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A sensitive electrochemical biosensor for detection of DNA methyltransferase activity by combining DNA methylation-sensitive cleavage and terminal transferase-mediated extension†

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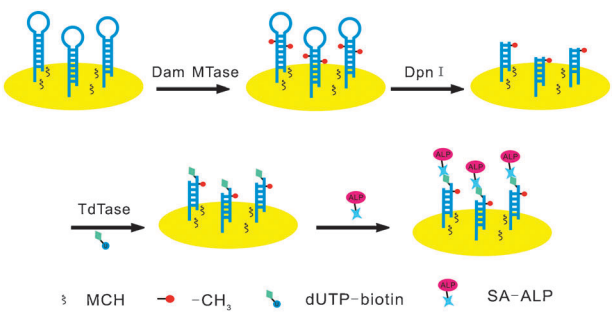
A novel electrochemical biosensor was developed for activity assay of DNA methyltransferase and its inhibitor based on methylation-sensitive cleavage, which activated a primer for terminal transferase-mediated extension of biotinylated dUTP followed by sensitive detection *via* enzymatic amplification.

DNA methylation plays an important role in many biological processes including transcription, genomic imprinting, cellular differentiation, chromatin structure and embryogenesis.[1–3] A number of human diseases have been found to be associated with aberrant gene methylation.[4,5] The DNA methylation process was regulated by DNA methyltransferase (MTase) catalyzing covalent addition of a methyl group to cytosine or adenine in DNA sequences.[6] The aberrant DNA MTase activity was reported to be related to pathogenesis of cancer, such relationship provides a potential target in disease diagnosis and therapy.[7] Therefore, it is critical to develop sensitive methods for quantitation, activity analysis and inhibitor screening of DNA MTase. Traditional methods for DNA MTase activity assay rely on radioisotope labeled substrates, various polymerase chain reaction (PCR)-based techniques, gel electrophoresis and high-performance liquid chromatography (HPLC).[8–11] Such methods are time-intensive, DNA-consuming, requiring laborious treatment and especially isotope labeling. Many of those limitations have been circumvented by using some new methods. For example, fluorescence assays based on hairpin fluorescence molecular beacon type DNA probes and cationic conjugated polymer–DNA complexes have been developed.[12,13] Au nanoparticle (AuNP) aggregation[14,15] and horseradish peroxidase (HRP) mimicking DNAzyme[16] were employed for the colorimetric assay of the MTase. AuNPs amplification and ferrocene-labeled DNA were used to detect MTase by electrochemical biosensors.[17,18] Although each technique has its own advantage, most of those methods utilized homogeneous reaction, the influence of an inhibitor on the methylation sensitive restrictive endonuclease activity could not be avoided. This limited the application of those methods to screen the MTase inhibitors. Therefore, it is still necessary to explore new sensitive methods for the detection of MTase activity and its inhibitors.

Herein, we developed a sensitive electrochemical biosensor based on methylation sensitive cleavage using terminal transferase (TdTase)-mediated extension for the detection of the MTase activity. In this assay, all the steps were performed on the surface of an electrode. When the biosensor was used to screen inhibitors, the DNA MTase and methylation sensitive restrictive endonuclease could be added, respectively, which would overcome the drawback in many previous methods. The TdTase-mediated base extension technique[19] was used to incorporate biotinylated dUTP (dUTP-biotin) into the 3′-OH terminal of the new primer produced by the cleavage of methylation sensitive restrictive endonuclease. Depending on the high exactitude of TdTase, the dUTP-biotin was only incorporated into the 3′-OH terminal, thus it would reduce the unspecific reaction and yield a lower background. The extended biotin could conjugate with the streptavidin-alkaline phosphatase (SA-ALP). ALP catalyzes conversion of electrochemically inactive 1-naphthyl phosphate (1-NP) into an electrochemically active phenol for the generation of an amplified electrochemical signal.[20–22] Taking the advantage of the TdTase-mediated dUTP-biotion extension principle, the amplified signal could be used to sensitively detect the activity of DNA MTase.

The schematic diagram of this DNA MTase activity assay is shown in Scheme 1 and the electrode modification process was



Scheme 1 Schematic illustration of electrochemical assay for the DNA methyltransferase activity based on methylation sensitive cleavage using TdTase-mediated extension with enzymatic amplification.

