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An ultrasensitive and specific electrochemical biosensor for DNA detection based on T7 exonuclease-assisted regulatory strand displacement amplification

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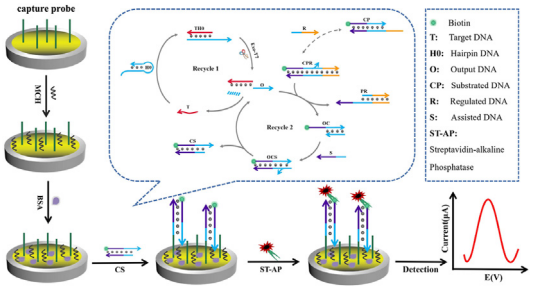
HIGHLIGHTS

- An ultrasensitive and specific electrochemical biosensor was proposed for the detection of DNA.
- The strategy was based on T7 exonuclease-assisted regulatory strand displacement to realize dual signal amplification.
- The designed biosensor was proved to rapid response, admirable stability and precision.
- This sensing method was successfully applied to detect DNA in 20% diluted human serum samples.

GRAPHICAL ABSTRACT



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ABSTRACT

An electrochemical biosensor for determination of DNA is developed based on T7 exonuclease-assisted regulatory strand displacement dual recycling signal amplification strategy. First, the hairpin probe recognized and bound the target DNA to form a double strand nucleotide structure, and then the T7 exonuclease was introduced. After be digested by T7 exonuclease, the target DNA was released and entered the next cycle of T7 exonuclease-assisted recycle amplification, while accompanied by a large number of mimic targets (output DNAs) into another cycle. Second, the mimic target reacted with double-chain substrated DNA (CP) by a regulated toehold exchange mechanism, yielding the product complex of detection probes with the help of assisted DNA (S). Finally, after many cycles, a large number of detection probes were produced for binding numerous streptavidin-alkaline phosphatases. The electrochemical biosensor showed very high sensitivity and selectivity with a dynamic response ranged from 0.1 fM to 10 pM with a detection limit of 31.6 aM. Furthermore, this proposed biosensor was successfully applied to the detection of target DNA in 20% diluted serum. The developed strategy has been demonstrated to have the potential for application in molecular diagnostics.

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1. Introduction

Recently, the highly sensitive and selective detection of DNA has received increasing attentions in clinical diagnosis, gene analysis, forensic analysis and pathogen detection [1–4]. In order to achieve DNA detection, researchers have made various efforts on different detection platforms. For example, fluorescent [5,6], colorimetric [7,8], chemiluminescent [9,10], surface plasmon resonance [11,12], electrochemical technique [13–15] and so on. Among these technologies, electrochemical technique affords many advantages such as simplicity of construction, rapid response, easy operation, low instrumentation cost and high sensitivity. Therefore, the electrochemical detection platform was used in this study to develop DNA detection.

Because enzyme-assisted recycle amplification strategy provides a firm foundation for improving the sensitivity of electrochemical biosensor, it has super extensive applications in detections based on electrochemical platform [16–20]. According to previous reports [21–23], enzyme-assisted recycle amplification strategy has the following advantages: first, due to the high efficiency and specificity of enzyme, the efficiency and specificity of the whole method are improved Significantly. Second, it can realize the conversion of bits of targets to substantial mimic targets due to its low cost and easy to use. Third, it is easy to combine with other amplification methods to achieve more accurate and sensitive detection.

Although the target molecule was amplified in these reported works, the amplification efficiency still needed to be improved since each input target could only be conversed to one mimic target in one recycle. Thus, it is very urgent to integrate an enzyme-assisted recycle amplification strategy with other amplification techniques for further improvement of sensitivity of electrochemical biosensors.

To increase the target conversion ratio and the ultimate amplification efficiency, enzyme-assisted dual recycle amplification strategies were developed in the construction of biosensors. So far, great efforts have been spurred in this because twice amplification in the same reaction can achieve cascade amplification of signal [24–26]. Although these works have achieved obviously additional amplification of signal, there are still some drawbacks including high environmental sensitivity, significant interference caused by different enzymes, and high cost because of a variety of enzymes. Therefore, a single-enzyme-assisted dual recycle amplification strategy might be the solution to the problems.

Recently, more and more studies on the regulatory strand displacement recycle amplification reaction have been developed in biosensor, because it possesses the advantages of simple structure, low cost and high reaction efficiency. According to our previous research [27], the regulatory strand displacement reaction has been successfully applied on the electrode surface. What's more, T7 exonuclease acts on double-stranded DNA and catalyzed the removal of single nucleotide from 5′ end to 3′ end. Beside starting digestion from the 5′ end, it can also start from the cut or gap of double-stranded DNA [28,29]. Compared with other exonucleases, T7 exonuclease does not require specific recognition site and has several advantages of reduction in interferential reaction, decrease in environmental sensitivity and cost saving [30,31]. Therefore, integrated T7 exonuclease-assisted cycle amplification with regulatory strand displacement recycle amplification is a promising way to achieve high sensitive detection of electrochemical biosensor at low cost and simple operation.

