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Ultrasensitive Electrochemical detection of Nucleic Acids Based on the Dual-signaling Electrochemical Ratiometric Method and Exonuclease III-Assisted Target Recycling Amplification Strategy

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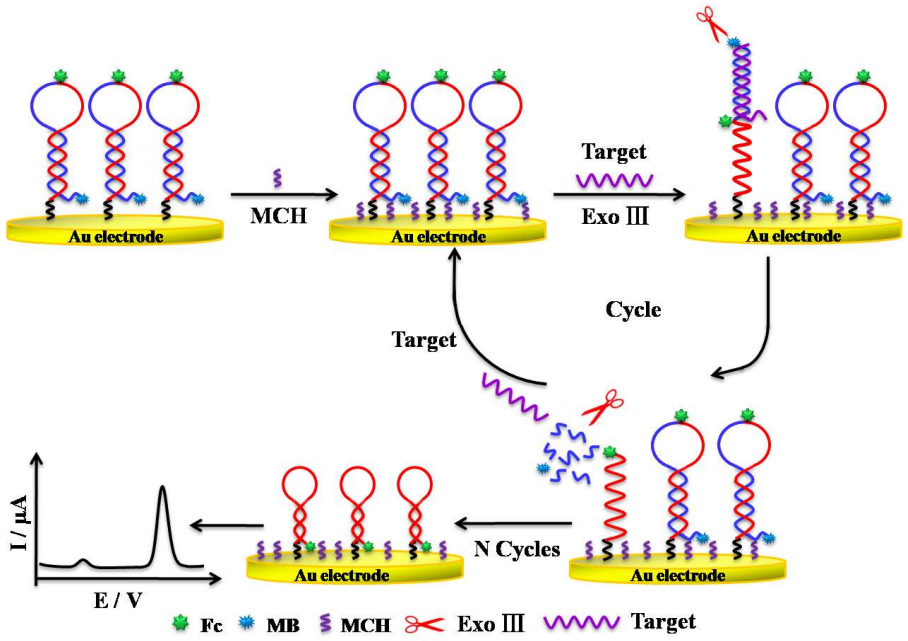
ABSTRACT: Because of the intrinsic importance of nucleic acids as bio-targets, the simple and sensitive detection of nucleic acids is very essential for biological studies and medical diagnostics. In this work, a novel, simple and selective electrochemical DNA biosensor for the sensitive detection of target DNA (T-DNA) has been developed based on dual-signaling electrochemical ratiometric method and exonuclease III (Exo III)-assisted target recycling amplification strategy. The assay strategy includes both "signal-on" and "signal-off" elements. The stem-loop (hairpin) DNA capture probe (HP), which was labeled by thiolated methylene blue (MB) at the 3′-protruding termini and ferrocene (Fc) in the middle of the loop, firstly self-assembled on the gold electrode surface via Au-S bond. In the presence of T-DNA, the T-DNA hybridized with HP, which triggered the Exo III cleavage process and accompanied with the releasing of T-DNA. As a result, the MB tags were away from and the Fc tags closed to the gold electrode surface, leading to the decrease of the oxidation peak current of MB (I_{MB}) and the increase of that of Fc (I_{Fc}). The value of $\Delta I_{Fc}/|\Delta I_{MB}|$ (ΔI_{Fc} and ΔI_{MB} are the change values of the oxidation peak currents of Fc and MB, respectively) is linear with the concentration of T-DNA from 0.01 pM to 0.8 pM. The detection limit (4.16 fM) of the developed method is much lower than that of the most reported electrochemical DNA biosensors. This strategy provides a simple and sensitive approach for the detection of T-DNA, and has promising applications in bioanalysis, disease diagnostics, and clinical biomedicine.

