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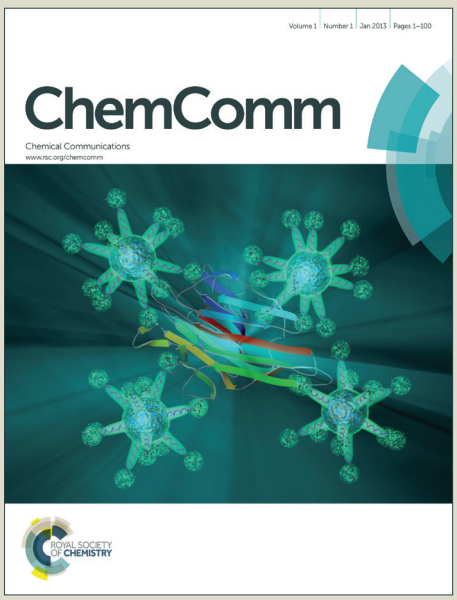

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ARTICLE TYPE

A Label-free Electrochemical Strategy for Highly Sensitive Methyltransferase Activity Assay

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A new electrochemical strategy for the simple and ultrasensitive evaluation of DNA methyltransferase (MTase) activity was developed based on electrocatalytic oxidation of ascorbic acid by graphene. In addition, the suitability of this sensing platform for MTase inhibitor screening was also demonstrated.

Deoxyribonucleic acid (DNA) methylation is one of the most pivotal epigenetic events that occurs in many biological processes such as gene transcription, genomic imprinting, cellular differentiation, chromatin structure and embryogenesis.[1] In eukaryotes, the DNA methylation is catalyzed by specific DNA methyltransferases (MTase) with a transfer of a methyl group from *S*-adenosyl-$_L$-methiolnine (SAM) to 5′-position of cytosine in the CpG dinucleotides.[2] It is reported that aberrant DNA methylation is recognized as potential biomarker in early diagnosis of cancer, and also regarded as hallmarks of other diseases.[3] Therefore, it is of great significance to develop simple and sensitive strategies for MTase activity assay.

In recent years, various methods have been proposed for the sensitive evaluation of MTase activity, including gel electrophoresis,[4a] real-time quantitative polymerase chain reaction,[4b] high performance capillary electrophoresis (HPCE),[4c] radioactive labeling,[4d] gas chromatography/mass spectrometry (GC/MS),[4e] colorimetry,[4f] fluorometry,[4g] and chemiluminescence.[4h] Although these methods were well established, most of them needed laborious sample treatment and/or expensive instrumental equipments.[5] Therefore, it is of great importance to develop simple, sensitive, and cost-effective strategies for MTase activity assay. Electrochemical methods have attracted great attention because of their low cost, simplicity, rapidity and high sensitivity.[6] Niwa et al.[7a] developed an electron cyclotron resonance (ECR) nanocarbon film electrode to sense DNA methylation events based on directly electrochemical oxidation of methyl-cytosine (mC). This method avoided bisulfite or labeling processes, but might not distinguish the accurate position of mC. Cai et al.[7b] used ferrocene labeled on 3′-terminus of the DNA as report signal to measure MTase activity. This method was further improved by replacing ferrocene with thionine/GO to amplify the signal.[7c] Ai et al.[7d] developed an electrochemically immune approach to evaluate the MTase activity and to monitor the DNA methylation at specific CpG sites with anti-5-methylcytosine antibody. To amplify the electrochemical response and to improve the sensitivity, anti-His-tag antibody and horseradish peroxidase-labeled immune globulin G functionalized gold nanoparticles (AuNPs-IgG-HRP) were used to recognize the mC and to amplify the signal.[7e] Those works needed complex label process, hence, a simple and label-free strategy for MTase activity assay is highly desired. Recently, employing the electrocatalytic oxidation of ascorbic acid (AA) by a special threading intercalator (N,N′-bis(3-propylimidazole)-1,4,5,8-naphthalene diimide (PIND) functionalized with electrocatalytic redox $Os(bpy)_2Cl^+$ moieties (PIND-Os)), a label-free electrochemical method was developed by Gao[5] et al for sensitive MTase activity assay. However, PIND-Os is a special compound synthesized in a special laboratory. The complex and multi-step synthesis route makes the use of PIND-Os be not convenient.

