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Terminal deoxynucleotidyl transferase induced activators to unlock the trans-cleavage of CRISPR/Cpf 1 (TdT-IU- CRISPR/Cpf 1): An ultrasensitive biosensor for Dam MTase activity detection

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ABSTRACT

DNA adenine methylation methyltransferase (Dam MTase) plays an important role in gene expression and cell development, and involves in some development of tumors. There are many methods to detect Dam MTase activity, yet they have some shortcomings such as high cost, limited detection limit and complicated operation. Here, a new fast and simple multiple amplification strategy based on terminal deoxynucleotidyl transferase induced activators to unlock the collateral cleavage activities (trans-cleavage) of CRISPR/Cpf 1 (TdT-IU-CRISPR/Cpf 1) was firstly established. Based on this, a new fluorescent biosensor for Dam MTase activity detection was proposed. Importantly, the proposed biosensor does not require strict control over the temperature changes, and has ultrasensitive detection with limit of detection as low as 1.26×10^{-3} U/mL. Moreover, the novel biosensor can not only be applied for screening the inhibitors for Dam MTase activity, but also can detect its activity in complex biological samples. The newly established multiple amplification strategy TdT-IU-CRISPR/Cpf 1 and the proposed biosensor for Dam MTase activity will be of great significance in biomedical testing and clinical diagnostics.

1. Introduction

DNA methylation, which plays a momentous role in gene expression, cell development, and chromatin structure modulation, is one of the crucial epigenetic modification in prokaryotes and eukaryotes (Cui et al., 2019). DNA methylation often occurs by transferring the methyl groups from donor Sadenosyl-L-methionine (SAM) to the N-6 position of adenine or the C-5/N-4 of cytosine (Xu et al., 2018; Wood et al., 2010). It's closely related to the activity of DNA MTase. As one of the crucial DNA MTase, Dam MTase can transform adenine into N6-methyladenine (m6A) in the sequence-specific 5′-GATC-3' (Feldheim et al., 2019). However, abnormal Dam MTase activity will induce the confusion of methylation regulation (Shukla et al., 2010), inhibit of the tumor suppressor genes (Spencer et al., 2017; Plumb et al., 2001; Wang et al., 2019), cause the generation of cell cytotoxicity (Xia et al., 2019) and involve in various types of cancer, such as colon, breast, lung, prostate and liver cancers (Vuong et al., 2020; Meng et al., 2020; Tsuboi et al., 2020; Hang et al., 2019). Moreover, abnormalities in Dam MTase activity generally occur far before other signs of malignancy and could be used as a potential biomarker and therapeutic target of early stage disease (Salvati et al., 2019; Luo et al., 2020). Therefore, developing a strategy that can simply, fast, and ultra-sensitively detect the Dam

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MTase activity is extremely important in both biochemical research and pharmacologies.

Conventional strategies such as radioactive labeling methylation approaches (Poh et al., 2016), high performance liquid chromatography (HPLC) (Torres et al., 2011), menthyaltion specific polymerase chain reaction (PCR) (Yang et al., 2004), and gel electrophoresis (Bergerat et al., 1991) have been proposed for Dam MTase activity detection. Regrettably, these methods suffer from many drawbacks. For example, some strategies like radioactive labeling methylation approaches, HPLC, and PCR always need bulky and expensive instruments. Besides, their operation procedures are usually time-consuming and complicated. While gel electrophoresis owns poor stability and limited detection, which limit their widespread applications. Recently, varieties emerging methods like electrochemical (Li et al., 2019), electrochemiluminescence (Zeng et al., 2013; Hou et al., 2019), fluorescent (Wang et al., 2019; J.S. Chen et al., 2018), bioluminescence (Jiang et al., 2012), and colorimetric (Li et al., 2017) have been developed to detect the Dam MTase activity. Those methods achieve more outstangding detection performance and adaptability. Among them, fluorescent approach preferred as a potential alternative tool due to its simplicity, low reagent cost, and flexibility in design. But the current fluorescent methods for detecting Dam MTase activity still need some elevations in detection limit, detection time, and simplicity of detection. In some studies, detection strategies combine with biological amplification techniques to amplify the signal. Some isothermal amplication techniques can get more sensitive detection performance of Dam MTase activity, for example, strand displacement amplification (SDA) (Zhang et al., 2019), rolling circle amplification (RCA) (Chen et al., 2019), and hybrid chain reaction (HCR) (Xu et al., 2019). But they have inherent flaws such as high cost and complicated operation. Using TdT to amplify the DNA fragments, there not only reduce the number of enzymes to reduce cost, but also simplify the process.

