the absence or presence of Dam MTase and DpnI. (a) Dam MTase; (b) DpnI endonuclease; (c) Dam MTase and DpnI endonuclease. The concentrations of Dam MTase and dsDNA were 10 U mL^{-1} and 1 μM, respectively. The amount of DpnI endonuclease was 5 U. The enzyme-coupled reaction was performed in methylase buffer (50 mM Tris-HCl, 60 μM SAM, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.4) at 37 °C for 30 min. The dsDNA-templated fluorescent CuNPs were obtained in the reaction solution containing 100 μM Cu^{2+} and 2 mM ascorbate for 10 min.

substrates were treated by Dam MTase or DpnI endonuclease alone, a strong fluorescence peak appeared at 570 nm, which is a typical fluorescent peak range of dsDNA-templated CuNPs.[23]

Optimization of experimental conditions

To elucidate the possible factors which influence the fluorescence response characteristics of the developed strategy, further experiments were performed. In this strategy, considering the fact that both the length and concentration of dsDNA could have an influence on fluorescent CuNP formation,[23,26] the number of base pairs and concentration of dsDNA were

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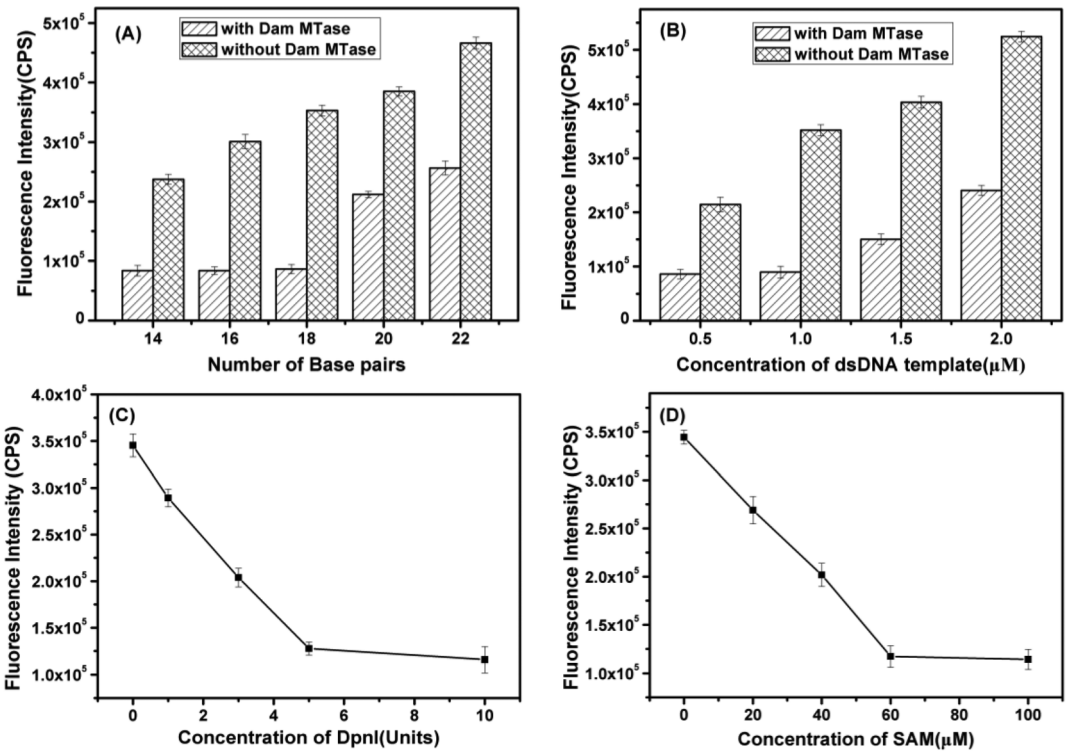

Fig. 3 Impact of (A) the number of base pairs in the dsDNA substrate and (B) the concentration of the dsDNA substrate on the fluorescence response of the dsDNA-templated fluorescent CuNPs in the presence and absence of Dam MTase; impact of (C) the amount of DpnI endonuclease and (D) the concentration of SAM on the fluorescence response of the dsDNA-templated fluorescent CuNPs. Other reaction conditions are as shown in Fig. 2. Error bars show the standard deviation of five experiments.

