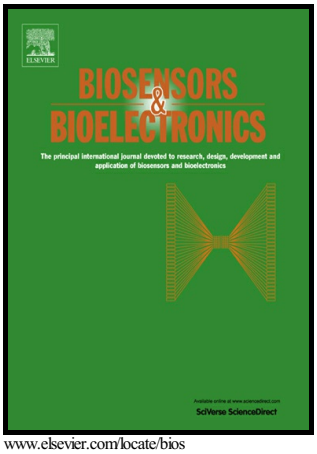

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An electrochemical strategy with tetrahedron rolling circle amplification for ultrasensitive detection of DNA methylation

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Abstract: Sensitive and specific detection of DNA methylation in genomic DNA is imperative for rapid epigenetic evaluations. Here, a novel sensitive electrochemical strategy was developed for ultrasensitive detection of DNA methylation in genomic DNA *via* padlock probe primer generating rolling circle amplification (RCA). Typically, after bisulfite treatment of methylated DNA, the methylation-specific linear padlock is only circularized in the presence of methylated DNA and subsequently

serves as a template containing a DNA tetrahedron for RCA. The DNA tetrahedron is utilized as a nanocarrier that can be immobilized on a gold electrode to generate RCA product to load hemin, an iron-containing porphyrin with chlorine, forming the G-quadruplex as a horseradish peroxidase like DNAzyme, which reduces methylene blue (MB) in the presence of H_2O_2 to yield a distinct current signal. Using the developed DNAzyme with the RCA signal amplification strategy, the DNA biosensor can achieve a detection limit as low as 0.1 fM for the ultrasensitive electrochemical detection of methylated DNA sequence with a detection range from 10^{-15} M to 10^{-9} M. At the same time, the satisfactory specificity, reproducibility, stability and recovery performances indicated its satisfied potentials for clinical diagnosis. Most importantly, this method can be further applied to analyse other genomic DNA also.

Keywords: DNA methylation; DNA tetrahedron; DNAzyme; DNA biosensor.

1. Introduction

DNA methylation is an essential epigenetic modification and plays significant regulatory roles in both prokaryotes and eukaryotes (Suzuki et al., 2008). Aberrant DNA methylation is responsible for the pathogenesis of many diseases, including diabetes (Ma et al., 2015; Kang et al., 2017),

coronary artery diseases (Sharma et al., 2014; Hao et al., 2018) and cancers (Paliwal et al., 2010; Jiang et al., 2013). Thus, detection of DNA methylation with high sensitivity and specificity is important for the early detection of human cancer. Recently, several methods have been developed for the detection of DNA methylation, such as PCR-based technologies (Xu et al., 2016; Pereira et al., 2017; Wu et al., 2017; Duan et al., 2018). However, a high-precision thermocycler is necessary for these methods. Bisulphite sequencing is a sensitive and quantitative approach (Lee et al., 2013), but a prerequisite for bisulphite sequencing is the accessibility of restriction sites to methylation-sensitive enzymes. Raman spectroscopy (Hu et al., 2012) is simple, but its sensitivity is limited to the detection of low-abundance methylated DNA. Therefore, development a new strategy for DNA methylation detection that overcomes the complexity and low sensitivity drawbacks of current methods is needed for early cancer diagnosis.

Electrochemical methods have attracted attention in recent years because of their simple operation, easy separation, high sensitivity and good selectivity (Dadmehr et al., 2014; Zhu et al., 2015; Xu et al., 2016); these methods are considered to be optimal for detecting DNA methylation. Zhu et al., 2015, utilized electrochemical impedance spectroscopy (EIS) to distinguish between the hybridization kinetics of a conducting polymer-bound probe in methylated and unmethylated

ds-DNA. Xu et al., 2016, used anodic stripping voltammetry, which produced sharp stripping signals in quantum dot-based assays, to sensitively detect DNA methylation. Liu et al., 2015, presented a DNA MTase activity detection method based on the voltammetric response of the electroactive labels thionine and graphene oxide. However, these methods require large amounts of time, exacting assembly of quantum dots, rigorous conditions for the DNA MTase reaction, and have low detection sensitivity. Thus, development of an electrochemical method with excellent sensitivity that is easy to operate to detect DNA methylation would be extremely valuable.