Clustered regularly interspaced short palindromic repeats (CRISPR) from *Prevotella and Francisella 1* (Cpf 1) is an effector endonuclease of the class 2 CRISPR-associated proteins gene editing system (Kim et al., 2016a, 2016b). Doudna found that the CRISPR/Cpf 1 could exhibit the indiscriminate collateral cleavage activities (trans-cleavage) after activated by specific recognition of target nucleic acids, which revealed the immense potential of CRISPR/Cas in analysis. By combining Cpf 1 ssDNase activation with isothermal amplification, they created a method termed DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR), which achieved attomolar sensitivity for DNA detection (Y. Chen et al., 2018). Subsequently, Liang developed a simple, supersensitive, fast and high-throughput platform for the detection of small molecules, CRISPR-Cas12a- and aTF-mediated small molecule detector (CaT-SMelor) designated by combining the single-stranded DNA cleavage ability of CRISPR-Cpf 1 and the competitive binding activities of aTFs for small molecules and double-stranded DNA. They used CaT-SMelor successfully achieve the nanomolar levels of various small molecules detection, including uric acid and p-hydroxybenzoic acid among their structurally similar analogues (Liang et al., 2019). Qian found Cpf 1 can also recognize another PAM sequence of UUUN resulting in activation of its ssDNA collateral cleavage effect. To make this finding useful, by combining with loop-mediated isothermal amplification (LAMP), they first realized CRISPR/Cpf 1 for directly visualized DNA detection at the single-copy level (Qian et al., 2019). All these findings prove that the CRISPR/Cpf 1 system has a great potential in analysis. Nevertheless, except for nucleic acids detection, few relevant research about other biomolecules detection based on CRISPR/Cpf 1 system is reported up to now. Therefore, it is needed to broaden the sensing applications of CRISPR/Cpf 1 system.

Here, a novel biosensor which can convenient, fast and ultrasensitive to detect the Dam MTase activity based on a new multiple amplification strategy TdT-IU-CRISPR/Cpf 1 was proposed for the first time. The schematic of the proposed biosensor was illustrated in Scheme 1. In the presence of Dam MTase and SAM, the palindromic sequence of 5′-GATC-3′ in hairpin substrate (HPs) stem are methylated to 5′-G-mA-T-C-3'. And the 3′-end of the HPs are modified with amino group to prevent TdT-induced nonspecific extension. Then the methylated HPs are recognized and cleaved at 'mA' by the endonuclease Dnp I, generating two DNA fragments which contained free 3'−OH end. The exposed free 3'−OH ends can be produced continuous poly-T tail in the presence of TdT and dTTP. Cleverly, the recognition region of crRNA was designed to contain a 23 bp poly-A sequence. Thus, the prolonged poly-T tail can act as activators which complements to crRNA to unlock the trans-cleavage of CRISPR/Cpf 1. In addition, the actived CRISPR/Cpf 1 intrinsically possesses the ability of significant signal amplification due that the actived CRISPR/Cpf 1 can indiscriminately cut the cleavage reporters added in the system. Thus a fast and simple multiple amplification strategy was achieved to ultra-sensitively detect the Dam MTase activity. Under the optimal experimental conditions, the proposed biosensor achieved Dam MTase activity detection as low as 1.26×10^{-3} U/mL in the range from 1.59×10^{-3} to 3.18×10^{-1} U/mL. The interference and inhibitor screening experiments indicated that the proposed biosensor possess excellent selectivity and can be used to screen the inhibitors of Dam MTase activity. The successful test of Dam MTase activity in complex biological samples demonstrated that the proposed biosensor owns the immense potential for detection of DNA MTase activity in complex biological samples.

