ANALYTICAL SCIENCES JULY 2020, VOL. 36

Amplified Analysis of DNA or Proteins by TdT-generated DNAzyme

Zhuoliang LIU,*, Tianxiong LIU,* Cheng-an TAO,* Xianzhe CHEN,* Jian HUANG,* Fang WANG,* and Jianfang WANG*†**

**College of Liberal Arts and Sciences, National University of Defense Technology, Changsha, Hunan, 410073, P. R. China*

***State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, Hunan, 410082, P. R. China*

Sensitive and specific detection of nucleic acids or proteins, which act as biomarkers, is of great importance in disease diagnosis. By combing the concept and operation of an endonuclease-assisted target-responsive amplification method and peroxidase-mimic DNAzyme generated by terminal deoxynucleotidyl transferase (TdT), a novel and facile colorimetric biosensor was developed for DNA and protein. Target DNA and thrombin were chosen as representative biomolecules. The production of cleavage fragments can only be triggered by specific target binding and the following nicking process, which do not occur spontaneously. In the signal collection part, numerous guanine-rich DNA were produced through the prolongation of cleavage fragments by TdT and formed highly effective DNAzyme with hemin. In this novel amplification method, we succeeded in realizing sensitive and specific detection of target DNA and thrombin. Under optimal conditions, target DNA can be detected as low as 1 pM, and thrombin with a detection limit of 100 pM. The method also proves the potential versatility and feasibility of TdT-generated DNAzyme in various bio-analyses.

Keywords DNAzyme, terminal deoxynucleotidyl transferase, DNA, thrombin

(Received October 16, 2019; Accepted January 11, 2020; Advance Publication Released Online by J-STAGE January 24, 2020)

Introduction

The early diagnosis of various diseases and mutation analysis rely on the specific and sensitive detection of trace amounts of DNA or protein.[1] DNA-based amplification methods were developed to realize the detection of these biomarkers with low expression levels. Polymerase chain reaction (PCR),[2] rolling circle amplification (RCA),[3,4] strand displacement amplification (SDA)[5] and hybridization chain reaction (HCR)[6,7] are widely applied in signal amplification in biomolecular analysis. While these signal amplification protocols are sensitive, they suffer from being complicated and expensive and have the need for rigorous experimental conditions due to the requirements of fluorescence labelled probes or nanomaterials. Thus, highly sensitive and flexible methods for the specific and sensitive detection of biomarkers must be developed.

G-Quadruplexes are four-stranded structures consisting of stacked planar G-tetrads. Once a G-quadruplex forms a complex with hemin (iron(III)-protoporphyrin IX), the cofactor of natural HRP can exhibit horseradish peroxidase (HRP)-mimic DNAzyme, which was first found by Sen's group in their selection of porphyrin-binding DNA aptamer *via* systematic evolution of ligands by exponential enrichment (SELEX) process.[8] The docking between hemin and G-tetrad of quadruplex creates a micro-environment that is conclucive to significantly improving the intrinsic peroxidase activity of hemin, and capable of efficiently catalyzing the oxidation of various substrates in the presence of H_2O_2.[9] Willner's group rearranged the sequence of PS2.M and innovatively employed this DNAzyme into bio-analysis as a signal producer, leading to the development of a number of colorimetric, fluorescent, chemiluminescent, and electrochemcial bioassays of metal ions, small molecules, nucleic acids, proteins, and living cells.[10-15] Owing to the relatively high catalytic ability and stability of this HRP-mimic DNAzyme, DNAzyme has been widely used as the signal output in amplification protocols as rolling circle amplification (RCA), strand displacement amplification (SDA) and hybridization chain reaction (HCR) instead of fluorescence labelled probes or nanomaterials.[16-20] In the aforementioned methods, the DNAzyme sequence that acts as the output is designed as an inactive form. The sequence of DNAzyme was designed in the signal probe in a blocked form or as a complementary sequence in the template probe. However, the unblocked DNAzyme after the incomplete pretreatment will result in a high background, which will lead to the fake signal. Though the synthesis of DNAzyme according to the template that contains the complementary sequence of DNAzyme will not suffer from the high background, the rigid sequence requirement creates it difficulties in template and primer probe design and limits its potential applications. To make the most use of the catalytic ability and stability of DNAzyme, and simplify the probe design process at the same time, a novel and practical protocol is needed.