On the other hand, graphene is a two-dimensional sp^2-bonded carbon, which has aroused great interests in recent years due to its excellent properties, such as large specific surface area, excellent biocompatibility, and extraordinary electronic transport properties.[8] More importantly, graphene can be synthesized easily and has wide applications in many fields, ranging from advanced sensors to highly efficient fuel cells and batteries.[9] Till now, many attempts have been made to design and construct electrochemical biosensors based on graphene.[10] Recently, taking graphene covalently linked to DNA strand as a signal carrier, electrochemical and electrogenerated chemiluminescence (ECL) methods have been developed for the MTase activity assay.[7c,11] However, to the best of our knowledge, there are no works to construct a sensing platform for MTase activity assay based on the electrocatalytic property of graphene although it was reported that graphene had excellent electrocatalytic properties towards the electrochemical oxidation and reduction of some small molecules, such as AA, oxygen and hydrogen peroxide.[12]

In this work, a new label-free electrochemical strategy for sensitive and selective evaluation of DNA MTase activity was developed based on the electrocatalytic oxidation of AA by graphene (Fig. 1). In this strategy, two strands of DNA (a long-chain single-stranded DNA (ss-DNA) (S1) and a short-chain ssDNA (S2)) were used. S1 included two fragments (complementary part and adsorption part), and the complementary part hybridized with S2 to form the ds-DNA which contained specific recognition sequence (5′-CCGG-3′) for both M.SssI MTase and HpaII. Then, the S1/S2 hybrid was immobilized on the gold (Au) electrode surface through the Au-S bond. The 6-Mercapto-1-hexanol (MCH) was used as the blocker

to prevent nonspecific adsorption from other interferents and the MCH/ds-DNA/Au electrodes were prepared (Fig. 1A). After incubated with M.SssI CpG methyltransferase (M.SssI MTase) which catalyzed the methylation of the specific CpG dinucleotides, the electrode was treated with a restriction endonuclease HpaII which recognized the 5′-CCGG-3′ sequence. Based on the fact that the HpaII endonuclease catalyzed cleavage of the immobilized ds-DNA would be blocked when the CpG dinucleotide site in the 5′-CCGG-3′ sequence was methylated, higher M.SssI MTase activity led to more CpG sites being methylated and consequently impeded more the restriction endonuclease HpaII digestion process. Thus, a larger amount of ds-DNA remained on the electrode surface after the HpaII treatment, leading to the larger amount of graphene adsorbed on the adsorption part of S1 and thus the larger oxidation current of AA catalyzed by graphene. The catalytic oxidation current of AA directly reflected the methylation event and M.SssI MTase activity (Fig. 1B). On the other hand, we also demonstrated the application of the developed method for a rapid evaluation and screening of the inhibitors of MTase, which might be helpful for the discovery of anticancer drugs.

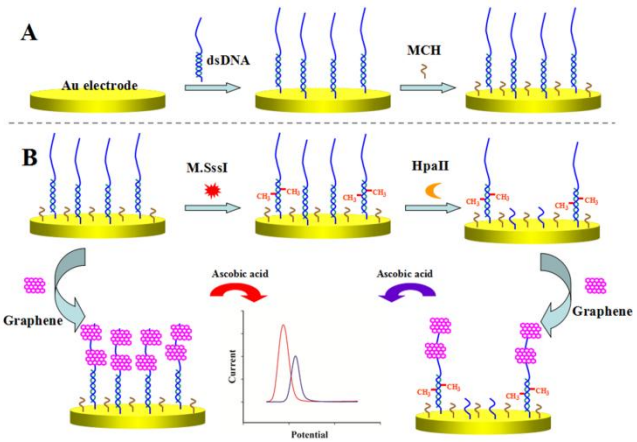

Fig. 1 Schematic illustration of the label-free electrochemical assay for methyltransferase activity based on the electrocatalytic oxidation of AA by graphene.