Herein, an ultrasensitive electrochemical biosensor has been constructed for target DNA detection based on T7 exonuclease-assisted regulatory strand displacement dual recycling signal amplification strategy. Randomly designed DNA molecules were chosen as the model target in this study. A hairpin probe was used to recognize the target DNA and distinguish other DNAs. Two cycles were designed to ensure cascade signal amplification. Thus, the electrochemical strategy exhibited excellent analytical performance towards target DNA detection with high specifity and sensitivity, which might be a great potential for bioanalysis, molecular diagnostic applications and environmental monitoring.

2. Experimental section

2.1. Chemicals and apparatus

All DNA oligonucleotides (Table 1) and T7 exonuclease we used were purchased and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China, http://www.sangon.com), and then dissolved in TE buffer. Bovine serum albumin (BSA), 6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP) and α-naphthyl phosphate (α-NP) were purchased from Sigma Aldrich (Saint Louis, Missouri, USA, http://www.sigmaaldrich.com). DNA Markers were obtained from Takara Biotech. Inc. (Dalian, China https://www.takarabiomed.com.cn). DNA hybridization buffer was phosphate-buffered saline. The washing buffer was 0.01 M PBS containing 137 mM NaCl, 2.5 mM KCl and 0.05% Tween-20 (pH 7.4). Diethanolamine (DEA) buffer contained 0.1 M DEA, 1 M $MgCl_2$ and 100 mM KCl (pH 9.6). The serums of healthy people were from First Teaching Hospital of Tianjin University of Traditional Chinese Medicine and stored at −80 °C before use. All reagents used in the experiments were of analytical grade, and prepared using Millipore-Q water (≥18 MΩ cm, Milli-Q, Millipore, http://www.mollipore.com).

Square wave voltammetry (SWV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS) were performed on a CHI660D electrochemical workstation (Chenhua Instru-ments Co.Ltd., Shanghai China, http://www.chinstruments.com). The gel electrophoresis analysis was performed by an electrophoresis analyzer (Bio-Rad, USA, https://www.bio-rad.com) and imaged on a ChemiDoc XRS system (Bio-Rad, USA).

2.2. Preparation of hairpin probes

Hairpin probes were designed based on target DNA. They were denatured at 95 °C for 5 min and cooled down slowly to room temperature. Then the probes were stored at 4 °C for further use.

2.3. Dual recycling signal amplification

Briefly, T7 exonuclease-assisted recycling amplification reaction was carried out by mixing 1 μL H0 (500 nM), 1 μL T7 exonuclease (0.3 U) with different concentrations of target DNA in hybridization buffer at a final volume of 50 μL. The mixture was incubated at 37 °C for 50 min, followed by inactivation of T7 exonuclease at 90 °C for 3 min and renaturation of residual H0 at 37 °C for 10 min. Then, 50 μL of hybridization buffer containing 200 nM double-chain substrated DNA, 200 nM regulated DNA and 200 nM assisted DNA were added to initiate regulatory strand displacement amplification. Finally, the resulted solutions were dipped on the modified electrode surface and the reactions were carried out at 37 °C for 40 min.

2.4. Preparation of electrochemical biosensor

The bare gold electrodes were freshly polished using 0.05 μm alumina powder to obtain mirror-like smoothness. Then they were ultrasonically treated with ultrapure water, ethanol, and ultrapure water sequentially to remove the surface impurities. Subsequently,

Table 1
Sequences of oligonucleotides (in 5′−3′ direction).

Oligonucleotide	Sequence (5′−3′)
Capture probe	SH−$(CH_2)_6$− TTTTTT AC CCG CA
Target oligonucleotide	CAC ACT CAA CTG GGA ATC CGC ATT
Hairpin probe 0 (H0)	AAT GCG GAT TCC CAG TTG AGT GTG ACC CGC ATT GAA GAT
Substrated DNA C (C)	biotin-T GAC ACA TCT TCA ATG CGG GT
Substrated DNA P (P)	GTC TCT CAC CCG CAT TGA AGA TGT GTC A
Regulated DNA (R) assisted DNA (S)	ATG CGG GTG AGA GAC C ACC GAATGT GTC A
Single-base-mismatched oligonucleotide	CAC ACT CAA CTG GGA ATG CGC ATT
Two-base-mismatched oligonucleotide	CAC ACT CAA CTG GGA AAG CGC ATT
Non-complementary oligonucleotide	ACT CGC AGG ACT TTG CAG TAA GGG

*The underlined sequence are mismatched bases.

in order to further clean the electrodes, the surface of electrodes was immersed with 10 μL piranha solution (98% concentrated sulfuric acid: 30% hydrogen peroxide = 3 : 1) for 10 min. After each step, the electrodes were rinsed with ultrapure water and dried naturally.