systematically investigated to obtain the optimum performance for Dam MTase activity assay. As can be seen in Fig. 3A, with the increase of the number of base pairs in dsDNA templates from 14 bp to 22 bp, the fluorescence intensity gradually increases, attributed to the longer dsDNA templates which induce generation of larger CuNPs with a higher quantum yield. The average diameter scales of CuNPs templated by dsDNA with 14 bp, 18 bp, and 22 bp are around 2.7 nm, 4.3 nm, 5.8 nm, respectively (Fig. S2 in the ESI†). The corresponding fluorescence quantum yields are 0.6%, 1.2%, 1.8%, respectively. These experimental results are consistent with the reported literature.[23,26] However, under the treatment of Dam MTase and DpnI endonuclease, longer dsDNA substrates would be separated into the cleaved dsDNA fragments with longer fragments, thus resulting in a high background response. Hence, in order to obtain the optimum signal-to-background ratio, 18 bp dsDNA was chosen as the optimal template. The concentration of the dsDNA substrate was further systematically investigated. As can be seen in Fig. 3B, the optimum signal-to-background ratio for DNA MTase activity assay appeared when the concentration of dsDNA was 1 μM. Thus, 1 μM dsDNA was applied for subsequent experiments.

We further investigated the effect of the concentration of Cu^{2+} on the fluorescence properties of CuNP formation (Fig. S3 in the ESI†). A peak-shaped dependency of the fluorescence response was observed within the concentration range from 0 nM to 800 μM. The maximum fluorescence response was obtained with a Cu^{2+} concentration of 100 μM. Hence, 100 μM Cu^{2+} was taken for subsequent experiments. Moreover, with a prolonged reduction time of Cu^{2+} by ascorbate, the observed fluorescence intensity gradually increased and became almost levelled off at 10 min (Fig. S3 in the ESI†). Thus, this time period was chosen as the optimized reduction time of Cu^{2+} for subsequent experiments.

DpnI endonuclease-amount-dependent methylation-responsive dsDNA cleavage was further investigated by utilizing various amounts of DpnI endonuclease. As can be seen in Fig. 3C, the fluorescence intensity dynamically decreased with the increase of the DpnI endonuclease amount. When the DpnI endonuclease amount was 5 U, the fluorescence intensity did not decrease significantly, suggesting that 5 U DpnI endonuclease could totally cleave the methylated dsDNA. Hence, this concentration was used for subsequent experiments. As a Dam MTase substrate, the concentration of SAM has also been systematically investigated. The fluorescence response decreased significantly as the concentration of SAM increased up to 60 μM, and then reached equilibrium at 60 μM, as shown in Fig. 3D.

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In this strategy, the duration of the enzyme reaction time is quite long compared to the methylation reaction and degradation of dsDNA substrates. The fluorescence intensity decreased with the increase of the enzyme reaction time, and tended to be constant at 30 min (Fig. S4 in the ESI†). In order to obtain a short enzyme reaction time, 30 min was used throughout in all further experiments.

Performance of this strategy

The ability of the developed biosensor strategy for Dam MTase activity quantitative assay was systematically investigated. Fig. 4A shows the corresponding fluorescence response curves for Dam MTase with different concentrations. It can be observed that the fluorescence intensity dynamically decreased on increasing the concentration of Dam MTase in the concentration range from 0 to 20 U mL^{-1}. This indicated that the gradual degradation of dsDNA happened and shorter dsDNA fragments could be obtained. The obtained fluorescence intensity was used as a quantitative analytical signal for Dam MTase. As shown in Fig. 4B, the plot of the fluorescence intensity *versus* the logarithm of Dam MTase concentrations exhibited a good linear relationship in the concentration range from 0.01 to 5 U mL^{-1} with a correlation coefficient of 0.996. The detection limit is estimated to be 0.01 U mL^{-1}, which is comparable to the reported strategies shown in Table S2 (ESI†). It can be concluded that the developed strategy exhibited a higher sensitivity, and a lower detection limit. These experimental results suggested that this novel label-free fluorescence strategy could be applied for conveniently and efficiently monitoring Dam MTase activity.

The assay reproducibility of the developed strategy was evaluated. Five different and fresh reaction solution systems of 1 U mL^{-1} Dam MTase yielded a reproducible fluorescence response with a relative standard deviation (RSD) of 3.6%. These results indicated that this label-free fluorescence strategy exhibited acceptable reproducibility for Dam MTase activity assay.

