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Ultrasensitive Electrochemical Biosensor for Detection of DNA from *Bacillus subtilis* by Coupling Target-Induced Strand Displacement and Nicking Endonuclease Signal Amplification

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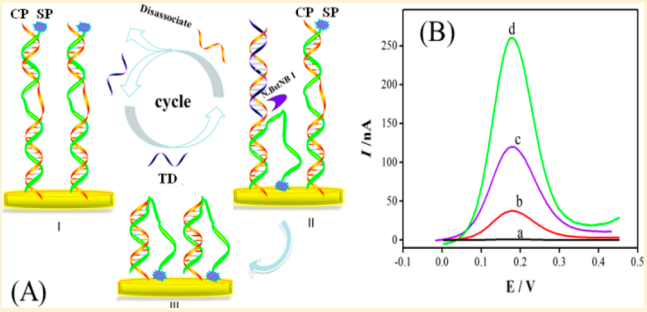
ABSTRACT: A simple, ultrasensitive, and specific electrochemical biosensor was designed to determine the given DNA sequence of *Bacillus subtilis* by coupling target-induced strand displacement and nicking endonuclease signal amplification. The target DNA (TD, the DNA sequence from the hypervarient region of 16S rDNA of *Bacillus subtilis*) could be detected by the differential pulse voltammetry (DPV) in a range from 0.1 fM to 20 fM with the detection limit down to 0.08 fM at the $3s_{blank}$ level. This electrochemical biosensor exhibits high distinction ability to single-base mismatch, double-bases mismatch, and noncomplementary DNA sequence, which may be expected to detect single-base mismatch and single nucleotide polymorphisms (SNPs). Moreover, the applicability of the designed biosensor for detecting the given DNA sequence from *Bacillus subtilis* was investigated. The result obtained by electrochemical method is approximately consistent with that by a real-time quantitative polymerase chain reaction detecting system (QPCR) with SYBR Green.



The *Bacillus subtilis* and related *Bacillus* strains, which can secrete many kinds of enzymes[1,2] and antibiotics,[3] are the dominant enzyme-producing microorganisms in applied and industrial microbiology. *Bacillus subtilis* has been widely applied in the area of medicine,[4] agriculture[5] and scientific research.[6−8] Some companies declare that the strains with high number have already been produced, but the percentage of contaminated microorganism usually restricts its application. In order to quantitatively detect *Bacillus subtilis* in the production, it is necessary to develop sensitive and selective methods for the determination of *Bacillus subtilis*. Current *Bacillus* spore detection methods include polymerase chain reaction (PCR),[9,10] immunoassays,[11] surface-enhanced Raman spectroscopy,[12,13] and standard microbial assays. These techniques suffer from several drawbacks including high consumption of reagents, long analysis time, and complex assay. In addition, the analysis results are usually vulnerable to environmental change and cross infection. Therefore, it is important to develop an efficient and selective method to determine *Bacillus subtilis*.

It has been reported that a hypervariant region of 16S rDNA of *Bacillus subtilis* is highly specific for other type strain. Goto et al. proposed a facile method to identify *Bacillus subtilis* based on the sequence difference of the hypervariant region.[14] In this report, a large number of 16S rDNA from many species were investigated to reveal that the hypervariant region was highly specific for each type strain and highly conserved within species. Because of the sizable database of the 16S rDNA gene that has been built, this method is reliable and easy to implement.

A nicking endonuclease N.BstNBI can recognize a simple asymmetric sequence, namely, 5′-GAGTC-3′, and cleave only one DNA strand, 4-bases away from the 3′ end of its recognition site. The Chen group reported an electrochemical assay for highly sensitive detection of the DNA sequence based on nicking endonuclease signal amplification, but this sensor was constructed based on the signal-off assay mode and a high background signal was needed for the as-prepared sensors.[15] In order to overcome this defection, a new experiment scheme, in which G-quadrupled hemin DNAzyme was introduced to catalyze signal enhancement, had been developed by the same group.[16] Although this method owned the character of low detection limit (0.02 fM), it needed complex operation procedures. Xiao et al. reported a signal-on electrochemical DNA sensor based on a target-induced strand displacement mechanism.[17] The above results demonstrate that the coupling method of target-induced strand displacement and nicking endonuclease signal amplification can be considered as a selective and versatile scheme for the detection of DNA sequence.