10 μL of 0.5 μM thiolated capture probe was added on the surface of gold electrode and incubated overnight at 4 °C. The next day, after rinse of washing buffer, the electrode was immersed into 100 μL of 1 mM MCH for 1 h to obtain well-aligned DNA monolayer which would occupy the left bare sites. The electrode was further rinsed by washing buffer and sealed by 1% BSA with 125 μg mL^{-1} salmon sperm DNA in turn for removing unbound structures.

2.5. Electrochemical measurements

After rinse of washing buffer, 10 μL of amplification products was dropped on the surface of gold electrode to hybridize with capture probes at 37 °C for 40 min. Following rinsed thoroughly with DEA buffer, 10 μL of 1.2 μg mL^{-1} ST-AP was dropped onto its surface and incubated at 37 °C for 30 min. Finally, the biosensor was washed with DEA buffer containing 0.05% Tween-20 thoroughly to perform DPV detection in 1 mg mL^{-1} α-NP dissolved by DEA buffer.

3. Results and discussion

3.1. Design of electrochemical biosensor

As illustrated in Scheme 1, we designed an electrochemical biosensor for the detection of target DNA. The assay system consists of T7 exonuclease-assisted recycle amplification and regulatory strand displacement amplification. In the presence of target DNA (T), hairpin DNA probe (denoted as H0) is opened by hybridizing with T to form DNA double stranded structure. Then, T7 exonuclease can bind to the double stranded structure and stepwise hydrolyze the mononucleotides from the 5′-terminus of H0 in the direction of 5′ to 3′, releasing the T and the residual sequence (called output DNA, denoted as O). The released T continuously hybridizes with H0 and triggers the cleavage process, forming more O, which can be used in regulatory strand displacement amplification.

The sequence of signal amplification could be explained as follow. First, the regulated DNA (R) cross with double strand substrated DNA (CP) to produce intermediate CPR. While this procedure was reversible, only in the presence of O, CPR binds with O by hybridization and initiates a branch migration leading to the production of two kinds of double stranded DNA complexes. It should be noted that one of the double strand complexes contains R (denoted as PR), the other contains O (denoted as OC). Because R activates as the role of enzyme during the overall reaction except catalytic center, so, the speed of the whole reaction is improved. With the help of assisted DNA (denoted as S), OC can react with S through a toehold exchange mechanism, yielding the product complex of detection double strand (denoted as CS). At the same time, O is released and free to catalyze the next cycle of toehold-exchange, forming more CS complexes.

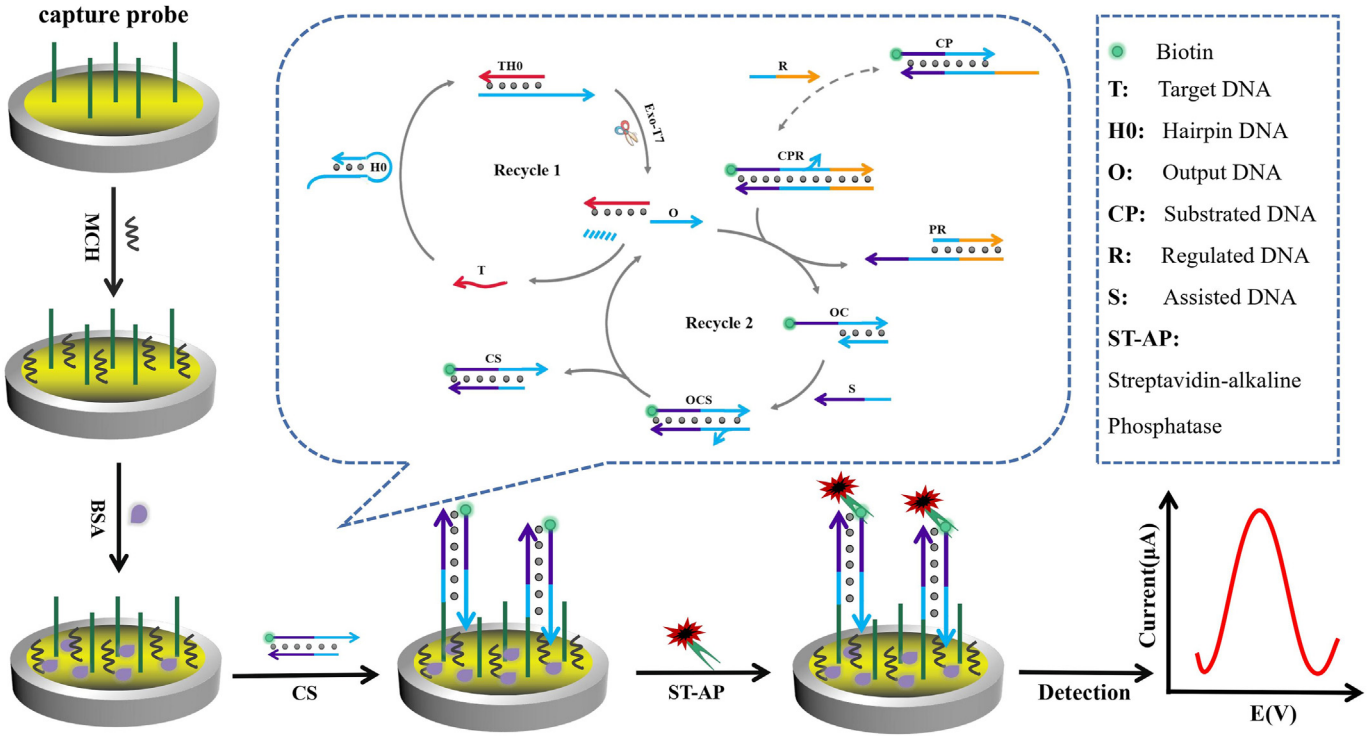
Finally, the capture probes immobilized on the gold electrode can specifically hybridize with CS complexes. Then streptavidin-alkaline phosphatase (ST-AP) is labeled on the electrode surface through streptavidin-biotin interaction, which produces a remarkable amplified enzymatic electrochemical signal readout. Based on the dual signal amplification strategy, an electrochemical biosensor was successfully developed for ultrasensitive and highly specific detection of target DNA.