2. Materials and methods

2.1. Reagents and instrumentations

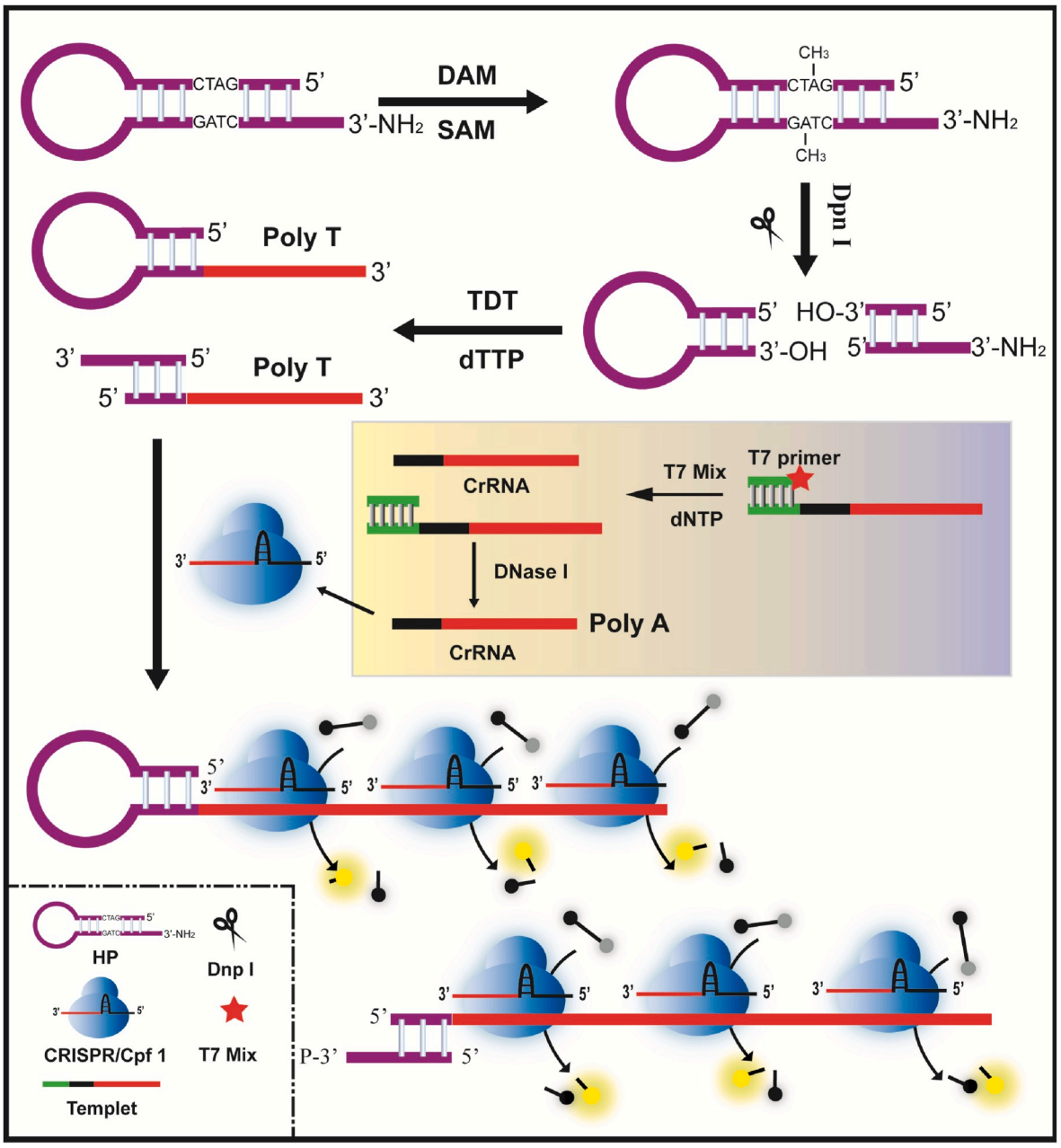
All oligonucleotides sequences designed in this study were synthesized by Sangon Biotech (Shanghai, China) and purified by high-performance liquid chromatography. The detailed information was seen in Table S1. HiScribe T7 Quick High Yield RNA Synthesis Kit, Dam MTase, 10 × Dam buffer, Sadenosyl-L-methionine (SAM), 10 × CutSmart, LbCas12a (Cpf 1), and 10 × NEB buffer 2.1 were ordered from New England Biotechnology Co., Ltd (Beijing, China). MiRcute miRNA Isolation Kit (DP501) was purchased from TIANGEN Biotechnology Co., Ltd (Beijing, China). Terminal deoxynucleotidyl transferase (TdT), 10 × TdT buffer, M.Sss I, phi 29, Hae III, and T4 PNK were bought from Sangon Biotech (Shanghai, China). Normal human serum was supplied by Solarbio Science & Technology Co., Ltd (Beijing, China). The fluorescence spectral measurements was implemented by exciting the system at 495 nm and measuring the emission at 556 nm for HEX on a LS-55 spectrophoto fluorometer (P. E. America). During the measurement process, the slits for excitation and emission were set at 12 nm and 8 nm, respectively. The gel was visualized by the Azure Biosystems C150 (America).