Here, we develop a simple and fast analytical electrochemical strategy for the sensitive detection of methylated genes based on signal amplification *via* rolling circle amplification (RCA) and a hemin/G-quadruplex horseradish peroxidase (HRP) mimic. We designed a DNA tetrahedron structure containing a free-standing probe DNA at one vertex and three thiol groups at the other three vertices (Pei et al., 2010). This nanostructure was expected to be readily and strongly anchored on Au surfaces by the three thiols at the base of the 'pyramid', leaving a freestanding probe at the top to adopt an upright surface orientation favouring target DNA hybridization (Ding et al., 2014; Li et al., 2015a; Li et al., 2015b). RCA is a simple and efficient isothermal enzymatic process in which short nucleic acid fragments can be extended

to a concatamer containing tens to hundreds of tandem repeats that are complementary to the circular template (Zeng et al., 2013; Ali et al., 2014; Li et al., 2016). By customizing the sequence of the circular template, RCA products can be tailored to include multifarious functional sequences, including a DNAzyme, aptamers and restriction enzyme sites (Bi et al., 2013; Cui et al., 2017). More importantly, the RCA-synthesized long DNA concatamer can carry a multitude of electroactive species or nanobiolabeled materials through intercalative binding, electrostatic adsorption or DNA hybridization to produce strong electronic and optical signals. To further improve the sensitivity of the electrochemical DNA biosensor, enzymes are used as signal amplification strategies. The G-quadruplex is a G-rich oligonucleotide sequence that can fold into a specific structure in the presence of Na^+ or K^+ (Gray et al., 2008; Yuan et al., 2013). Upon complexation with hemin, G-quarduplex DNAzymes can be generated with enhanced horseradish peroxidase (HRP)-mimicking activity (Yuan et al., 2013; Golub et al., 2013; Cui et al., 2015).

In this study, we employed DNA tetrahedral probes as signal primers to bind to circular templates for RCA amplification. With the assistance of RCA, the primer was elongated and formed a tandem multi-G-quadruplex. In the presence of hemin, a hemin/G-quadruplex was produced to form a HRP-mimicking enzyme that catalysed H_2O_2-mediated oxidation of MB, producing a significantly enhanced

electrochemical signal for the sensitive detection of DNA methylation. Impressively, the designed strategy was also suitable for the simple and ultrasensitive detection of other DNA sequences, opening a new avenue for clinical diagnosis.

2. Experimental

2.1. Reagents

Taq DNA ligase, Bst DNA polymerase large fragment, and their corresponding buffers were purchased from Shanghai Sangon Biotechnology Co. (China). The deoxynucleotide solution mixture (dNTPs), Tris (hydroxymethyl) methyl aminomethane (THAM), dimethyl sulfoxide (DMSO), hemin, exonuclease I, exonuclease III, SYBR Green I and their corresponding buffers were obtained from Bio Basic, Inc. (Canada). The Bisulfite Conversion Kit was purchased from Tiangen Biochemical Technology Co., Ltd. (Beijing, China). All other materials were of analytical grade and purchased from Sigma Chemical Co., USA. Ultrapure water was used in all experiments. All of the oligonucleotide (Table S1 in Supplementary material) sequences are listed below (from 5' to 3' end) and were synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

2.2. Apparatus

Cyclic voltammetric (CV), electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) measurements were

performed using a Zennium electrochemical workstation (ZAHNERElektrik GmbH & Co. KG, Germany) with a three-electrode system composed of a platinum wire as the counter electrode, a saturated silver chloride electrode (Ag/AgCl) as the reference electrode and a gold electrode (diameter 2 mm) as the working electrode, respectively.

2.3. Experiment principle

As shown in Scheme 1, the DNA methylation assay involved three principal components: (1) the DNA tetrahedron structure, (2) an RCA reaction at 60°C, and (3) electrochemical DNA biosensor detection. In the first step, upon treatment with sodium bisulfite, cytosine was converted to uracil, but C5-methylcytosine (mC) remained unchanged. Therefore, a distinguishable difference in the DNA sequence was generated between methylated and unmethylated DNA. In the second step, for the purpose of both the discrimination of methylated DNA and initiation of RCA, a linear 60-nucleotide (nt) padlock probe containing distinct regions, two target-complementary sequences located at the 5′ and the 3′ ends and two special regions that initiated RCA, was used. When the two ends of the linear padlock probe perfectly hybridize with methylated DNA, the 5′ and the 3′ ends are brought into close proximity, generating a circular padlock probe in the presence of Taq DNA ligase. The unclosed proximity, methylated DNA and unmethylated DNA were removed by Exonuclease I and Exonuclease III. Therefore, the solution