It has been reported that randomly arrayed guanine rich DNA sequence prepared by TdT polymerization could form a complex with hemin exhibiting enhanced peroxidase activity, which

† To whom correspondence should be addressed.
E-mail: wangjianfang@nudt.edu.cn

presents a novel approach to preparing peroxidase-mimic DNAzyme.[21] After optimizing the composition of the dNTP pool, stable and functional G-quadruplexes folded by the long DNA chain were applied in enzyme detection, apoptotic cell analysis and photodynamic therapy.[22,23] Considering the production of DNAzyme relies on a "tail synthesis" method, it seems that telomerase can accomplish this task as well in a template-free manner.[15,24] Because telomerase is not commercially available, the further application of telomerase-generated DNAzyme is hampered. However, there is no such restriction for TdT and the following examples would show the versatility of TdT-yielded DNAzyme in bio-analysis. TdT-generated DNAzyme may provide a novel methodology to develop biosensors using its peroxidase-mimic character without considering the sequence of DNAzyme in the probe design, which considerably facilitates the design process and shortens the probe sequence.

Here, we developed a versatile sensing system based on TdT-generated DNAzyme. This novel TdT-generated DNAzyme amplification protocol is totally different from that of conventional DNAzyme-based methods. Traditional methods require a sophisticated probe design to arrange the specific HRP-mimic DNAzyme sequence in caged form, split form, or complementary template form,[25-28] which can be released, reunited, or replicated to activate DNAzyme in response to the target. However, using the TdT-generated DNAzyme, both the DNAzyme sequence and complementary template can be avoided in biosensor composition, which remarkably simplifies the probe design process. Recently, specific DNA sequences and proteins have been recognized as biomarkers in early diagnosis.[29,30] Herein, target DNA and thrombin were chosen as two typical biomolecules to demonstrate the sensitive and specific detection by TdT-generated DNAzyme. By integrating restricted nicking endonuclease and aptamer in the bio-sensing strategy, a novel signal amplification protocol based on the DNAzyme generation approach by TdT was developed for highly sensitive detection of DNA and protein, respectively. This protocol avoids the complicated, expensive, and rigorous experimental conditions that are needed due to the requirements of traditional amplification methods. The development of simple, sensitive and convenient methods for the detection of target DNA and thrombin would expand the application of TdT-generated DNAzyme and enhance its potential biological role.

Experimental

Reagents and chemicals

Terminal deoxynucleotidyl transferase (TdT) was purchased from Fermentas Inc. (Vilnius, Lithuania). 2,2′-Amino-di(2-ethyl-benzothiazoline sulfonic acid-6) ammonium salt ($ABTS^{2-}$), dATP, dTTP, dGTP, the oligonucleotides, the oligonucleotides with 3′ phosphorylated end, SYBR Green II, chymotrypsin, 2-(4-morpholino) ethanesulfonic acid (MES) and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES) were obtained from Sangon (Shanghai, China). Amplex red, BSA, lysozyme, and chymotrypsin, hemin and thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nt.AlwI and Nb.BbvCI were purchased from New England Biolabs (NEB) Inc. (Ipswich, MA, USA). All other chemicals were of analytical reagent grade and used without further purification. Ultrapure water with an electric resistance of 18.3 MΩ was obtained from a Millipore filtration system and used throughout.

UV-vis absorption spectra were obtained on a Beckman DU-800 spectrophotometer. Chemiluminescence was recorded by the Synergy Mx multimode microplate reader (BioTek).

The DNA sequences were as follows:

DNA-primer	5′-AAT ACA ACC TCT CA-3′
DNA-primer-P	5′-AAT ACA ACC TCT CA-PO_4^{3-}-3′
Probe	5′-AAA GGA TCG AAT AAG AGG TCC TTC-PO_4^{3-}-3′
Target	5′-GAA GGA CCT CTT ATT CGA TCC TTT-3′
Mismatch-1	5′-GAA GGA CCT CTT ATT CGA CCC TTT-3′
Mismatch-2	5′-GAA GGA CCT CTT ATT CGC CCC TTT-3′
Mismatch-3	5′-GAA GGA CCT CTT ATT CTC CCC TTT-3′
Unrelated-DNA	5′-CTT CTG GTG GAA GCG TGA TCT GTT-3′
17-DNAzyme	5′-GGG TAG GGC GGG TTG GG-3′
Aptameric probe	5′-AGTCCGTGGTAGGGCAGGTT-GGGGTGACTGCTGATTTTTGA-GCCTCAGCAGTCACCC-PO_4^{3-}-3′
Amplification probe	5′-TTTTTAGACTGCTGAGGC-PO_4^{3-}-3′

Randomly arranged G-rich sequence generated by TdT

The typical polymerization experiment was performed in 10 μL of TdT buffer (5×, 1 M potassium cacodylate, 0.125 M Tris, 0.05% (v/v) Triton X-100, 5 mM $CoCl_2$, pH 7.2) containing 1 μM DNA-primer (or DNA-primer-P), 1 mM dNTP (40% dATP and 60% dGTP for AG-DNAzyme), and 4 U TdT at 37°C for 2 h and terminated by heating the solution at 75°C for 10 min.