2.2. Synthesis of crRNA

2 μL of 100 μM crRNA-template, 2 μL of 100 μM T7 promoter and 14 μL of RNase-free water were added in 200 μL centrifuge tube. Then the mixture was maintained for 5 min at 95°C, following by cooled to room temperature at a rate of 0.1 °C/S and kept for 20 min. After that, 10 μL of 100 mM dNTP buffer and 1 μL of T7 Mix were added. Then, the mixture was maintained at 37 °C for 16 h to obtain adequate targets crRNA. The single and heterozygous DNA was digested by DNase I. Finally, the transcription products were purified with miRcute miRNA Isolation Kit and redissolved in DEPC-treated water. The concentration of obtained crRNA was measured using NanoDrop 2000C and stored at −20°C. The gel electrophoresis of crRNA was shown in Fig. S1.

2.3. Dam MTase activity detection

40 μL of mixture contained 500 nM HPs, 160 μM SAM, 1 × Dam buffer (50 mM Tris-HCl, 10 mM EDTA, 5 mM 2-Mercaptoethanol, pH 7.5@25C°), 1 × CutSmart (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$,



Scheme 1. Schematic diagram of Dam MTase activity detection.

100 μg/mL BSA, pH 7.9@25C°), 1 × TdT buffer (200 mM potassium cacodylate, 0.025 M Tris, 0.05%(v/v)Triton X-100, 1 mM $CoCl_2$, pH 7.2@25C°), 2 U/mL Dnp I, 0.3 U/mL TdT, 1.25 mM dTTP, and different concentrations of Dam MTase. Then the mixture was incubated for 120 min at 37 °C. After that, 2 μL of 1 μM Cpf 1, 2 μL of 1 μM crRNA, 2 μL of 10 μM cleavage reporters, and 5 μL of 10 × NEB buffer 2.1 (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 1000 μg/mL BSA, pH 7.9@25C°) were added into the system. After incubating for 30 min at 37 °C, the mixture were terminated reaction by adding another 50 μL of ddH_2O. The fluorescence spectrum of the reaction mixture was recorded by exciting the system at 495 nm and measuring the emission at 556 nm on a LS-55 spectrophoto fluorometer (P. E. America). During the measurement process, the slits for excitation and emission were set at 12 nm and 8 nm, respectively.

2.4. Selectivity and inhibition assay

To verify the selectivity of the proposed biosensor, four interfering enzymes (M.Sss I, phi 29, Hae III, and T4 PNK) were chose to study its selectivity. During the experiment, the target Dam MTase was insteaded by the other interfering enzymes, and the following process were performed the same as that in Dam MTase activity assay.

To observe the inhibition of 5–fluorouracil and gentamycin on Dam MTase activity, different concentrations of inhibitors were added to the system. The inhibition effect of inhibitors on Dam MTase activity was evaluated by the relative activity (*RA*) of Dam MTase. The *RA* of the Dam MTase was formulated as: $RA = (F' - F_0)/(F - F_0)$, where F', F, and F_0 represent the fluorescence intensity in the presence of different concentrations of inhibitors (5–fluorouracil or gentamycin), in the absence of inhibitors and in the absence of Dam MTase, respectively.

2.5. Gel electrophoresis analysis

The urea-denaturing PAGE gel analysis was performed to verify the generation of target activators. It was executed with 20% urea-denaturing PAGE gel in 1 × TBE buffer at 100 V for 60 min. An Azure Biosystems C150 was used to obtain gel images.

2.6. Real samples detection

Different concentrations of Dam MTase (0.003, 0.01 and 0.03 U/mL) were spiked in 10% normal human serum to demonstrate that the biosensor can be used to detect Dam MTase activity in real samples. The procedure for the fluorescent measurement was the same as that described above.

3. Results and discussions

3.1. Feasibility of the proposed biosensor for the Dam MTase activity detection

As illustrated in Fig. 1A, the fluorescence intensity of the system was intense in the presence of Dam MTase (curve a). While in the absence of Dam MTase, the HPs can't be methylated, resulting in the subsequent steps cannot be performed normally. As a result, the fluorescence intensity of the system was extremely weak (curve b). Additionlly, an urea-denaturing PAGE gel analysis was implemented to confirm the generation of activators. In Fig. 1B, the lane 1, lane 2, lane 3 and lane 4 respectively represented the HPs, the products in the presence of Dam and Dnp I, in the presence of Dam, Dnp I and TdT, in the presence of Dnp I and TdT. That is because in the presence of Dam and Dnp I, the HPs were sheared at its palindromic sequence of 5′-GATC-3′, generating two DNA fragments (lane 2). In lane 3, some large fragments DNA were appeared at the upper part of urea-denaturing PAGE gel, mainly attribute to the exposed free 3'−OH ends of two DNA fragments can be extended continuous poly-T tail in the presence of TdT and dTTP. In lane 4, there was no Dam, so the HPs can't be methylated, and can't be recognized and sheared by Dnp I. All those results indicated that the proposed biosensor could be used to detect the activity of Dam MTase.