3.2. Feasibility of the electrochemical biosensor

In order to clearly demonstrate the feasibility of this proposed biosensor for sensitive detection of target DNA, the reaction was examined by DPV under same conditions (n = 3). As exhibited in Fig. 1A, in the absence of T7 exonuclease, the current signal value of the biosensor (curve b) was almost the same as that of the blank (curve a), which indicated that there was no O and the regulatory strand displacement reaction cannot be triggered. However, in the presence of T7 exonuclease, a significantly enhanced current signal was observed, and the target DNA signal value at high concentration (curve d) was higher than that of the target DNA at low concentration(curve c). The result showed that regulatory strand displacement reaction was triggered by O, which was produced by T7 exonuclease digestion. Furthermore, this strategy can distinguish different concentrations of target DNA signals. In addition, the whole reaction was verified by 3% agarose gel electrophoretic. As can be seen in Fig. 1B, H0 was observed in lane 1, while target DNA cannot be seen in lane 2 because of its short sequence. As shown in lane 3 and lane 4, H0 was opened by hybridizing with target DNA to form a new band. CP strands and CPR strands were observed in lane 5 and lane 6, respectively, showing that CP strands and R strands combined to form the intermediate CPR. Comparing lane 7 with lane 8, more CS marked with red box were generated with the assistance of S strands, suggesting that successful T7 exonuclease digestion and excellent regulatory strand displacement reaction could be achieved. These results illustrated this strategy had feasibility for the detection of target DNA.

3.3. Characterization of electrode assembly process

To monitor the stepwise assembly process of this constructed biosensor, EIS and SWV measurements were performed to characterize the different phase of electrode surface modification in the electrolyte solution embracing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. 1 pM target DNA was adopted. According to previous reports [36],



Scheme 1. Schematic illustration of the electrochemical biosensor for DNA detection base on exonuclease T7-assisted regulatory strand displacement amplification.

