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Highly sensitive fluorescence assay of DNA methyltransferase activity by methylation-sensitive cleavage-based primer generation exponential isothermal amplification-induced G-quadruplex formation

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ABSTRACT

Site-specific identification of DNA methylation and assay of MTase activity are imperative for determining specific cancer types, provide insights into the mechanism of gene repression, and develop novel drugs to treat methylation-related diseases. Herein, we developed a highly sensitive fluorescence assay of DNA methyltransferase by methylation-sensitive cleavage-based primer generation exponential isothermal amplification (PG-EXPA) coupled with supramolecular fluorescent Zinc(II)-protoporphyrin IX (ZnPPIX)/G-quadruplex. In the presence of DNA adenine methylation (Dam) MTase, the methylation-responsive sequence of hairpin probe is methylated and cleaved by the methylation-sensitive restriction endonuclease Dpn I. The cleaved hairpin probe then functions as a signal primer to initiate the exponential isothermal amplification reaction (EXPAR) by hybridizing with a unimolecular DNA containing three functional domains as the amplification template, producing a large number of G-quadruplex nanostructures by utilizing polymerases and nicking enzymes as mechanical activators. The G-quadruplex nanostructures act as host for ZnPPIX that lead to supramolecular complexes ZnPPIX/G-quadruplex, which provides optical labels for amplified fluorescence detection of Dam MTase. While in the absence of Dam MTase, neither methylation/cleavage nor PG-EXPA reaction can be initiated and no fluorescence signal is observed. The proposed method exhibits a wide dynamic range from 0.0002 to 20 U/mL and an extremely low detection limit of 8.6×10^{-5} U/mL, which is superior to most conventional approaches for the MTase assay. Owing to the specific site recognition of MTase toward its substrate, the proposed sensing system was able to readily discriminate Dam MTase from other MTase such as M.SssI and even detect the target in a complex biological matrix. Furthermore, the application of the proposed sensing strategy for screening Dam MTase inhibitors was also demonstrated with satisfactory results. This novel method not only provides a promising platform for monitoring activity and inhibition of DNA MTases, but also shows great potentials in biological process researches, drugs discovery and clinical diagnostics.

1. Introduction

DNA methylation, an epigenetic modification in human genomes, plays a critical regulatory role in gene transcription, cellular differentiation, and pathogenesis of various human diseases (Bernstein et al., 2007; Frauer and Leonhardt, 2011; Zhang et al., 2014a). It has been well documented that DNA methylation is closely associated with the activity of DNA methyltransferase (MTase) (Li et al., 2012; Rajendran et al., 2011; Zhang et al., 2014b). The DNA methylation process is catalyzed by DNA methyltransferase (MTase) which can transfer a methyl group from sadenosyl methionine (SAM) to target adenine or cytosine residues in the recognition sequences (Cheng and Roberts, 2001). Recent researches demonstrate that DNA methylation and DNA MTases have become predictive biomarkers for early clinical diagnosis and a novel family of pharmacological targets for anticancer therapy (Laird and Jaenisch, 1996; Mutze et al., 2011). Thus, sensitive assay and inhibitor (anti-methylation drugs) screening for DNA MTases

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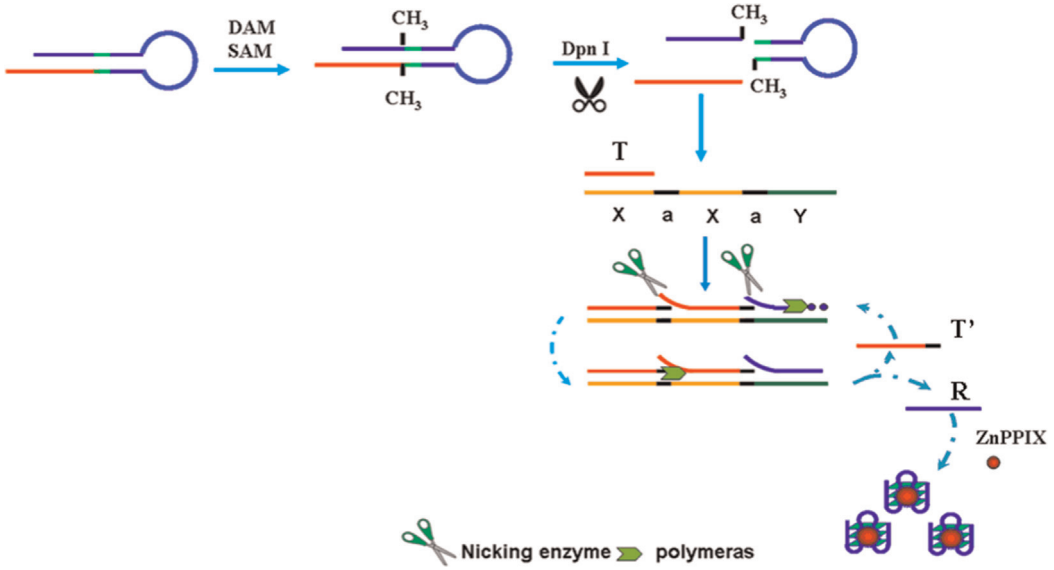
have become powerful tools for clinical diagnosis of cancer and therapeutics. In addition, effective assay of MTase activity can help in understanding how MTase activity contributes to cancer initiation and progression.

