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# Highly Sensitive Electrochemical Aptasensor Based on a Ligase-Assisted Exonuclease III-Catalyzed Degradation Reaction

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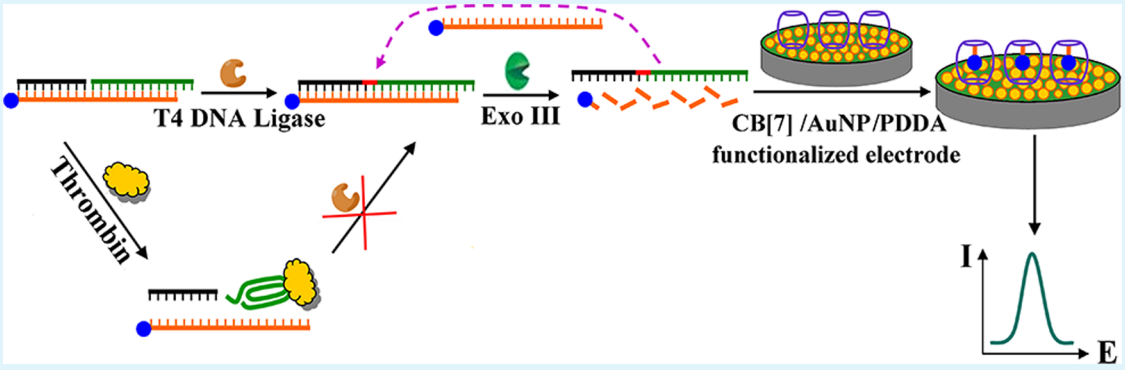


**ABSTRACT:** In this paper, we have proposed a new electrochemical aptasensor based on a novel ligase-assisted Exo III-catalyzed degradation reaction (LAECDR), which consists of DNA ligase-catalyzed ligation of thrombin-binding aptamer (TBA) with an extension strand (E-strand) and Exo III-catalyzed selective degradation of probe DNA, by using an improved target-induced strand displacement strategy. As a result of LAECDR, methylene blue (MB)-labeled mononucleotides can be released from the 3′-terminal of probe DNA and captured by cucurbit[7]uril-functionalized electrode to induce noticeable electrochemical response. Nevertheless, in the presence of the target protein, thrombin, the TBA that is partially complementary to probe DNA is preferentially binding with the target protein, thereby inhibiting LAECDR from taking place. The remaining intact probe DNA will prevent the terminal-attached MB from approaching to the electrode surface due to strong electrostatic repulsion, so the electrochemical response will be changed by thrombin. By tracing the electrochemical response of adsorbed MB, our aptasensor can exhibit high sensitivity for thrombin detection with a wide linear range from 100 fM to 1 nM and an extremely low detection limit of 33 fM, which can also easily distinguish thrombin in the complex serum samples with high specificity. Therefore, our aptasensor might have great potential in clinical applications in the future.

**KEYWORDS:** *electrochemical aptasensor, cucurbit[7]uril, DNA ligase, exonuclease III, thrombin*

## 1. INTRODUCTION

Aptamer, usually a single-stranded artificial oligonucleotide (RNA or ssDNA), can specially bind to a target molecule with high affinity that is comparable to an antibody.[1] Since the first report by Gold's and Szostak's group in 1990, many aptamers have been screened against different targets of interest including small molecules, proteins, and even whole cells.[2−4] So, a new type of biosensor by making use of an aptamer as a biological recognition element has been developed as an alternative to the conventional immunosensor, which is called aptasensor.[5,6] Coupled with different detecting techniques, such as fluorescence, electrochemistry, surface plasmon resonance, etc., many kinds of aptasensors have been fabricated.[7−9] Among them, the electrochemical aptasensor has held great promise and exhibited several noticeable advantages, such as simple instrumentation, rapid response, low cost, and high sensitivity.[10−12]

It has been known that aptamers can fold into unique intramolecular structures when binding to the targets, which may alter the distance between the electrode surface and the electroactive molecules (e.g., methylene blue (MB) and ferrocene) anchored at the aptamer terminus. So, some conformational switch-based electrochemical aptasensors have been developed.[13−15] However, this type of aptasensor relies too much on the three-dimensional structures of aptamers, and the immobilization of target molecules (especially the biological macromolecules) onto the electrode surface may affect the

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electron transfer process, both of which will severely restrict its further application. Therefore, others have proposed another kind of electrochemical aptasensor on the basis of target-induced strand displacement strategy, which has lower requirement in aptamer spatial conformation and more flexibility in sequence design.[16−18] Because of the relatively higher binding affinity between an aptamer and its target, the addition of target molecules can dissociate the aptamer from its complementary DNA and thus induce the changes in the electrochemical signal for the target detection.