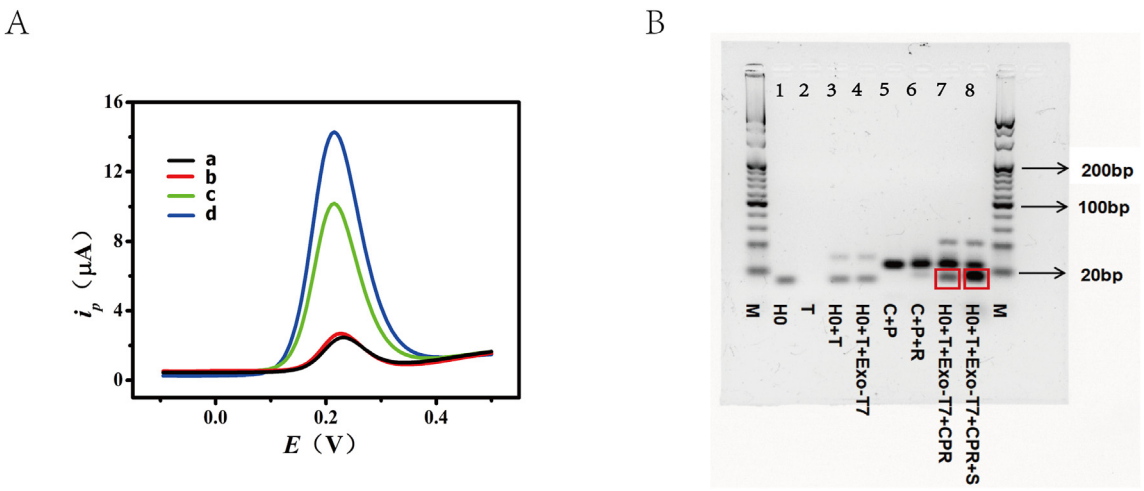


Fig. 1. Verifying the feasibility of the assay. Typical DPV signal of Fig. 1A: (a) balnk, (b) H0+100 fM target DNA + CP + R + S, (c) H0+10 fM target DNA + Exo-T7+CP + R + S, (d) H0+100 fM target DNA + Exo-T7+CP + R + S, respectively. (B) Verification of whole reaction by PAGE: (1) H0, (2) T, (3) H0+T, (4) H0+T + Exo-T7, (5) C + P, (6) C + P + R, (7) H0+T + Exo-T7+CPR, (8) H0+T + Exo-T7+CPR + S, respectively. The reaction time was set at 40 min. The final concentration of all nucleotides is 1 μM.

the semicircle diameter of each EIS curve represents the charge transfer resistance (Rct). As depicted in Fig. 2A, the bare Au electrode presented a approximately straight line (curve a), indicating the superior conductive capability of the bare electrode. When the thiolated capture probe was immobilized on the bare electrode surface covalently via Au—S bond, the Rct increased to 574 Ω (curve b), which was primarily attributed to the electrostatic repulsion between $[Fe(CN)_6]^{3-/4-}$ and the negatively charged phosphate backbone of the oligonucleotides. After the electrode was further modified with MCH and BSA, it can be visually monitored that the Rct increased to 1906 Ω (curve c). The reasons might be ascribed to that the biomacromolecules could obstruct the transmission of electrons and enhanced the charge transfer resistance. Followed that, the capture probe hybridized with amplification products of the T7 exonuclease-assisted regulatory strand displacement recycling amplification (Exo T7-RSD), the Rct further increased to 5326 Ω (curve d). This could be attributed to the generation of a large number of CS complexes in the existence of target DNA, which hindered the transfer of electrons on the electrode interface. Finally, the binding of ST-AP onto the electrochemical sensor surface via biotin-streptavidin interaction accounted for the result that Rct continuously increased to 7316 Ω (curve e). These results were in accordance with the outcomes obtained from SWV (Fig. 2B), where the peak currents varied upon the assembly, binding and

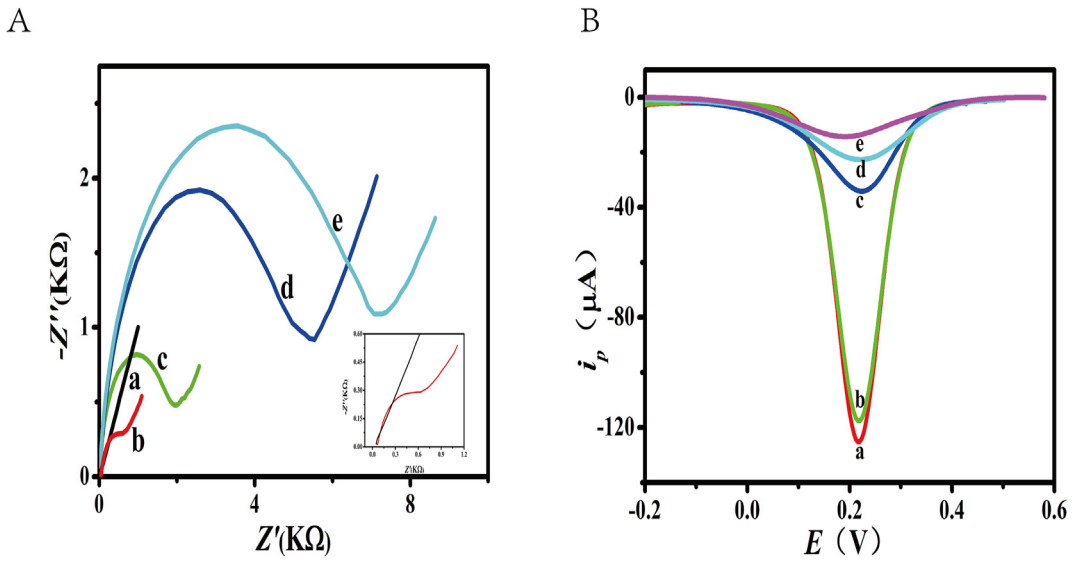


Fig. 2. EIS (A) and SWV (B) in 0.4 M KCl containing 0.5 mM $Fe[(CN)_6]^{3-/4-}$ at bare electrode (a), capture DNA modified electrode (b), capture DNA modified electrode after sealed with MCH and BSA (c), after complex CS (d) and ST-AP (e), respectively. Inset of panel A: an enlarged superposition of curve a and b.

amplification procedure. All these results proved that the biosensor indeed operated as designed working principle.

