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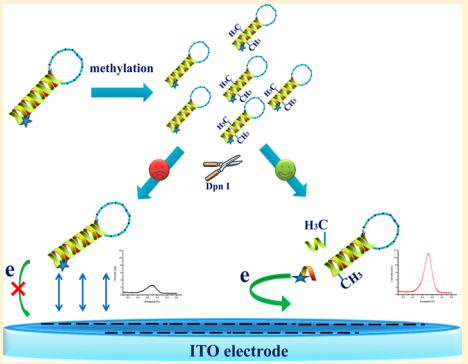
# DNA Methylation Detection and Inhibitor Screening Based on the Discrimination of the Aggregation of Long and Short DNA on a Negatively Charged Indium Tin Oxide Microelectrode

Xiaofeng Wei, Xiaoming Ma, Jian-jun Sun, Zhenyu Lin,* Longhua Guo, Bin Qiu, and Guonan Chen

MOE Key Laboratory of Analysis and Detection for Food Safety, Fujian Provincial Key Laboratory of Analysis and Detection Technology for Food Safety, Department of Chemistry, Fuzhou University, Fuzhou, Fujian, 350002, China

**ABSTRACT:** On the basis of the different diffusivity and existence of electrostatic repulsion between long and short DNA on the negatively changed indium tin oxide (ITO) microelectrode, a simple but sensitive immobilization free solution-phase electrochemical method for DNA methylation detection and inhibitor screening has been developed. Electroactive substance (methylene blue) tagged at the penultimate base T close to the 3′-terminal first, in the absence of DNA methylation, methylene blue-labeled electroactive fragments cannot be generated by Dpn I and results in a weak electrochemical response being detected on the ITO electrode. On the contrary, a remarkable electrochemical response can be achieved by the cleavage in the presence of DNA methylation since methylene blue-labeled electroactive fragments can be generated and aggregate on the ITO electrode. The proposed system does not need complex operation procedures such as bisulfitetreatment, PCR amplification, and electrode immobilization. Six ITO microelectrodes had been assembled on the same microchip, which can achieve the parallel detection of the same sample and improve the experimental efficiency of drug screening. The system was used to conveniently and specifically monitor the change of the DNA methylation level with high sensitivity and selectivity. The proposed system has the potential application to screen the drugs as inhibitors on the activity of methyltransferase in the clinic.




The methylation of DNA is catalyzed by methyltransferase (MTase) with a transfer of a methyl group from conjunct addition to adenine or cytosine in the specific DNA palindromic sequence. It is one of the essential epigenetics events and plays a crucial role in the regulation of gene transcription, genome imprinting, and embryogenesis in all mammals, eukaryotes, and prokaryotes. It is reported that DNA methylation is an important biological activity related to various pathogenic mechanisms to humans such as some cardiovascular diseases and carcinomas.[1−7] In most eukaryotic and prokaryotic cells, DNA methylation possesses the function of gene protection. However, for human, DNA aberrant methylation represses gene transcription, deregulates gene expression, and causes various human diseases.[8−11] Hence, detecting the DNA aberrant methylation level and the activity of MTase benefits the early diagnosis of some tumors and the epigenetic therapy for DNA methylation-related diseases.