Although the aptasensors based on the target-induced strand displacement strategy have shown much wider applicability beyond the conformational switch-based electrochemical aptasensors, their practical application has also suffered from several problems. On the one hand, this kind of aptasensor is not well suited to aptamers with inherent low dissociation constant. On the other hand, despite that some aptamers have exhibited high binding affinity in the screening system, the environmental factors in the real detection system, such as the immobilization approaches, the required labeling, and even the reaction buffer (ionic strength, electrolyte, and pH), may cause a loss in the binding affinity and recognition specificity of aptamer that may decrease the sensitivity of the aptasensors.[19,20] For example, increased NaCl concentration can weaken the binding of thrombin to the aptamer, while high NaCl concentration is believed to stabilize the DNA duplex; thus, the steady hybridization of an aptamer and its complementary DNA in such a case may prevent the target-induced strand displacement and result in a lower sensitivity for target detection. Consequently, lengthy optimization and sequence alteration are quite desirable in the strand displacement-based detection to ensure the high sensitivity of the aptasensors by eliminating the adverse effects from the detection environment.[21,22]

As is well-known, signal amplification strategy has been commonly involved in the design and construction of aptasensors, which can greatly enhance the detection sensitivity.[23] Tool enzymes, which are usually employed for gene engineering, are very compelling in the introduction of signal amplification nowadays, and they have already had several examples of successful application in DNA-based detection.[24] Moreover, since the aptamer-target recognition event can be converted to the quantitative determination of probe DNA, nuclease-assisted signal amplification strategies, especially the exonuclease III (Exo III)-catalyzed degradation reaction of probe DNA, have become attractive in the fabrication of aptasensors, which allows one target to interact with multiple DNA probes and contributes to ultrahigh detection sensitivity.[25−29] Meanwhile, DNA ligase that facilitates the annealing of two independent DNA strands on a DNA template by catalyzing the formation of a phosphodiester bond at a nick junction has also been conducive to achieving signal or target amplification in the detection of DNA, RNA, and proteins, which offers more options for nucleic acid sequence optimization.[30−32]

In this paper, we report a highly sensitive electrochemical aptasensor fabricated by using an improved target-induced strand displacement strategy, based on a novel ligase-assisted Exo III-catalyzed degradation reaction (LAECDR), which contains a DNA ligase-assisted ligation reaction for sequence optimization and an Exo III-catalyzed degradation reaction for sensitivity enhancement. Moreover, by adopting a cucurbit(7)uril (CB[7])/gold nanoparticle (AuNP)/PDDA functionalized electrode for selective capture of the released MB, the whole LAECDR process can be carried out in the solution rather than on the electrode surface, which can not only well maintain the high binding affinity of unmodified aptamer but also accelerate DNA interaction in the enzymatic reaction for reduced space steric hindrance. Therefore, the proposed aptasensor has exhibited excellent performance in the electrochemical detection of thrombin with ultrahigh sensitivity and specificity.

## 2. EXPERIMENTAL SECTION

**Materials and Reagents.** CB[7], thrombin, poly(diallyldimethylammonium chloride) (PDDA), trisodium citrate, and chloroauric acid ($HAuCl_4$) were purchased from Sigma. Exo III and T4 DNA ligase were purchased from New England Biolabs. Bovine serum albumin (BSA) and bovine serum were purchased from Dingguo Biotech. Co. Other chemicals were of analytical grade and used without further purification. For all experiments, Milli-Q water (>18.0 MΩ) was used, purified by a Milli-Q Plus 185 ultrapure water system (Millipore purification pack).