3.4. Optimization of the experimental conditions

To achieve the desired sensing performance of target DNA detection, several crucial experimental parameters were explored. Considering the concentration of H0 was directly related to the smooth progress of the whole amplification process, the effect of H0 concentration was investigated in the first place. As the concentration of H0 increased, the DPV response enhanced progressively and then reached a steady value at 150 nM (Fig. 3A). Therefore, 150 nM was employed as the optimal concentration of H0 in the subsequent experiments. Futhermore, the concentration of T7 exonuclease was also a critical factor affecting the reaction efficiency in the Exo T7-RSD system. There was a remarkable increase of the DPV peak with the augment of T7 exonuclease concentration from 0.1 U to 0.5 U, and the electrochemical signal arrived relatively stable at 0.3 U (Fig. 3B). Thus, 0.3 U was adopted as the appropriate concentration. Finally, the effect of incubation time on the response of the biosensor was inspected. As exhibited in Fig. 3C, the peak current increased obviously as the increasing incubation time and the signal presented no further conspicuous variation after more than 40 min. Consequently, 40 min was selected for the optimized incubation time. As shown in Fig. 3D, with the increase concentration of capture probe, the DPV signals enhance gradually and reach the peak value at 400 nM. Therefore, 400 nM was chosen as the optimized concentration. Because incubation time of H0+T7+target DNA mixture has a great impact on the whole analysis, its was investigated. Fig. 3E illustrated the change in DPV signal with incubation time of H0+T7+target DNA mixture, the signal of DPV would not increase when the time reaches 45min. Therefore, 45 min was selected for the optimal time. These optimized reaction conditions were used to subsequent experiments.

3.5. Analytical performance of the biosensor

Under the optimal experimental conditions, the DPV responses at 0.18 V increased with the increment of synthetic target oligonucleotides concentration (Fig. 4A). As shown in Fig. 4B, the calibration plots showed a good linear relationship between the peak currents and the logarithm of target DNA concentrations in the range from 0.1 fM to 10 pM with detection limit of 31.6 aM calculated from three times the standard deviation corresponding to the blank sample detection. The resulting linear equation was ip (μA) = $5.33\log C_{DNA}+5.34$ with a correlation coefficient of 0.995. To further highlight the merits of the designed biosensor, the analytical properties were compared with those of other reported methods shown in Table 2. It shows that this biosensor has higher sensitivity than those reports and relatively wide detection range. This is primarily attributed to T7 exonuclease-assisted amplification and regulatory strand displacement amplification. Moreover, the binding nucleotide molecules can be removed completely from the sensor surface without irreversible loss of probes activity by sodium hydroxide. Up to 3 cycles of binding/regeneration processes on the same sensor, the signal only decreased less than 10%. These results indicated that the established detection platform was capable of testing the target DNA with a low detection limit.

3.6. Specificity, repeatability and stability of the biosensor

Specificity, repeatability and stability of the biosensor were great important properties to verify the detection performance of the designed sensor. To appraise the specificity of the established biosensor toward target DNA, the biosensor was applied to analyze the complementary target DNA, the single-base mismatched DNA (SM), the double-base mismatched DNA (DM), the non-complementary mismatched DNA (NC) and a blank sample without target DNA. According to the detection outcomes. Fig. S1A displayed the comparation of DPV peak current after hybridization with 10 fM and 1 pM of the four different oligonucleotides and the background. It could be clearly observed that current value of the target DNA were much higher than other interference mismatch sequences, which implied the fabricated biosensor exhibited high accuracy faultlessly in distinguishing target DNAs and the mismatched DNAs. After that, to further assess the repeatability of this exploitative electrochemical biosensor, target DNA samples at the concentrations of 10 pM and 1 fM were measured with 5 individual electrodes, respectively. As depicted in Fig. S1B, the coefficient of