At present, the electrochemical biosensor has been recognized as a promising tool for DNA detection. Herein, an ultrasensitive and selective signal-on electrochemical biosensor for detecting specific DNA sequence (a sequence was selected from the hypervariant region of 16S rDNA of

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Bacillus subtilis) had been developed by coupling target-induced strand displacement and nicking endonuclease signal amplification. The established biosensor has high specificity and will be expected to identify a single-base mismatch and single nucleotide polymorphisms (SNPs). In addition, it can be efficient for the quantitative determination of DNA from the real sample.

EXPERIMENTAL SECTION

Materials. Ferrocenecarboxylic acid (FeCOOH) was purchased from Alfa Aesar (Britain). N.BstNBI and 10× NEBuffer 3 (containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9)) were purchased from New England BioLabs (America). Mecatohexanol (MCH) was purchased from Tokyo Chemical Industry Co. Ltd. (Japan). Tris(hydroxymethyl)methyl aminomethane (Tris) was purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Ethylenediaminetetraacetic acid (EDTA) and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (America). SYBR Premix Ex Taq (Tli RNaseH Plus) was purchased from Takara Biotech. Co. Ltd. (Dalian, China). The 2× Taq PCR Master Mix was purchased from Tian Gen Biotech. Co. Ltd. (Beijing, China). The agarose M., GeneRuler Ultra Low Range DNA Ladder, 1-ethyl-3-(3-dimenthyl-aminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysul-fosuccin imide (NHS), and the synthesized DNA were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Gel stock solution (30%, 29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)), ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). All the other chemicals were of analytical grade and used without further purification. Ultrapure water obtained from a Mliilpore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all runs.

Buffer 1: 1 M NaCl, 10 mM TCEP, 1 mM EDTA, and 300 mM Tris-HCl (DNA immobilization buffer, pH 7.4).

Buffer 2: 1 M NaCl, 1 mM EDTA, 1 mM $MgCl_2$, and 300 mM Tris-HCl (DNA hybridization buffer, pH 7.4).

Buffer 3: 10 mM Tris, 1 mM EDTA (pH 8.0).

Buffer 4: 10 mM phosphate buffered saline (PBS), 100 mM NaCl, and 1.0 M $NaClO_4$ (electrochemical detection solution, pH 7.4).

10× TBE stock solution (500 mL): 54.0 g Tris, 0.5 M EDTA 20 mL (pH 8.0), and 27.5 g of boric acid (electrophoresis buffer, pH 8.3).

The sequences of synthesized DNA are as follows:

(a) Capture probe (CP): 5′-HS-$(CH_2)_6$-GCA GGT ATG CAC AGT GAG TCT GGG CCG TGT CTC AGT-3′ (The underline refers to the recognition sequence of nicking endonuclease N.BstNBI).

(b) NH_2 modified signal probe: 3′-CGT CCA TAC GTG TCA AAA CCC TAG GCA CAG AG-NH_2-5′;

(c) Target DNA (TD): 3′-CTC AGA CCC GGC ACA GAG TCA-5′;

(d) Single-base mismatched DNA: 3′-CTC AGA CCC GAC ACA GAG TCA-5′;

(e) Double-base mismatched DNA: 3′-CTC AGA CCC AAC ACA GAG TCA-5′;

(f) Noncomplementary DNA: 3′-AGG CAC ATG ACA TTT AGT AGC-5′;

(g) Forward primer: 5′-TGT AAA ACG ACG GCC AGT GCC TAA TAC ATG CAA GTC GAG CG-3′;

(h) Reverse primer: 5′-CAG GAA ACA GCT ATG ACC ACT GCT GCC TCC CGT AGG AGT-3′;

(i) Specific DNA sequence from hypervariant region of 16S rDNA of *Bacillus subtilis*: 5′-GCC TAA TAC ATG CAA GTC GAG CGG ACA GAT GGG AGC TTG CTC CCT GAT GTT AGC GGC GGA CGG GTG AGT AAC ACG TGG GTA ACC TGC CTG TAA GAC TGG GAT AAC TCC GGG AAA CCG GGG CTA ATA CCG GAT GGT TGT TTG AAC CGC ATG GTT CAA ACA TAA AAG GTG GCT TCG GCT ACC ACT TAC AGA TGG ACC CGC GGC GCA TTA GCT AGT TGG TGA GGT AAC GGC TCA CCA AGG CAA CGA TGC GTA GCC GAC CTG AGA GGG TGA TCG GCC ACA CTG GG A CTG AGA CAC GGC CCA GAC TCC TAC GGG AGG CAG CAG T-3′ (319 bp).