The conventional methods for the DNA MTase assay, including radioactive labeling (Som and Friedman, 1991), gel electrophoresis (Rebeck and Samson, 1991), high performance liquid chromatography (Reenila et al., 1995) and immune reaction (Wang et al., 2012), have been well-established. Unfortunately, these methods are time-intensive, laborious, not sensitive, or require isotope labeling. To overcome these disadvantages, alternatives techniques including colorimetry (Li et al., 2010; Song et al., 2009), fluorescence (Ouyang et al., 2012; Tian et al., 2012), chemiluminescent (Bi et al., 2013), surface enhanced Raman spectroscopy (SERS) (Marz et al., 2011), electrochemical approach (Li et al., 2012; Wang et al., 2012; Wu et al., 2012), etc. have been developed for MTase activity assay. Due to the unique optical, electronic, and mechanic properties of nanomaterials such as gold nanoparticles, carbon nanotubes, graphene oxide, a series of approaches based on nanomaterial amplification have been developed for gene-specific methylation detection and MTase activity assay. Although these methods are well-established, most of them are laborious with the involvement of complicated, tedious handling and labeling procedures. In addition, the heterogeneous formats at the surface of nanomaterials slow reaction rate and enzyme kinetics compared with homogeneous assays. Consequently, the development of a homogeneous, sensitive, economic and simple method for the detection of DNA methylation and assay of MTase activity still remains a great challenge.

Recently, the emerging research field of isothermal amplification-based DNA MTase assay has been developed to improve the detection sensitivity and flexibility, such as strand displacement amplification (SDA)-based colorimetric assay (Li et al., 2010; Liu et al., 2010), nicking enzyme signal amplification (NESA)-based fluorescence assay (Zhao et al., 2013), and rolling circle amplification (RCA) -based assay (Bi et al., 2013; Cao and Zhang, 2012; Zeng et al., 2013), etc. The SDA-based colorimetric method allows simple visualization of MTase activity, but the detection sensitivity of the colorimetric method is limited. The NESA-based fluorescence method exhibits improved sensitivity, but it needs labeled DNA probes and is facilely affected by external nonspecific events. Recently, a variety of RCA methods have been introduced to develop new highly sensitive assays of DNA MTase such as the hyper-branched rolling circle amplification (HRCA) (Bi et al., 2013), primer generation rolling circle amplification (PG-RCA) (Zeng et al., 2013), and hybridization chain reaction based branched rolling circle amplification (HCR-BRCA) (Cao and Zhang, 2012). Although these RCA-based methods exhibit high sensitivity, they involve multiple assay steps and require the addition of many exogenous reagents, which might increase nonspecificity. For example, the preparation of circular probes by ligation reaction involves many exogenous reagents such as ligase, exonuclease I and exonuclease III. In addition, it has been proven that the HRCA process is difficult to control and often generates circle-independent amplification by-products. And, it needs two primers and might suffer from the nonspecificity (Cao and Zhang, 2012). Consequently, the development of highly sensitive, specific, simple and low-cost methods for the DNA MTase assay still remains a great challenge. Recently, the exponential isothermal amplification reaction (EXPAR) has found growing interest for detecting DNAs and RNAs with high amplification efficiency (10^6–10^9-fold amplification) under a constant temperature (Jia et al., 2010). Although many EXPAR-based methods have been reported for sensing of various targets such DNAs and RNAs, no such methods are currently available for DNA MTase assay.

Inspired by the signal amplification strategy, herein, we developed a facile, label-free, highly sensitive and selective method for the DNA MTase assay by methylation-sensitive cleavage-based primer generation exponential isothermal amplification (PG-EXPA) coupled with supramolecular fluorescent Zinc(II)-protoporphyrin IX/G-quadruplex (Scheme 1). The scheme takes advantage of the intrinsically extreme fidelity of MTase toward its substrate and specific methylation-sensitive restriction endonuclease, the powerful signal amplification capability of exponential isothermal amplification reaction (EXPAR) and the significant increase of the fluorescence signal from the interaction between Zinc(II)-protoporphyrin IX (ZnPPIX) and G-quadruplexes. We aim to improve the sensitivity of MTase activity detection via EXPAR, and the selectivity by the intrinsically extreme fidelity of MTase toward its substrate and specific methylation-sensitive restriction endonuclease. The proposed method exhibits a wide dynamic range from 2×10^{-4} to 20 U/mL and an extremely low detection limit of 8.6×10^{-5} U/mL. In addition, owing to the specific site recognition of MTase toward its substrate, the proposed



Scheme 1. Schematic illustration of Dam MTase fluorescence assay using methylation-sensitive cleavage-based primer generation exponential isothermal amplification (PG-EXPA) coupled with supramolecular fluorescent ZnPPIX/G-quadruplex.