continue to just retain the close proximity related with the methylated DNA. Then, the DNA tetrahedron probe functions as a signal primer that binds with the circular template and initiates a cascade RCA reaction in the presence of Bst (exo-) polymerase, producing the long repeat sequence of the circular probe. Meanwhile, MB, as an electrochemical indicator, was abundantly adsorbed onto oligonucleotides *via* specific interactions of MB with guanine bases and served as an electron mediator. Finally, hemin was intercalated into the G-quadruplex to obtain the hemin/G-quadruplex structure as a DNAzyme, yielding a biocatalytic G-quadruplex/hemin/ DNAzyme structure and generating a current signal in the presence of H_2O_2 and MB. While, the unmethylated DNA could not generate RCA and produced a weak current signal. By monitoring the change in current, we were able to indirectly determine the concentration of DNA methylation in the sample.

2.4. Construction of the DNA tetrahedron

The DNA tetrahedron was constructed according to a previously published protocol with modifications (Pei et al., 2010). First, 1 μL of DNA S1 (100 μM/L), 1 μL of DNA S2 (100 μM/L), 1 μL of DNA S3 (100 μM/L), 1 μL of DNA S4 (100 μM/L), and 6 μL of TM buffer solution (10 mM/L Tris, 5 mM/L $MgCl_2$) were mixed for 2 min, incubated at 95°C for 2 min and cooled to 4°C for 30 sec. Then, gel electrophoresis was used to evaluate the DNA tetrahedron construction.

2.5. Bisulfite treatment of DNA

Bisulfite treatment of DNA was performed according to the guidelines of the Bisulfite Conversion Kit. The resulting DNA was resuspended in buffer and stored at -20°C.

2.6. Ligation and Exonuclease treatment

Hybridization of the linear padlock probe with the target was carried out in 20 μL of pH 7.6 Tris-HCl buffer, 25 mM potassium acetate, 10 mM dithiothreitol, 10 mM magnesium acetate, 1 mM nicotinamide adenosine dinucleotides, 100 nM linear padlock probe, different concentrations of methylated DNA and unmethylated DNA, and 12 U of Taq DNA ligase at 95°C for 5 min, and then, the mixture was incubated at 65°C for 1 h. After ligation, 10 μL of products was added to 10 μL of Exonuclease mixture containing distilled water, 10 U of Exonuclease I, and 20 U of Exonuclease III. The mixture was incubated at 37°C for 1 h, followed by inactivation at 95°C for 10 min.

2.7. RCA reaction

After ligation and purification, another step was performed to amplify the circular probes in the presence of Bst DNA polymerase. The RCA reaction was performed in 30 μL of buffer containing 1× polymerase buffer, 1 μM DNA tetrahedron, 400 μM deoxynucleoside triphosphate mixtures, 10 μL of postdigestion mixture, and 8 U of Bst DNA polymerase. The circular probes were amplified at 63°C for 1 h and

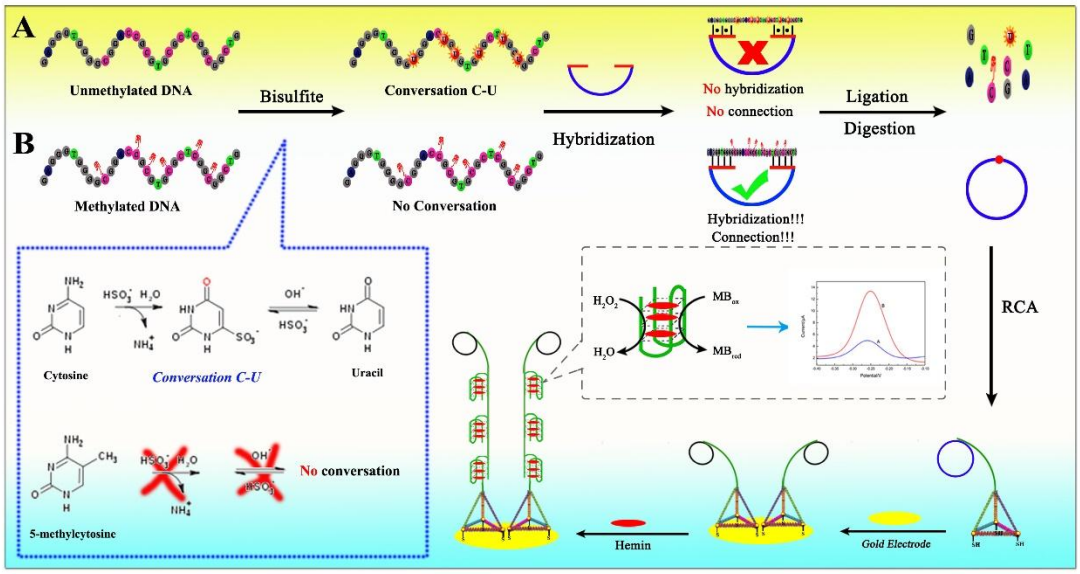
terminated by incubation at 95°C for 10 min.