So far, various methods have been exploited to detect DNA methylation, mainly including methylation-specific polymerase chain reaction (MSP),[12−15] bisulfite sequencing polymerase chain reaction (BSP),[16−18] and methylation-specific restriction enzyme polymerase chain reaction (MS-RE-PCR).[19] However, all these methods are based on bisulfite treatment and PCR amplification with the time-consuming and tedious process. Recently, some modified methods are developed to avoid the shortcomings of traditional approaches. For instance, Ma et al. presented a DNA methylation detection method based on the quartz crystal microbalance (QCM) with low DNA sample consumption.[20] Zhang et al. developed a fluorescent method to detect DNA methylation by ligation-mediated hyperbranched rolling circle amplification, which obtained a low detection limit with a solution-phase probe.[21] Another method based on the single base extension reaction and surface enhanced Raman spectroscopy for DNA methylation had also been developed by the same group.[22] Kazunori et al. reported a chemiluminescent method to measure DNA methylation levels by using methyl CpG-binding protein and luciferase-fused zinc finger protein in a homogeneous assay.[23] However, these approaches still need the alternative between bisulfite treatment and PCR amplification with complex operations. Besides, Shiyun Ai et al. lately proposed an electrochemical biosensing method for the detection of DNA methylation.[24] Zeng et al. suggested a colorimetric detection of DNA methylation.[25] Cai et al. also developed two kinds of DNA probe with ferrocene acetic acid and thionine/graphene oxide to conduct electrical signal via electrode modification for the detection of DNA methylation.[26,27] These methods do not need the process of bisulfite treatment or PCR amplification but require a fussy immobilization to the electrode or nanoparticle. Therefore, it is still

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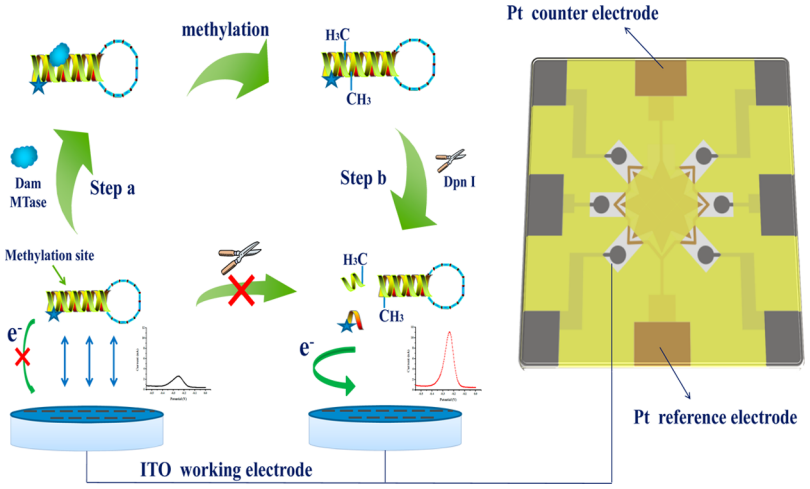


**Figure 1.** Mechanism of the proposed solution-phase electrochemical molecular beacon biosensor based on an ITO microelectrode chip for DNA methylation detection. Inset: ITO microelectrode chip with six ITO working electrode spots (diameter 1 mm), a Pt counter electrode, and a Pt pseudoreference electrode.

valuable to develop a simple, efficient, and economical method for the detection of DNA methylation.

Besides the simplicity, parallel detection is also vital for real sample assays to reduce the error, e.g., a successive assay of DNA methylation is necessary for the selection of drugs for methylation-related diseases.[28,29] In view of the large amount of samples for drug screening, a fast and efficient method is extremely preferred. In this regard, it is necessary to introduce a parallel system for drug screening of DNA methylation. In this study, an electrochemical molecular beacon biosensor based on an ITO microelectrode chip was constructed for DNA methylation detection. The proposed system does not need complex operation procedures such as bisulfite treatment, PCR amplification, and electrode immobilization. The ITO microelectrode chip (see Figure 1) was designed with several working electrodes, which can achieve the parallel detection to improve the experimental efficiency. This system is proposed to be used for conveniently and specifically monitoring the change of DNA methylation level in clinical samples and is also suitable for MTase inhibitors screening.

## EXPERIMENTAL SECTION

**Reagents and Apparatus.** Methylene blue-modified oligodeoxynucleotides (eLO) was purchased from Sangon Biotech (Shanghai) Co. Ltd., and the sequence was 5′-AGGATCCCGCTTCTTTTGAAGCGGGATCCCT-(methylene blue)-C-3′. Methylene blue was modified at the italic *T*. The DNA adenine methylation (Dam) MTase (*Escherichia coli*), Dpn I endonuclease, *S*-adenosyl-L-methiolnine (SAM), and the corresponding buffer solution were purchased from New England Biolabs Inc. Double-distilled water was used throughout the whole process. Other chemicals were of analytical grade and were used without further purification. Differential pulse voltammetry (DPV) experiments were conducted with a VMP3 multi-channel and multi-users electrochemical station (Bio Logic Science Instruments).