3.2. Optimization of the proposed biosensor

To make the proposed biosensor have better detection performance, the reaction time of the system, the shear time of the CRISPR/Cpf 1, and the concentrations of Dnp I and TdT were observed. As shown in Fig. 2A, the net difference in fluorescence intensity ($\triangle F$, which represents the net difference of fluorescence intensity in the presence or absence of Dam MTase) increased gradually as the reaction time of the system increased, but reached plateau till to 120 min. So 120 min was chosen as the optimal reaction time of the system. This is because when the reaction time of the system is too short, the generated polar T sequences are limited, resulting in a weak fluorescence intensity of the system. However, when the reaction time of the system is too long, non-specific amplification occurs, resulting in the fluorescence intensity of the control group increasing rapidly. The length of the reaction time of the system determines the length of the polar T sequence. While the length of the polar T sequence represents how many activators can activate the trans-cleavage of CRISPR/Cpf 1, which is directly related to the amplification efficiency of the proposed technology. Therefore, the length of the reaction time of the system plays an extremely important role in limiting the speed of the proposed technology. In Fig. 2B, the value of $\triangle F$ increased significantly when the shear time of the CRISPR/Cpf 1 rised from 10 min to 30 min, while it kept relatively steady when the shear time of the CRISPR/Cpf 1 increased continuously. Therefore, 30 min was chosen as the optimal shear time of the CRISPR/Cpf 1. When the shear time of the CRISPR/Cpf 1 is too short, the cleavage reporters in the system are not completely sheared off. When the shear time of the CRISPR/Cpf 1 is too long, the cleavage reporters became instability and are non-specific shearing, making the control group also appears fluorescence. As the concentration of Dnp I increased, the value of $\triangle F$ was improved, but when the concentration of Dnp I reached to 2.0 U/μL, the value of $\triangle F$ was relatively highest. So the optimized concentration of Dnp I was 2.0 U/μL (Fig. 2C). This is because 2.0 U/μL Dnp I is enough to recognized and cut the methylated HPs. The concentration of Dnp I can affect the identification and cut-off of the methylated HPs. If the concentration of Dnp I is sparing, it will limit the smooth shearing of methylated HPs and affect the detection speed of the proposed biosensor. Fig. 2D demonstrated that the ΔF was increased accompanied by the augment of TdT, but when the concentration of TdT reached to 0.3 U/μL, its value instead decreased. Thus, the optimized concentration of TdT was 0.3 U/μL. Excessive TdT will increase the signal value of non-specific recognition. Under fixed the reaction time of the system, the concentration of TDT will affect the rate of synthesis of polar T sequences.

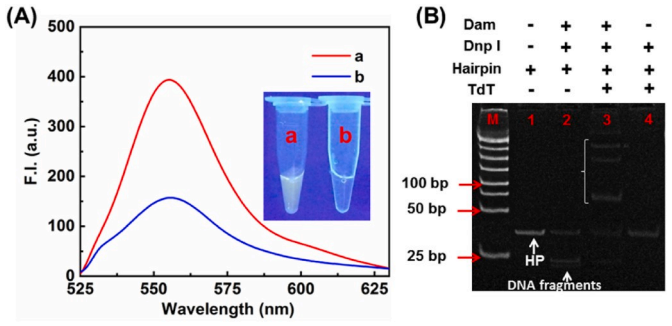


Fig. 1. The feasibility of the proposed biosensor for the Dam MTase activity detection. (A) Shown the fluorescence spectrum of the system in the present of Dam MTase (curve a) and in absence of Dam MTase (curve b). (B) Shown the urea-denaturing PAGE gel under different circumstances, in which the lane from left to right represented maker, HPs, HPs+Dam+Dnp I, experimental group and control group.

