Label-free molecular beacon-based quadratic isothermal exponential amplification: a simple and sensitive one-pot method to detect DNA methyltransferase activity†

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We developed a one-pot label-free molecular beacon-mediated quadratic isothermal exponential amplification strategy (LFMB-QIEA) for simple, rapid and sensitive DNA methyltransferase (MTase) activity detection.

DNA methylation, a crucial epigenetic modification of the genome, plays a pivotal role in gene expression, cellular differentiation, and pathogenesis of various human diseases such as cancer.1 Recent studies have revealed that DNA methylation is closely associated with the activity of DNA methyltransferase [MTase].2 The DNA methylation process is catalyzed by DNA methyltransferase [MTase] which can transfer a methyl group from S-adenosylmethionine (SAM) to target adenine or cytosine residues in the recognition sequences. Alterations of MTase activity may lead to aberrant DNA methylation patterns. Notably, DNA methyltransferases have become predictive biomarkers and potential therapeutic targets in various types of cancer.3 Moreover, abnormalities in MTase activity usually occur far before other signs of malignancy and could thus be used for early cancer diagnosis. Therefore, the development of novel approaches for simple, rapid and sensitive activity assay and inhibitor screening for MTases is of paramount importance.4

Currently, PCR-assisted methods,4a radioactive labeling techniques,4b gel electrophoresis,1c high performance liquid chromatography and immune reaction4d,e have been established for MTase activity analysis. However, these assays are usually time-consuming with laborious operations. For overcoming the above limitations, various new strategies have been recently developed to improve the MTase activity detection sensitivity and adaptability such as nanomaterial-based assay,5 enzymatic amplification-based strategies,6 bioluminescence-based assay,7 exponential amplification reaction (EXPAR) assay,8 rolling circle amplification (RCA)9 assay, strand displacement assay,10 and enzyme-based cyclic amplification.11a-f Among these methods, the enzyme (endonuclease or exonuclease)-based cyclic amplification has become increasingly popular in the MTase activity detection due to its simplicity, specificity, and high sensitivity.11a-f Especially, molecular beacon (MB)-assisted enzyme cyclic isothermal amplification, with its inherent stability and specificity, has recently emerged as a potential technique for rapid and sensitive detection of MTase activity.11b Here, through rational design of a functional molecular probe with high sequence specificity that takes advantage of the aforementioned limitations. First, a hairpin recognition probe (HRP) with the 5′-G-A-T-C-3′ sequence in the stem part, which functions as the reaction substrate, is catalyzed to a methylated hairpin probe (5′-G-mA-T-C-3′) in the presence of Dam MTase. Then, the methylated hairpin probe is cleaved into two parts by the methylation-sensitive restriction endonuclease Dpn I. One is a new short hairpin DNA, and the other one is a 10-bp hybrid duplex with a 3′-overhang. However, this duplex is unstable.
This duplex will be separated into two independent single-stranded DNA (ssDNA), one of which is a 19-mer ssDNA as the trigger signal primer (TSP). Subsequently, the TSP hybridizes to the label-free molecular beacon (LFMB), and the LFMB, which integrates the trigger signal primer (the purple red part), nicking site (the green loop region) and the signaling sequence (a “caged” inactive G-quadruplex sequence) (the mandarin blue part) within one multifunctional design, is ingeniously designed as the amplification substrate and signal reporter. In the LEMB design, the G-quadruplex sequence, the specific sequence for nicking and the trigger signal primer fragment are incorporated into a hairpin probe in an initially locked format by hybridizing with their partially complementary sequences. When the LFMB is challenged with the liberative TSP, the LFMB was opened, resulting in the formation of the double-stranded recognition site for Nb.BbvCI. Nb.BbvCI can then cut the hybrid strand of LFMB in the duplex DNA hybrid, releasing the TSP and liberating the quadruplex-forming oligomer. Thus, the fluorescent supramolecular structure, ZnPPIX/G-quadruplex, can be formed to give a fluorescence response with the help of ZnPPIX. Furthermore, the released TSP is free to bind to another LFMB to trigger a new cleavage process, which constitutes cycle I in Scheme 1. At the same time, the trigger signal primer fragment in LFMB (the purple red part) is also released during each cleavage process, which can be used as a new trigger signal primer (N-TSP) to activate the successive cleavage process. The N-TSP is the same as the 19-mer ssDNA trigger signal primer (TSP). After its hybridization with the LFMB, the Nb.BbvCI also cuts the hybrid strand of LFMB in the duplex DNA hybrid, releasing again the trigger signal primer fragment in LFMB (the purple red part) as a new trigger signal primer (N-TSP) for the next cleavage cycle and liberating the quadruplex-forming oligomer for amplified fluorescence response. This constituted cycle II in Scheme 1. On the basis of the autonomous cleavage in cycles I and II, the target Dam MTase can be exponentially produced. Therefore, the current label-free molecular beacon-mediated quadratic isothermal exponential amplification strategy (LFMB-QIEA) by a simple Nb.BbvCI cleavage process is hopeful for sensitively monitoring the DNA methylation catalyzed by Dam MTase.