2.8. Measurement procedure

Before the electrochemical measurements, the gold electrode was polished using 0.5 μm and 0.3 μm aluminium slurries and ultrasonicated in acetone, ethanol and ultrapure water, in that order. The gold electrode was immersed in piranha solution [30% H_2O_2/ 98% H_2SO_4 (1:3) v/v] for 15 min, rinsed with distilled water and dried with nitrogen.

Subsequently, the cleaned gold electrode was modified with the RCA mixture. After rinsing the electrode surface with 10 mM PBS buffer, the electrode was immersed in 10 mL of 20 mM Tris-HCl buffer (pH 7.4) containing 20 mM MB and 20 mM KCl, allowed to equilibrate for 30 min at room temperature and washed thoroughly again. To form the G-quadruplex, the electrode was dripped in hybrid buffer (containing 200 mM/L THAM, 40 mM KCl, 400 mM NaCl, 0.1% Triton-100 and 1% DMSO). After reacting for 1 h, 5 μL of hemin was added and the electrode was incubated at 37°C for another hour. Finally, the electrode was washed with 10 mM buffer for 5 min. To detect the electrochemical responses, the electrode was immersed in 15 mL of 100 mM phosphate buffer (pH 7.4, containing 0.1 M KCl and 2.5 mM H_2O_2) and differential pulse voltammetry (DPV) was performed from -0.5 to 0 V with a 50 mV pulse amplitude and 5 ms pulse width to measure the reduction peak current of MB. Cyclic voltammetry measurements were performed in a 2

mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution containing 0.1 M KCl at a scan rate of 50 mV/s.



Scheme 1. Schematic diagram and detection of the prepared DNA biosensor.

3. Results and discussion

3.1. Construction of DNA tetrahedron probes

DNA tetrahedrons were constructed to serve as scaffolds for methylated target DNA. Gel electrophoresis was used to evaluate the successful assembly of the four well-designed DNA strands following the principle of complementary base pairing. Control studies are shown in Fig. 1A, where a DNA marker is provided on the left as a reference. Single-stranded DNA is shown in lines a-d (close to50 bp), double-stranded DNA is shown in line e-h (close to 100 bp), and the DNA tetrahedron, which moves slowest, is shown in line i (close to 200 bp). Small DNA molecules move faster and migrate farther than larger

ones due to the greater hindrance of larger molecules through the pores of the agarose gel. The results confirmed that the DNA nanostructures were assembled successfully.