Amplified detection of DNA based on TdT-generated DNAzyme

Each sample contained 1.5 μM probe and a specific amount of target DNA (10 nM, 7.5 nM, 5 nM, 4 nM, 100 pM, 10 pM) in 10 μL of 1 × NBE buffer 4 (20 mM Tris–Ac, 10 mM $Mg(Ac)_2$, 50 mM KAc, pH 7.9). Upon incubation at 58°C for 5 min, 8 U of Nt.AlwI was added to the solution to start the nicking reaction. After being incubated for 2 h, the resulting mixture was heated at 80°C for 20 min to stop the reaction. Then the TdT-elongation reaction was initiated by incubation of the nicking reaction product with 1 mM dNTP (40% dATP and 60% dGTP) and 6 U of TdT in 15 μL of TdT buffer at 37°C for 2 h, and terminated by heating the solution at 75°C for 10 min. After the polymerization reaction, 1 μL of 10 μM hemin (prepared in DMSO), 50 μL of 2.5 × MES buffer (final concentration was 100 mM MES–Tris, 40 mM KCl, and 0.05% Triton X-100, pH 5.5), and 15 μL of H_2O were added to the sample. Half an hour later, 10 μL of 20 mM $ABTS^{2-}$ and 10 μL of 20 mM H_2O_2 were added to initiate the bio-catalytic oxidation of $ABTS^{2-}$. UV-vis absorption spectra were obtained at 415 nm in 4 min at 27 ± 2°C.

Amplified detection of thrombin based on TdT-generated DNAzyme

The aptameric probe (1 μM) was prepared by being heated to 95°C for 5 min, and slowly cooled to 4°C in 2 h. For thrombin detection, the aptameric probe (20 nM), the amplification probe (6 μM) and varying concentrations of thrombin or other proteins were incubated in 5 μL of 1 × Tris–Ac buffer (20 mM Tris–Ac, 100 mM NaAc, 20 mM KAc, 2 mM $Mg(Ac)_2$, pH 8.0) for 1 h at 37°C. Then, the nicking reaction was started by addition of 4 U of Nb.BbvCI in 10 μL of 1 × NBE buffer 4 for 45 min at 37°C, and consequently terminated by heating the solution at 80°C for

20 min. After that, the TdT polymerization procedure was carried out under the same process mentioned in the target DNA detection experiment section.

Results and Discussion

Randomly arranged G-rich sequences generated by TdT can form functional G-quadruplexes similar to the reported fixed sequence G-quadruplex, which can form peroxidase mimic DNAzyme and enhance the fluorescence of thioflavin T (ThT). To investigate the substrate generality of TdT-generated DNAzyme, we chose amplex red (AR) and luminol as the organic probes for fluorescent and chemiluminescent detection, respectively. The three different dNTP contents were as follows: dGTP only, 60% dGTP + 40% dATP, and 60% dGTP + 40% dTTP, and were employed as fuel to synthesize G-rich sequences. These TdT-generated DNAzyme were called G-DNAzyme, AG-DNAzyme, and TG-DNAzyme, respectively. Similar to the results using $ABTS^{2-}$ as the substrate, all TdT-yielded DNAzymes (100 nM) showed better catalytic activity than 17-DNAzyme (100 nM) on oxidation of AR or luminol (Fig. S1, Supporting Information). The activity ratios of AG-DNAzyme, G-DNAzyme, and TG-DNAzyme to 17-DNAzyme were 2.9, 2.2, and 2.1 for the AR-based fluorescent system, and 2.0, 2.0, 1.9 for the luminol-based chemiluminescent detection, respectively. Meanwhile the activity ratio of AG-DNAzyme, G-DNAzyme, and TG-DNAzyme, to 17-DNAzyme were 3.1, 1.3 and 1.9 for ABTS-based colorimetric system. The substrate-dependent variation of relative activity might result from the different affinity of DNAzymes to these substrates. The activity of TdT was quantitatively measured using an AR-based fluorescent system with a linear range from 0.3 to 4 U which further demonstrated the catalytic ability and stability of TdT-generated DNAzyme (Fig. S2, SI). After comparing the performance of the three typical substrates, we chose $ABTS^{2-}$ as the substrate in the following detection experiment.