Fig. 2A shows the electrochemical impedance results of the different electrodes (bare Au electrode, MCH/ds-DNA/Au electrode, MCH/ds-DNA/Au electrode treated by M.SssI MTase/HpaII, and MCH/ds-DNA/Au electrode treated by HpaII) in 0.1 M KCl aqueous solution containing 5 mM (1:1) $[Fe(CN)_6]^{3-/4-}$as the redox probe. In the Nyquist plots of impedance spectra, the diffusion-limited process results in a linear portion at the lower frequencies, and the charge-transfer process performs in semicircle portion of the plot at the higher frequencies. The increase of the diameter of the semicircle reflects the increase of the interfacial charge-transfer resistance (R_{ct}). It is noted that the bare Au electrode shows a very small semicircle domain (R_{ct}=150 Ω, Fig. 2A, curve a), implying a very fast electron-transfer process of $[Fe(CN)_6]^{3-/4-}$. The self-assembly mixed layer of the MCH and negatively-charged ds-DNA on the Au electrode surface effectively repels the $[Fe(CN)_6]^{3-/4-}$ anions and thus leads to a significantly enhanced charge-transfer resistance (R_{ct}=26000 Ω, Fig. 2A, curve b). When the MCH/ds-DNA/Au electrode treated with M.SssI MTase/HpaII, some of the ds-DNA are cleaved to short ss-DNA due to the dehybridization of the remnant short segment of ds-DNA (5 base pairs) at the restriction endonuclease incubation conditions, resulting in an obvious decrease of R_{ct} (R_{ct}=18000 Ω, Fig. 2A, curve c). Also the R_{ct} of the M.SssI MTase/HpaII-treated electrode (curve c) is larger than that of the HpaII-treated electrode (R_{ct}=10000 Ω, Fig. 2A, curve d), this is attribute to the cleavage of the unmethylated ds-DNA by the restriction endonuclease HpaII. These results are in good accordance with that obtained by cyclic voltammetry (CV) (Fig. 2B) and imply that the procedure for the preparation of the MCH/ds-DNA/Au electrode is appropriate and the ds-DNA at the electrode surface is fully functional with respect to M.SssI MTase and HpaII.