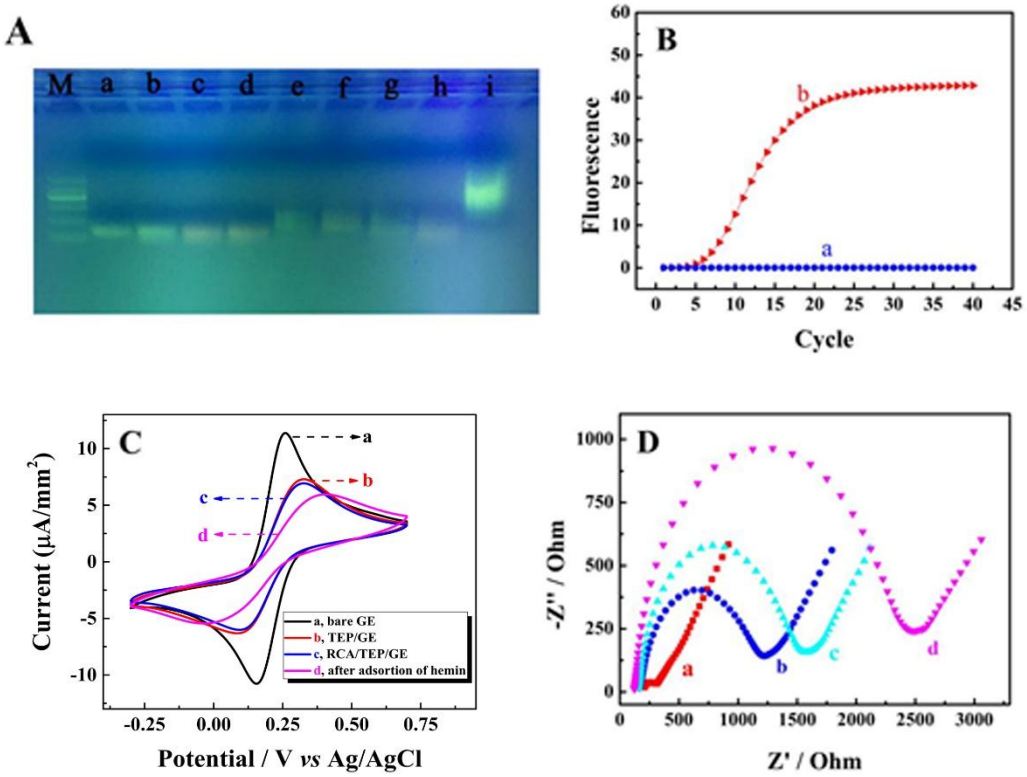


Fig. 1. (A) Agarose gel electrophoresis characterization of the DNA tetrahedron: single-stranded samples (lane a: S1, lane b: S2, lane c: S3, lane: S4) and partially mixed strands samples (lane e: S1+S2, lane f: S1+S3, lane g: S2+S4, lane h: S3+S4), TEP sample (lane i). (B) Real-time fluorescence monitoring of the RCA reaction with SYBR Green I as the fluorescent indicator in the presence of 1 nM unmethylated DNA (curve a) and 1 nM methylated DNA (curve b). (C) CV and (D) EIS characterizations obtained from different electrodes in a phosphate buffer solution (pH 7.0) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl (scan

rate of CV: 50 mV/s).

3.2. Characterization of rolling loop amplification products

The DNA methylation assay is based on the principle that upon treatment of DNA with sodium bisulfite, cytosine is converted to uracil, but C5-methylcytosine (mC) remains unchanged. Therefore, a significant difference is generated between methylated and unmethylated DNA in the DNA sequence. To distinguish methylated DNA from unmethylated DNA and initiate RCA, a linear padlock probe with two target-complementary sequences located at the 5′ and the 3′ ends and two special regions was used to initiate RCA. Real-time fluorescence measurements were used to evaluate the RCA reaction, and the results are shown in Fig. 1B. In the presence of unmethylated DNA, linear padlock probes cannot be circularized due to the defectively hybridization between linear padlock probes and the DNA sequence converted from the unmethylated DNA via the conversation from cytosine to uracil; neither RCA nor fluorescence enhancement is observed (curve a). When the two ends of the linear padlock probe perfectly hybridize with methylated DNA, the 5′ and the 3′ ends are brought into proximity, generating a circular padlock probe in the presence of Taq DNA ligase. Using Exonucleases I and III, RCA will take place and produce a large number of double-stranded DNA (dsDNA) fragments of variable length. In this case, the fluorescence signal is observed (curve b).

3.3. Electrochemical characterization of the biosensor

CVs were performed step by step in 2 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ containing 0.1 M KCl with a potential range from -0.3 V to 0.7 V at a scan rate of 50 mV/s (Fig. 1C). The bare gold electrode exhibited a pair of well-defined redox peaks of $[Fe(CN)_6]^{3-/4-}$ (curve a). By contrast, the redox peak currents of $[Fe(CN)_6]^{3-/4-}$ obtained from the DNA tetrahedron-modified electrode (curve b) decreased, indicating that the DNA tetrahedron was successfully immobilized onto the gold electrode surface. After the rolling circle amplification mixture was incubated on the electrode, the peak currents decreased because the insulation effect of the G-quadruplex retarded electron transfer on the electrode surface (curve c). When the fabricated gold electrode conjugated hemin formed the DNAzyme, an obvious decline in the redox current was obtained and the peak-to-peak separation increased (curve d). The results of the CV measurements confirmed the successful fabrication via the proposed strategy.

EIS as an important electrochemical technology was also used to monitor the features of the modified gold electrode. EIS in 0.1 M PBS with 5 mM $[Fe(CN)_6]^{3-/4-}$ at a frequency range from 5×10^{-2} to 1×10^{6} Hz and an open circuit voltage with an amplitude of 10 mV is shown in Fig. 1D. Electron transfer resistance (R_{et}) was employed to quantitatively demonstrate the electron transfer kinetics, which were similar to the

semicircle diameter in the EIS response in the form of a Nyquist plot. For the bare gold electrode (curve a), a normal EIS response was obtained with a R_{et} of approximately 300 Ω. Increasing the R_{et} to approximately 1200 Ω for the DNA tetrahedrons immobilized electrode (curve b) revealed that the DNA tetrahedrons hindered electron transport from the solution to the electrode surface, suggesting successful assemblage of DNA tetrahedrons. During hybridization with the rolling circle amplification mixture, an increased R_{et} of approximately 1550 Ω was obtained (curve c) due to the formation a large amount of G-quadruplexes, which blocked electron transfer at electrode surface. Furthermore, the R_{et} increased to approximately 2500 Ω after conjugation of the hemin (curve d). The EIS changes were in agreement with those from the CV method, which further demonstrated the success of each modification step.