DNA (a) was dissolved in buffer 1. DNA (b), (c), (d), (e), and (f) were dissolved in buffer 2. DNA (g), (h) and (i), which were used for QPCR, were dissolved in buffer 3.

Preparation of FC Labeled Signal Probe (SP). FeCOOH was conjugated to DNA (b) by the EDC-NHS method.[18,19] Briefly, 10 μM DNA (b), 1 mM FeCOOH, 0.1 M EDC, and 0.1 M NHS were incubated together according to the ratio of 1:1:1:1 (volume ratio) at room temperature for 3 h. After removing the excessive FeCOOH by ultrafiltration for 12−18 times, the mixture was then stored at 4 °C as the SP.

Biosensor Preparation. A gold electrode (GE, 2 mm in diameter, CH Instruments, Shanghai, China) was polished with 0.3 and 0.05 μm alumina powders on a polishing microcloth, respectively, and then rinsed with distilled water for 5 min. The GE was then electrochemically cleaned by consecutively cycling between 0 and +1.6 V in a fresh prepared 0.5 M H_2SO_4 solution until a stable and repeatable cyclic voltammogram reached. After that, the GE reacted with 0.5 μM CP in buffer 1 for 2 h at 37 °C and kept in the dark (held upside-down). And then the CP/GE was immersed into 1 mM MCH for 2 h to prevent the nonspecific adsorption of endonuclease on the electrode surface and displace the weaker adsorption between CP and GE. Next, the CP/GE hybridized with 2.5 μM FC modified SP in buffer 2 for 16 h at 37 °C to obtain the final SP/CP assembly on the electrode.[17] Finally, the SP/CP/GE reacted with various concentration of TD in 1× NEBuffer 3 (including appropriate nicking endonuclease N.BstNBI) for 5.5 h at 37 °C. (Note: after each reaction step, the electrode should be rinsed with PBS to eliminate physical adsorption and dried by N_2.)

Electrochemical Measurements. All electrochemical measurement were performed with a CHI 660A electrochemical Workstation (CH Instrument, Shanghai, China) by using a modified GE as the working electrode, a platinum wire as the auxiliary electrode, and a Ag−AgCl as the reference electrode. The DPV that had a potential interval of 0 to +0.6 V was carried out in buffer 4. The DPV peak current was collected and registered as the sensor signal. All measurements were conducted at room temperature.

Polyacrylamide Gel Electrophoresis. A volume of 5.0 mL of 30% gel stock solution, 1.0 mL of 10× TBE buffer, 100 μL of APS (10%), 10 μL of TEMED, and 3.89 mL of deionized water were mixed to prepare the hydrogel. The gel was polymerized for 50 min at room temperature and then soaked in 1× TBE buffer for use. A volume of 4.0 μL of each reacted sample was mixed with 2.0 μL of 6× loading buffer and was subjected to the 15% nondenaturing polyacrylamide gel electrophoresis (PAGE). The PAGE was carried out in 1×

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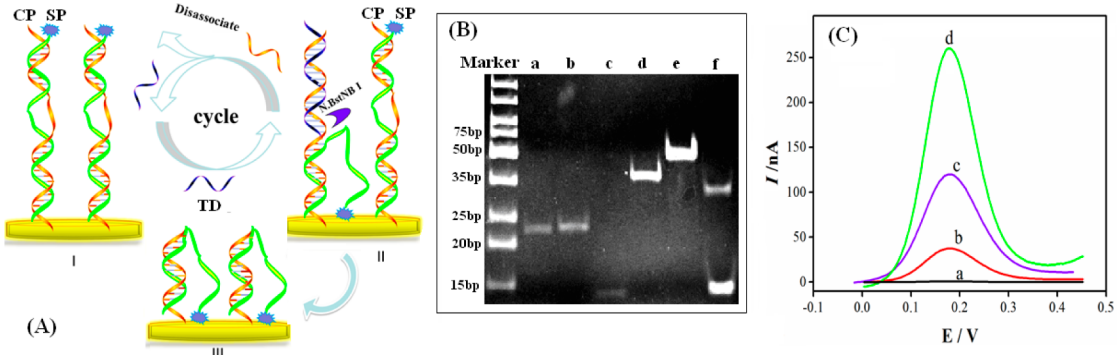