It is interesting to compare TdT with telomerase, which is the other enzyme capable of tailing DNA to generate G-quadruplex-based DNAzyme without the requirement of a template. Unlike TdT which catalyzes polymerization to form random G-rich sequences, the telomerase synthesizes ordered "TTAGGG" repetitive sequences, namely tolemeric sequences, through reverse transcription according to its endogenous small RNA template. In respect of their analytical applications, the DNAzyme-based assay of telomerase activity has been established based on the G-quadruplex formed by the tolemere sequence,[15,31] whereas the further application of telomerase-generated DNAzyme is hampered by no commercially available telomerase product. However, there is no such restriction for TdT and the following examples show the versatility of TdT-yielded DNAzyme in bio-analysis.

The repeatability of the catalytic capability and stability of TdT-generated DNAzyme suggest it can be a prominent signal output candidate in bioanalytical analysis. We aimed to develop a versatile sensing system suitable for detection of various targets based on the TdT-generated DNAzyme. Because there is no specific sequence required for TdT-generated DNAzyme, the design criteria of this new sensing system are quite distinct from that of conventional DNAzyme-based methods. Owing to the fact that the 3′ OH end is essential for initial addition of TdT, the basic idea of our design criteria is to place the 3′ OH end into a passivated form which could be released to start TdT-mediated polymerization as a response to the target. Fortunately, we found that the 3′ phosphorylated end of DNA could completely prohibit the TdT-mediated tailing (shown in Fig. 1), which is perfectly suitable for the passivation of the 3′ end. Scheme 1A shows the basic design principle for a signal on detection by TdT-generated DNAzyme. The probe with caged phosphate group at the 3′ end stays in the "off" state. Target-triggered cleavage of DNA removes the strand with the phosphate end and releases the new 3′ OH end, serving as a primer to switch on TdT elongation, which is the key for signal transduction in this method. The random G-rich sequence was tailed on the 3′-OH end by TdT catalysis in the presence of optimized dNTP pool (60% dGTP and 40% dATP), followed by incorporation with hemin to form HRP-mimic DNAzyme. The concentration of target was finally read out by colorimetric signal of TdT-generated DNAzyme in $ABTS^{2-}/H_2O_2$ system. Using nicking endonuclease as a site-specific cleavage tool, a novel bio-sensing system has been developed and its versatility was proven by using it for two important applications: target DNA detection and thrombin aptasensor.

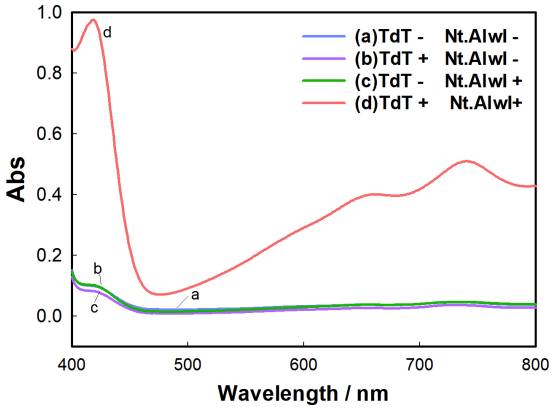
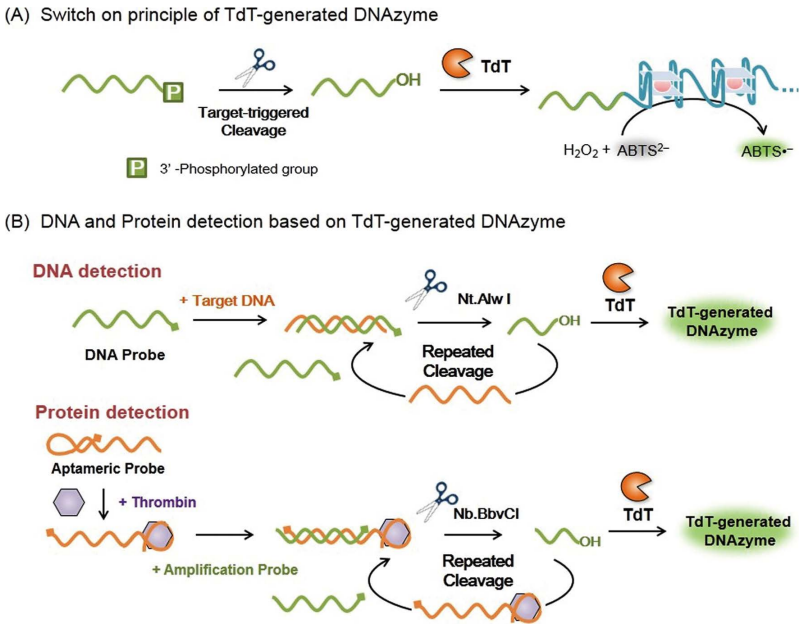


Fig. 1 UV-vis absorbance spectra of the samples employing (a) 14 nt single-stranded DNA without 3′ PO_4^{3-} group as the TdT elongation primer; (b) 14 nt single-stranded DNA with 3′ PO_4^{3-} group as the TdT elongation primer; (c) hemin only without DNA and TdT. The polymerization reactions were conducted with 1 μM primer, 4 U TdT and 1 mM dNTP (40% dATP and 60% dGTP).