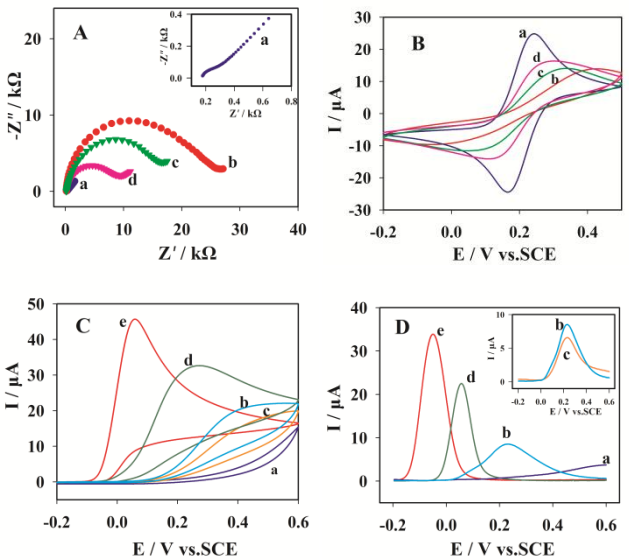

Fig. 2 (A) Electrochemical impedance spectra (Nyquist plots) and (B) cyclic voltammograms of the bare Au (a), MCH/ds-DNA/Au (b), MCH/ds-DNA/Au electrode treated by M.SssI MTase/HpaII (c), and MCH/ds-DNA/Au electrode treated by HpaII (d) in 0.1 M KCl aqueous solution containing 5.0 mM (1:1) $[Fe(CN)_6]^{3-/4-}$ as the redox probe. (C) Cyclic voltammograms and (D) DPV curves of the different electrodes in PBS (20 mM phosphate, 0.15 M NaCl, pH 7.4) containing 5.0 mM AA. (a) MCH/ds-DNA/Au electrode, (b) MCH/ds-DNA/Au electrode treated with 50 U mL^{-1} HpaII and then incubated with 0.5 mg mL^{-1} graphene, (c) MCH/ds-DNA/Au electrode only treated with 50 U mL^{-1} HpaII, (d) MCH/ds-DNA/Au electrode treated with 10 U mL^{-1} M.SssI MTase and 50 U mL^{-1} HpaII and then incubated in 0.5 mg mL^{-1} graphene, (e) MCH/ds-DNA/Au electrode incubated in 0.5 mg mL^{-1} graphene.

In order to evaluate the feasibility of the MTase Activity and inhibitor detection based on the MCH/ds-DNA/Au electrode, the CV responses to the electrocatalytic oxidation of 5.0 mM AA at the modified electrodes with different treatments were investigated. As shown in curve a of Fig. 2C, no oxidation peaks of AA can be observed at the MCH/ds-DNA/Au electrode due to the large R_{ct} value of the MCH/ds-DNA/Au electrode (Fig. 2A, curve b). When the MCH/ds-DNA/Au electrode was only treated

with 50 U mL^{-1} HpaII and then incubated with graphene (Fig. 2C, curve b), a broad and irreversible oxidation peak of AA appears at about 0.4 V due to the low R_{ct} value of the HpaII-treated electrode and possible adsorption of a few graphene on the short ss-DNA (5 bases). This is further confirmed by AA oxidation on the MCH/ds-DNA/Au electrode only treated with HpaII (Fig. 2C, curve c). However, for the MCH/ds-DNA/Au electrode treated with 10 U mL^{-1} M.SssI MTase and then 50 U mL^{-1} HpaII (Fig. 2C, curve d), the oxidation peak current of AA increases obviously and the related peak potential shifts negatively to about 0.26 V. Furthermore, for the non-treated MCH/ds-DNA/Au electrode, the electrode produces a largest and sharpest oxidation peak of AA, accompanied by a substantially negative shift of the peak potential to about 0.06 V (Fig. 2C, curve e). These imply that the oxidation peak current of AA is directly associated with the amount of the remnant ds-DNA on the electrode surface due to the impeding of the HpaII digestion process by the methylation of the ds-DNA at the CpG sites. A larger amount of ds-DNA remained on the electrode surface after the HpaII treatment, the larger amount of graphene adsorbed on the adsorption part of S1, leading to the larger oxidation peak current and more negative shift of the oxidation peak potential of AA due to the good electrocatalytic property of graphene. The above results, which were further confirmed by the differential pulse voltammetry (DPV) results (Fig. 2D), indicate that the developed strategy shown in Fig. 1 can be used to evaluate the activity of M.SssI MTase. Considering that well-defined oxidation peak of AA is beneficial to the sensitive assay of MTase Activity and inhibitor, the DPV method would be used in the following investigation.