Figure 1. (A) Schematic representation and assay principle of the electrochemical biosensor: CP, orange; SP, green; TD, blue. (B) The PAGE analysis of the strategy: (a) 5 μM SP, (b) 5 μM CP, (c) 5 μM TD, (d) 5 μM SP + 5 μM CP, (e) 5 μM SP + 5 μM CP + 5 μM TD, (f) 5 μM SP + 5 μM CP + 5 μM TD + N.BstNBI (appropriate). (C) The DPV responses of the different electrode in buffer 4: (a) bare GE, (b) SP/CP/GE, (c) SP/CP/GE after reacting with 15 pM TD without nicking endonuclease, (d) SP/CP/GE after reacting with 10 fM TD including a certain nicking endonuclease.

TBE at 150 V for 50 min at room temperature and ethidium bromide staining.

Amplification of the DNA Extractive of *Bacillus subtilis* by PCR. DNA (g) and DNA (h) were constructed as the forward primer and reverse primer, respectively. The DNA extractive of *Bacillus subtilis*, which was extracted by the method of phenol−chloroform extraction,[20] was the DNA template. PCR amplification was performed in a total volume of 20 μL, containing 0.4 μL of 10 μM each primer, 10 μL of 2× Taq PCR Master Mix, 2.0 μL of DNA template, and 7.2 μL of distilled water. The cycling conditions were as follows: initial denaturation at 97 °C for 5 min, followed by 40 cycles of 97 °C for 30 s, 45 °C for 1 min, and 72 °C for 1 min. The PCR products were detected by 2% agarose gel electrophoresis with 1× TBE as a running buffer and ethidium bromide staining.

RESULTS AND DISCUSSION

Principle of Electrochemical DNA Biosensor. The principle of signal-on electrochemical biosensor is based on the target-induced strand-displacement mechanism and nicking endonuclease signal amplification. As shown in Figure 1A, the biosensor contains two components (CP and SP). CP is modified on the GE surface through thiol−Au interaction and keeping upright due to the immobilized MCH. The FC modified SP is immobilized on the GE surface through partly hybridizing with CP. In the absence of TD, FC is far away from the electrode surface and only a week faradaic current can be obtained. In the presence of TD, target hybridization displaces the nine hybridized bases at the 5′ terminus of SP. This displacement liberates the FC-modified end of SP, allowing FC to collide with the electrode surface and transfer electrons. Meanwhile, the nicking endonuclease N.BstNBI can nick the nicking position after forming the recognition site. As a result, more and more FC quickly approach the electrode surface to obtain a significant current. After nicking, CP is cleaved into two pieces and TD is liberated from CP. The free TD can then hybridize with another double-strand formation and provide the second cycle of cleavage. Finally, each TD can go through many cycles and interact with many CP. As a result, a significant current of FC can be detected with a low concentration of TD. The enhancement of signal depends on the TD concentration in the solution, which can be used for the quantitative analysis of TD.

Polyacrylamide gel electrophoresis (PAGE) was used to investigate the viability of our strategy. As shown in Figure 1B, the bands in lanes a, b, and c corresponded to SP, CP, and TD, respectively. When CP incubated with SP for 16 h at 37 °C, one bright band was observed on lane d which was different from lanes a and b, indicating that CP had hybridized with SP. When TD was introduced in the system to react with CP/SP without N.BstNBI, there was one bright band (lane e) which was different from lane d, implying that TD had hybridized with CP and displaced the 9 hybridized bases at the 5′ terminus of SP. When N.BstNBI was added to the reaction system of CP/SP/TD, there were two bands in lane f. One of bands lied in the position of TD, the other lied in a new position which corresponded to the nicking product of the CP/SP. These results demonstrated that N.BstNBI could nick the nicking position after forming the recognition site and CP was cleaved into two pieces and TD was liberated from CP.

A simple electrochemical experiment had also been performed to verify our assumption. As shown in Figure 1C, no electrochemical response was detected on the bare GE (curve a), but a weak electrochemical signal was obtained on the SP/CP/GE (curve b). When TD reacted with the biosensor without nicking endonuclease, the electrochemical response increased (curve c). This suggests that FC is close to the electrode surface due to the strand displacement. When a certain nicking endonulease N.BstNBI was added into the reaction system, the current increased greatly (curve d). At this stage, TD could go through many cycles after the nicking position was nicked by nicking endonuclease, resulting in the current amplification.