In physiological samples, coexisting methyltransferases may interfere with the methylated reaction of DNA substrates catalyzed by Dam MTase.[35] Hence, in order to verify the selectivity of the developed strategy, control samples using some potential interfering methyltransferases including M.SssI and AluI were tested with the same experimental procedures of Dam MTase. M.SssI MTase could specifically methylate the cytosine residues within a double-stranded DNA recognition sequence of 5′-C-G-3′, and AluI MTase could methylate the cytosine residues in the double-stranded symmetric 5′-AGCT-3′ sequence. Under the treatment of these potential coexisting methyltransferases, the obtained fluorescence responses were comparable to that of the blank sample (Fig. S5 in the ESI†). In contrast, Dam MTase could cause a remarkable fluorescence decrease. These results indicated that the developed strategy based on dsDNA-templated CuNPs exhibited high selectivity for Dam MTase activity assay.

In order to verify the reliability of the developed strategy for a real sample analysis, the sensor was applied to determine Dam MTase in diluted human serum (1%). As shown in Fig. S6 in the ESI,† the fluorescence intensity increased with the increase of Dam MTase concentration from 0 to 2 U mL^{-1} in serum. Satisfactory recoveries in the range of 95.0%–102.0% were obtained by adding Dam MTase of different concentrations into the serum samples (Table S3 in the ESI†), indicating that the developed strategy holds great potential in complicated real samples.

Assay of the inhibition on Dam MTase activity

Since aberrant DNA methylation is closely related to diseases, especially cancer, screening DNA MTase inhibitors plays an important role in the early diagnostics of cancer and disease treatment. The validity of our strategy in assaying the inhi-

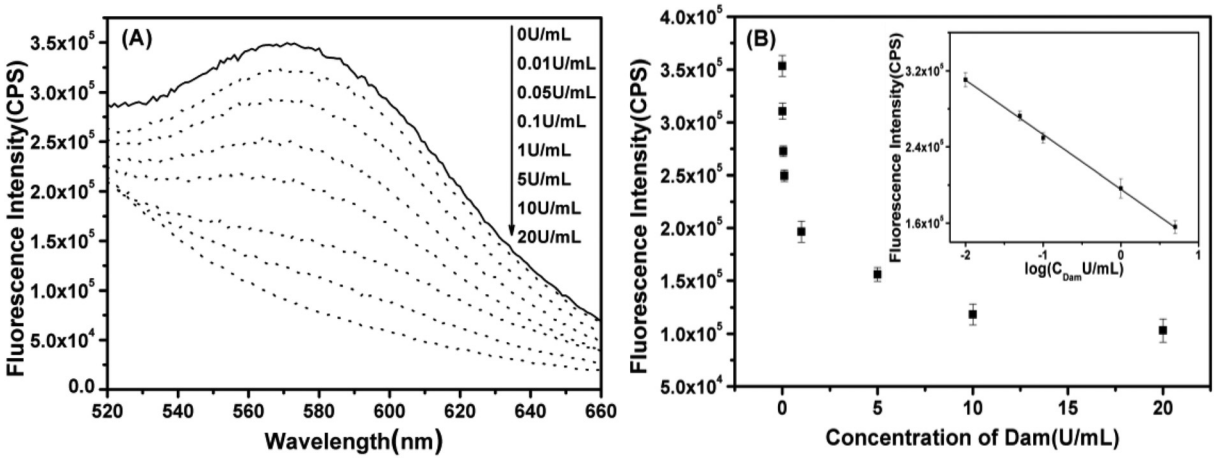

Fig. 4 (A) Typical fluorescence response curves for different Dam MTase concentrations from 0 to 20 U mL^{-1}. (B) Calibration curves of the fluorescence response *vs.* the Dam MTase concentrations for this developed strategy. Other reaction conditions are as shown in Fig. 2. Error bars show the standard deviation of five experiments.