As we all know, the development of DNA biosensor capable of amplified detection is of great importance in biomedical studies, environmental analysis, forensic investigations, disease and molecular diagnostics.[1-4] Until now, various techniques such as colorimetry,[5-8] electrochemistry,[9-11] spectroscopy,[12-14] molecular biology[15] have been utilized toward the detection of DNA. Especially, the signal amplification strategies have drawn increasing concerns and usually been applied in the fabrication of DNA biosensors due to the significant improvement of the detection sensitivity toward targets,[16-18] such as DNA-based strategies (polymerase chain reaction (PCR),[19-21] strand displacement amplification (SDA),[22-24] rolling circle amplification (RCA), [25-31] and ligase chain reaction (LCR),[32,33] etc.) and nanomaterials-based methods (gold nanoparticles (GNPs),[34] quantum dots (QDs),[35] carbon nanotubes (CNTs),[36] graphene (GN)[37] and magnetic nanoparticles (MNPs),[38] etc.). Although these DNA-based signal amplification strategies have received more and more concerns due to their high sensitivity and easy designability, sensing protocols are limited because of the drawbacks of complex handing procedures, high cost, vulnerability to contamination.[39] Recently, a new class of signal amplification strategies based on nuclease (exonuclease and endonuclease)-assisted target recycling were especially attractive for the trace level detection of target DNA (T-DNA).[27,40-45] However, unlike nicking endonuclease which requires a specific recognition site, exonuclease does not require any specific recognition sequence, thus, has received more and more attention in DNA amplification detection. For example, Exonuclease III (Exo III) is a kind of sequence-independent enzyme which has a high exodeoxyribonuclease activity for duplex DNAs in the direction from 3′ to 5′ terminus and limited activity on single-stranded DNA or duplex DNAs with blunt or recessed 3′-termini.[39,46-48] Thus, Exo III provides a more versatile platform for amplified detection of DNA. In recent years, many groups have carried out related researches. For instance, Li et al. and Liu et al. reported the fluorescence detection of DNA based on coupling Exo III-assisted target recycling strategy.[49,50] Li et al. and Lu et al. developed the DNA biosensors based on colorimetry and chemiluminescence method which combined with Exo III-assisted signal amplification strategy.[51,52] However, these DNA biosensors often confronted with relatively expensive instruments and complicated sample preparation processes.

By comparison, electrochemical methods are particularly attractive for the detection of DNA because of its advantages of simplicity, high sensitivity, good selectivity and low cost.[53-56] Thus, the development of sensitive electrochemical detection of DNA is high desirable. Ju et al. and Tang et al. reported the electrochemical biosensors for the detection of DNA based on Exo III-assisted signal amplification strategy and obtained a

Scheme 1. Schematic illustration of the electrochemical biosensor for target DNA detection.



low detection limit.[48,57] However, most of these electrochemical biosensors have only one signal, either the type of "signal-on" or "signal-off". Recently, to circumvent the limitation of "signal-off" strategy and to make use of the superiority of "signal-on" strategy, the ratiometric electrochemical assay, combining "signal-on" and "signal-off" strategies, has been developed due to its good analytical performances (such as low detection limit, wide linear range, good reliability and reproducibility, and low background noise),[58-62] which inspires us to construct a new electrochemical biosensor for DNA assay by combining the advantages of the dual-signaling electrochemical ratiometric method and Exo III-assisted target recycling amplification strategy.

In this work, a simple and sensitive electrochemical biosensor for the detection of T-DNA has been developed based on the dual-signaling electrochemical ratiometric method and Exo III-assisted target recycling amplification strategy (Scheme 1). The stem-loop (hairpin) DNA capture probe (HP), which labeled by thiolated methylene blue (MB) at the 3′-protruding termini and ferrocene (Fc) in the middle of the loop, firstly self-assembled on the gold electrode surface via Au-S bond. In the presence of the T-DNA (here, *K-ras* gene, which derived from the colorectal tumor, was chosen as the model), the T-DNA hybridized with HP and a probe-target duplex with a blunt end at the 3′-termini of HP is formed, which triggered the Exo III cleavage process and accompanied with the releasing of T-DNA. As a result, the MB tags were away from the gold electrode surface and the Fc tags closed to the electrode surface because the remaining single-stranded HP with tailor-made complementary bases at both ends could form a hairpin structure in the presence of Mg^{2+}.[63,64] These changes led to the decrease of the oxidation peak current of MB (I_{MB}), accompanied with the increase of that of Fc (I_{Fc}). Based on the value of $\Delta I_{Fc}/|\Delta I_{MB}|$ (ΔI_{Fc} and ΔI_{MB} are the change values of the oxidation peak currents of Fc and MB, respectively), T-DNA was detected sensitively and selectively. This method provides a versatile tool in nucleic acid assay and holds a great potential for the development of an ultrasensitive biosensing platform for early diagnosis in gene-related diseases.