Optimization of Experiment Conditions. Referred to the reported literature,[15−17,21] the different kinds of buffers were used for the biosensor preparation. TCEP included in the buffer 1 is used to reduce disulfide-bonded oligomers and to immobilize CP to the gold electrode easily. $MgCl_2$ included in the buffer 2 can facilitate the hybridization between CP and SP. Since NEBuffer 3 can ensure the optimal activity of N.BstNBI, 1× NEBuffer 3 is used as the hybridization buffer and nicking buffer. $NaClO_4$ (1.0 M) included in buffer 4 is used to avoid the instability of ferrocenium.

Surface density of CP on the electrode is a key factor that influences the analytical properties of the biosensor. The concentration and the self-assembly time of CP, which influenced the density of CP on the electrode, were tested.

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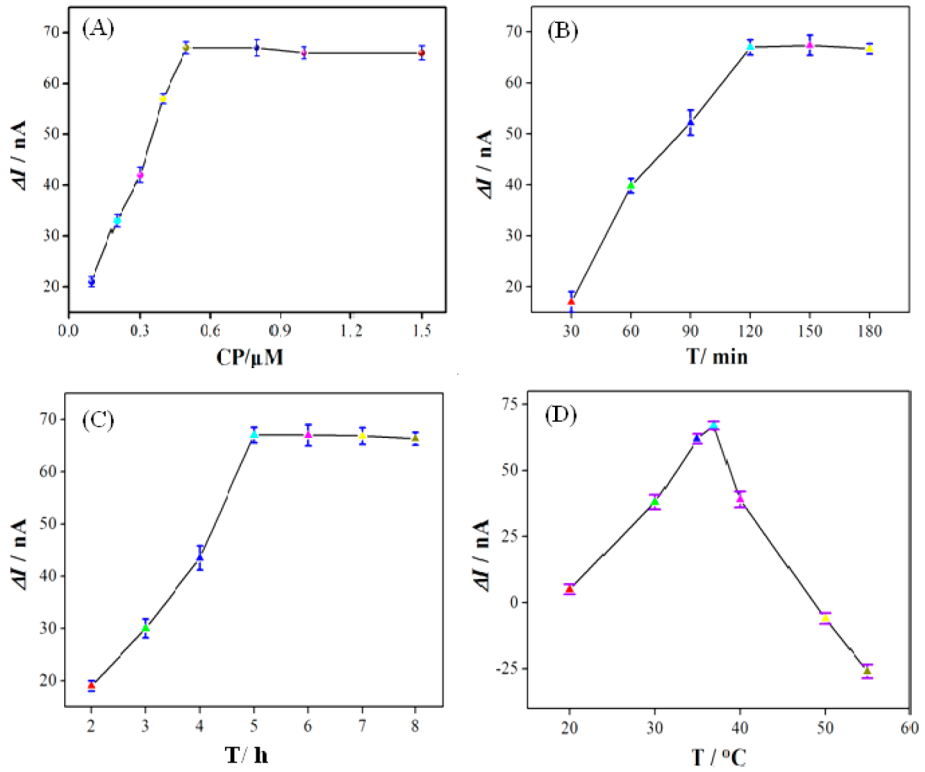

Figure 2. (A) Effect of CP concentration on the enhanced current. (B) Effect of CP self-assembly time on the enhanced current. (C) Effect of reaction time between as-prepared biosensor and TD on the enhanced current. (D) Effect of incubation temperature of N.BstNBI on the enhanced current (10 fM TD, as an example). The error bars represent the standard deviation (S.D.) of three measurements conducted.