**Electrode Fabrication and Preparation.** The ITO coated glass chip is prepared in our laboratory according to the reported procedures.[30] One Pt counter electrode, one Pt pseudoreference electrode, and several patterned circular ITO spots (sinked into the insulated photoresist grooves) as working electrodes are integrated on the chip. The active surface of each ITO working electrode spot is a circle with 1 mm of the diameter. The potential of the Pt pseudoreference electrode in the 1× NEBuffer was determined to be +0.36 V with respect to an Ag/AgCl reference electrode. Before electrochemical detection, we sonicated the chip in an Alconox solution (10 g of Alconox/L of double-distilled water) for 15 min, propan-2-ol for 15 min, and twice in double-distilled water for 15 min in sequence. A negatively charged working electrode surface was achieved after these treatment processes.[31]

**Incubation of Molecular Beacon DNA.** Hybridization was conducted by incubating eLO (ultimate concentration of 1 $\mu$M) at 37 °C in 100 $\mu$L of 10 mM Tris-HCl buffer (pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl). Our results indicated that 2 h was long enough to guarantee complete hybridization, obtaining methylene blue-modified molecular beacon DNA (eMB).

**DNA Methylation and Cleavage of Dpn I Endonuclease.** The methylation of eMB was executed at 37 °C for 2 h in 10 mM Tris-HCl buffer (pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl) containing 160 $\mu$M SAM, 1 mM dithiothreitol (DTT), and diversified concentration of Dam MTase (from 0 to 70 U/mL) (step a in Figure 1).

After the above operation, Dpn I (ultimate concentration of 20 U/mL) was added in different samples. Cleavage was executed at 37 °C for 2 h to ensure complete reaction (step b in Figure 1). Then, for differential pulse voltammetric (DPV) scanning of each concentration, 2 $\mu$L of the sample solution was severally pipetted into grooves to cover the Pt counter electrode, the Pt pseudoreference electrode, and the ITO working electrodes. In other words, the same sample was detected several times by ITO working spots in the meantime. Then, the mean of DPV signals was as the final result.

**Inhibition of Some Drugs on DNA Methylation.** The inhibition of the drugs to the activity of Dam MTase was studied in our system. Herein, 2 $\mu$M disparate drugs were separately mixed with eMB by incubating for 2 h in Tris-HCl buffer (pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl) containing 160 $\mu$M SAM, 1 mM DTT, and superfluous Dam MTase (50 U/mL). Then, Dpn I was added in the samples to perform the cleavage reaction. Finally, different samples were pipetted several times into different grooves to achieve parallel screening.


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## RESULTS AND DISCUSSION

**Principle of Electrochemical DNA Biosensor.** Figure 1 illustrates the principle of site-specific DNA methylation detection and Dam MTase activity assay by an electrochemical MB-based DNA biosensor. The configuration of the ITO microelectrode chip is shown in the right inset in Figure 1, which owns several working electrode spots. After special dispose, the ITO working spots surface can be negatively charged, which can inhibit the aggregation of the negatively charged long DNA on the ITO working electrode.[30] This character was utilized to displace the traditional electrode modification.[32,33] That is to say, the different diffusivity and electrostatic repulsion exists between eLO and short-oligonucleotides (eSO), resulting in different DPV signal. The designed eMB DNA possesses a 31-mer DNA strand and an electroactive substance (methylene blue) tagged at penultimate base T close to the 3′-terminal. The eLO self-hybridizes into a hairpin eMB structure with a palindromic sequence of 5′-G-A-T-C-3′ on the stem, which is specifically recognized by Dam MTase and Dpn I. In the absence of Dam MTase, the reaction of DNA methylation has not been initiated, without release of methylene blue-labeled electroactive eSO by the cleavage of Dpn I. Hence, the eMB DNA electrostatically repulses the negative ITO working spots, so an appreciable DPV signal can be detected. In the presence of Dam MTase, it can recognize the site-specific palindromic sequence 5′-G-A-T-C-3′ of eMB DNA and facilitate the exchange of a methyl group from SAM to the target adenine A, forming 5′-G-Am-T-C-3′. Then, the methylated eMB DNA is cleaved into two parts by Dpn I through the recognition site. One part is a neo-hairpin structural DNA including the loop and the fractured terminal. The other is a new hybrid including a 4-base ssDNA linking methylene blue and a 5-base ssDNA. According to the $T_m$ value calculation equation ($T_m$ = 4 °C × G/C pair + 2 °C × A/T pair), the newly emerged hybrid only had a $T_m$ of 10 °C and will be detached into eSO and another short-oligodeoxynucleotides at the reaction temperature (37 °C). Therefore, the eSO was liberated by Dpn I with higher diffusivity. The electrostatic repulsion from the negatively charged ITO working spots to eSO is much littler than the eMB DNA,[31] so an obvious enhancement of the DPV signal can be detected.