EXPERIMENTAL SECTION

Materials. The HPLC-purified oligonucleotide sequences were purchased from Takara Biotechnology Co., Ltd. (Dalian, China) and listed below: the hairpin DNA capture probe, 5′-SH-$(CH_2)_6$-CCA TCA CGC TGG TGA GTT (Fc) TAT GGC ACC AGC TCC AA-MB-3′; the T-DNA sequence, 5′-TTG GAG CTG GTG GCG TA-3′; the single-base mismatched DNA (Sm-DNA) sequence, 5′-TTG GAC CTG GTG GCG TA-3′; the three-bases mismatched DNA (Tm-DNA) sequence, 5′-TTG GAC GAG GTG GCG TA-3′; the non-complementary DNA (N-DNA) sequence, 5′-AGT ACT ACA ACC GCG TA-3′. Exo III was purchased from New England Biolabs Co., Ltd. (Beijing, China). 6-Mercaptohexanol (MCH) and tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Tris(hydroxymethyl) aminomethane (Tris) was purchased from Solarbio Science & technology Co., Ltd. (Beijing, China). Metal salts (NaCl, KCl, $MgCl_2$, K_2HPO_4, KH_2PO_4, $K_3Fe[CN]_6$ and $K_4Fe[CN]_6$) were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analytical reagent grade and used without further purification. Aqueous solutions used throughout were prepared with ultra pure water (>18 MΩ cm) obtained from a Millipore system. Human serum was provided by the Affiliated Hospital of Hunan University and stored at 4 ^{o}C.

Apparatus. All electrochemical measurements, including differential pulse voltammetry (DPV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), were performed on a CHI 660D Electrochemical Workstation (Chenhua Instrument Company of Shanghai, China) at room temperature. A conventional three-electrode cell was used with a planar gold electrode (2 mm in diameter) as the work-

ing electrode, a platinum wire as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode. All the potentials were referred to SCE.

Preparation of the MCH/HP/Au electrode. Gold electrode was firstly polished to a mirror-like surface with 0.5 and 0.05 μm alumina slurries, followed by ultrasonication in ultra pure water and methanol. The electrodes were then pretreated by electrochemical oxidation and reduction in 0.5 M H_2SO_4 aqueous solution by potential cycling in the potential between -0.3 and 1.5 V with a scan rate of 100 mV s^{-1} until the cyclic voltammogram characteristic for a clean gold electrode was obtained. Then, the gold electrode was washed with copious amounts of ultra pure water and dried under nitrogen gas.

Before immobilization onto the gold electrode surface, the HP was dissolved in 20 mM Tris-HCl buffer solution (containing 50 mM NaCl, 10 mM $MgCl_2$, 10 mM TCEP, PH 8.0) and incubated in the dark for 1 h to reduce disulfide bunds. 10 μL of 1 μM HP solution was placed onto the cleaned gold electrode surface for 16 h at room temperature to obtain the HP/Au electrode, and then the HP/Au electrode was further immersed into 10 mM Tris-HCl buffer solution with 2 mM MCH for 1 h to inhibit the nonspecific DNA adsorption.[65] The obtained electrode was labeled as the MCH/HP/Au electrode and investigated by DPV. To monitor each immobilization step, the electrochemical impedance measurements were performed in 0.1 M KCl aqueous solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ in the frequency range from 0.1 Hz to 100 kHz with 5 mV as the amplitude at a potential of 0.22 V (vs. SCE).

Electrochemical detection of T-DNA. 10 μL of 10 mM Tris-HCl solution (50 mM NaCl, 10 mM $MgCl_2$, pH 8.0) containing 1 unit/μL Exo III and different concentration of T-DNA was dripped on the surface of the MCH/HP/Au electrode, and then the electrode was kept at 37 °C for 2 h.[57] Then, the obtained electrode was thoroughly rinsed with 10 mM Tris-HCl and investigated by DPV in 4 mL of 10 mM PBS (pH 8.0) containing 50 mM NaCl and 10 mM $MgCl_2$. Each measurement was repeated at least 3 times. The control experiments for base mismatched or noncomplementary DNA were also performed under the same conditions.

