Microplate based assay for thrombin detection using an RNA aptamer as affinity ligand and cleavage of a chromogenic or a fluorogenic peptide substrate

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Abstract The authors report on a microplate based thrombin assay by using an RNA aptamer (Toggle-25) as the affinity ligand. The aptamer is nuclease-resistant because it contains nucleobases modified with 2'-deoxyfluoro saccharides. The aptamer is coated on the surface of the wells of microplates where it captures thrombin from a sample. Following a washing step, a chromogenic or a fluorogenic peptide substrate is added which is cleaved by the captured thrombin to form a yellow product (with absorbance at 405 nm) or a blue fluorescent product (with an emission maximum at 444 nm) after incubation at 37 °C for 2 h. The assay enables thrombin to be determined with a 10 fM detection limit when using the fluorogenic substrate. The assay is selective in that several other proteins do not interfere. The assay can be applied to the determination of thrombin in diluted human serum and plasma.

Keywords Photometry · Fluorescence · Toggle-25 · Enzyme substrate · 2'-deoxyfluoro sugars · Microtiter plate · Kinetic assay · DNA aptamer · Nuclease resistance

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Introduction

Thrombin is a serine protease that selectively cleaves peptide substrates at Arg residues [1]. With crucial roles in physiological and pathological coagulation, thrombin is involved in various diseases [2, 3]. Thrombin is rare in blood under normal conditions, only after vascular injury, thrombin is rapidly generated from inactive zymogen prothrombin by a series of enzyme cleavages. The concentration of thrombin may vary from pM to μM levels during the coagulation [4]. Thus sensitive and specific detection of thrombin assays are important for therapy and diseases diagnostics.

Since the discovery of aptamers [5, 6], many aptamer based assays have been developed for thrombin [7]. Aptamers are the artificially generated single-stranded DNA and RNA by an in vitro process called SELEX (systematic evolution of ligands by exponential enrichment), specifically binding to targets with high affinity [5, 6]. They have some unique analytical features in assay developments [7–12], such as ease of generation, synthesis, and labeling with functional groups, and they have been applied in many assays for a variety of targets [7–16]. Two DNA aptamers are widely used in these aptamer-based assays for thrombin. A 15-nt aptamer (Apt-15, 5'-GGT TGG TGT GGT TGG-3') [17] binds with the fibrinogen binding site of thrombin with a dissociation constant (K_d) around 100 nM, and another 29-nt aptamer (Apt-29, 5'-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3') [18] can bind to the heparin-binding site of thrombin with a dissociation constant (K_d) around 0.5 nM. White et al. reported an RNA aptamer (Toggle-25, 5'-GGG AAC AAA GCU GAA GUA CUU ACC C-3') in 2001 [19]. Toggle-25 contains fluoro modification on 2' position of sugar ring of C and U, showing much higher nuclease resistance and better biostability than the unmodified RNA aptamers that can be fast degraded by nuclease in biological fluids. Toggle-25 has high binding affinity to

thrombin ($K_d \sim 0.5$ nM), much stronger than the earlier selected RNA aptamers without containing modified bases [20].