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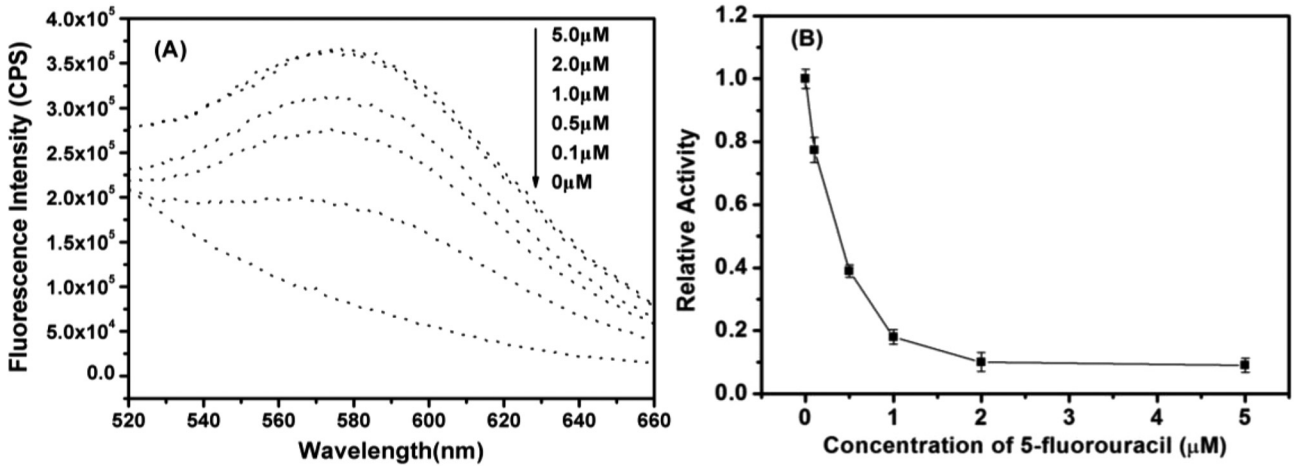

Fig. 5 (A) Typical fluorescence response curves for different inhibitor 5-fluorouracil concentrations from 0 to 5 μM. (B) Effect of 5-fluorouracil concentrations on the relative activity of Dam MTase. Other reaction conditions are as shown in Fig. 2. Error bars show the standard deviation of five experiments.

bition of Dam MTase was tested by using 5-fluorouracil as a model inhibitor, and it has been confirmed that it could effectively inhibit Dam MTase activity in physiological samples.[36] Considering that there are two enzymes involved in this developed strategy, it is preliminarily necessary to evaluate the influence of 5-fluorouracil on DpnI. The control experiment results indicate that 5-fluorouracil has no influence on the activity of DpnI when the concentration of 5-fluorouracil is not more than 10 μM (Fig. S7 in the ESI†). Fig. 5 depicts the effect of the concentration of 5-fluorouracil on the activity of Dam MTase. It can be observed that the fluorescence intensity of CuNPs increased significantly as the inhibitor concentration increased up to 2 μM, and then reached equilibrium at 2 μM. The corresponding fluorescence intensity was comparable to that of the blank sample, indicating that Dam MTase activity can be effectively blocked by the inhibitor. Moreover, several other reported Dam MTase inhibitors (benzylpenicillin, gentamycin, and ampicillin sodium) have also been used in the developed strategy. Experimental results show that ampicillin sodium has almost no effect on methylation. However, benzylpenicillin, gentamycin, and 5-fluorouracil could strongly inhibit the activity of Dam MTase and gentamycin was the most effective inhibitor, achieving an inhibition ratio of 70% (Fig. S8 in the ESI†). The results are in good agreement with the previous reports,[3,9,33,36] indicating that our strategy can be used to study the MTase inhibitors and be employed for MTase inhibitor screening.

Conclusions

In summary, we have developed a label-free fluorescence strategy for DNA MTase activity based on dsDNA-templated CuNPs coupled with endonuclease assisted signal transduction. This novel strategy takes advantage of the specific recognition of Dam MTase and methylation-sensitive restriction endonuclease DpnI as well as the excellent fluorescence properties of dsDNA-templated CuNPs. Compared to routine methods for monitoring Dam MTase activity, this developed label-free fluorescence strategy was easily performed, requiring no complicated design and labelling technologies, and exhibited high reproducibility and high selectivity. In addition, we have also demonstrated that this strategy could be applied for the rapid and sensitive assay of Dam MTase activity and screening of the inhibitors. Such excellent performances indicated the great potential of this label-free fluorescence strategy in clinical diagnostics and drug discovery.

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