As shown in the DNA detection part of Scheme 1, target DNA hybridized with the specific probe, and a nicking endonuclease cleaves the specific nicking site on the probe to produce a free 3′ OH end. The probe cleavage can be repeated since the cleaved probe would be readily displaced by another entire probe, significantly amplifying the 3′ OH generation event. Subsequently, the probe fragments with 3′-OH end served as the primers to yield TdT-generated DNAzyme as a read-out.

To verify the feasibility of our sensing strategy in DNA detection, a 24-nt single-stranded target DNA was probed and the nicking endonuclease Nt.AlwI was chosen according to the target sequence. Figure 2A shows the typical colorimetric detection of the 2 nM target DNA. The TdT-inactive probe induces no signal, while the addition of a target causes a significant absorbance change with the aid of dual enzymes. The sole TdT or Nt.AlwI with the probe leads to no response to the target. Nondenaturing polyacrylamide gel electrophoresis was carried out to validate the target DNA analysis process. As we can see from Fig. S3 (SI), when the target was incubated with the probe in the prescene of nicking endonuclease Nt.AlwI, a small fragment was observed. While the TdT and Nt.AlwI both worked in the DNA detection system, a long and consecutive DNA chain was clearly observed. The results of PAGE analysis were in accordance with the colorimetric



Scheme 1 Schematic representations of (A) general signal on principle of TdT-generated DNAzyme and (B) target DNA and thrombin detection based on TdT-generated DNAzyme assay.

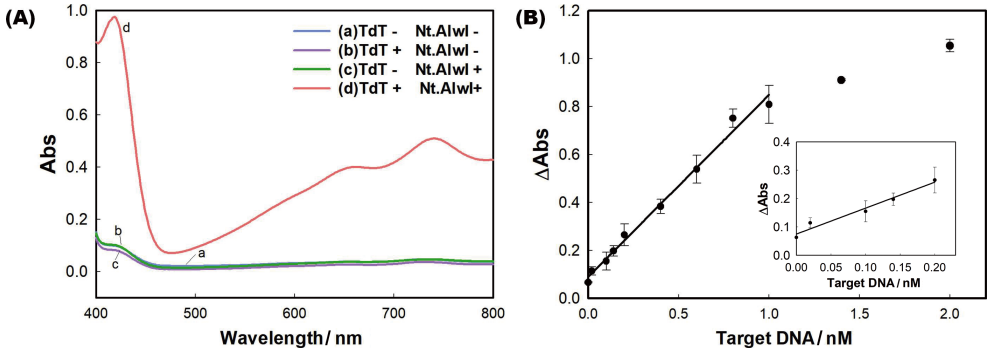


Fig. 2 Colorimetric detection of DNA by nicking nuclease-assisted target-responsive activation of TdT-generated DNAzyme. (A) UV-vis absorption spectra of the samples: (a) probe and 2 nM target DNA; (b) addition of TdT into sample (a); (c) addition of Nt.AlwI into sample (a); (d) addition of TdT and Nt.AlwI into sample (a). (B) Absorbance changes in 4 min observed upon the analysis of different concentrations of DNA based on TdT-generated DNAzyme.

detection, which demonstrated the feasibility of the designed strategy for DNA analysis. The efficiency of repetitive nicking nuclease cleavage relies on the balance between the probe-target pairing and the cleaved fragment release, which is strongly associated with the reaction temperature. Hence, the reaction temperature for nicking cleavage cycles was optimized and the maximal signal of the sensing system was obtained at 58°C (Fig. S4, SI). As a result, the reaction temperature of nicking nuclease was set at 58°C for DNA detection.

Figure 2B shows the absorbance changes observed upon the activation of TdT-generated DNAzyme by different concentrations of target DNA (0 – 2 nM). It suggests that a concentration of DNA primer lower than 10 nM is unable to provide observable activity of TdT-generated DNAzyme (data not shown). It was determined that target DNA concentrations lower than 10 nM can avoid the interference from TdT tailing of the target itself. As the target concentration increases, more probes are cleaved to yield more primers for preparation of TdT-generated DNAzyme, leading to produce more colored product.