DNA oligonucleotides were synthesized by Sangon (Shanghai, China), and the sequences are as follows. Thrombin binding aptamer (TBA): 5′-pGAGGTTGGTGTGGTTGG-3′; Extended strand (E-strand): 5′-ATATCGCT-3′; Probe DNA: 5′-AAAAAACCAACCT-CAGCGATAT-MB-3′; ET-strand: 5′-ATATCGCTGAGGTTG GTGTGGTTGG-3′.

The buffer solutions used in this work are as follows. TE buffer for DNA: 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4). Thrombin preparation solution: 10 mM Tris-HCl, 50 mM NaCl, 50 mM KCl, 0.1% BSA (pH 7.5). The thrombin binding buffer: 10 mM Tris-HCl, 100 mM NaCl, 5 mM KCl (pH 7.4). The enzyme reaction buffer: 10 mM Bis Tris propane-HCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT (pH 7.0). Buffer for preparing the test solutions is 10 mM Tris-HCl (pH 7.4).

**Ligase-Assisted Exo III-Catalyzed Degradation Reaction.** Before the experiments, TBA was incubated at 90 °C for 5 min and then cooled slowly to room temperature so that the aptamer could fold correctly when binding to the target protein.[33] Subsequently, 100 nM MB-labeled probe DNA, 1 nM E-strand, and 1 nM TBA were first incubated with 1.6 unit/μL T4 DNA ligase at 16 °C for 0.5 h to initiate the ligation reaction, which was then incubated at 65 °C for 10 min to denature DNA ligase. After that, 0.4 unit/μL of Exo III was added into the solution and further incubated at 37 °C for 20 min, terminated by being heated to 70 °C for 20 min. For the detection of thrombin, TBA was reacted with a desired concentration of thrombin for 1 h before LAECDR. The thrombin detection was repeated by using the LAECDR procedure at least three times.

**Preparation of CB[7]/AuNP/PDDA-Functionalized Electrode.** A 3 mm disk pyrolytic graphite (PG) electrode that was wrapped by polytetrafluoroethylene was used in this experiment, and a CB[7]/AuNP/PDDA-functionalized PG electrode has been prepared by layer-by-layer self-assembly. First, a pyrolytic graphite electrode was separately polished on fine sand paper and aluminum oxide (particle size of about 0.05 μm)/water slurry on silk to achieve a smooth, mirror-like surface. Then, the electrode was thoroughly washed by ultrasonicating in both doubly distilled water and ethanol for 5 min, respectively. Afterward, the electrode was dipped in PDDA solution (3.5 mg/mL, containing 0.05 M NaCl) for 20 min to form a positive charged PDDA film (~6 nm in the thickness) on the electrode surface. After washing with double-distilled water, 13 nm circular AuNPs prepared by citrate reduction of $HAuCl_4$ (0.01%) were immobilized onto the electrode surface through the electrostatic interaction with PDDA film for 1 h.[34] Finally, the electrode was incubated with 1 mM CB[7] for another 1 h at room temperature, so that CB[7] can be firmly attached to AuNPs through the interaction between carbonyls and gold. After thoroughly washing with double-distilled water and drying by nitrogen, the CB[7]/AuNP/PDDA-functionalized electrode was finally prepared for further use.