A simple experiment has been performed to verify our presumption. The DPV signal of eLO and eMB with/without methylation under the cleavage reaction of Dpn I were detected, respectively. As shown in Figure 2, the DPV signal of eLO and eMB were negligible (see line a and line d), which indicates that the methylene blue-labeled oligonucleotides before/after self-hybridization are hardly close to the negatively charged ITO working spots surface on account of the electrostatic repulsion. Before methylation initiation, Dpn I could not cleave the unmethylated specific site of eLO and eMB, leading to a weak DPV response (see line b and line e). Comparing line c and line d, only the DPV signal of methylated eMB after the cleavage was distinct enhanced. It demonstrates that eLO without the palindromic sequence of 5′-G-A-T-C-3′ was not methylated in the presence of SAM and Dam. From these facts, it is credible that our proposal can be effectively conducted.

**Optimization of Reaction Conditions and Exonuclease Interference Assay.** The optimization of Dpn I concentration and cleavage time was conducted for the research of methyltransferase activity. As shown in Figure 3, the DPV signal increased with the augment of Dpn I concentration from 0 to 20 U/mL (see line a to line e). Then, the saturation was reached by persistently increasing Dpn I (see line e to line g). Afterward, the effect of cleavage time was studied also. We measured the DPV signal at 0−8 h after adding 20 U/mL Dpn I. The DPV signal increased gradually until 2 h and then reached a stabilized platform. Consequently, 20 U/mL Dpn I and 2 h cleavage time were chosen as the optimized conditions.

As shown in Figure 4, Dpn I (20 U/mL) was replaced by Exo I (100 U/mL) or Exo III (100 U/mL) to perform the cleavage reaction at the common conditions. Compared with the blank solution which contains no cleavage, the DPV signal increased

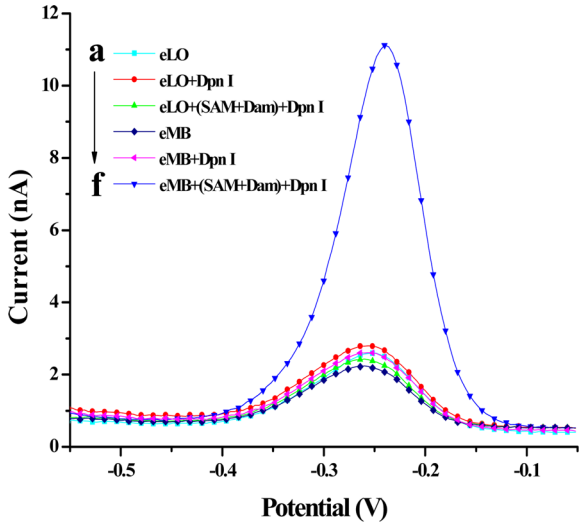


**Figure 2.** DPV responses of eLO (a), eLO + Dpn I (b), eLO + (SAM + Dam) + Dpn I (c), eMB (d), eMB + Dpn I (e), eMB + (SAM + Dam) + Dpn I (f) in Tris-HCl (10 mM, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT) after incubation at 37 °C for 4 h. [eLO] = 1 $\mu$M, [eMB] = 1 $\mu$M, [SAM] = 160 $\mu$M, [Dam] = 50 U/mL, [Dpn I] = 20 U/mL.