The microplate-based assays offer benefits in terms of fast sample handling, analysis of multiple samples, small volume, continuous monitoring and convenience, which are beneficial to the practical application [21, 22]. A few groups reported the assays for thrombin by using microplates as solid support and DNA aptamers as affinity ligands in different formats [23–28]. Edwards et al. described an aptamer sandwich fluorometric assay [24]. Apt-15 is immobilized on the microplates firstly to capture thrombin, and then a fluorescein-labeled Apt-29 is added to form a sandwich complex. The assay enables the detection of thrombin around 63.8 nM by measuring the fluorescence intensity of fluorescein-labeled Apt-29 [24]. Baldrich et al. developed a displacement aptamer based assay for thrombin [25]. Biotinylated Apt-15 is immobilized on streptavidin-coated plates. The enzyme-labeled thrombin is firstly captured and then the unmodified thrombin will displace the enzyme-labeled thrombin. By measuring the signal changes of enzyme labeling, thrombin at 4.5 nM is detected [25]. He et al. developed an electrochemical sandwich assay for thrombin by using antibody coated microplate for capture and aptamer modified gold nanoparticles as reporter [26]. The gold nanoparticles are modified with an oligonucleotide containing Apt-15 and 25 adenine (A) bases. The detection of thrombin is achieved by recording the redox signals of A bases cleaved from the aptamer sequence, and the detection limit reaches 2.7 pM [26]. Müller et al. reported an affinity capture based assay for thrombin by using a DNA oligonucleotide containing Apt-15 and Apt-29, which are linked with a polyA spacer [27]. The oligonucleotide is coated on microplate for thrombin capture, and the thrombin hydrolyzes the fluorogenic peptide substrates to products. The detection limit is about 0.47 pM [27]. Shimada et al. described a competitive assay for thrombin on a microplate. A DNA oligonucleotide containing Apt-15 sequence and a complementary fragment of detection probe (DNA-enzyme conjugate) is attached on microplates [28]. In the absence of thrombin, the oligonucleotide hybridizes with a DNA-enzyme conjugate, and labeled enzyme will generate signals. The presence of thrombin causes conformation change of the oligonucleotide and the release of DNA-enzyme conjugate, leading to decreased signals. This competitive assay achieves the detection of thrombin at 11 nM [28]. Most of these microplate assays use a pair of two DNA aptamers or a pair of an antibody and a DNA aptamer to constitute a sandwich assay [23, 24, 26]. The assays usually require a reporter (fluorescent dye or enzyme)-labeled aptamer or enzyme-labeled thrombin.

The use of RNA aptamers for thrombin detection is still limited [29–31], and the RNA aptamer Toggle-25 for thrombin has not been applied to the microplate based assay for thrombin. Here, we report a microplate based assay for thrombin by using Toggle-25 as affinity ligand, on the basis of

previous study [27, 32]. We coat Toggle-25 on the surface of microplate. The thrombin is specifically captured from the mixture by Toggle-25 on microplates, and then the enriched thrombin catalyzes the conversion of the small chromogenic or fluorogenic peptide substrate to detectable products. By measuring the generated products, detection of thrombin is achieved (Fig. 1). The microplate based assay using fluorogenic substrate led to detection of 10 fM. thrombin. The assay also allowed for the detection of thrombin in dilute human serum or plasma samples, benefiting from the good biostability of Toggle-25 with 2'-fluoro modification in sugar rings of bases.

Experimental

Reagents and apparatus

Human serum albumin (HSA), hemoglobin from bovine blood, lysozyme, and bovine serum albumin (BSA) were ordered from Sigma (USA, <http://www.sigmaaldrich.com>). Human α -thrombin and human plasma sample were purchased from Haematologic Technologies Inc. (Essex Junction, VT) (<http://www.haemtech.com/>). Human serum was obtained from Zhongke Chenyu Biotechnology (Beijing, China). The chromogenic substrate of thrombin (N-p-tosyl-Gly-Pro-Arg-p-nitroanilide acetate) and the fluorogenic substrate of thrombin (N-p-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin hydrochloride) were ordered from Sigma (USA, <http://www.sigmaaldrich.com>). The clear 96-well plates were purchased from Corning (Costar 2592) (USA, <http://www.corning.com>). The high-binding black 96-well NUNC Maxisorp plates were purchased from Thermo Scientific (USA, <http://www.thermofisher.com>).

RNA oligonucleotides were synthesized and purified by Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China, <http://www.takara.com.cn>) The biotinylated thrombin-

Fig. 1 Schematic diagram of the assay for thrombin by using RNA aptamer Toggle-25 coated microplates. Toggle-25 is immobilized on the surface of microplate. Thrombin is specifically captured by Toggle-25, and then catalyzes the conversion of the chromogenic or fluorogenic peptide substrate to detectable products, generating signals