**Electrochemical Measurements.** For the electrochemical measurements, 20 μL of LAECDR mixture was dropped onto the

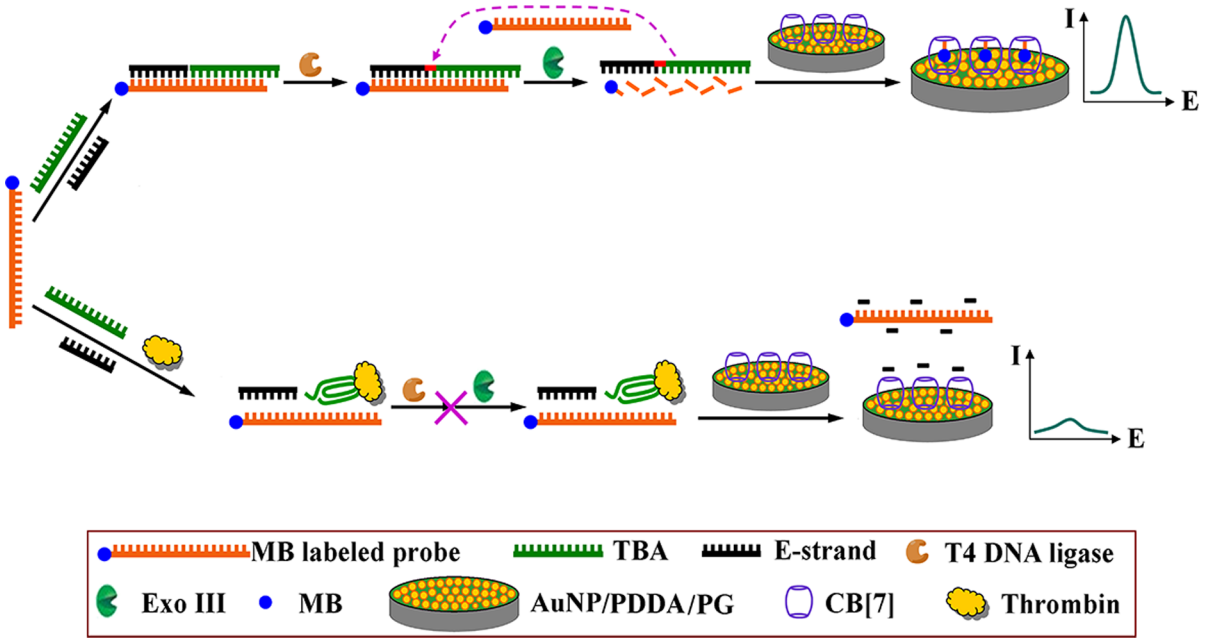


**Figure 1.** Schematic illustration of the electrochemical aptasensor based on ligase-assisted Exo III-catalyzed degradation reaction.

surface of the CB[7]/AuNP/PDDA-functionalized PG electrode. After 1 h, the electrode was thoroughly rinsed with double-distilled water. Electrochemical measurements were carried out on a model 660c Electrochemical Analyzer (CH Instruments) with a conventional three-electrode cell at room temperature. The three-electrode system consisted of CB[7]/AuNP/PDDA-modified PG electrode, a saturated calomel electrode (SCE), and a platinum wire electrode. All test solutions were thoroughly deoxidized by high-purity nitrogen before the experiments, and a stream of nitrogen was used to keep the solution anaerobic throughout the experiments. The parameters for square wave voltammetry (SWV) were as follows: potential scan range, 0 ~ −0.6 V; potential step, 4 mV; amplitude, 25 mV; frequency, 15 Hz.

## 3. RESULTS AND DISCUSSION

**Principle of the Electrochemical Aptasensor Fabricated with LAECDR.** Thrombin, a vital enzyme in the process of hemostasis and blood coagulation, which has also been known as a tumor marker for its essential role in tumor growth and metastasis, has been chosen as a model target for illustrating the principle to fabricate this kind of electrochemical aptasensor.[33] As is shown in Figure 1, a ligatable nick is produced from the separate hybridization of the E-strand and TBA with the probe DNA, which can be joined to form a long ET-strand (E-strand plus TBA strand) by the catalysis of DNA ligase. After the strand joining reaction, Exo III can recognize the thermostable duplex and selectively degrade the probe DNA from its 3′-end. Subsequently, the ET-strand dissociates from the digested probe DNA and then binds to another intact probe DNA to trigger the next round of degradation reaction. With the cycle of ET-strand formation and digestion of the probe DNA, numerous MBs that are labeled at the probe DNA will be released. After LAECDR, the released MB molecules are captured by the CB[7]/AuNP/PDDA-functionalized electrode through the formation of stable host−guest complexes with carbonyl-fringed portals and the hydrophobic cavities of CB[7]. Meanwhile, AuNPs may facilitate the subsequent electron transfer between the adsorbed MB and the electrode surface, leading to a noticeable electrochemical response.[35−38]

In the presence of thrombin, the TBA strand prefers to form a stable thrombin−TBA complex through the interaction with the target protein, instead of hybridizing with the probe DNA, due to the relatively higher affinity between the aptamer and its target. So, the ligase-catalyzed strand-joining reaction is inhibited due to the lack of free TBA. Since the short E-strand with only eight complementary bases cannot form stable duplex DNA with the probe DNA at the reaction temperature, Exo III is thus not active on single-stranded DNA. Consequently, the Exo III-catalyzed degradation reaction cannot take place. In a word, the E-strand without ligation reaction cannot initiate Exo III-catalyzed degradation of the probe DNA. Since the negatively charged phosphodiester backbone of intact probe DNA strongly repels the negatively charged carbonyl portals of CB[7], which may prevent the interaction of terminal-attached MB and CB[7] on the electrode surface, the generation of electrochemical response is inhibited. Therefore, highly sensitive detection of thrombin can be realized by tracing the changes of electrochemical response produced by the MB molecules.

