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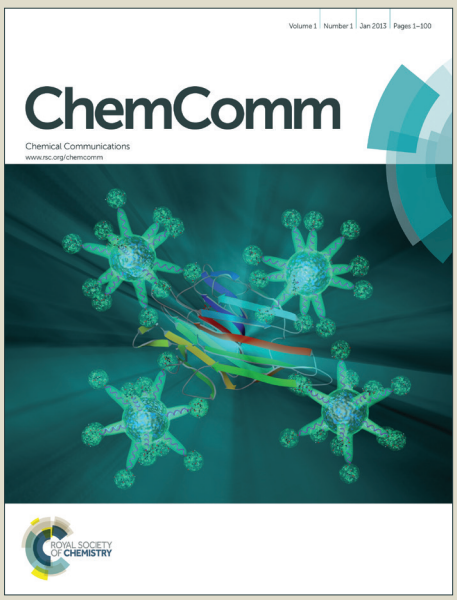

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ARTICLE TYPE

Highly sensitive methyltransferase activity assay and inhibitor screening based on fluorescence quenching of graphene oxide integrating with the site-specific cleavage of restriction endonuclease

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We report a sensitive and selective approach for DNA methyltransferase (MTase) activity assay and MTase inhibitor screening by coupling the fluorescence quenching of graphene oxide with site-specific cleavage of a restriction endonuclease.

DNA methylation, which plays a critical role in the regulation of gene transcription, cellular differentiation, and pathogenesis of various human diseases such as cancers,[1-5] is closely associated with the activity of DNA methyltransferase (MTase).[1d,2,3] Alterations of MTase activity may lead to aberrant DNA methylation patterns.[4] Notably, MTase has become predictive biomarkers and potential therapeutic targets in a variety types of cancers,[5] particularly useful for disease diagnosis and prognostics as well as to study the response of various organisms to environmental conditions.[6] Moreover, abnormalilties in MTase activity usually occur far before other signs of malignancy and could thus be used for early cancer diagnosis.[1d,7] Thus, sensitive activity assay and inhibitor (anti-methylation drugs) screening for MTases represent a valuable strategy to both clinical diagnostics and therapeutics. In addition, effective assay of MTase activity can help to understanding how MTase activity contributes to cancer initiation and progression.

Various assays have been developed for MTase activity assay. While radioactive labeling is the current standard for assaying MTase activity,[8] the desire to avoid radioactive reagents has motivated the development of diverse alternatives including high-performance liquid chromatography (HPLC),[9] colorimetry,[10] gel electrophoresis,[11] chemiluminescence,[12] and electrochemical methods,[3a,b,4a,13] etc. Although these methods are well-established, most of them are laborious with the involvement of expensive equipments and complicated, tedious labeling procedures.[3a,b,13] Consequently, the development of highly sensitive, specific, and low-cost methods for MTase assay still remains a great challenge.

This work reports a rapid, sensitive, and selective MTase (using M.SssI as an example) activity assay by coupling the fluorescence quenching of graphene oxide (GO) with site-specific cleavage of a restriction endonuclease (HpaII) for improving selectivity (Figure 1). We designed a single-stranded probe DNA (P1. The sequences of P1 and targets are depicted in Experimental section, ESI) that carries both a binding region and a sensing region. The binding region (64 bases) provides an anchoring function to facilitate the interaction between GO and P1, inducing fluorescence quenching of the 5′-terminus-labeled fluorophore (FAM, 6-carboxyfluorescein) (step a). The sensing region (32 bases) specifically recognizes the target (T1, a 32-mer DNA sequence from the promoter region of the *Homo sapiens* *p*53 gene) and hybridizes with it to form P1/T1 duplex (step b). This duplex contains the specific sequence recognized by HpaII endonuclease, which recognizes the duplex symmetrical sequence 5′-CCGG-3′ and catalyzes its cleavage between the cytosines.[14] After digested by HpaII, FAM is subject to be released from the GO surface, resulting in the recovery of fluorescence intensity of the fluorophore and producing a readily detectable signal (step c). However, after the duplex was methylated by M.SssI (step d), this cleavage is blocked (step e), and therefore no recovered fluorescence signal can be detected. Thus, the recovered fluorescence signal after HpaII cleavage is related to MTase activity, which establishes the basis of MTase activity assay.