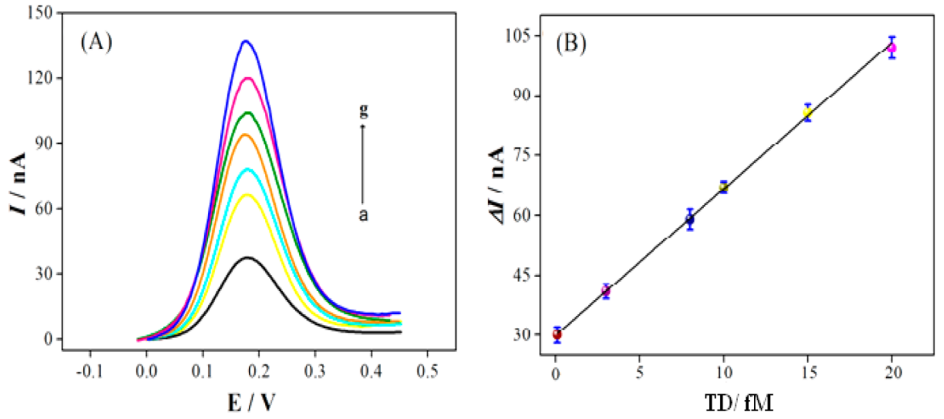

Figure 3. (A) DPV responses for the different concentration TD: (a) 0 fM, (b) 0.1 fM, (c) 3 fM, (d) 8 fM, (e) 10 fM, (f) 15 fM, (g) 20 fM. (B) The calibration curve between the current increment and the concentration of TD. The error bars represent the standard deviation (S.D.) of three measurements conducted.

As expected in Figure 2A, the ΔI (DPV current increment, $\Delta I = I_1 - I_2$, I_1 is the current that the proposed biosensor reacts with TD including a certain nicking endonuclease. I_2 is the current that the proposed biosensor reacts with the 1× NEBuffer 3 and a certain nicking endonuclease) increased with the CP concentration in the range from 0.1 to 0.5 μM and then reached the saturated condition after 0.5 μM. So 0.5 μM CP was chosen for the following experiments. Figure 2B showed that the ΔI increased with the self-assembly time in the range of 0−120 min and then tended to reach a plateau after 120 min. These results indicated that a self-assembly time of 120 min was the ideal reaction time for the electrochemical sensor.

The effect of the reaction time between the biosensor and TD was also investigated. As shown in Figure 2C, the ΔI increased with the increasing of reaction time and reached a plateau after 5.0 h. Therefore, 5.5 h had been chosen as the optimized condition for the following study.

The effect of the incubation temperature of nicking endonuclease on the detected signal had also been studied. As shown in Figure 2D, the ΔI increased with an increment of incubation temperature up to 37 °C. However, the ΔI decreased to negative from 48 °C. The reason lies in that CP and SP rapidly melt when the incubation temperature reached the unwinding condition. Thus, the temperature of 37 °C was selected as a compromise.

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Analytical Performance of the Biosensor. The sensitivity and dynamic range of the as-prepared electrochemical biosensor was evaluated. The results showed that electrochemical signal increased with the increasing of TD concentration (see Figure 3A). With higher TD concentration, more capture probes on the electrode were cleaved, and more cycles proceeded until the end, resulting in increasing faradic current. Moreover, the ΔI displayed a good linear relationship with TD concentration in the range from 0.1 fM to 20 fM (see Figure 3B).The regression equation is

$$\Delta I/\mathrm{nA} = 29.99 + 3.667\mathrm{Cx/fM}, \quad R = 0.9993$$

where ΔI is DPV current increment, Cx is TD concentration, and R is the regression coefficient. The detection limit (LOD) was 0.08 fM at the $3s_{blank}$ level, which was lower than that of the reported target-induced strand displacement strategy.[17] Compared to the existing electrochemical DNA biosensors based on the enzyme-amplification,[22,23] the other nuclease assisted target recycling amplification and signal-off mechanism[24,25] and other strategies,[26,27] this DNA biosensor showed higher sensitivity due to the nicking endonuclease signal amplification and signal-on mechanism.

To investigate the repeatability of the electrochemical biosensor, five biosensors had been fabricated for paralleling assay and the relative standard deviation (RSD) for the determination of 0.4 fM TD was 6.0%. Additionally, after reacting with 0.4 fM TD, the biosensor was scanned by DPV at least 5 times repeatedly and the RSD was 5.1%. This result indicates that the prepared biosensor has good repeatability. When the biosensor was stored at 4 °C over 1 weeks, the electrochemical response did not change significantly (RSD = 4.7%), indicating that the manufactured biosensor has good stability.