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confirmed by electrochemical impedance spectroscopy (ESI†, Fig. S1). The DNA adenine methylation (Dam) MTase was selected as a DNA MTase model. A hairpin DNA probe labeled with 3′-SH (for sequence see S1, ESI†) as the substrate was self-assembled on the surface of a gold electrode by means of facile gold–thiol chemistry. The S1 probe contained the sequence 5′-G-A-T-C-3′ which could be recognized by Dam MTase. In the presence of Dam MTase, a methyl group of S-adenosylmethionine (SAM) was transferred to the N6 position of the adenine residues in the specific sequence. And the sequence 5′-G-Am-T-C-3′ formed was specifically recognized and cleaved by methylation sensitive restrictive endonuclease Dpn I (Dpn I), which produced a new primer with a 3′-OH terminal on the electrode surface. Subsequently, the dUTP-biotin was incorporated into the 3′-OH terminal of the new primer by TdTase-mediated extension. Then, the SA-ALP was added and bound with the labelled biotin on the primer. The amplified electrochemical signal was obtained by ALP catalyzing 1-NP conversion. Differential pulse voltammetry (DPV) was employed to detect the signal which can be used for quantifying the Dam MTase activity and the concentration of its inhibitor.

Fig. 1 shows the DPV curves of the biosensor obtained in response to Dam MTase and the control experiments, respectively. In the presence of Dam MTase, a very strong current peak (curve a in Fig. 1) was observed, while otherwise, there was a rather small oxidation peak (curve b in Fig. 1). The control DNA probe (for sequence see S2, ESI†), which does not contain the specific recognition sequence of Dam MTase, was also studied. Compared to the S1 probe containing a specific recognition sequence of Dam MTase, an inconspicuous oxidation peak was obtained (curve c in Fig. 1). In the inhibitor screening experiment, the 5-fluorouracil was added into the Dam MTase containing sample, compared to the sample without the inhibitor, the oxidation peak recorded was sharply lower (curve d in Fig. 1). These results indicated that the electrochemical assay for Dam MTase activity and its inhibitor could be carried out.

Herein, the time of TdTase-mediated dUTP-biotin extension, the reaction time of DNA methylation and the concentration of dUTP-biotin were optimized. Maintaining longer time, the current does not increase significantly (Fig. S2, ESI†). The current signal reached a plateau after 60 min. The optimum response of DNA methylation was obtained after incubating for 40 min (Fig. S3, ESI†). Even with longer time, there was no significant current change. Therefore, 60 min of extension time and 40 min of methylation reaction time were used for all the subsequent experiments. Then, the ratios of dUTP-biotin : dATP were also studied, the concentration of 10 μM dUTP-biotin was chosen (Fig. S4, ESI†).

Under the optimum conditions, the activity of MTase was investigated with different concentrations of Dam MTase (0 to 80 U mL^{-1}). The DPV response of the electrochemical biosensor is shown in Fig. 2A, the current increased with the increase of Dam MTase concentration. Fig. 2B shows the curve of the peak current with different Dam MTase concentrations. The DPV current is proportional to the logarithmic value of Dam MTase concentration ranging from 0.1 to 20 U mL^{-1}. According to the rule of three times standard

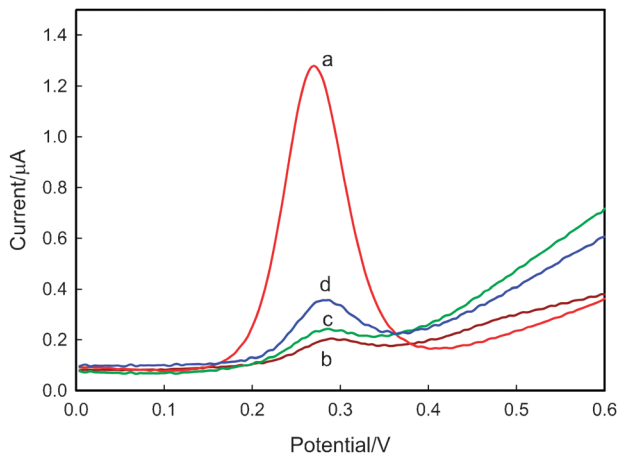

Fig. 1 Differential pulse voltammograms between 0 and 0.6 V *versus* SCE in response to (a) 80 U mL^{-1} Dam MTase, (b) 0 U mL^{-1} Dam MTase, (c) control DNA sequence, (d) 80 U mL^{-1} Dam MTase and 600 μM 5-fluorouracil. The DPV experiments were performed in 100 mM tris-HCl (pH 9.8), 1 mM Mg^{2+} and 4 mM 1-NP.