3.4. Detection of methylated DNA and unmethylated DNA

To validate the feasibility of the proposed method, we detected a DNA sequence homologous to part of the PL6 tumour suppressor gene under optimum reaction conditions (Fig. S1 in the Supplementary material). The RCA/TEP/GE on the electrode surface was first self-assembled and then MB signal molecules were loaded. The hemin/G-quadruplex/DNAzyme formed with the help of the hemin and K^+. Finally, the current response of MB, produced by the reaction between MB and H_2O_2, was recorded by DPV. Fig. 2A shows DPV

curves of different electrodes. The bare GE had almost no oxidation peak (curve c in Fig. 2A). In the absence of methylated DNA, a weak current peak was detected (curve b in Fig. 2A) because the DNA methylation loop was digested, there was no RCA amplification and only the DNA tetrahedron existed, allowing only a few MB molecules to be loaded. However, a strong current peak was observed with the further loading of MB onto the RCA/GE (curve a in Fig. 2A). Similarly, the electrochemical response to methylated DNA at the same concentration was significantly higher than that of unmethylated DNA (Fig. 2B). An obvious peak current produced by 1 nM methylated DNA occurred at about 4.3 $\mu A/mm^2$, 3 times higher than that produced by 1 nM unmethylated DNA with a current response of about 1.3 $\mu A/mm^2$, suggesting that the proposed method can be used to distinguish methylated DNA from unmethylated DNA.