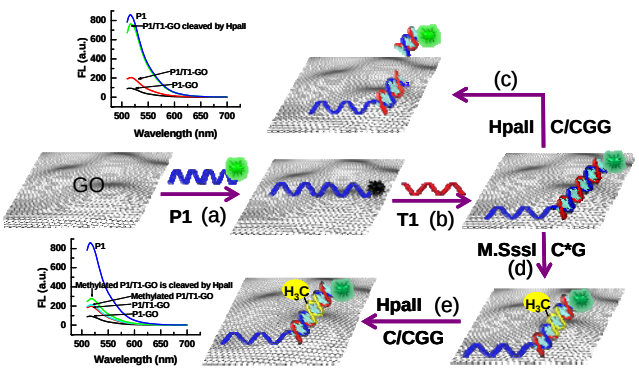

Fig. 1 Ilustration of MTase activity assay based on coupling the fluorescence quenching of GO with a restriction endonuclease.

P1 (10 nM) exhibits a strong characteristic fluorescence signal of FAM at ~520 nm (a, Fig. 2A. Please refer to ESI for experimental details). This fluorescence is very stable because no time-dependent fluorescence changes can be observed in solution at least within 10 h (Fig. S1, ESI). Addition of GO (20 μg/mL) into solution causes the complete quenching of the fluorescence of FAM (b, Fig. 2A), indicating high quenching efficiency of GO to FAM. The detailed results on GO concentration- and quenching time-depdendent quenching efficiency of GO to FAM are presented in ESI (Fig. S2 and the related discussion, ESI).

Addition of T1 (10 nM) into the solution induces a slight recovery of the fluorescence intensity of the quenched FAM (c, Fig. 2A). This is attributed to the formation of P1/T1 duplex, leading FAM to be away from GO surface because of the weaker interaction between GO and the duplex. However, the recovered fluorescence intensity is very low, implying that nearly no FAM is released to the solution. The P1/T1 duplex at GO surface is stable because the alteration of the fluorescence intensity is negligible in 4 h (Fig. S3, ESI), which is the total time span of our measurements. The fluorescence intensity can be greatly recovered by adding HpaII into the solution (20 U/mL) (d, Fig. 2A, 2 h cleavage) because of cleavage of HpaII between the cytosines in P1/T1 duplex symmetrical sequence of 5′-CCGG-3′. Of note, the dependences of the fluorescence inetnsity on HpaII dose and cleavage time are depicted in Fig. S4A, B (ESI).

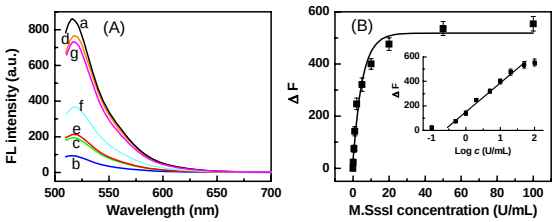

Fig. 2 (A) Fluorescence emission spectra of P1 in solution (a) and at GO surface (b), of P1/T1 at GO surface before (c) after (d) 2-h cleavage of HpaII, and of methylated P1/T1 before (e) and after (f) cleavage of HpaII. (g) is the fluorescence spectrum of the P1/T1 after 2-h treatment with 160 μM SAM (without M.SssI) and 2-h cleavage with HpaII. (B) Dependence of ΔF of the methylated P1/T1 on M.SssI concentration. The inset shows the plot of ΔF vs the logarithm of the M.SssI concentration. Error bars were based on five measurements.