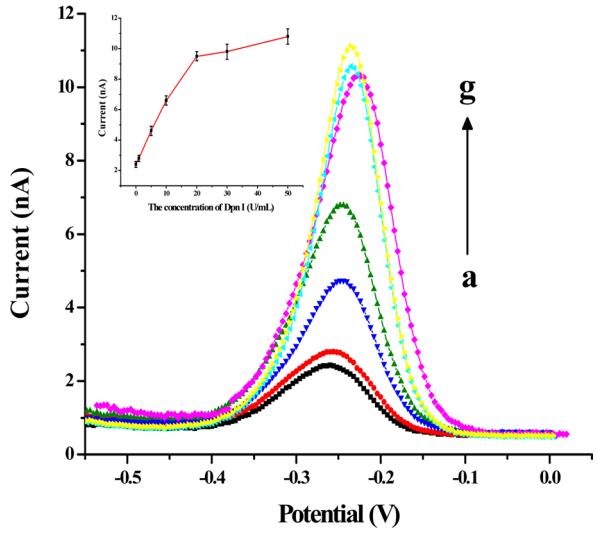


**Figure 3.** DPV responses with different concentrations of Dpn I in Tris-HCl (10 mM, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT) after incubation at 37 °C for 2 h. [eMB] = 1 $\mu$M, [SAM] = 160 $\mu$M, [Dam] = 50 U/mL. From a to g: 0 U/mL, 1 U/mL, 5 U/mL, 10 U/mL, 20 U/mL, 30 U/mL, and 50 U/mL. Inset: DPV peak currents plotted against concentration of Dpn I. The error bar was calculated from six detection points.


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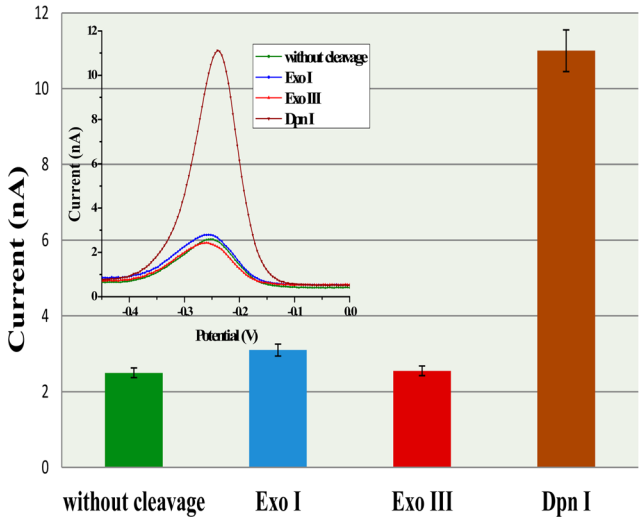


**Figure 4.** Histograms of DPV peaks changed by Exo I, Exo III, and Dpn I in Tris-HCl (10 mM, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT) after incubation at 37 °C for 2 h. Inset: DPV responses after cleavage by different enzymes. [eMB] = 1 $\mu$M, [Dam] = 50 U/mL, [SAM] = 160 $\mu$M, [Dpn I] = 20 U/mL, [Exo I] = [Exo III] = 100 U/mL. The error bar was calculated from six detection points.