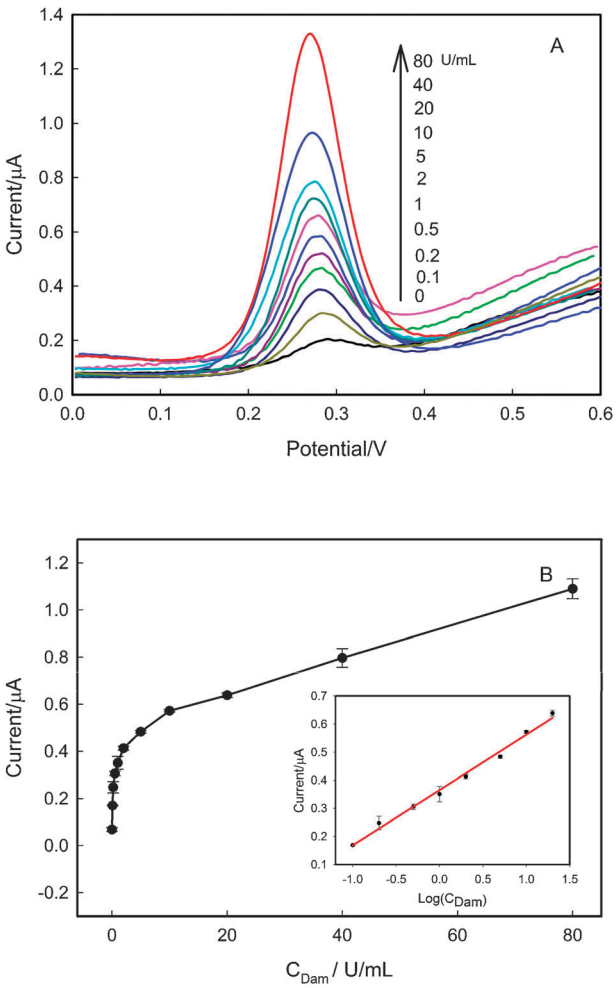

Fig. 2 (A) DPV response of the modified electrodes for different concentrations of Dam MTase: 0 (control), 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 40, 80 U mL^{-1}. (B) The response current obtained with different concentrations of Dam MTase from 0 to 80 U mL^{-1}. Inset: linear response from 0.1 to 20 U mL^{-1}.

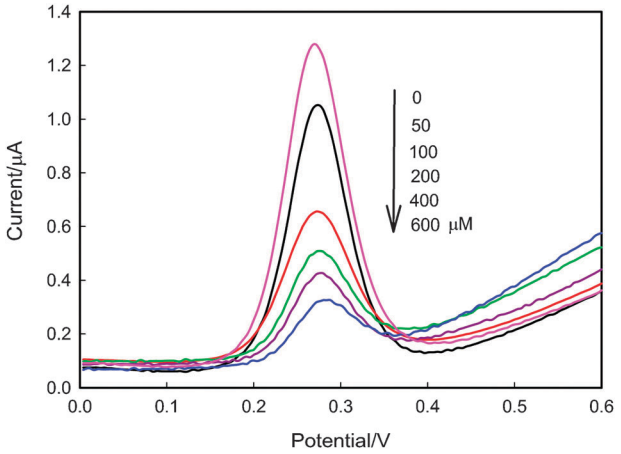

Fig. 3 Differential pulse voltammograms between 0 and 0.6 V *versus* SCE of inhibition assay for Dam MTase, 5-fluorouracil as a function of inhibitor concentration: 0, 50, 100, 200, 400, 600 μM.

deviation over the blank, the limit of detection for Dam MTase was calculated to be 0.04 U mL^{-1}, which is lower than most of previously reported assays.[12–18] This indicates that the proposed electrochemical method to monitor DNA MTase activity is highly sensitive.

To further demonstrate the potential application of this biosensor in MTase inhibitor screening, the experiments were performed in the presence of a Dam MTase inhibitor. As shown in Fig. 3, the 5-fluorouracil as a model inhibitor was used to study the influence on Dam MTase activity, the current peak decreased with the increase of the 5-fluorouracil concentration. This indicated that the activity of Dam MTase was inhibited by 5-fluorouracil. The result shows that the biosensor also has the potential ability to screen the inhibitors to the DNA MTase.

In conclusion, we developed a sensitive biosensor with TdTase-mediated extension and enzymatic signal amplification for the detection of MTase activity. This method does not need sophisticated instrumentation, the detection limit was achieved down to 0.04 U mL^{-1}. Furthermore, the developed sensor has eliminated the influence of inhibitors on methylation sensitive restrictive endonuclease activity and shows potential applications in DNA MTase inhibitor screening.

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