binding RNA aptamers had the following sequence, 5'-GGG AAC AAA GCU GAA GUA CUU ACC C-3' (named as Toggle-25), and C and U bases contained 2'-deoxyfluoro sugars [19]. The biotinylated control RNA had the following sequence: 5'-GGG AAC AAA GCU GAA GUA CAA ACC C-3' (C and U bases contained 2'-deoxyfluoro sugars, denoted as Control-25). The biotinylated DNA aptamers were synthesized and purified by Sangon Biotech (Shanghai, China, <http://www.sangon.com>). The DNA aptamers for thrombin had the following sequences, 5'-A GTC CGT GGT AGG GCA GGT TGG GGT GAC T-3' (Apt-29) and 5'-GGT TGG TGT GGT TGG-3' (Apt-15) [17, 18]. Biotin was labeled on 5' end in all oligonucleotides. Solvents and other reagents were in analytical grade. The following buffer solutions were used. Buffer A contained 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 68.5 mM NaCl, 2 mM MgCl₂, and 1 mg·mL⁻¹ BSA (pH 7.5). Buffer B contained 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 68.5 mM NaCl, 2 mM MgCl₂, and 0.1 % Tween20 (pH 7.5). Buffer C contained 50 mM Tris-HCl and 1 M NaCl (pH 8.5).

Preparation of aptamer coated microplates

Aptamer coated microplates were prepared by the following procedure. In the assay using chromogenic peptide substrate, a clear microplate was used. In the assay using fluorogenic peptide substrate, a black microplate was used instead. First, 100 µL of 10 µg·mL⁻¹ of streptavidin in 0.1 M Na₂CO₃ solution (pH 9.6) was added in the wells of microplate, and incubated at 4 °C overnight. After washing three times with 150 µL of buffer B, the wells were blocked by 200 µL of buffer containing 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2.7 mM KCl, 68.5 mM NaCl, 2 mM MgCl₂, and 10 mg·mL⁻¹ BSA (pH 7.5) at 37 °C for 1 h under shaking, and then the wells were washed one time with 200 µL of buffer B. Then, 100 µL of 10 nM of biotinylated aptamer in the buffer A was added in the well of streptavidin coated plates, and incubated at room temperature for 30 min under shaking. The biotinylated aptamers were conjugated on the microplates through the interaction between biotin and streptavidin. After that, the wells were washed three times with 150 µL of buffer B.

Detection of thrombin

100 µL of thrombin sample was added in the wells coated with Toggle-25, and incubated at room temperature for 30 min under shaking to capture thrombin. After washing three times with 150 µL of buffer B, the wells were loaded with 100 µL of enzyme reaction buffer containing 375 µM chromogenic substrate of thrombin or 52.8 µM fluorogenic substrate of thrombin, and incubated at 37 °C for 2 h under shaking. Finally, 20 µL of 20 % acetic acid was added to wells

to stop the reaction. In the assays using chromogenic substrate, the absorbance was measured at the wavelength of 405 nm by a plate reader (Varioskan Flash, Thermo Fisher Scientific, Inc.). In the assays using fluorogenic substrate, we measured fluorescence intensity by the plate reader with an excitation at 370 nm and an emission at 440 nm. Three measurements were made in determination of thrombin. When we compared the performance of Apt-15, Apt-29, and Toggle-25 in the microplate based assays, the corresponding oligonucleotides were coated on the microplate with the same coating step, and the same experimental procedure described above for thrombin detection was used.

Specificity test

To investigate the assay specificity, other proteins (human serum albumin, hemoglobin, and lysozyme) were analyzed by using the same procedure described above for the detection of thrombin. In the assay using the fluorogenic substrates, thrombin at 10 pM was tested, and the other proteins at 2 µM were tested. Thrombin (10 pM) in the presence of human serum albumin (2 µM), hemoglobin (2 µM), or lysozyme (2 µM) was also tested. We also tested the sequence specificity of the Toggle-25 by using RNA Control-25 coated microplates.

Detection of thrombin in complex sample matrix

Human serum and human plasma samples were diluted with binding buffer solution, and the diluted samples were used as the complex sample matrix. Thrombin was spiked in the 20-fold or 100-fold diluted complex samples. Thrombin was analyzed with the assay using Toggle-25 coated microplate and the fluorogenic substrate.