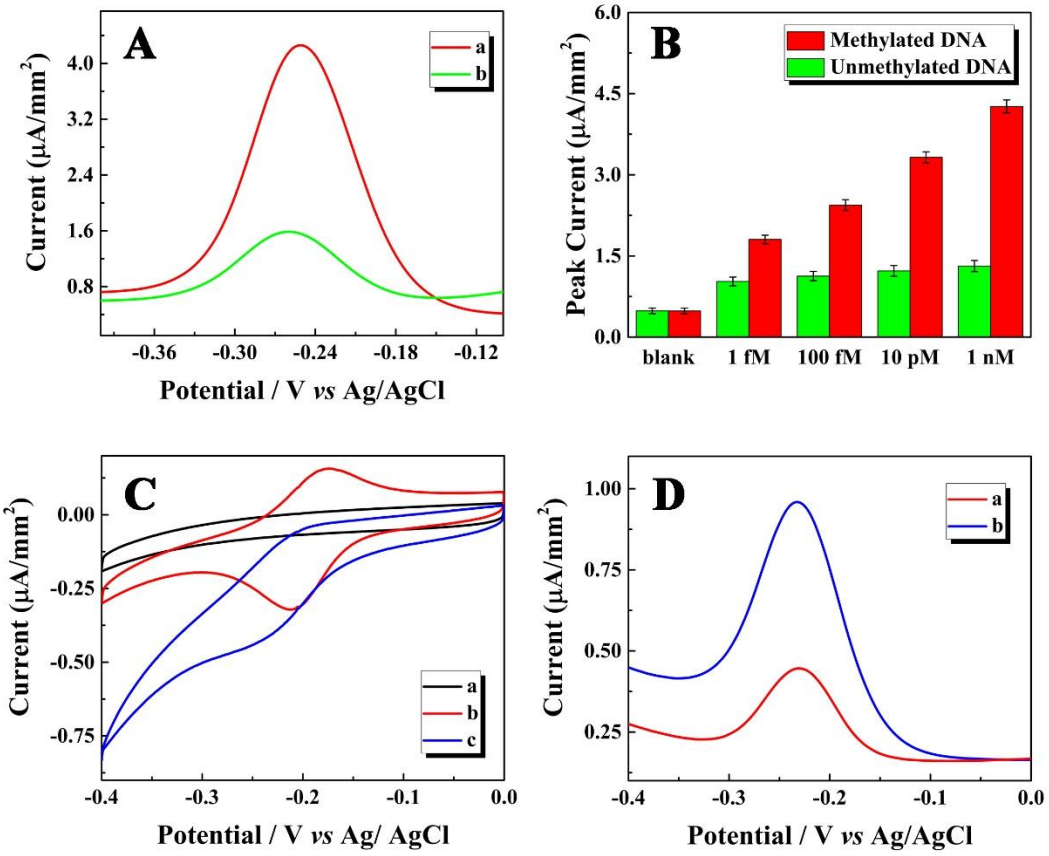


Fig. 2. (A) DPV current signal in response to 10 nM methylated (red) and 10 nM unmethylated (green) DNA. (B) Bar chart of the DPV peak currents recorded for the DNA biosensor fabricated with different concentrations of methylated DNA (red bar) and unmethylated DNA (green bar), and the error bars are the standard deviations of three repetitive measurements. (C) CVs of the as prepared RCA/TEP/GE: interacted with hemin (curve a); incubated with hemin and MB in the absence (curve b) or presence of 2 mM H_2O_2 (curve c). (D) DPV of the fabricated DNA biosensor: incubated with MB (curve a); incubated with hemin and MB (curve b).

3.5. The characterization of the signal amplification strategy

To investigate whether the formed G-quadruplex-hemin DNAzyme could catalyse the reduction of H_2O_2 in solution to amplify the electrochemical signal of the adsorbed MB, we studied the electrochemical behaviour of RCA/TEP/GE of methylated DNA. As shown in Fig. 2C., no redox peaks were observed after the biosensor was incubated with only hemin (curve a). When the resulting biosensor was incubated with hemin and MB, a pair of redox peaks was observed (curve b). Notably, upon addition of 2 mM H_2O_2 in Tris-HCl, an obvious catalytic characteristic appeared with a dramatic increase of the peak current (curve c). This result indicated that the formed G-quadruplex-hemin DNAzyme exhibited an obvious enzymatic catalytic behaviour. The increase of the peak current was mainly derived from the G-quadruplex-hemin DNAzyme toward the catalytic reduction of H_2O_2 with the help of MB electron mediators. Simultaneously, to demonstrate the dramatically amplified electrochemical signal induced reactions, a sensitive electrochemical technique, DPV, was utilized to obtain the direct electrochemical signal. As observed in Fig. 2D, the electrochemical response to the generated G-quadruplex/hemin DNAzyme (curve b) was significantly higher than that without hemin (curve a). Hence, the G-quadruplex/hemin DNAzyme can be used for signal amplification in this biosensor system.

3.6. Sensitivity of proposed electrochemical DNA biosensor

The performance of the integrated biosensor was evaluated by DPV under optimized experimental conditions. Fig. 3 presents the steady-state response of the proposed biosensor to different concentrations of methylated DNA. Meanwhile, with the increasing concentration of target DNA, more MB molecules were loaded on the electrode, thereby increasing the positive current response (curves a–k). Notably, the DPV peak current increased when the concentration of methylated DNA was increased from 10^{-17} M to 10^{-8} M, indicating that the assembly of the current signal was dependent on the concentration of target DNA. Fig. 3B shows that the DPV peak currents of the fabricated DNA biosensor exhibited a good linear correlation to the logarithm of the concentrations of methylated DNA ranging from 10^{-15} M to 10^{-8} M. The regression equation can be expressed as ΔI ($\mu A/mm^2$) = 3.78 + 0.24 log C (M) with a coefficient of determination (r) of 0.988, where ΔI is peak current subtracted the blank current and C is the methylated DNA concentration (n = 3). We also provide a data table to compare the present results with previous studies on methylation recognition by different detection techniques. Comparing the linear range and detection limit, our proposed method has advantages over the previous reports (Table S2), which maybe contributed to the high efficient DNA amplification.