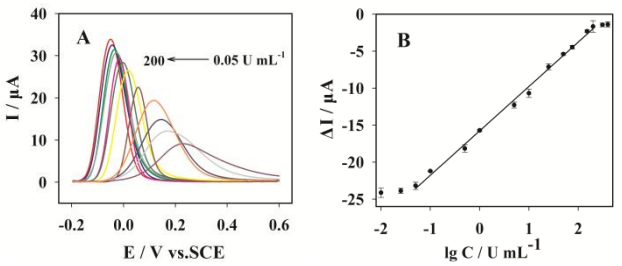

Fig. 3 (A) DPV responses of AA (5.0 mM) oxidation in PBS (20 mM phosphate, 0.15 M NaCl, pH 7.4) obtained on the MCH/ds-DNA/Au electrode treated in different concentrations of M.SssI MTase, 50 U mL^{-1} HpaII and 0.5 mg mL^{-1} graphene. (B) The relationship between the peak current difference (ΔI) and the logarithm of M.SssI MTase concentration (0.01 - 400 U mL^{-1}). ΔI = I - I_0, I_0 is the AA oxidation peak current on the MCH/ds-DNA/Au electrode incubated with 0.5 mg mL^{-1} graphene, I is the AA oxidation peak current on the MCH/ds-DNA/Au electrode treated with a definite concentration of M.SssI MTase, 50 U mL^{-1} HpaII and 0.5 mg mL^{-1} graphene.

Fig. 3A shows the DPV responses of AA oxidation on the MCH/ds-DNA/Au electrode treated with various concentrations of M.SssI MTase (0.05 − 200 U mL^{-1}) in the methylation step and followed by the incubation with 50 U mL^{-1} HpaII and 0.5 mg mL^{-1} graphene successively. It is noted clearly that the oxidation peak current of AA increases with the increase of the concentration of M.SssI MTase, and the corresponding oxidation peak potential shifts negatively. The above results should be resulted from the following fact: the higher the M.SssI MTase activity, the higher the methylation level, the more ds-DNA remaining on the electrode surface, and thus, the larger amount of graphene bound to the adsorption part in S1, which in turn, generates a higher catalytic oxidation peak current of AA at more negative potential. The relationship between the oxidation peak current of AA and the concentration of M.SssI MTase is shown clearly in Fig. 3B. It is noted that the calibration plot exhibits a linear range from 0.05 to 200 U mL^{-1} and the detection limit is 0.025 U mL^{-1} (S/N= 3) .The corresponding linear equation is ΔI (μA)=5.9969-15.7744lgC (U mL^{-1}) (R=0.9979). The detection range of this method is much wider than that of the previously reported MTase assays based on colorimetric approaches (6 - 100 U mL^{-1} or 2.5 - 40 U mL^{-1}) [4f,13] and also wider than that of the recently reported label-free electrochemical method with PIND-Os as catalyzer (0 - 120 U mL^{-1})[5]. The detection limit (0.025 U mL^{-1}) is also much lower than that of the previously reported colorimetric methods based on cross-linking AuNPs aggregation (2.5 U mL^{-1})[4f] and methylation-responsive DNAzyme (6 U mL^{-1})[12] strategies, and electrochemical methods with GO nanomaterial-based (0.05 U mL^{-1})[7c] and enzymatic HRP-based signal amplification systems (0.1 U mL^{-1})[7d]. This should be mainly due to the excellent electrocatalytic property of graphene towards AA oxidation.

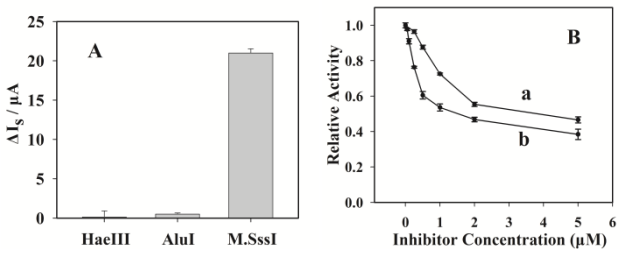

Fig. 4 (A) Selectivity of the MCH/ds-DNA/Au electrode. $\Delta I_s = I_s - I_{s0}$, I_s is the AA oxidation peak current obtained on the MCH/ds-DNA/Au electrode treated with 100 U mL^{-1} HaeIII MTase (AluI MTase or M.SssI MTase), 50 U mL^{-1} HpaII and 0.5 mg mL^{-1} graphene. I_{s0} is the AA oxidation peak current obtained on the MCH/ds-DNA/Au electrode treated with 50 U mL^{-1} HpaII and 0.5 mg mL^{-1} graphene. Solution for AA oxidation, PBS (20 mM phosphate, 0.15 M NaCl, pH 7.4) containing 5.0 mM AA. (B) The effect of the inhibitor (5-Aza (a) or 5-Aza-dC (b)) on the M.SssI MTase activity.