Results and discussion

Choice of aptamers

Aptamers are selected for inhibition of thrombin activity in hydrolysis of fibrinogen to control the function of thrombin in blood clotting [17, 18]. The specific binding of aptamers to thrombin also allows the use of aptamers as affinity ligands in assay developments for thrombin detection [7]. Many aptamer based assays for thrombin used DNA aptamers, either Apt-15 or Apt-29 [7]. Apt-15 or Apt-29 can also be used as a pair of affinity ligands in sandwich assays as they bind to different binding sites of thrombin [7]. Apt-15 has lower binding affinity than Apt-29, so Apt-29 usually gives better sensitivity in homogenous assays for thrombin [7]. The selected RNA aptamers for thrombin also show high binding affinity to thrombin [19, 20, 29–31], which is comparable to or higher

than that of DNA aptamers. One weakness of RNA aptamer is the easier degradation by nuclease than DNA aptamer because RNA has 2' hydroxyl in sugar ring of bases [19]. RNA aptamer meets challenge in analytical applications, especially for real sample analysis in biological fluids containing nuclease, because of poor stability and high cost of production, and more cares are usually needed in sample storage and preparation and assay developments. Among the RNA aptamers of thrombin, Toggle-25 is attractive as it shows higher binding affinity and stability, whose K_d (0.5 nM) is close to that of Apt-29 DNA aptamers [19]. Toggle-25 can circumvent the limitation of unmodified RNA aptamers for thrombin in stability [20], due to the 2'-fluoro modification in sugar rings of bases, which greatly increases the nuclease resistance of oligonucleotides [19]. The lifetime of Toggle-25 in biological fluid is even higher than DNA aptamers [19]. The aptamers with increased biostability will be favorable for their applications to complex sample matrix (e.g. blood), so Toggle-25 with high binding affinity and stability is more desired than the unmodified RNA aptamers of thrombin [20, 29–31]. Compared with the DNA aptamer based assays for thrombin [7–9, 11, 12, 15, 16, 23–28], the RNA aptamer based assays are relatively limited [29–32]. Here, we report a microplate based assay for thrombin using Toggle-25 as affinity ligands, to further expand its analytical applications and draw attentions to the RNA aptamers with modified bases as it can be good alternative to DNA aptamers, and we also compared its assay performance with that of DNA aptamers of thrombin.

Absorbance assay using Toggle-25 coated microplate

We firstly achieved the detection of thrombin by using Toggle-25 coated microplate and a chromogenic substrate, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide. We conjugated biotinylated Toggle-25 on the surface of streptavidin coated microplate through the interaction between biotin and streptavidin. Thrombin was captured by the Toggle-25 on

Fig. 2 Effect of the concentration of applied Toggle-25 for aptamer immobilization on the obtained signal in the assay for thrombin using chromogenic substrate. The concentration of thrombin was 25 pM

microplates. Then, the enriched thrombin catalyzed the cleavage of the chromogenic peptide substrate to generate p-nitroaniline. Finally, we measured the absorbance of p-nitroaniline at the wavelength of 405 nm to achieve the detection of thrombin. Though the maximum absorbance of p-nitroaniline was at 380 nm, we measured the absorbance at 405 nm to reduce the interference from p-nitroaniline.

The ionic strength of the buffer usually has a significant effect on the binding of the aptamer to a protein. We examined the influence of the concentration of total NaCl and $MgCl_2$ on the thrombin assay (Fig. S1 in Electronic Supplementary Material (ESM)). It shows that the concentration of total NaCl about 68.5 mM is favorable for the affinity binding

Fig. 3 Detection of thrombin by the assay using chromogenic peptide substrates. **a** The recorded absorbance spectra of the generated product corresponding to varying concentrations of thrombin in the assay. 2-h enzyme reaction at 37 °C for cleavage of the chromogenic peptide substrate was applied. **b** The relationship between the absorbance signal (Abs) at 405 nm and the concentration of thrombin. The inset shows the obtained absorbance signal corresponding to low concentration of thrombin

Fig. 4 Detection of thrombin by the assay using fluorogenic peptide substrates. **a** The recorded fluorescence spectra of the generated product corresponding to varying concentrations of thrombin (0–50 pM). (RFU was relative fluorescence unit). The inset shows the obtained spectra corresponding to low concentrations of thrombin. 2-h enzyme reaction at 37 °C for cleavage of the peptide substrate was applied. **b** The relationship between fluorescence signal and the concentration of thrombin. The inset shows the obtained fluorescence signal corresponding to low concentration of thrombin

between thrombin and the Toggle-25. Higher concentration of NaCl caused decreased signals. The effect of MgCl_2 on obtained signals was not significant at low concentrations.