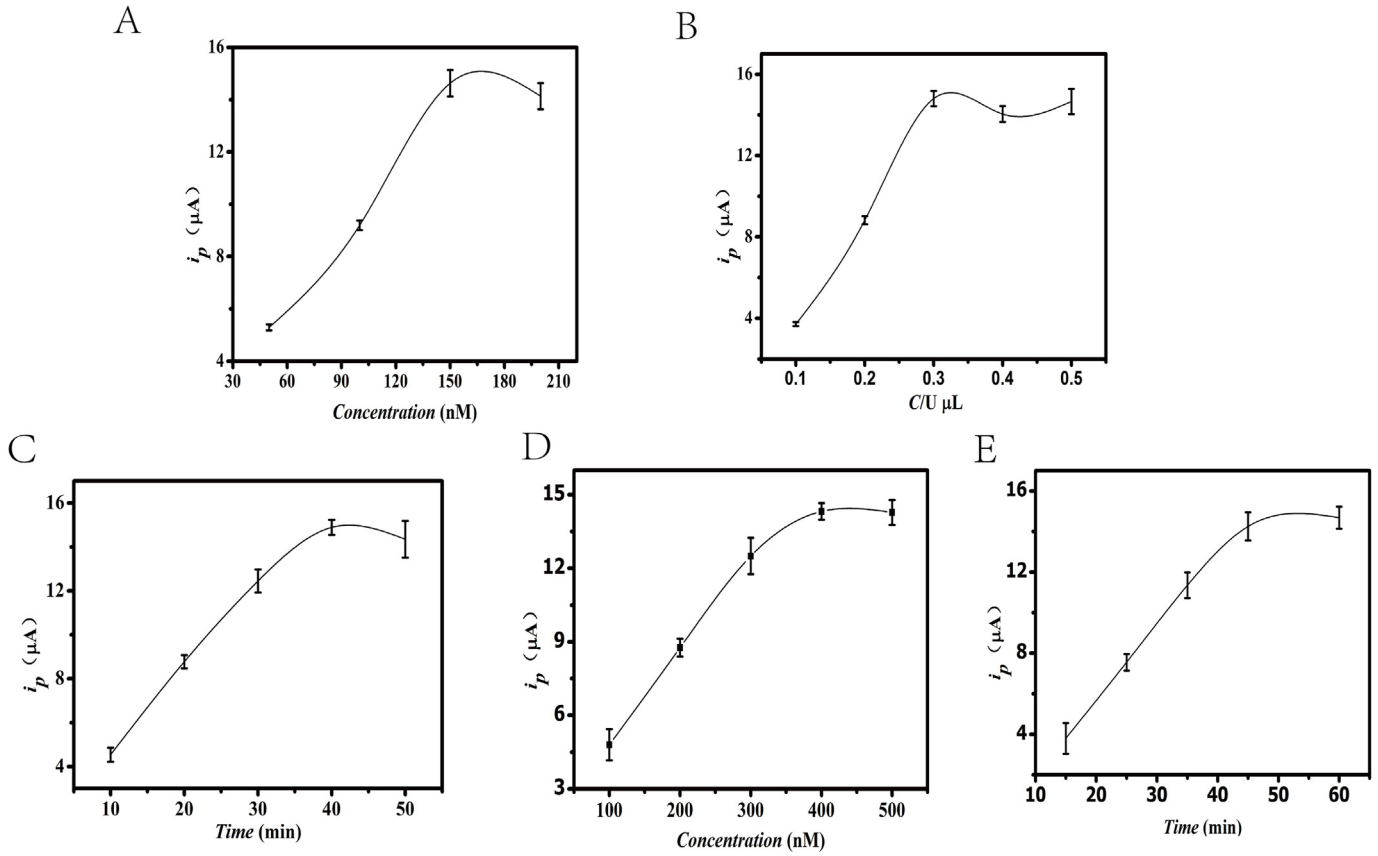


Fig. 3. Optimizations of experimental parameters. The effect of the (A) H0 concentration, (B) exonuclease T7 concentration, and (C) reaction time of complex CS on electrochemical biosensor, (D) capture probes concentration, and (E) incubation time of H0+T7+target DNA mixture. The concentrations of target DNA was 100 fM, Data are expressed as mean ± standard deviations, sample replicates n = 3.