**Electrochemical Studies of LAECDR.** To prove the principle of our aptasensor, we have first employed the electrochemical technique SWV to study the stepwise reaction in LAECDR. As shown in Figure 2, a high SWV response of MB molecules can be observed if the CB[7]-modified electrode has been incubated with the LAECDR-induced solution, while low electrochemical response will be obtained for the cases that not all the enzymes have been used. The results are quite consistent with our expectation. On one hand, intact probe DNA can prevent the terminal-attached MB from approaching CB[7] on the electrode surface through the strong electrostatic repulsion, while released MB molecules resulting from LAECDR can much more easily approach the electrode surface and interact with CB[7]. On the other hand, if the DNA strands have been treated by only one enzyme (DNA ligase or Exo III), the obtained electrochemical response is almost the same as that without any enzyme treatment. The reason is quite clear. Although DNA ligase can catalyze the ligation of TBA

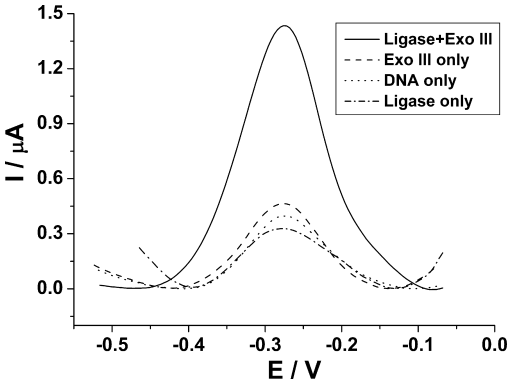


**Figure 2.** SWV response obtained at the CB[7]/AuNP/PDDA-functionalized PG electrode for the cases that the DNA strands have been treated by both T4 DNA ligase and Exo III, or T4 DNA ligase alone, or Exo III alone, or neither. Potential scan range, 0 ~ −0.6 V; potential step, 4 mV; amplitude, 25 mV; frequency, 15 Hz. Test solution: 10 mM Tris-HCl (pH 7.4). The reported SWV curves have been baseline corrected.

and E-strand, digestion of the mononucleotides from the 3′ terminal of probe DNA and the release of the labeled MB cannot happen in the absence of Exo III. Similarly, the short E-strand without ligation reaction cannot initiate Exo III-catalyzed cyclic degradation of probe DNA, so release of MB is also inhibited in the absence of DNA ligase.

**Electrochemical Detection of Thrombin by Using the Aptasensor.** Detailed studies have been conducted for quantitative detection of thrombin. As shown in Figure 3A, the electrochemical response decreases monotonically with the addition of thrombin, and a much lower peak current can be obtained with high concentration of thrombin (10 nM). The result has clearly shown that the addition of thrombin can inhibit the generation of the release of MB due to LAECDR. To confirm the principle of our design and to further demonstrate the superiority of our aptasensor, we have performed control experiments by using an intact synthesized ET-strand instead of the ET-strand obtained from the ligation reaction. As shown in Figure 3B, a higher electrochemical response can also be obtained due to Exo III-catalyzed degradation of the probe DNA, quite similar to that obtained after LAECDR. So, the control experiments have confirmed the production of the ET-strand from the DNA ligase-catalyzed strand joining reaction in our strategy as well as the digestion of MB-labeled mononucleotides due to the Exo III-catalyzed degradation of the probe DNA. The peak current is also found to decrease along with the increase of thrombin concentration, indicating that the occurrence of strand displacement resulting from binding of thrombin to the aptamer part within the ET-strand can prevent Exo III-catalyzed degradation of probe DNA. However, the detection by using the synthesized ET-strand has displayed much less sensitivity compared with our aptasensor since the control can only distinguish thrombin at the pM level, while our aptasensor can easily reveal the presence of target protein at the fM level. The result may be attributed to the inherent defect of the strand displacement-based aptasensor as mentioned above. The extension of the aptamer strand may reduce its binding affinity and recognition ability, while the steady DNA hybridization with longer complementary sequences may prevent target-induced strand displacement, thereby resulting in the reduced detection sensitivity. So, the control experiment has demonstrated the superiority and feasibility in the use of DNA ligase for our strategy, which can advance both aptamer−target interaction and target-induced strand replacement.