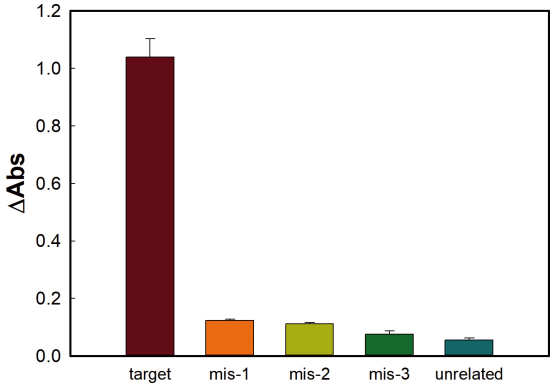


Fig. 3 The absorbance responses of the proposed DNA assay based on TdT-generated DNAzyme to target DNA, one base mismatched (mis-1), two bases mismatched (mis-2), three bases mismatched target DNA (mis-3), and an unrelated DNA. All the DNA concentrations are 2 nM.

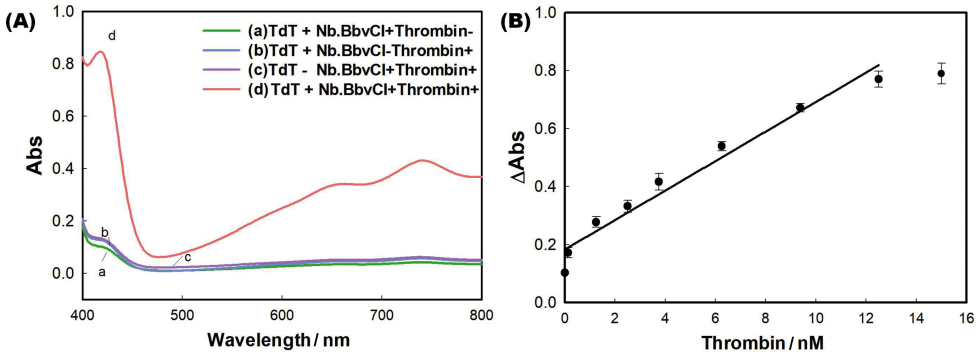


Fig. 4 Colorimetric detection of thrombin by nicking nuclease-assisted target-responsive activation of TdT-generated DNAzyme. (A) UV-vis absorption spectra of the samples: (a) probe with coexisted Nt.BbvCI and TdT; (b) addition of 12.5 nM thrombin and TdT to probe; (c) addition of 12.5 nM thrombin and Nt.BbvCI to probe; (d) addition of 12.5 nM thrombin into sample (a). (B) Absorbance change in 4 min observed during the analysis of different concentrations of thrombin based on TdT-generated DNAzyme.

The calibration curve indicates that the linear range of this DNA assay is from 20 pM to 1 nM. This detection limit is less than or comparable with those of some conventional DNAzyme-based DNA assays integrating with various nucleic acid amplification techniques.[32,33] Moreover, the sensitivity of this assay can be further improved by increasing the TdT concentration and prolonging the peroxidation reaction time (Fig. S5, SI). With the increasing concentration of DNA target, a gradual increasing intensity was observed (1 – 20 pM) and can detect target DNA as low as 1 pM could be detected. Additionally, due to the precise base pairing and recognition of nicking nuclease, our DNA assay can efficiently discriminate target DNA from the single base mismatched DNA and two bases mismatched DNA, corroborating its strong specificity (Fig. 3). All the results described above implied that the proposed method based on TdT-generated DNAzyme provides high sensitivity and selectivity for low level DNA detection.

Scheme 1 depicts the detailed mechanism of the nicking nuclease-assisted detection of thrombin based on TdT-generated DNAzyme. All the DNA probes used here made inactive for TdT by coupling the phosphate group on the 3′ end. For thrombin detection *via* aptamer recognition, two DNA probes, an aptameric probe with hairpin structure and an amplification probe, were involved (The detailed mechanism and DNA sequence design is shown in Scheme S1 and Scheme S2, respectively). The aptameric probe contains two main function areas: one part is the thrombin aptamer which plays the recognition role, the other part consists of the recognition and cleavage site of endonuclease that triggers the amplification process. The binding of thrombin by the aptamer causes the hairpin structure of the aptameric probe to open to release the sequence capable of pairing with the amplification probe. The resulting hybrid of two probes provided a whole duplex nicking site, triggering the similar enzymatic cleavage cycles to massively produce DNA fragments with 3′-OH end. Consequently, the catalytic activity of the randomly arrayed DNAzyme yielded by TdT polymerization was exploited to realize highly sensitive detection of thrombin. Notably, all the probes did not include any specific DNAzyme sequence and the sophistical activity-switching mechanism of DNAzyme was avoided, revealing the advantage of our strategy in facile biosensor design.