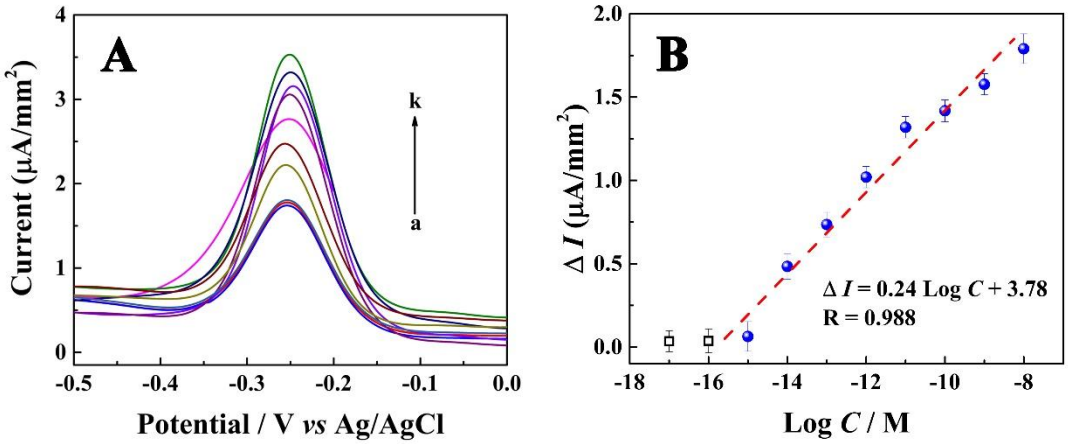


Fig. 3. (A) DPV responses of the fabricated DNA biosensor toward the detection of different concentrations (a–k: 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M, 10^{-12} M, 10^{-13} M, 10^{-14} M, 10^{-15} M, 10^{-16} M and 10^{-17} M, respectively) of methylated DNA; (B) linear relationship between the peak currents and the logarithm of the methylated DNA concentration.

3.7. Specificity, reproducibility, and stability

The specificity of the proposed biosensor was verified by exposing the biosensor to four types of target sequences, including methylated DNA (T1), unmethylated DNA (T2), and non-complementary DNA (T3) (inset in Fig. S2). Among the three types of DNA sequences, the current change of methylated DNA was much greater than those of the other mismatched sequences. As shown in Fig. S2, the currents of T2 and T3 were much less than that of methylated DNA (T1). Thus, the electrochemical biosensor was effective at discriminating between the complementary sequence and mismatched DNA. These results suggest that the proposed DNA biosensor exhibited good selectivity for

methylated DNA.

The electrode to electrode repeatability was also evaluated. Five integrated biosensors prepared with the same procedure were tested for methylated DNA concentrations of 100 pM (Table S3). The relative standard deviations (RSD) were 6.6% (Fig. S3). The reproducibility of the electrode was measured by three people in different laboratories, and the RSD was 9.1% (Fig. S4). The excellent repeatability and reproducibility strongly confirmed that this electrochemical strategy using tetrahedron rolling circle amplification remarkably minimized the biosensor to biosensor deviation. Stability is another important factor of the proposed biosensor and was investigated by long-term storage. After a storage period of 30 days at 4 ^{o}C, the current response retained 92.9% of its initial response (Fig. S5), suggesting the satisfactory stability of the as-proposed biosensor.

3.8. Recovery of the prepared DNA biosensor

Accurate assessment of methylation in serum or plasma is significant for early diagnosis of cancer. A recovery experiment was used to verify the validity of the proposed assay after a certain amount of methylated DNA was added into the DNA biosensor. As shown in Table 3, some results obtained through testing the proposed biosensor are acceptable, with recovery between 92% and 104.4% with the RSD values ranged from 4.8% to 7.3% (n = 3), indicating that this sensor satisfied the

needed measurement accuracy.

Table 3 Recovery of the prepared DNA biosensor.

Sample number	Add (pM)	Found (pM)	Recovery (%)
1	1	1.04	104.00
2	5	4.85	97.00
3	10	10.44	104.40
4	20	18.4	92.00
5	40	37.6	94.00
6	60	61.4	102.33
7	80	79.83	99.79
8	100	103	103.00

4. Conclusion

In summary, a simple, highly sensitive electrochemical DNA biosensor was developed for the detection of methylated DNA based on DNA tetrahedron primer generation rolling circle amplification. The highlights of this work can be summarized as follows: (1) DNA tetrahedron probes can serve as signal primers to initiate RCA; (2) the hemin/G-quadruplex that functions as a bioelectrocatalytic DNAzyme mimic could improves the sensitivity of the biosensors for methylated DNA. Notably, taking advantage of the exponential amplification efficiency of RCA and the high sensitivity of the DNAzyme catalytic electrochemistry, the proposed method exhibits excellent sensitivity, with

a detection limit of 1×10^{-16} M. This method holds great promise for further applications in the study of epigenetic modification and early diagnosis of methylation-related diseases.

Acknowledgments

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Highlights

- An sensitive electrochemical strategy was developed for DNA methylation detection.
- DNA tetrahedron probes serve as signal primers to initiate RCA reactions achieve signal amplification.
- The hemin/G-quadruplex simultaneously acting as a mimicking DNAzyme for amplifying system could improve the sensitivity of methylated DNA.