Figure 4 depicts the peak current obtained for the measurement of different concentrations of thrombin from 0

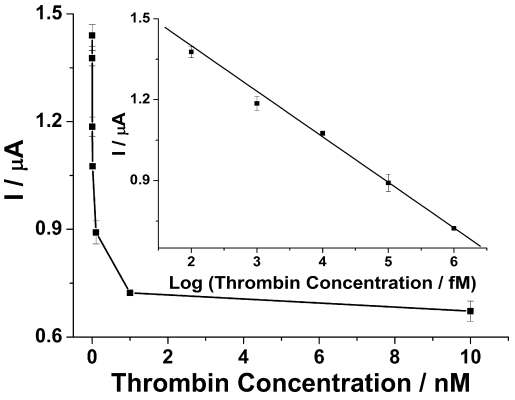


**Figure 4.** Peak currents obtained in the presence of different concentrations of thrombin from 0 to 10 nM. Inset shows a linear relationship between peak current and log of the thrombin concentration from 100 fM to 1 nM. Error bars represent the standard deviations of three independent measurements.

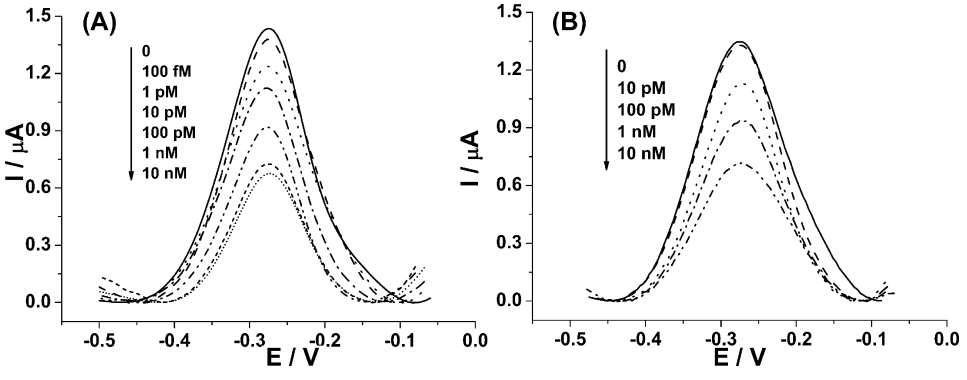


**Figure 3.** (A) SWV responses obtained at the CB[7]/AuNP/PDDA-functionalized PG electrode for the detection of different concentrations of thrombin. (B) SWV responses obtained at the CB[7]/AuNP/PDDA-functionalized PG electrode by using 1 nM synthesized ET-strands, instead of the ligated ET-strands, for the Exo III-catalyzed degradation reaction in the presence of different concentrations of thrombin. Others are the same as in Figure 2.

to 10 nM, and the inset of Figure 4 shows a linear correlation of the peak currents versus the thrombin concentrations in the range from 100 fM to 1 nM. The regression equation is $y = 1.738 - 0.1689 \times \lg x$, where y is the peak current ($\mu$A); $x$ is the concentration of thrombin (fM); and $R^2 = 0.997$. The detection limit, defined as three times the signal-to-noise ratio, is 33 fM. We have also compared the detection limits of the present work with some other reported aptasensors. As is shown by Table 1, our aptasensor can have a much better detection limit in comparison to the reported methods, which has reconfirmed its high sensitivity.[39−42] To evaluate the reproducibility of the aptasensor, each concentration has been separately measured at least three times. The relative standard deviation can all be within 5%, implying the quite good reproducibility of our aptasensor.