Finally, we chose the binding buffer containing 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 2.7 mM KCl, 68.5 mM NaCl, 2 mM MgCl_2 , and 1 $\text{mg}\cdot\text{mL}^{-1}$ BSA.

We also tested the effect of the amount of aptamer used for immobilizing aptamer onto microplate on the obtained signals (Fig. 2). The result shows the amount of aptamer for immobilization in the tested range did not cause significant influence on the obtained absorbance signal. We applied 10 nM aptamer (100 μL) to prepare the aptamer coated microplate for the further study.

Under optimized conditions, thrombin at varying concentrations was successfully detected by measuring the absorbance at 405 nm. Figure 3a shows the obtained absorbance spectra of generated product corresponding to different concentrations of thrombin. Figure 3b shows that thrombin can be detected in the 2.5 pM to 250 pM concentration range. A good linear relationship between the net absorbance increase (ΔAbs , obtained by subtracting the blank signal from the absorbance signal from thrombin sample) and the concentrations of thrombin in the range from 2.5 pM to 100 pM was obtained ($y = 0.0044x$, $R^2 = 0.999$). When the concentration of thrombin was higher than 250 pM, the obtained signal reached the plateau level. This can be attributed to that the chromogenic substrate was totally converted into product by thrombin at high concentrations. The limited amount of aptamer coated on the plate surface can also affect the detected concentration range of thrombin.

Fluorescence assay using Toggle-25 coated microplate

To further improve the sensitivity of the assay, we also developed Toggle-25 coated-microplate based assay for thrombin by using the fluorogenic peptide substrates. In the enzyme reaction step, thrombin hydrolyzed a fluorogenic peptide substrate, N-p-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin, to generate 7-amido-4-methylcoumarin. We measured fluorescence by the plate reader with an excitation at 370 nm and an emission at 440 nm to achieve the final detection of thrombin. Due to high sensitive fluorescence measurement, a greatly improved sensitivity was obtained. Figure 4a shows the

Table 1 Comparison of various microplate based assays for thrombin using aptamers with respect to methods and limit of detection (LOD)

Methods	LOD (pM)	Ref.
Fluorescence sandwich assay using aptamer-tagged liposome as label	64	[23]
Fluorescence sandwich assay using fluorescein-labeled Apt-29 for reporter	63,800	[24]
DNA oligonucleotide-based enzyme capture assay	0.47	[27]
Displacement enzyme linked aptamer assay	4500	[25]
Electrochemical assay using aptamer modified gold nanoparticle as barcode	2.7	[26]
Competitive assay for thrombin using DNA-enzyme conjugate as reporter	11,000	[28]
Absorbance assay using RNA Toggle-25 coated microplate	2.5	This work
Fluorescence assay using RNA Toggle-25 coated microplate	0.01	This work

obtained fluorescence spectra of generated fluorescent products corresponding to different concentrations of thrombin. Figure 4b shows that thrombin can be detected in the 0.01 pM to 50 pM concentration range by using this fluorogenic peptide substrate. The net fluorescence increase (Δ RFU, obtained by subtracting the blank signal from the fluorescence signal from thrombin sample) shows a good linear relationship with the concentration of thrombin in the range from 0.01 pM to 25 pM ($y = 43.31x$, $R^2 = 0.999$). A plateau signal was observed when the concentration was higher than 50 pM, and this is due to that the fluorogenic substrate was totally cleaved. Increasing the amount of the fluorogenic substrate can extend the detected concentration range of thrombin.

Compared with a few reported aptamer-based assays for thrombin by using microplates as solid support (Table 1) [23–28], our assay using fluorogenic substrate has lower limit. Our assay using fluorogenic substrate also showed higher sensitivity than the recently reported assays for thrombin using quantum dots (LOD: 12 pM) [15] or gold nanoparticles (LOD: 0.02 pM) [16]. Though the sensitivity of our absorbance assay is lower, it is still better than many other microplate based assays [23–25, 28], and it can meet the demand of some assays for detection of high concentration of thrombin.