3.3. Analytical performance for the Dam MTase activity

Under the optimal conditions, different concentrations of Dam MTase were implemented to evaluate the analytical performance of the proposed biosensor. Fig. 3A showed the fluorescence spectra of the system under the different concentrations of Dam MTase (0–317.46 U/mL). In Fig. 3B, the fluorescence intensity at 556 nm increased gradually with increasing the amount of Dam MTase. But when the most of the HPs were methylated and consumed at high concentration of Dam MTase, the $\triangle F$ became weakened. The dynamic range of the biosensor in Dam MTase quantitative analysis was presented in the illustration of Fig. 3B. A good linear relationship, which the correlation coefficient was 0.98286, could be obtained between the $\triangle F$ and the logarithm of Dam MTase concentrations from 1.59×10^{-3} to 3.18×10^{-1} U/mL. The corresponding regression equation was $\triangle F = 108.07906 logC + 328.05255$. In this mathematical model, the $\triangle F$ was affected only by the concentration of Dam MTase. According to the rule of 3σ/k, the limit of detection (LOD) was evaluated to be 1.26×10^{-3} U/mL. The more Dam MTase, the more methylated HPs will be obtained. As a result, the more prolonged DNA fragments which own lots of regions (activators) that can complementary paired with the recognition area of crRNA were obtained after being cleaved and extended by Dnp I and TdT. Then the generated activators complementary paired with crRNA, activating the trans-cleavage of CRISPR/Cpf 1 to cut the cleavage reporter added in the system. Thus, a two-step amplification endowed the proposed biosensor an excellent performance in detection of Dam MTase activity. In comparison, this biosensor is more outstanding (Table S2).

To study the selectivity of this proposed biosensor, four interfering enzymes were chosen. As displayed in Fig. 4A and B, the fluorescence

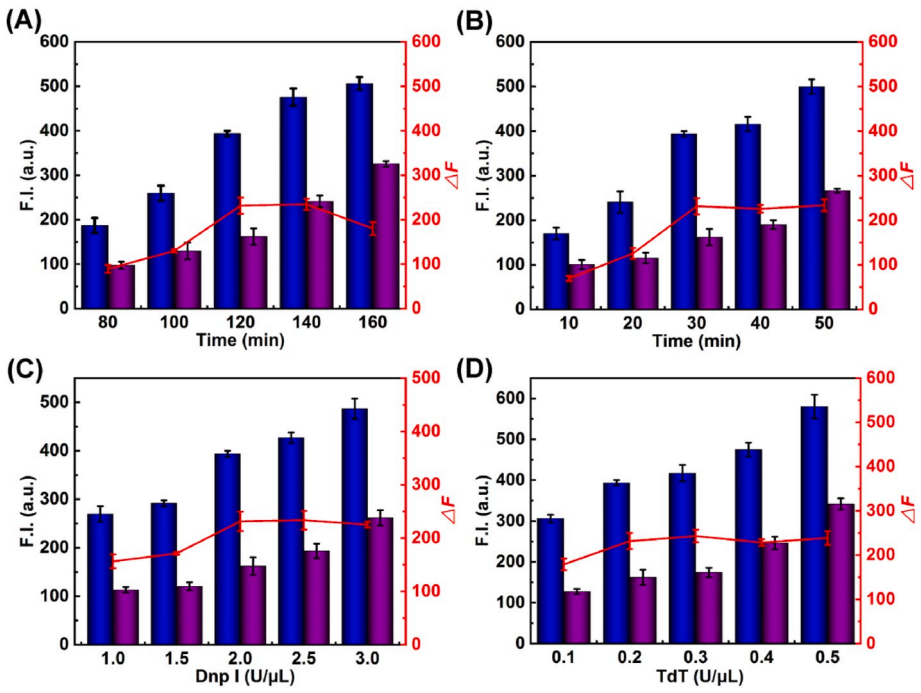


Fig. 2. Effects of the reaction time of the system (A), the shear time of the CRISPR/Cpf 1 (B), the concentrations of Dnp I (C), and the concentrations of TdT (D) on the proposed biosensor. Note: the blue bars in all graphs represent the fluorescence value of the system at 556 nm in the presence of Dam MTase, while the purple bars represent the fluorescence value of the system at 556 nm in the absence of Dam MTase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