**Table 1. Thrombin Detection by Using Different Aptasensors**

| methods | detection limit | ref |
|---|---|---|
| amplified fluorescence aptamer-based sensors using Exonuclease III amplification | 89 pM | 39 |
| host−guest-recognition-based electrochemical aptasensor | 4.6 pM | 40 |
| amperometric aptasensor using enzyme-mediated direct electrochemistry and DNA-based signal amplification strategy | 120 fM | 41 |
| electrochemical aptasensor using functionalized mesoporous silica@multiwalled carbon nanotubes as signal tags and DNAzyme signal amplification | 50 fM | 42 |
| electrochemical aptasensor based on a ligase-assisted exonuclease III-catalyzed degradation reaction | 33 fM | our work |

We have also conducted experiments to check the specificity of our assay by using BSA as the control. Figure 5A shows the SWV curves obtained in the presence of BSA (10 nM) and thrombin (1 nM and 100 fM). As is shown by Figure 5A, the peak current obtained in the presence of BSA is nearly the same as if no target protein exits. So, the studies have clearly shown that BSA has no effect on the termination of LAECDR, proving the specificity of our aptasensor. Furthermore, taking the fetal calf serum as an example, we have also demonstrated the applicability of our aptasensor in the complex biological samples. As shown in Figure 5B, the presence of thrombin in the serum samples can also achieve satisfactory electrochemical responses, which can be well comparable to that in the reaction buffer, so the proposed method may be clinically applicable in the future. Notably, the signals obtained in the serum are slightly lower than those obtained in buffer, which may be ascribed to the presence of thrombin in the serum.

## 4. CONCLUSIONS

In summary, we have proposed an improved strand displacement-based electrochemical aptasensor by making use of a novel ligase-assisted Exo III-catalyzed degradation reaction. The use of DNA ligase can well maintain the high binding affinity of the unmodified aptamer for aptamer−target interaction and decrease the interference of DNA hybridization for target-induced strand displacement, while the Exo III-assisted signal amplification strategy can provide significant help to enhance the detection sensitivity. Additionally, the adoption of CB[7]/AuNP/PDDA-functionalized electrode can greatly facilitate the signal acquisition for electrochemical detection, which not only takes advantage of good conductivity and large surface-to-volume ratio of AuNPs but also profits from the strong and selective supramolecular recognition ability of CB[7]. Therefore, our aptasensor has displayed excellent performance in the electrochemical detection of thrombin in both reaction buffer and complex serum samples, including a broad linear range, quite low detection limit, and high specificity. In the future, coupling with different signal molecules and tumor marker aptamers, our aptasensors may have great potential for combined detection of tumor markers in accordance with clinical demand.

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### Notes

The authors declare no competing financial interest.

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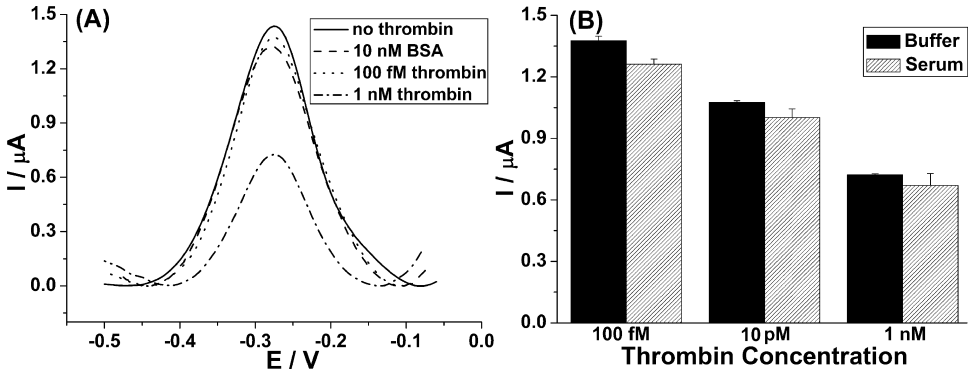


**Figure 5.** (A) SWV responses obtained in the presence of thrombin (1 nM and 100 fM) or BSA (10 nM). Others the same as in Figure 2. (B) Results obtained from the detection of thrombin in buffer and serum (thrombin concentrations are 100 fM, 10 pM, and 1 nM, respectively).

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