RESULTS AND DISCUSSION

Characterization of the electrochemical DNA biosensor. As one of the most powerful tools for interfacial investigation, EIS is usually adopted to investigate the modification process of an electrode.[66] In a typical electrochemical impedance spectrum, the semicircle portion observed at higher frequencies corresponds to the charge-transfer limited process and the increase of the diameter of the semicircle reflects the increase of the interfacial charge-transfer resistance (Rct).[67] Figure 1A shows the EIS results of the Au electrode at different modification stages. Obviously, the bare gold electrode exhibits a very small semicircle domain (Rct = 130 Ω, curve a), indicating a very fast charge-transfer process. The self-assembly layer of negatively charged HP on the Au electrode surface effectively repels the $[Fe(CN)_6]^{3-/4-}$ anions and thus leads to an enhanced charge-transfer resistance (Rct = 4200 Ω, curve b), subsequent surface blocking with MCH further results in an obvious enhancement of the Rct value of the electrode (6500 Ω, curve c). After the MCH/HP/Au electrode is treated with T-DNA in the presence of Exo III, the diameter of the semicircle decreases dramatically and the corresponding Rct value is about 3700 Ω (curve d), which attributes to the fact that considerable HP strands are digested, and indicates the implement of Exo III-assisted target recycling strategy. In order to further verify the stepwise modification process, the electrochemical biosensor was also characterized by CV. As shown in Figure 1B, compared with the bare gold electrode (curve a), the peak current of the HP/Au electrode (curve b) decreases obviously and the peak-to-peak separation increases. When the surface of the HP/Au electrode is blocked by MCH, the peak current decreases correspondingly (curve c). After further treatment with T-DNA in the presence of Exo III, the peak current increases and the peak-to-peak separation decreases (curve d), proving the implement of the designed Exo III-assisted target recycling strategy, similarly. These results are in good accordance with that observed in EIS investigation (Figure 1A) and demonstrate that the electrochemical biosensor has been fabricated successfully according to Scheme 1.

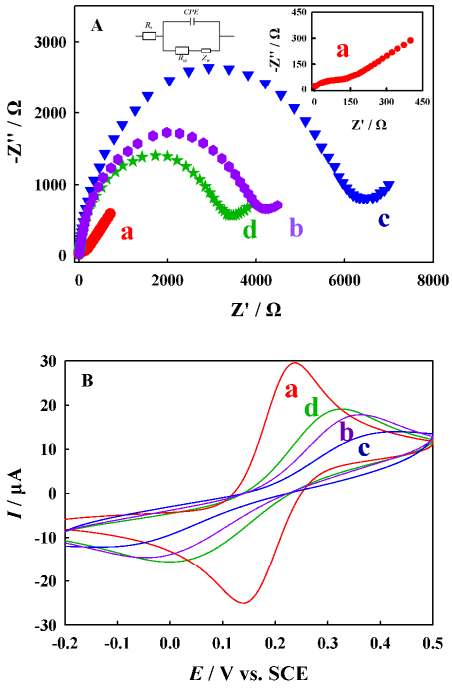


Figure 1. (A) Electrochemical impedance spectra (Nyquist plots) of the different modified electrodes in 0.1 M KCl aqueous solution containing 5 mM (1:1) $[Fe(CN)_6]^{3-/4-}$ in the frequency range from 0.1 Hz to 100 kHz with 5 mV as the amplitude at a potential of 0.22 V. Inset shows the related equivalent circuit. (B) Cyclic voltammograms obtained in 0.1 M KCl aqueous solution containing 5 mM (1:1) $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 100 mV s^{-1}. (a) bare Au electrode, (b) HP/Au electrode, (c) MCH/HP/Au electrode, (d) MCH/HP/Au electrode after incubated with T-DNA and Exo III.