Aptamers are single-stranded nucleic acids that selectively bind to various targets with high affinity.[34,35] As a potent molecular recognition element, aptamers have emerged as a promising tool in biosensing and diagnostics.[14,36] Our sensing strategy is also feasible for fabricating a thrombin-targeted aptasensor by introduction of a corresponding aptamer and the nicking nuclease Nb.BbvCI. As illustrated in Fig. 4A, an evident absorbance change was observed upon addition of 12.5 nM thrombin, indicating the thrombin-triggered activation of DNAzyme preparation by TdT polymerization. The control experiments proved that the co-existence of Nb.BbvCI and TdT is essential for thrombin-responsive DNAzyme preparation. The quantitative measurement of thrombin is shown in Fig. 4B. The absorbance change against the concentration of thrombin was linear over the concentration range of 0.1 to 12.5 nM, and the detection limit was 0.1 nM, which is comparable to some amplified thrombin aptasensors using conventional DNAzyme as a read-out.[37] The selectivity of the thrombin aptasensor was further examined (Fig. 5). Only thrombin induces a remarkable signal while the non-target proteins, such as BSA, lysozyme, and chymotrypsin, give a negligible signal comparable to control. Therefore, our TdT-generated DNAzyme strategy can be readily integrated with the specific aptameric recognition to develop novel aptasensors.

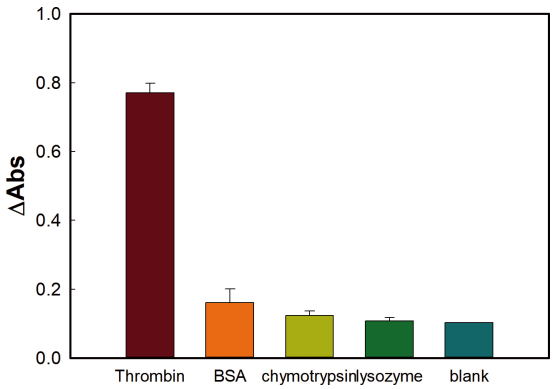


Fig. 5 The absorbance responses of the proposed thrombin aptasensor based on TdT-generated DNAzyme to thrombin and other non-target proteins. The concentration of thrombin was 12.5 nM, while the concentration of BSA, lysozyme, or chymotrypsin was 250 nM.

Conclusions

Herein, we present a novel approach to apply TdT-generated DNAzyme in the amplification detection of target DNA and thrombin. Unlike conventional DNAzyme-related assays based on the DNA probes containing the specific DNAzyme sequence, this TdT-generated DNAzyme provides a novel methodology to develop biosensors without considering the sequence of DNAzyme in the probe design, which considerably facilitates the design process and shortens the probe sequence. With the assistance of nicking endonuclease, a new signal transduction mechanism eligible for target-responsive TdT-generated DNAzyme was developed to realize DNA detection and aptameric sensing for thrombin. Furthermore, our sensing system can be readily extended to a great variety of targets, such as metal ion, small molecules, transcription factors, enzymes, and even cancer cells, by integrating the cognate DNA recognition element with nuclease-mediated activation of TdT-generated DNAzyme.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (21705163, 21601204), Natural Science Foundation of Hunan Province (2019JJ50737), the Research Project of the National University of Defense Technology (ZK18-03-39) and the Open Subject of State Key Laboratory of Chemo/Biosensing and Chemometrics (2017011).

Supporting Information

This material (figures and experiment description) is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