To investigate the selectivity of the proposed method, HaeIII MTase and AluI MTase were introduced as the interference enzymes. HaeIII MTase can methylate the internal cytosine residues within the double-stranded tetranucleotide recognition sequence of 5′-GGCC-3′, and AluI MTase can methylate the cytosine residues in the double-stranded symmetric recognition sequence of 5′-AGCT-3′. As shown in Fig. 4A, the response signal (ΔI_s) caused by HaeIII MTase or AluI MTase is very small, while the M.SssI MTase sample gives obvious change of the electrochemical signal. This is due to the fact that the CpG dinucleotide site in the HpaII endonuclease recognition sequence (5′-CCGG-3′) cannot be methylated by the HaeIII and AluI cytosine MTase. As a result, the unmethylated ds-DNA strands were cleaved off from the electrode surface after treated with HpaII. The above results indicate that the proposed method has

an excellent selectivity for M.SssI activity assay.

On the other hand, it is well-known that aberrant DNA methylation is closely related to cancer and diseases. Therefore, it is very important to evaluate and screen the DNA MTase inhibitors. Here, 5-Aza and 5-Aza-dC were selected as the model inhibitors. Both of the compounds are nucleoside analogs and prevent M.SssI MTase to catalyze the transfer of the methyl group in SAM to the basic group in DNA. The inhibition effects of 5-Aza and 5-Aza-dC on M.SssI MTase are illustrated in Fig. 4B. It is noted that the relative activity (Please see ESI†) of M.SssI MTase decreases with the increase of the concentration of 5-Aza (5-Aza-dC). The inhibiting efficiency of the inhibitors can be expressed as the half maximal inhibitory concentration (IC_{50}) which represents the inhibitor concentration required for 50% decrease in enzyme activity. From Fig. 4B, it can be obtained that the IC_{50} values for 5-Aza and 5-Aza-dC are 3.8 μM and 1.5 μM, respectively. These results suggest that the developed method can be used to rapidly evaluate and screen the inhibitors of DNA MTase and has great potential applications in anticancer drug discovery.

The reproducibility of the MCH/ds-DNA/Au electrode was also investigated. Five modified electrodes were used to detect the same concentration of M.SssI MTase (100 U mL^{-1}) and the relative standard deviation (RSD) is 4.7 %. Furthermore, the MCH/ds-DNA/Au electrode stored at 4 ^{o}C for two weeks still remains bioactive and the related response signal of AA at the graphene-treated MCH/ds-DNA/Au electrode has no significant change. These demonstrate that the developed electrochemical method has satisfactory reproducibility and stability for M.SssI MTase assay.

In conclusion, we developed a new electrochemical method for simple and highly sensitive evaluation of the M.SssI MTase activity. This strategy takes HpaII endonuclease to improve selectivity, and employs signal amplification of graphene to enhance the assay sensitivity based on the π-π stacking interaction between graphene and ssDNA strand and excellent electrocatalytic property of graphene toward AA oxidation. A linear relationship between the catalytic oxidation current of AA and the activity of M.SssI MTase ranged from 0.05 to 200 U mL^{-1} with a detection limit of 0.025 U mL^{-1} (S/N= 3) is obtained. In addition, the developed sensing platform shows good ability for MTase inhibitor screening and should be a potentially useful tool for anticancer drug discovery.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [TEM and AFM images of graphene, and detailed experimental methods]. See DOI: 10.1039/b000000x/

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