Feasibility of the electrochemical biosensor for T-DNA detection. To further verify the feasibility of the proposed method for the detection of T-DNA, DPV measurements were performed on the MCH/HP/Au electrodes in the presence/absence of Exo III with/without T-DNA. As shown in Figure 2, in the absence of both T-DNA (1 pM) and Exo III (1unit/μL) (curve a), the oxidation peak current of MB is very

large and that of Fc is small. In the presence of only Exo III, no obvious changes of the oxidation peak currents of MB and Fc are observed (curve b). However, in the presence of T-DNA without the addition of Exo III, the oxidation peak current of MB decreases and that of Fc increases, slightly (curve c). Such changes are basically due to the hybridization of T-DNA and HP and the formation of the probe-target duplex with a blunt end at the 3′-termini of HP. When the MCH/HP/Au electrode is incubated with T-DNA and Exo III, there are significant changes of the oxidation peak currents of MB and Fc (curve d), owing to the digestion of the probe-target duplex and the recycling of T-DNA. These results shown here clearly demonstrate that the designed autocatalytic electrochemical strategy could pave a new way to detect T-DNA.

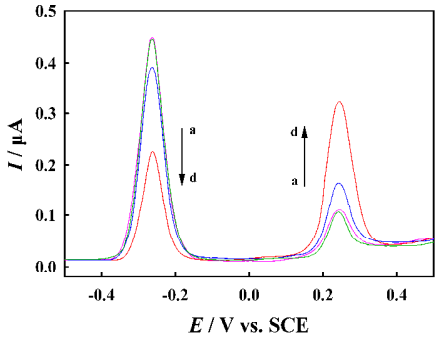


Figure 2. DPV responses of the developed DNA biosensor at different experimental conditions: (a) no T-DNA, no Exo III; (b) no T-DNA, 1 unit/μL Exo III; (c) 1 pM T-DNA, no Exo III; (d) 1 pM T-DNA, 1 unit/μL Exo III.

Electrochemical assay of T-DNA. Under the optimized experimental conditions, the analytical performance of the fabricated autocatalytic sensing system was investigated. As shown in Figure 3A, it is noted that the oxidation peak current of MB decreases and the oxidation peak current of Fc increases with the increase of T-DNA concentration. Based on the individual Fc or MB signal, the dependence of the change of the oxidation peak current of Fc (ΔI_{Fc}) or MB (ΔI_{MB}) on the T-DNA concentration is shown in Figure 3B or 3C. It can be observed clearly that the value of ΔI_{Fc} (or ΔI_{MB}) increases (or decreases) with the increase of the T-DNA concentration in the range of 0.01 pM to 10 nM. Both of them exhibit linear relationship in the range of 0.1 pM to 0.8 pM, and the detection limits are 32 fM (based on Fc signal) and 45 fM (based on MB signal) (S/N = 3), respectively. The Figure 3D shows the calibration plot of the electrochemical ratiometric assay of T-DNA using $\Delta I_{Fc}/|\Delta I_{MB}|$ as signal. It is noted that the value of $\Delta I_{Fc}/|\Delta I_{MB}|$ is linear with the concentration of T-DNA in the range from 0.01 pM to 0.8 pM with the detection limit of 4.16 fM (S/N = 3), and the linear regression equation is $\Delta I_{Fc}/|\Delta I_{MB}| = 0.1440\ C_{T\text{-}DNA} + 0.8519$ ($R^2 = 0.9851$). It is worthy to note that the electrochemical ratiometry exhibits a lower detection limit and a wider linear range than that obtained by using ΔI_{MB} or ΔI_{Fc} as the response signal alone. Also, to the