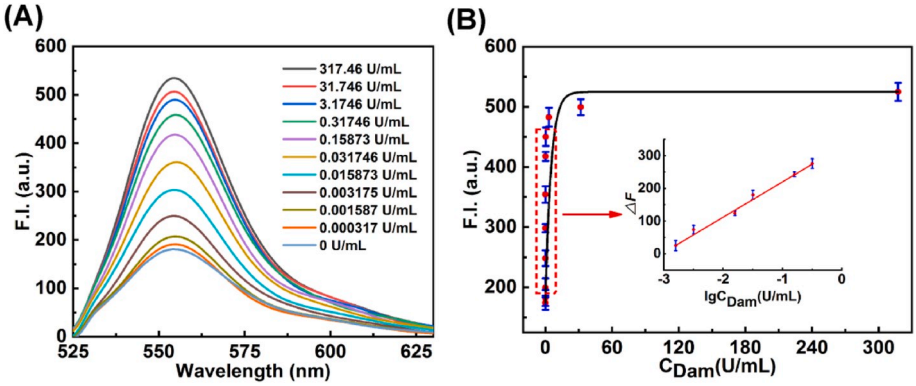


Fig. 3. Detection of Dam MTase activity by the proposed biosensor. Fluorescence spectra (A) and corresponding fluorescence response at 556 nm of the system (B) under the different amount of Dam MTase from 0 U/mL to 317.46 U/mL.

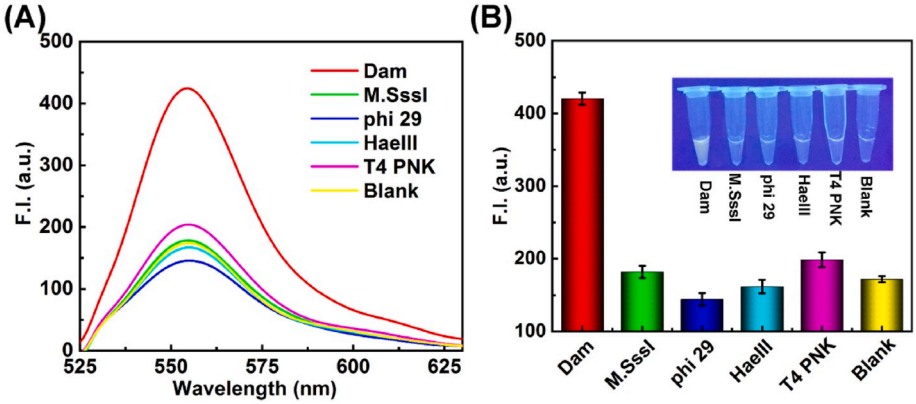


Fig. 4. The fluorescence spectra (A) and corresponding responses (B) of Dam MTase and different analogues with the same concentrations of 0.16 U/mL (the illustration is the physical photograph).

responses to M.Sss I, phi 29, Hae III, and T4 PNK were weak, nearly the same as the blank sample, while Dam MTase was obviously higher than the interference groups. Moreover, the illustration in Fig. 4B shown the actual photograph of selective experiments. Clearly, the experiment group was obviously brighter than blank group and all interfering groups. All illustrated that the proposed biosensor owned excellent selectivity.

3.4. Inhibition evaluation of Dam MTase activity

It is of significance to screen pharmacological inhibitors of Dam MTase activity. According researches, we chose the 5–fluorouracil and gentamycin as model inhibitors. Fig. 5A and C presented the fluorescence spectrum of system that have added different concentrations of 5–fluorouracil and gentamycin, respectively. The suppression efficiency of 5–fluorouracil and gentamycin to Dam MTase activity were evaluated by the *RA*. Therein, the half maximal inhibitory concentration (IC_{50}) represents the inhibitor concentration required for 50% decrease in enzyme activity. Fig. 5C and D demonstrated that the inhibition of 5–fluorouracil and gentamycin to Dam MTase was in a dose-dependent manner, and their IC_{50} for Dam MTase were estimated to be 2.019 μM and 9.69 mM, respectively. These results illustrated that the proposed strategy could be used for Dam MTase inhibitors screening and the further research of the interaction between Dam MTase and its inhibitors.

3.5. Dam MTase activity assay in complex biological samples

To investigate the practical applicability and stability of the proposed biosensor in complex biological samples, different concentrations of Dam MTase (0.003, 0.01, and 0.03 U/mL) were spiked into a 10% serum sample. The performance index of the proposed biosenor was evaluated by the recovery rate, which was calculated by comparing the measured Dam MTase activity and the amount of Dam MTase added into the system. As presented in Table S3, the recovery rates of the spiked samples were in the range of 92.9–105.2%, with relative standard deviations (RSD) ranging from 5.27% to 7.57%. Those results revealed that the proposed biosensor owns the immense potential for detection of DNA MTase activity in complex biological samples and have stability.