To demonstrate the methylation process of Dam MTase, a nondenaturating PAGE experiment was carried out (Fig. 1A). Only one band is observed for the original probe when either Dpn I or Dam MTase is absent (Fig. 1A, lanes 1 and 2), indicating that no 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 occurs, and the methylated HRP is then cut into cleavage segments.

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 LFMB and ZnPPIX, a negligible fluorescence signal was observed (curve a) due to the “caged” inactive G-quadruplex sequence in LFMB. With the addition of HRP, Dpn I and Nb.BbvCI, the fluorescence signal barely changed (curve d), because in the absence of the target (Dam MTase), the methylation of HRP 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 HRP, the cleavage of the methylated HRP, as well as the two subsequent cycling cleavage processes illustrated in Scheme 1. However, in the absence of Dpn I (curve b) or Nb.BbvCI (curve c), small fluorescence signals were observed, which clearly demonstrated that both the cleavage of the methylated HRP by Dpn I and the subsequent nicking enzyme assisted target recycling were necessary to achieve significantly amplified signals. In our strategy, to ensure low background, it is crucial for LFMB to guarantee the stabilization. Thus, the stabilization of LFMB was further investigated by gel electrophoresis experiments (Fig. S1†). The results demonstrated that the LFMB is fairly stable, further improving the accuracy and sensitivity of detection. Therefore, these results clearly demonstrated the feasibility of our LFMB-QIEA strategy for MTase activity assay.

In order to achieve the system’s best sensing performance, several experimental conditions, e.g. the HRP sequence (HRP3),
distinct fluorescence signal is observed in the presence of MTase. As shown in Fig. S6, can easily discriminate Dam MTase from M.Sss I and HhaI.

Under the optimal conditions, the sensitivity and dynamic range of the LFMB-QIEA strategy were evaluated toward Dam standards with various concentrations by using the designed route. As shown in Fig. 2, the fluorescence increased with the increasing target Dam concentration from 0.0005 to 5 U mL$^{-1}$ in the sample. An exponential curve is obtained between the fluorescence intensity and concentration of Dam MTase from 0.0005 to 5 U mL$^{-1}$. Meanwhile the fluorescence intensity was linear to the concentration of Dam in the range from 0.0005 to 0.01 U mL$^{-1}$ with a regression equation of $F = 60.09 + 63057.9 C$ and a correlation coefficient of 0.992, where $F$ and $F_0$ are the fluorescence intensities of the biosensing system in the presence and absence of Dam MTase, respectively. The limit of Dam MTase detection, based on three times the signal-to-noise level, was about $1.5 \times 10^{-4}$ U mL$^{-1}$, which was lower than that of previously reported assays (Table S2†). The results indicated that the signal amplification effect of the proposed LFMB-QIEA strategy was indeed realized.

Such extremely high sensitivity of the proposed method can be attributed to the following two factors: the high amplification efficiency of the LFMB-QIEA strategy and lowered background signal interference due to elaborately designed LFMB as the signal reporters. In addition, the relative standard deviation (RSD) for five replicate determinations of Dam MTase from the same batch at the 0.5 U mL$^{-1}$ level was 3.6% and the RSD for the interday was 4.2%, indicating the acceptable repeatability of the proposed method. These results suggest that the proposed method can be used for quantitatively analyzing MTase activity conveniently and efficiently.