To compare Toggle-25 with DNA aptamers (Apt-15 and Apt-29) in assay performance, we also detected the thrombin by using the DNA aptamers coated microplates (Fig. 5). The assay using Toggle-25 shows a slightly higher sensitivity than that using Apt-29, which is consistent with the similar K_d values of Toggle-25 and Apt-29. Apt-15 has a lower binding affinity than Toggle-25, so the obtained sensitivity is lower on the Apt-15-coated microplates. The results suggest Toggle-25 shows better assay performance than Apt-29 and Apt-15 on the microplate assay format.

Fig. 5 Comparison of assays for thrombin using fluorogenic substrates with Apt-15, Apt-29 or Toggle-25 coated microplate. Apt-15 or Apt-29 was immobilized on the microplate following the same procedure for immobilization of Toggle-25 on the microplate. The same assay procedures were applied

Fig. 6 **a** Specificity analysis of the assay using fluorogenic substrates. The concentration of tested human thrombin was 10 pM, and the concentration of human serum albumin (HSA), hemoglobin, and lysozyme was all 2 μ M. Thrombin (10 pM) in the presence of HSA (2 μ M), hemoglobin (2 μ M) or lysozyme (2 μ M) was also tested. **b** Sequence specificity test by using the RNA Control-25

Specificity test

We tested the assay selectivity by analyzing other proteins, including human serum albumin (HSA), hemoglobin, and lysozyme. Because of the higher sensitivity of the assay using

Fig. 7 Detection of thrombin spiked in 100-fold or 20-fold diluted human serum with the assay using fluorogenic substrates in comparison with the result of thrombin detection in the binding buffer solution

fluorogenic substrate, we analyzed the assay specificity by the assay using the fluorogenic substrate. Figure 6a shows that only thrombin (10 pM) gave high fluorescence signals, but 200,000 times of all the other tested proteins did not cause significant interference. This result shows our assay is highly specific.

The sequence specificity of the RNA aptamer was also tested by assessing thrombin with RNA Control-25 coated microplate. In the Control-25, two U bases of Toggle-25 were replaced by two A bases. We did not observe significant signal increase in the presence of thrombin when Control-25 coated microplate was used (Fig. 6b). This shows that thrombin is not captured in wells coated with Control-25. The result shows that the binding between Toggle-25 and thrombin is disrupted by the mutation of two U bases and the binding between Toggle-25 and thrombin highly depends on the sequence of Toggle-25 [19].

Detection of thrombin in complex sample matrix

To further assess the applicability of our assay in complex sample matrix, thrombin spiked in diluted human serum sample was tested in the assay by using Toggle-25 coated microplate and the fluorogenic substrates. As Fig. 7 shown, the recoveries for thrombin detection ranged from 90 to 108 % in 100-fold diluted human serum, while in 20-fold diluted human serum, the assay achieved lower recoveries about 40 %. We also analyzed thrombin spiked in diluted human plasma sample. The recoveries in 100-fold diluted human plasma was close to 100 %, however the recovery in 20-fold diluted plasma sample reached about 55 % (Fig. S2 in ESM). It shows our assay can be used for the detection of thrombin in complex samples. As comparison, Toggle-25 without 2'-fluoro modification in C and U bases failed in analysis of thrombin in diluted serum or plasma samples when they were coated on the microplate although they allowed for thrombin detection in binding buffer solution. The failure is because nuclease in serum or plasma can fast degrade the RNA Toggle-25 without 2'-fluoro modification in bases. Toggle-25 with 2'-fluoro modification in sugar ring is desired in analysis of blood samples or other biological fluids samples due to the higher stability resulted from the 2'-fluoro modification in bases [19].