1. G. Shipp, *Biotechnology Healthcare*, **2006**, *3*, 35.
2. K. J. Livak and T. D. Schmittgen, *Methods*, **2001**, *25*, 402.
3. P. M. Lizardi, X. H. Huang, Z. G. Zhu, P. Bray-Ward, D. C. Thomas, and D. C. Ward, *Nat. Genet.*, **1998**, *19*, 225.
4. A. Y. Chen, S. Y. Ma, Y. Zhuo, Y. Q. Chai, and R. Yuan, *Anal. Chem.*, **2016**, *88*, 3203.
5. G. T. Walker, M. S. Fraiser, J. L. Schram, M. C. Little, J. G. Nadeau, and D. P. Malinowski, *Nucleic Acids Res.*, **1992**, *20*, 1691.
6. N. Ying, C. J. Ju, Z. Y. Li, W. S. Liu, and J. Y. Wan, *Talanta*, **2017**, *164*, 432.
7. N. Ying, T. F. Sun, Z. B. Chen, G. P. Song, B. Y. Qi, S. J. Bu, X. W. Sun, J. Y. Wan, and Z. H. Li, *Anal. Biochem.*, **2017**, *528*, 7.
8. P. Travascio, A. J. Bennet, D. Y. Wang, and D. Sen, *Chem. Biol.*, **1996**, *6*, 779.
9. P. Travascio, P. K. Witting, A. G. Mauk, and D. Sen, *J. Am. Chem. Soc.*, **2001**, *123*, 1337.
10. T. Li, S. J. Dong, and E. K. Wang, *Anal. Chem.*, **2009**, *81*, 2144.
11. D. Li, A. Wieckowska, and I. Willner, *Angew. Chem. Int. Ed.*, **2008**, *120*, 3927.
12. J. Elbaz, B. Shlyahovsky, and I. Willner, *Chem. Commun.*, **2008**, *13*, 1569.
13. G. Pelossof, R. Tel-Vered, J. Elbaz, and I. Willner, *Anal. Chem.*, **2010**, *82*, 4396.
14. D. Li, B. Shlyahovsky, J. Elbaz, and I. Willner, *J. Am. Chem. Soc.*, **2007**, *129*, 5804.
15. T. Niazov, V. Pavlov, Y. Xiao, R. Gill, and I. Willner, *Nano Lett.*, **2004**, *4*, 1683.
16. Z. L. Ge, M. H. Lin, P. Wang, H. Pei, J. Yan, J. Y. Sho, Q. Huang, D. N. He, C. H. Fan, and X. L. Zuo, *Anal. Chem.*, **2014**, *86*, 2124.
17. L. H. Tang, Y. Liu, M. M. Ali, D. K. Kang, W. A. Zhao, and J. H. Li, *Anal. Chem.*, **2012**, *84*, 4711.
18. H. Xu, S. X. Zhang, C. H. Ouyang, Z. M. Wang, D. Wu, Y. Y. Liu, Y. F. Liu, and Z. S. Wu, *Talanta*, **2019**, *192*, 175.
19. X. Hun, Y. Meng, S. Wang, Z. Mei, and X. Luo, *Sens. Actuators, B*, **2017**, *246*, 734.
20. W. Li, Z. L. Liu, H. Lin, Z. Nie, and J. H. Chen, *Anal. Chem.*, **2010**, *82*, 1935.
21. Z. L. Liu, W. Li, Z. Nie, F. F. Peng, Y. Huang, and S. Z. Yao, *Chem. Commun.*, **2014**, *50*, 6875.
22. Z. L. Liu, X. Y. Luo, Z. Li, Y. Huang, Z. Nie, H. H. Wang, and S. Z. Yao, *Anal. Chem.*, **2017**, *89*, 1892.
23. T. H. Shi, M. L. Wang, H. Li, M. Wang, X. Y. Luo, Y. Huang, H. H. Wang, Z. Nie, and S. Z. Yao, *Sci. Rep.*, **2018**, *8*, 5551.
24. Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler, and I. Willner, *J. Am. Chem. Soc.*, **2004**, *126*, 7430.
25. C. Teller, S. Shimron, and I. Willner, *Anal. Chem.*, **2009**, *81*, 9114.
26. M. G. Deng, D. Zhang, Y. Y. Zhou, and X. Zhou, *J. Am. Chem. Soc.*, **2008**, *130*, 13095.
27. T. Li, S. J. Dong, and E. K. Wang, *Chem. Commun.*, **2007**, *41*, 4209.
28. Y. Weizmann, M. K. Beissenhirtz, Z. Cheglakov, R. Nowarski, M. Kotler, and I. Willner, *Angew. Chem. Int. Ed.*, **2006**, *45*, 7384.
29. Y. Q. He, K. Zeng, A. S. Gurung, M. Baloda, H. Xu, X. B. Zhang, and G. D. Liu, *Anal. Chem.*, **2010**, *82*, 7169.
30. Y. Xu, Y. H. Liu, Y. Wu, X. H. Xia, Y. Q. Liao, and Q. G. Li, *Anal. Chem.*, **2014**, *86*, 5611.
31. L. Stefan, F. Denat, and D. Monchaud, *J. Am. Chem. Soc.*, **2011**, *133*, 20405.
32. J. Elbaz, M. Moshe, B. Shlyahovsky, and I. Willner, *Chem. Eur. J.*, **2009**, *15*, 3411.
33. D. M. Köster, D. Haselbach, H. Lehrach, and H. Seitz, *Molecular BioSystems*, **2011**, *7*, 2882.
34. M. Famulok and G. Mayer, *Nature*, **2006**, *439*, 666.
35. S. E. Osborn and A. D. Ellington, *Chem. Rev.*, **1997**, *97*, 349.
36. C. F. Zhu, Y. Q. Wen, D. Li, L. H. Wang, S. P. Song, C. H. Fan, and I. Willner, *Chem. Eur. J.*, **2009**, *15*, 11898.
37. Y. Huang, J. Chen, S. L. Zhao, M. Shi, Z. F. Chen, and H. Liang, *Anal. Chem.*, **2013**, *85*, 4423.