To validate the observed fluorescence recovery is caused by HpaII cleavage, three control experiments were designed and performed. One control was made by replacing T1 with a 32-mer modified *p*53 gene containing one mismatch base (T2). After P1 on GO surface was hybridized with T2 to form P1/T2 and subsequently cleaved by HpaII for 2 h, the fluorescence signal did not increase because HpaII cannot cleave P1/T2 due to the lack of the specific recognition sequence of in P1/T2 (c, Fig. S5, ESI). The second control was made by heating the HpaII at 90 °C for 10 min to undergo an irreversible inactivation. We found that the activity of heat-treated HpaII reduced greatly because the fluorescence intensities were not observed to increase upon 2 h cleavage (a, b, Fig. S5). The third control was made by recording the fluorescence changes of P1-functionalized GO after 2-h cleavage with HpaII in the absence of T1. The results indicated that the interaction of P1 with HpaII did not induce a significant change of fluorescence (d, e, Fig. S5). These results confirm that the observed fluorescence recovery in Fig. 2A (d) originates only from the cleavage of P1/T1 duplex with HpaII.

The recovered fluorescence also depends on M.SssI dose in solution. After P1/T1 was methylated by 10 U/mL M.SssI (in the presence of 160 μM SAM, S-adenosyl-L-methionine) for 2 h (the optimization of methylation time is is depicted in Fig. S4C, ESI) and then cleaved by HpaII (20 U/mL) for 2 h, the recovered fluorescence (f, Fig. 2A) is significantly lower than that for unmethylated duplex (d, Fig. 2A). This is due to that the cleavage of HpaII is blocked by CpG methylation,[14] and therefore no FAM is released to solution. Note that the methylation of the duplex does not affect fluorescence features of the FAM because the fluorescence spectrum of methylated duplex (e, Fig. 2A) is almost identical with that for unmethylated duplex (c, Fig. 2A). To confirm the decrease in the recovered fluorescence intensity is caused by the methylation of P1/T1, a control experiment was conducted with a buffer only containing SAM (160 μM, in absence of M.SssI). As expected, a high fluorescence recovery was observed (g, Fig. 2A) because methylation cannot occur without M.SssI. These results suggest that the proposed method can be used for M.SssI activity assay.

The analytical performance was evaluated by 2-h treatment of P1/T1 with various concentrations of M.SssI (0−100 U/mL) in methylation step and followed by 2-h cleavage with 20 U/mL HpaII. The recovered fluorescence intensity depends on the concentration of M.SssI (Fig. S6, ESI). We employed the value of ΔF as the analytical signal and plotted the relationship between ΔF and the concentration (activity) of M.SssI (Fig. 2B). ΔF was defined as $\Delta F = F_0 - F_1$, where F_0 and F_1 are the recovered fluorescence intensity of the unmethylated P1/T1 and the methylated P1/T1 (methylated with different concentration of M.SssI), respectively, after cleaved by HpaII. As depicted in Fig. 2B, ΔF increases with the concentration of M.SssI at lower concentration and then levels off at higher concentration. This is because almost all of P1/T1 duplex at GO surface are methylated at a high concentration of M.SssI and subsequently cannot be cleaved by HpaII. The plot of ΔF versus the logarithm value of the concentration of M.SssI (in U/mL) displays a linear relationship in the range from 0.1 to 100 U/mL with a limit of detection (LOD) of ~(0.03 ± 0.01) U/mL (at a signal/noise of 3) (inset, Fig. 2B). The assay range is comparable with that obtained based on electrochemical approach (0−120 U/mL)[13] and is much wider than that of previously reported one based on colorimetric approach (1.0−10 U/mL),[15] and HPR-based immunosensing method (0.5−50 U/mL).[16] The detection limit is also much lower than that obtained using cross-linking Au nanoparticles aggregation (2.5 U/mL),[17] methylation-responsive DNAzyme methods (6 U/mL),[18] or hairpin-structured fluorescent probe coupled with enzume-linked reactions (0.8 U/mL).[19] Ten repetitive measurements with 10 U/mL M.SssI was used for evaluating the precision of the proposed method, and a relative standard deviation (RSD) of ~2.4% was obtained, demonstrating good reproducibility of the assay. These results suggest the method can be used for quantitatively analyzing MTase activity conveniently and efficiently.