We further evaluated the selectivity of this assay for Dam MTase activity. To investigate the selectivity of the proposed method, M.Sss I and HhaI that function as methyltransferases with the recognition sequences 5’-CG-3’ and 5’-GC-3’, respectively, were selected as interference methyltransferases 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. S6,‡ significant fluorescence enhancement is observed in the presence of Dam MTase. In contrast, no distinct fluorescence signal is observed in the presence of M.Sss I and HhaI. These results demonstrated that the amplified strategy for Dam MTase exhibited a good selectivity.

A big challenge for an excellent enzyme activity assay is its ability to be applied in real biological samples. To determine whether this method could be applied in real samples, we investigated the endogenous DAM activity of E. coli cells using our developed method. As shown in Fig. S7A,† the fluorescence intensity increased with the increased amounts of the total protein of E. coli cells, and we can readily observe the fluorescence signal even when 0.1 ng of the total protein was used, suggesting the high sensitivity of this assay. These results indicated that the proposed approach held great potential for the accurate quantification of Dam MTase in complex biological samples. The DAM activity in different growth stages of E. coli cells was then compared. The result showed that the activity of DAM in the exponential growth stage (from 1.5 to 3 h) is slightly higher than that in the stationary growth stage (after 12 h) (Fig. S7B†) with the DAM activity being $0.61 \pm 0.04$ U mg$^{-1}$ and $0.42 \pm 0.05$ U mg$^{-1}$, 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. In addition, we further examined the inhibition of DAM from E. coli cell lysate by gentamicin, benzylpenicillin and 5-fluorouracil, and evaluated DAM activity in DAM-negative E. coli of JM110 (Fig. S7B†). The results showed that DAM activity can be efficiently inhibited and no obvious DAM activity was observed (Fig. S7B†). These results suggested that the obtained signal is derived from DAM activity, and not from the nonspecific cleavage of LFMB. In conclusion, the developed assay can be optimistically applied to evaluate DAM activity in complex biological samples.

We also demonstrated the validity of our strategy in evaluating and screening the inhibitor DNA MTase using two antibiotics (benzylpenicillin and gentamicin) and one anticancer drug (5-fluorouracil) as model inhibitors. Given Dpn I and Nb.BbvCl enzymes involved in the whole process, it was essential to ensure whether these drugs have no influence on them. The result indicated that all the inhibitors exhibited negligible influence on the activity of both Dpn I and Nb.BbvCl when the concentration of each inhibitor was 1.0 μM (Fig. S8†). As a result, 1.0 μM of different inhibitors was used to estimate the influence on Dam MTase activity. In comparison with other inhibitors, 5-fluorouracil, a well-known broad spectrum anticancer drug, exhibited the strongest inhibitory effect at the same concentration (Fig. S9A†). We further investigated the concentration dependent inhibitory effect of 5-fluorouracil on the activity of Dam MTase. It is observed in Fig. S9B† that the calculated IC$_{50}$ for 5-fluorouracil was 0.75 ± 0.047 μM. These findings demonstrated that the proposed magnetic sensor can be used to study the MTase inhibitor and applied for screening of MTase inhibitors.

In conclusion, we have developed a one-pot label-free molecular beacon-mediated quadratic isothermal exponential amplification strategy (LFMB-QIEA) for simple, rapid and sensitive DNA methyltransferase (MTase) activity detection. In comparison with the molecular beacon (MB)-assisted enzyme cyclic isothermal amplification for the MTase assay† and the reported quadratic isothermal...
amplification strategy, the LFMB-QIEA strategy does not require the expensive and labor-intensive fluorescence modification, and avoids complex polymerization process and experimental operation in quadratic signal amplifications, the whole reaction can be performed under isothermal conditions within a relatively short time of 60 min in one step, making the proposed method more simple, rapid and cost-effective. Due to the significant quadratic signal amplifications, the proposed method exhibits excellent sensitivity with a detection limit of 1.5 × 10^{-4} \text{ U mL}^{-1}. Owing to the specific site recognition of Dam, this new approach could discriminate Dam from other methyltransferases with high selectivity. In addition, the application in E. coli cell extracts further demonstrated that the assay has great potential for sensitive detection of Dam activity in complex biological samples. Importantly, the inhibition effects of several antibiotics and anticancer drugs were also investigated, indicating the excellent performance of the developed method in DNA MTases inhibitor screening. With minor modification of the hairpin recognition probe and the change in the 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 the attractive analytical characteristics, the sensing strategy might find many important applications in early clinical diagnosis and biomedical research.

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Notes and references