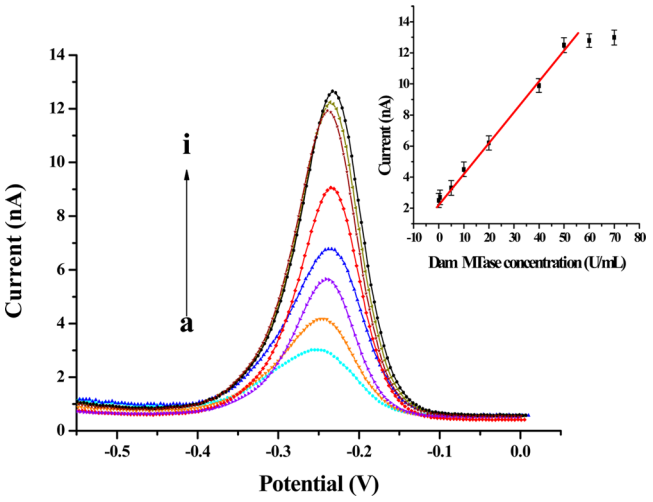


**Figure 5.** DPV responses with different concentrations of Dam in Tris-HCl (10 mM, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT) after incubation at 37 °C for 2 h. From a to i: 0 U/mL, 0.5 U/mL, 5 U/mL, 10 U/mL, 20 U/mL, 40 U/mL, 50 U/mL, 60 U/mL, and 70 U/mL. Inset: DPV peak currents plotted against concentration of Dam. [eMB] = 1 $\mu$M, [SAM] = 160 $\mu$M, [Dpn I] = 20 U/mL. The error bar was calculated from six detection points.

little in the presence of Exo I and Exo III, but remarkable enhancement had been detected in the presence of Dpn I. That is because Exo I and Exo III just, respectively, degrade the 3′-terminal of single-strain DNA and 3′-terminal of double-strain DNA. Our beacon structure has more one single oligonucleotide at the 3′-terminal, which can inhibit the cleavage of Exo I and Exo III. This consequence definitely certifies that the cleavage of Dpn I selectively occurred for the methylated peculiar sequence without the interference from other enzymes.

**Dynamic Studies of Methyltransferase Activity.** In order to test the reproducibility of the signal reached from the different working spots with the same sample. The results showed that the relative standard deviations (RSD) of DPV signals are in the range of ~2.56−4.97% at the concentration of ~0−70 U/mL. These results indicate that there is a good reproducibility from the six detection points. So in the following study, the average value from each working spots has been employed for quantitative analysis.

To estimate the analytical performance of our biosensor, eMB were methylated with various concentrations of Dam MTase from 0 to 70 U/mL for 2 h and then cleaved by Dpn I for 2 h, and then detected by the proposed system. The DPV peak current gradually increased with the increasing of Dam MTase concentration (Figure 5). This phenomenon is in accord with the fact that higher concentration of Dam MTase, more eMB methylated. Inset in Figure 5 is the linear relationship between the DPV signal and the Dam MTase concentration in the range of 0.5−50 U/mL. The limit of detection was estimated to be 0.18 U/mL (S/N=3). The DPV response leveled off at higher concentration after 50 U/mL. It is because that almost all eMBs were methylated at a high concentration of Dam MTase and no more methylated-eMBs could be generated by increasing Dam MTase. So, the saturated methylated-eMBs were cleaved by Dpn I with an invariable DPV signal finally.

**Quantification of DNA Methylation Level.** The proposed system was employed to monitor the DNA methylation level in the course of time. As shown in Figure 6A, the DPV signal increases with the increasing methylation time. This suggests the Dam MTsae catalyzes the methyltransfer activity as time progresses. However, the rate of increasing the process gradually decreased with the prolonging of the methylation time, indicating that the efficiency of the Dam MTase catalysis is reduced. This characteristic is due to the gradual consumption of substrates. Finally, the DPV signal reached a terrace when the methylation time was prolonged. It means the substrates are exhausted and the methyltransfer activity ends.

**Investigation of Inhibitor on the Activity of Methyltransferase.** For testing the potential of the proposed system to evaluate and screen the inhibitor of Dam MTase, four kinds of antibiotics and two kinds of anticancer drugs as model inhibitors had been chosen as models. As shown in Figure 6B, the benzylpenicillin sodium, gentamycin sulfate, and fluorouracil could distinctly inhibit the methylation. The inhibition of gentamycin sulfate was the most efficient with the inhibition ratio of about 50%. The benzylpenicillin sodium and fluorouracil reached the inhibition ratio of about 65%. Nonetheless, the rest of the antibiotics and anticancer drugs had hardly an effect on the methylation. These results indicate that the proposed system can be applied to screen the drugs as inhibitors on the activity of Dam MTase.