The specificity of the proposed assay was evaluated by selecting two other cytosine MTases (HaeIII and AluI) as the potential interfering M.SssI. HaeIII methylates the internal cytosine residues in the sequence of symmetric tetranucleotide 5′-GGCC-3′, and AluI methylates the cytosine residues in the double-stranded symmetric 5′-AGCT-3′ sequence.[13] As shown in Fig. S7 (ESI), the proposed assay displays high specificity to M.SssI. This is evidently due to that the CpG dinucleotide site in the HpaII recognition sequence (5′-CCGG-3′) cannot be methylated by HaeIII or AluI. As a result, the P1/T1 were cleaved off in the HpaII cleavage step. Therefore, the proposed assay exhibit good selectivity toward M.SssI activity assay.

We also demonstrated the validity of our method in evaluating and screening the inhibitors of M.SssI with use of 5-Aza (5-

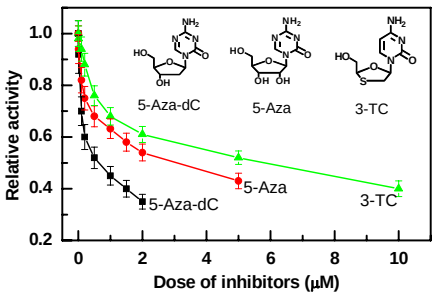

Fig. 3 Dose-dependent inhibition of M.SssI activity by anticancer drugs. Error bars were based on three measurements. The inset shows the chemical structures of 5-Aza-dC, 5-Aza, and 3-TC.

azacytidine), 5-Aza-dC (5-aza-2′-deoxycytidine), and 3-TC (2′,3′-dideoxy-3′-thiacytidine) as model inhibitors. These compounds are nucleoside analogs and representative anticancer drugs used in a large number of clinical trials and in the majority of methylation inhibition experiments.[20] They have been proven to be able to be incorporated into DNA to trap and inactivate MTase.[20] We evaluated the inhibited effects of the three drugs on M.SssI activity by comparing the fluorescence signal under various dose of the drug. The activity of the M.SssI decreases with the increasing dose of 5-Aza, 5-Aza-dC, and 3-TC (Fig. 3), showing significant dose-dependent inhibition. It is worth noting that 5-Aza-dC exhibited the highest inhibition efficiency in the three selected drugs. This is in accordance with the fact that 5-Aza-dC does not need to be modified to a deoxy form and can be more readily incorporated into DNA.[21] The inhibition of DNA methylation by M.SssI (about 10%) can be observed even treated with trace 5-Aza-dC as low as 0.01 μM. The IC_{50} value, the inhibitor concentration required to reduce enzyme activity by 50%, is acquired from the plots of relative activity of M.SssI versus the inhibitors concentration and is found to be ~0.63, 3.1, and 6.3 μM for 5-Aza-dC, 5-Aza, and 3-TC, respectively. It should be noted that the three drugs have negligible influence on the activity of HpaII when the dose of 3-TC, 5-Aza and 5-Aza-dC is lower than 15, 10, and 5 μM, respectively. These results indicate that the developed method has a potential application in studying the inhibited effects of anticancer drugs on MTase and for screening MTase inhibitors. It is noteworthy that such kind of study on the pharmacological inhibition of DNA MTase can provide a broad spectrum of therapeutic applications such as antibiotics and anticancer therapeutics.

In summary, we have described a rapid, sensitive, and selective method for M.SssI activity assay and inhibitors screening. The extraordinarily high quenching efficiency of GO (resulting in a high signal-to-background ratio) and the high site-specific cleavage of HpaII (resulting a high selectivity) make this method a promising assay for MTase activity with high sensitivity and selectivity. In comparing with these previously reported methods, our method operates via detecting the recovered fluorescence signal, which is a combined result of the specific methylation and the site-specific endonuclease cleavage; thus, it should be relatively impervious to false signals arising due to the nonspecific adsorption of interferants. In addition, it does not require bisulfite conversion, PCR amplification, separation, or radioactive labeling. The developed method may find potential use in the discovery of anticancer drugs.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details, and seven Figures. See DOI: 10.1039/b000000x/

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