4. Conclusions

In summary, we established a new fast and simple multiple amplification strategy TdT-IU-CRISPR/Cpf 1 to ultra-sensitively detect the Dam MTase activity, which couples the isothermal polymerization capability of TdT with the trans-cleavage of CRISPR/Cpf 1 to amplifying signal. As a result, the proposed biosensor for Dam MTase activity detection possesses several innovations. Firstly, detection object innovation: using the TdT-IU-CRISPR/Cpf 1 to detect non-nucleic acid substance, Dam MTase activity, which broadens the sensing applications of CRISPR/Cpf 1 system. Secondly, triple signal amplification: (1) The HPs were cut into two fragments by Dpn I, and each fragments can participate in the subsequent process, thus achieving a double signal amplification. (2) In the presence of TdT and dTTP, the exposed 3'−OH ends of DNA fragments can be produced continuous poly-T tails to bind with the 23 bp recognition region of multiple crRNA. (3) the actived CRISPR/Cpf 1 intrinsically possesses the ability of significant signal amplification. Thirdly, ultrasensitive detection: under the optimal experimental conditions, the proposed biosensor can achieve the detection of Dam MTase activity as low as 1.26×10^{-3} U/mL in the range from 1.59×10^{-3} to 3.18×10^{-1} U/mL. Finally, practical application: the proposed biosensor can be used to screen the inhibitors of Dam MTase activity and owns the immense potential for detection of Dam MTase activity in complex biological samples. Although the established multiple amplification strategy TdT-IU-CRISPR/Cpf 1 and the proposed biosensor for Dam MTase activity detection achieved excellent results. There have no mature testing products up to now. In the future, we will further study the established multiple amplification strategy and explore the development of detection test strips or kits based on the proposed biosensor.

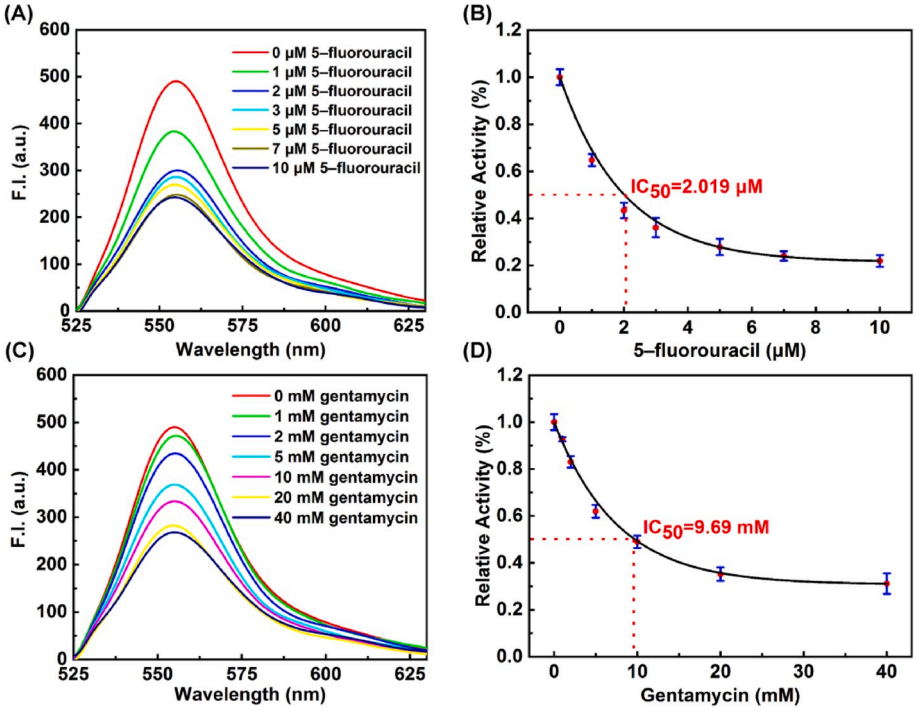


Fig. 5. (A) Fluorescence spectra of different concentrations of 5–fluorouracil in 20 U/mL Dam MTase. (B) The inhibition effect of different concentrations of 5–fluorouracil on Dam MTase activity (20 U/mL). (C) Fluorescence spectra of different concentrations of gentamycin in 20 U/mL Dam MTase. (D) The inhibition effect of different concentrations of gentamycin on Dam MTase activity (20 U/mL).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Xiaolong Chen: Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Gaihua Cao:** Validation, Methodology, Formal analysis. **Xianfeng Wang:** Methodology, Software. **Zhong Ji:** Resources. **Faliang Xu:** Project administration. **Danqun Huo:** Supervision, Project administration. **Xiaogang Luo:** Project administration, Funding acquisition. **Changjun Hou:** Writing - review & editing, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2020.112271.

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