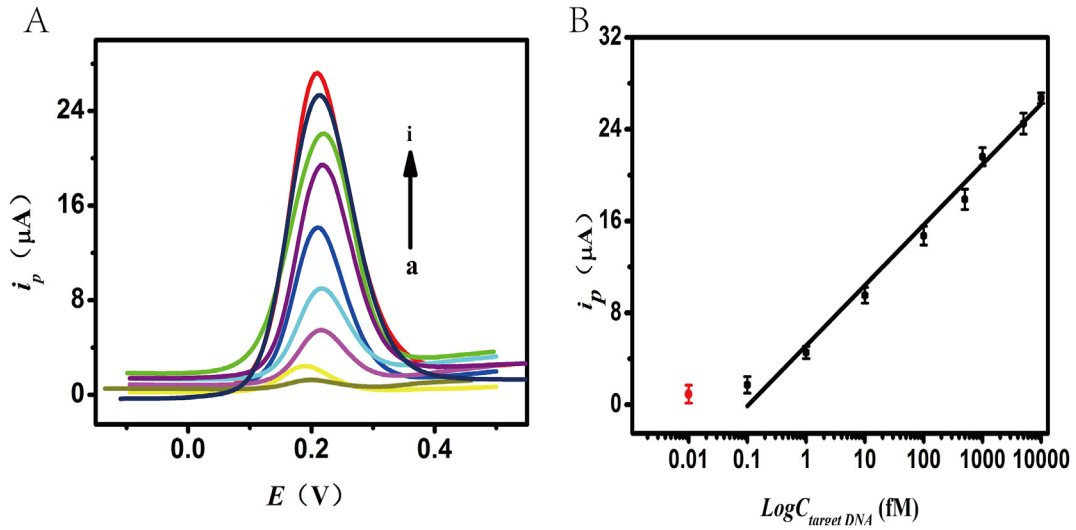


Fig. 4. Evaluation of the sensitivity of the strategy. (A) DPV corresponding to different concentrations of target DNA. From a to g: 0, 0.1 fM, 1 fM, 10 fM, 100 fM, 500 fM, 1 pM, 5 pM, 10 pM. (B) Linear relationship between the logarithm of the target DNA concentration ranging from 0.1 fM to 10 pM and current value. The error bars represent the standard deviations calculated from three different spots.

variation (CV) value of two different concentrations were calculated to be 3.15% and 1.26%, respectively, testifying that the biosensor possessed excellent reproducibility. Moreover, the stability of this biosensor was further estimated. As shown in Fig. S1C, the DPV responses has no remarkable variation even if the components of the developed biosensor were stored for ten days at 4 °C, which manifested that this method had an acceptable stability.

All the results presented above indicated that the proposed biosensor could effectively detect the target DNA with admirable selectivity, satisfactory repeatability and acceptable stability.

Table 2
Comparison between the developed assay and other reported methods for DNA detection.

Signal amplification method	Linear range	Detection limit	References
Allosteric DNA toehold	1 fM to 1 nM	0.83 fM	[27]
Dendritic gold nanostructure	1 fM to 1 nM	1 fM	[32]
CSRP/MNPs	1 pM to10 nM	0.5 pM	[33]
CSDPR/HCR	10 fM to 1 nM	8 fM	[34]
III Exonuclease/CHA	100 fM to 5 nM	92 fM	[35]
IIIexonuclease/SDR/TDT	0.1 fM to 1 nM	0.05 fM	[36]
T7 Exonuclease/RCR	0.1 fM to 10 pM	31.6 aM	This work

3.7. Real sample analysis

In order to evaluate the potential application of the fabricated biosensor for the detection of actual samples, various known concentrations of DNA were spiked into 20% diluted healthy human serum to conduct the recovery test. Human serums were got from the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. Then, the designed biosensor was used to detect added target DNA in these serum samples. As everyone knows, the smaller difference between the assayed value of this method and the added target DNA value, the higher the accuracy of this method will be. Table 3 shows the recovery rates were between 99.9% and 103%, implying that the biosensor was not be interfered in complex mixtures. However, there are still some problems when the method was directly used in blood samples. So we will continue to give impetus to resolve this issue in the following work. Although the biosensor had a certain defect, the recoveries were acceptable. Therefore, this biosensor might become a potential approach for DNA test in real clinical samples analysis.

4. Conclusion

In summary, an ultrasensitive and specific biosensor was developed for detection of DNA by employing T7 exonuclease-assisted regulatory strand displacement amplification. It was proved that both efficient catalytic rate of T7 exonuclease and excellent regulatory strand displacement reaction can be achieved. Consequentially satisfied analytical sensitivity is produced. Moreover, the biosensor can be regarded as a universal detection platform conveniently applied for the detection of any DNA sequences by programming the target-specific sequences and providing a universal detection platform. In addition, this strategy can also be combined with other amplification methods to establish higher sensitive electrochemical biosensor in the future. In a word, this proposed biosensing strategy would provide a great potential for DNA detection in the area of clinical diagnosis and therapy, pathogen detection and environmental monitoring in the future.

Compliance with ethical standards

The author (s) declare that they have no competing interests.

Table 3
The recoveries determined using the proposed method via spiking synthetic DNA into human serum samples.

Sample No.	Spiking value (fM)	Assayed value (fM)	Recovery	RSD(n = 3)
No.	C DNA (fM)	C DNA (fM)[a] ±SD[b]	(%)	(%)
1	10	10.3 ± 0.49	103	4.76
2	100	101.1 ± 2.20	101.1	2.18
3	1000	999.9 ± 1.35	99.9	0.14

[a] The data represent the average value of three independent determinations.
[b] SD, standard deviation.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "**An ultrasensitive and specific electrochemical biosensor for DNA detection based on T7 exonuclease-assisted regulatory strand displacement amplification** ".

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338988.

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