## ■ CONCLUSION

In conclusion, a simple, solution-phase electrochemical detection method for DNA methylation has been developed. Our approach utilized the electrostatic repulsion between DNA probe and the negative ITO working spots to achieve the solution-phase measurement, which averts complex operation procedures such as bisulfate treatment, PCR amplification, and electrode immobilization. The use of an ITO microelectrode chip achieved parallel detection and promoted the stability of electrochemical detection. In addition, this microelectrode chip only consumes a small amount of sample with the additional advantage of portability. The mechanism of the biosensor was meticulously constructed for the detection of DNA methylation


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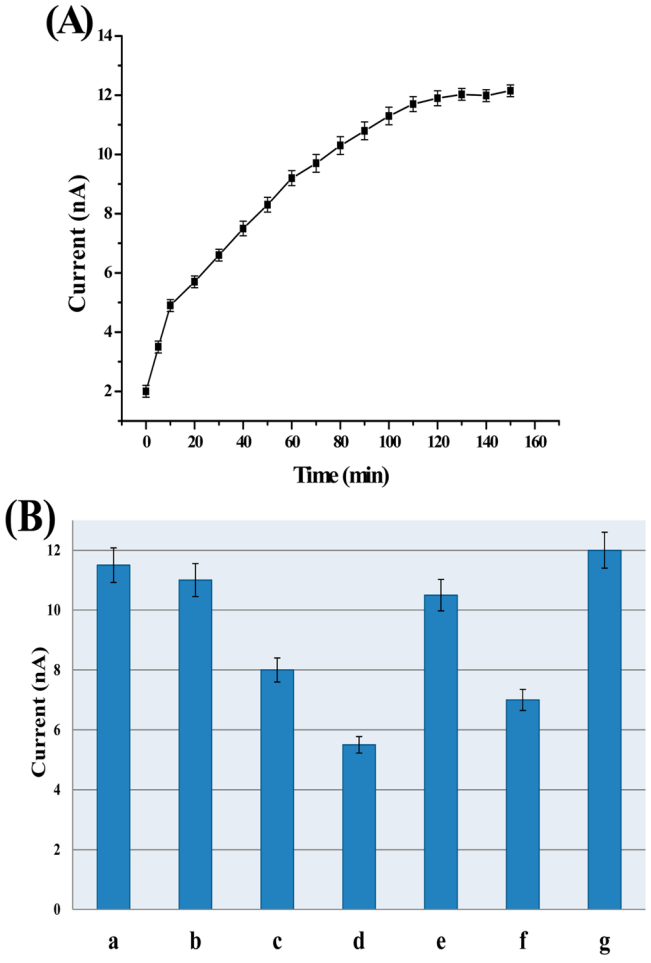


**Figure 6.** (A) Effects of methylation time on DPV peak responses of methylene blue in Tris-HCl (10 mM, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT) after incubation at 37 °C. [eMB] = 1 $\mu$M, [Dam] = 50 U/mL, [SAM] = 160 $\mu$M, [Dpn I] = 20 U/mL. The error bar was calculated from six independent experiments. (B) Inhibition of different drugs on the activity of Dam MTase: (a) no drug, (b) amoxicillin, (c) benzylpenicillin sodium, (d) gentamycin sulfate, (e) ofloxacin, (f) fluorouracil, (g) mitomycin. The error bar was calculated from six detection points.

with decent specificity and sensitivity. In view of the simplicity, portability, and parallel detection ability of the system, which has a great potential application for DNA methylation related clinical diagnosis and drug screening.

## AUTHOR INFORMATION

### Corresponding Author

*E-mail: zylin@fzu.edu.cn. Phone and fax: 86-591-22866135.

### Notes

The authors declare no competing financial interest.

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