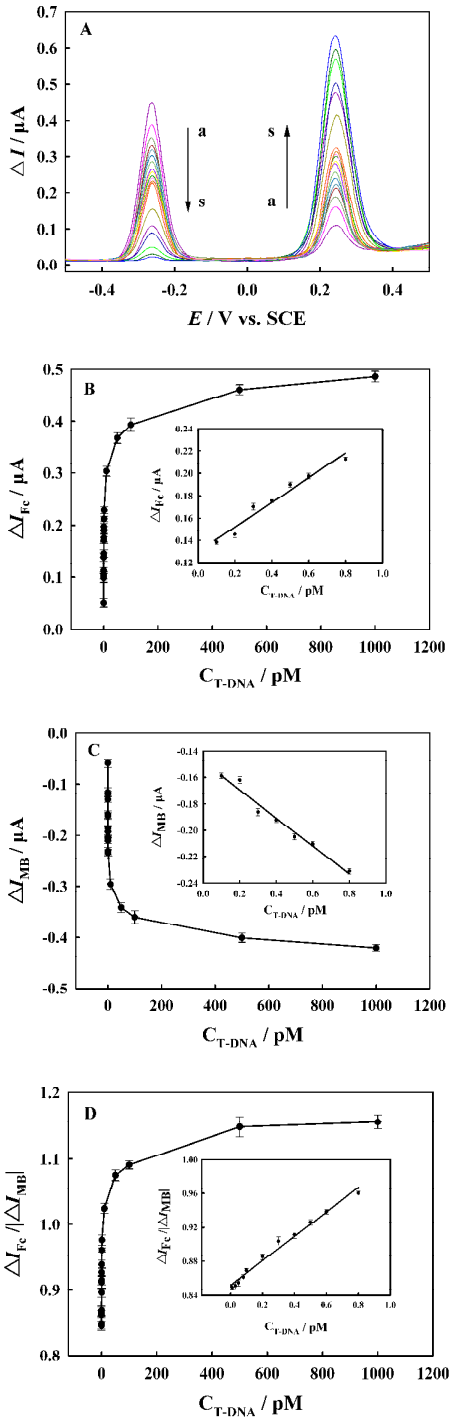


Figure 3. (A) DPV curves for the detection of different concentrations of T-DNA. The concentrations are (from a to s) 0 M, 0.01 pM, 0.03 pM, 0.05 pM, 0.08 pM, 0.1 pM, 0.2 pM, 0.3 pM, 0.4 pM, 0.5 pM, 0.6 pM, 0.8 pM, 1 pM, 10 pM, 50 pM, 100 pM, 500 pM, 1 nM, 10 nM. The dependence of ΔI_{Fc} (B) and ΔI_{MB} (C) on T-DNA concentration. (D) The dependence of $\Delta I_{Fc}/|\Delta I_{MB}|$ on T-DNA concentration. Insets show the linear fit plots of ΔI_{Fc}, or ΔI_{MB} and $\Delta I_{Fc}/|\Delta I_{MB}|$ as function of the T-DNA concentration. Error bar represents the standard deviation of three parallel experiments.

best of our knowledge, the proposed method has a much lower value of detection limit than the most other electrochemical methods only based on the single signal response mechanism and Exo III-assisted signal amplification strategy (Table 1).

Table1. Comparison of some electrochemical biosensors for the detection of target DNA.[a]

Method	Probe	Detection limit	Reference
CV	RuHex	33 pM	44
EIS	–	10 fM	68
SWV	RuHex	20 fM	69
DPV	ST-AP	8.7 fM	48
DPV	Hemin	10 fM	57
DPV	MB	20 fM	70
DPV	MB	20 pM	71
DPV	Fc	120 fM	72
DPV	MB and Fc	4.16 fM	this work

Stability, reproducibility and specificity of the electrochemical biosensor. The stability of the MCH/HP/Au electrode has been checked. Three independent experiments demonstrated that the MCH/HP/Au electrode could retain about 95.4% of its initial response toward T-DNA after its storage in the refrigerator at 4 °C over two weeks. The reproducibility of the MCH/HP/Au electrode has also been evaluated. Five modified electrodes were used to detect T-DNA (1 pM) and the relative standard deviation is 4.3%. These indicate that the developed electrochemical biosensor has satisfactory stability and reproducibility.

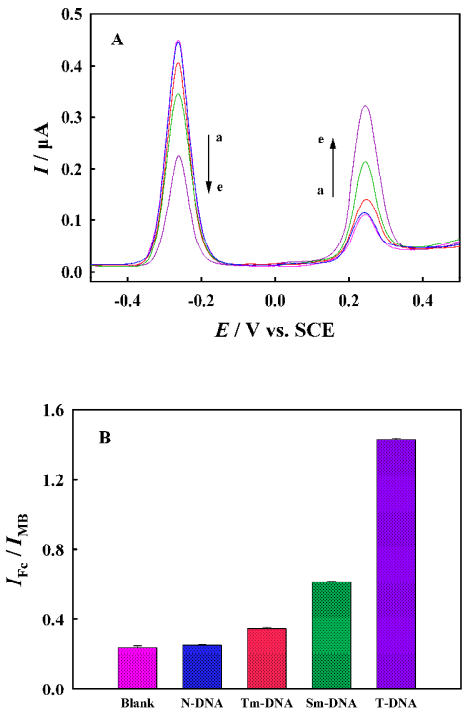


Figure 4. (A) DPV curves of the MCH/HP/Au electrode in 10 mM Tris-HCl solution without (a), and with N-DNA (b), or Tm-DNA (c), Sm-DNA (d), and T-DNA (e). (B) The values of I_{Fc}/I_{MB} for the blank solution and different DNA sequences. Error bar represents the standard deviation of three parallel experiments.