Interference Study. Specificity is another important factor to evaluate a biosensor. Here, we verified the specificity of the electrochemical biosensor toward single-base mismatched DNA, double-base mismatched DNA, and the noncomplementary DNA. As shown in Figure 4, the addition of interfering

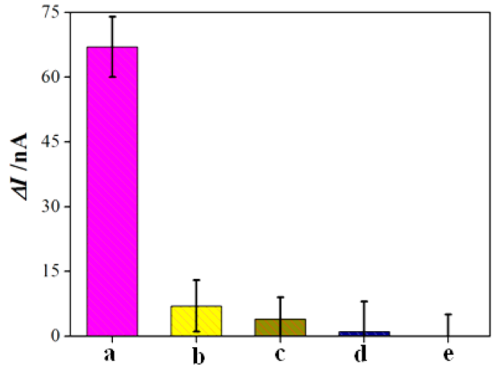

Figure 4. Specificity of the electrochemical biosensor: (a) 10 fM TD, (b) 25 nM single-base mismatched DNA, (c) 25 nM double-bases mismatched DNA, (d) 25 nM noncomplementary DNA, and (e) blank.

agent at a concentration of 25 nM did not cause significant electrochemical signal changing. However, it was found that the designed biosensor had an obvious response with respect to 10 fM TD. The results clearly demonstrated that the electrochemical biosensor had excellent discrimination for single-base mismatch and noncomplementary DNA sequences. The incredibly high selectivity of the strategy was due to the high specificity of nicking endonuclease to special DNA sequence and target recycling,[15,16,21] indicating the developed electrochemical biosensor here could be applied for the ultrasensitive detection of TD from *Bacillus subtilis* with high specificity. Moreover, this strategy is expected to detect a single-base mismatch and single nucleotide polymorphisms (SNPs).

Real Sample Detection. The DNA extractive of *Bacillus subtilis* was used to test the applicability of the proposed biosensor for real sample determination. First, the DNA extractive of *Bacillus subtilis* was amplified by PCR. The agarose gel electrophoresis of the PCR products showed that there was only one band (approximately 320 bp, lane a) lying in the position of the standard DNA (i) (lane b) (see Figure 5).

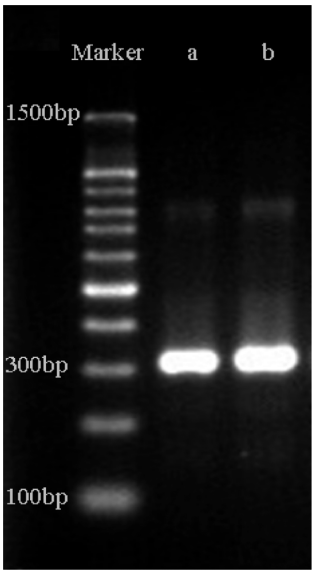

Figure 5. Agarose gel electrophoresis analysis of the PCR product: (a) PCR products and (b) the standard DNA (i).

Second, the accurate quality of the DNA extractive was detected by QPCR with SYBR Green (according to the specification of SYBR Premix Ex Taq (Tli RNaseH Plus)) and the value is 7.40×10^{-7} ng/uL (16.7 fM). Third, the DNA extractive took the place of TD to react with the proposed biosensor. According to the above-mentioned calibration curve, the concentration of the DNA extractive is 15.4 fM, which is approximately consistent with the results obtained by QPCR. The relative error is about −7.8%. These results indicate that the proposed biosensor can be applied to test the real sample with satisfied results. Compared with QPCR,[10] the electrochemical instrument is simpler and cheaper than QPCR instrument. The developed biosensor here will facilitate the construction of inexpensive and hand-held electrochemical instrument to detect the given DNA sequence from *Bacillus subtilis*.

CONCLUSIONS

An ultrasensitive and selective electrochemical biosensor for the determination of the given DNA sequence from *Bacillus subtilis* by coupling target-induced strand displacement and nicking endonuclease signal amplification has been designed in this paper. The proposed signal-on biosensor has high selectivity to TD and can distinguish TD from single-base mismatch, double-bases mismatch, and noncomplementary DNA sequence.

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Moreover, the applicability of the designed biosensor for detecting real sample was investigated. The result obtained by electrochemical method is approximately consistent with that by QPCR with SYBR Green. The relative error is about −7.8%. Thus, the designed biosensor represents an expectation to detect single nucleotide polymorphisms (SNPs) and can be efficient for the quantitative determination of DNA from the real sample.

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Notes

The authors declare no competing financial interest.

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