To further investigate the specificity of the proposed method, the sensor was exposed to four kinds of DNA sequences, including T-DNA, Sm-DNA, Tm-DNA, and N-DNA at the same concentration of 1 pM, and the corresponding DPV results are shown in Figure 4A. It is noted that no significant changes of the oxidation peak currents of MB and Fc are observed when the MCH/HP/Au electrode is incubated in the blank solution (curve a) and the solution with N-DNA (curve b). However, the increase of the oxidation peak current of Fc and the decrease of the oxidation peak current of MB can be observed when the MCH/HP/Au electrode is exposed to the solution with Sm-DNA, or Tm-DNA and T-DNA (curves c, d, and e). These results are further shown in Figure 4B more clearly. The values of I_{Fc}/I_{MB} for Sm-DNA and Tm-DNA are only about 43.0% and 24.2% of that for T-DNA at the same concentration, respectively. The N-DNA shows almost the same value with the blank solution. This could be ascribed to the fact that Exo III has a limited digestion property towards the mismatched DNA duplex. These results indicate clearly that the developed electrochemical biosensor possesses good specificity for the detection of T-DNA.

Recovery test. In order to evaluate the applicability and reliability of the developed electrochemical biosensor, the recovery experiments for different T-DNA concentrations were carried out in human serum samples which were obtained from healthy individuals. T-DNA with different concentrations (0.1 pM, 0.3 pM, 0.5 pM) was added in 10-fold diluted human serum sample solutions which diluted with 20 mM PBS. As shown in Table 2, the recoveries for the added T-DNA are 97%, 103% and 106%, respectively. This indicates that possible interferences from the serum on T-DNA detection are negligible. These results demonstrate that the developed electrochemical biosensor can be successfully applied to detect T-DNA in complex biological samples.

Table 2. Recovery assays of T-DNA in 10-fold diluted human serum samples.

Sample (Nos.)	Added (pM)	Found (pM)	Recovery (%)
1	0.1	0.097 ± 0.016	97
2	0.3	0.31 ± 0.09	103
3	0.5	0.53 ± 0.04	106

CONCLUSION

In summary, based on the dual-signaling electrochemical ratiometric method and Exo III-assisted target recycling strategy, a novel, simple and sensitive electrochemical DNA biosensor has been developed. The presence of T-DNA switches the structure of the HP on the sensor surface and generates nicking sites for Exo III to proceed with cyclic cleavage of the HP strands, which results in the changes of the peak currents of MB and Fc. The developed method greatly lowers the detection limit towards T-DNA down to 4.16 fM. What's more, since Exo III does not require specific recognition sequences, the developed electrochemical biosensor may offer much better flexibility for choosing a target sequence and would pro-

vide a potential platform for DNA detection in the area of bioanalysis, disease diagnostics, and clinical biomedicine.

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Notes

The authors declare no competing financial interest.

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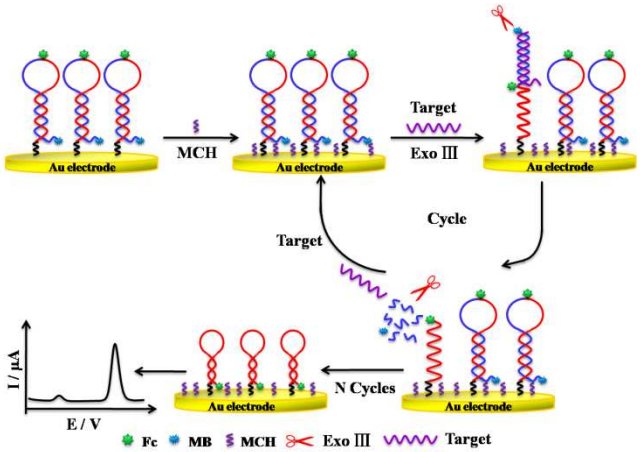
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