Conclusion

We reported a sensitive and selective microplate-based thrombin assay by using RNA aptamer Toggle-25 as affinity ligands on microplate. Toggle-25 has good biostability due to the 2'-fluoro modification on sugar ring of bases. This property allows Toggle-25 to be used for thrombin detection in diluted human serum and plasma sample, overcoming the limitation

of RNA aptamer without modified bases in ease of degradation by nuclease in biological fluids. Toggle-25 can be used as affinity ligand for thrombin detection in other assay formats showing potential in wide applications. Our assay requires a 2 h-enzyme reaction to amplify signals and may not meet the demand of fast analysis in some applications, but rapid assay is also feasible by reducing reaction time. The use of peptide substrate with better kinetic parameter will further improve the assay. Although the cost of synthesis of RNA aptamer with modified bases is relatively high now, the required amount of aptamer in our assay is small, and the progress of chemical synthesis will eliminate the cost. The application of RNA aptamer with modified bases is promising for development of assays for a variety of targets.

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References

1. Maragoudakis ME, Tsopanoglou NE (2009) Thrombin: physiology and disease. Springer Science, New York
2. Brummel-Ziedins KE, Vossen CY, Butenas S, Mann KG, Rosendaal FR (2005) Thrombin generation profiles in deep venous thrombosis. *J Thromb Haemost* 3:2497–2505
3. Sokolova E, Reiser G (2008) Prothrombin/thrombin and the thrombin receptors PAR-1 and PAR-4 in the brain: localization, expression and participation in neurodegenerative diseases. *Thromb Haemost* 100:576–581
4. Franchini M, Mannucci PM (2012) Thrombin and cancer: from molecular basis to therapeutic implications. *Semin Thromb Hemost* 38:95–101
5. Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature* 346:818–822
6. Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249:505–510
7. Deng B, Lin Y, Wang C, Li F, Wang Z, Zhang H, Li XF, Le XC (2014) Aptamer binding assays for proteins: the thrombin example—A review. *Anal Chim Acta* 837:1–15
8. Li F, Zhang H, Wang Z, Newbigging AM, Reid MS, Li XF, Le XC (2015) Aptamers facilitating amplified detection of biomolecules. *Anal Chem* 87:274–292
9. Mairal T, Ozalp VC, Lozano Sanchez P, Mir M, Katakis I, O'Sullivan CK (2008) Aptamers: molecular tools for analytical applications. *Anal Bioanal Chem* 390:989–1007
10. Wu J, Zhu Y, Xue F, Mei Z, Yao L, Wang X, Zhang L, Liu J, Liu G, Peng C, Chen W (2014) Recent trends in SELEX technique and its application to food safety monitoring. *Microchim Acta* 181:479–491
11. Cho EJ, Lee JW, Ellington AD (2009) Applications of aptamers as sensors. *Annu Rev Anal Chem* 2:241–264
12. Liu J, Cao Z, Lu Y (2009) Functional nucleic acid sensors. *Chem Rev* 109:1948–1998
13. Singh P, Gupta R, Sinha M, Kumar R, Bhalla V (2016) MoS₂ based digital response platform for aptamer based fluorescent detection of pathogens. *Microchim Acta*. doi:10.1007/s00604-016-1762-2
14. Duan N, Wu S, Dai S, Miao T, Chen J, Wang Z (2015) Simultaneous detection of pathogenic bacteria using an aptamer

- based biosensor and dual fluorescence resonance energy transfer from Quantum dots to carbon nanoparticles. *Microchim Acta* 182:917–923
15. Lin Z, Pan D, Hu T, Liu Z, Su X (2015) A near-infrared fluorescent bioassay for thrombin using aptamer-modified CuInS₂ Quantum dots. *Microchim Acta* 182:1933–1939
  16. Zhang L, Li L (2016) Colorimetric thrombin assay using aptamer-functionalized gold nanoparticles acting as a peroxidase mimetic. *Microchim Acta* 183:485–490
  17. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ (1992) Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* 355:564–566
  18. Tasset DM, Kubik MF, Steiner W (1997) Oligonucleotide inhibitors of human thrombin that bind distinct epitopes. *J Mol Biol* 272:688–698
  19. White R, Rusconi C, Scardino E, Wolberg A, Lawson J, Hoffman M, Sullenger B (2001) Generation of species cross-reactive aptamers using "toggle" SELEX. *Mol Ther* 4:567–573
  20. Kubik MF, Stephens AW, Schneider D, Marlar RA, Tasset D (1994) High-affinity RNA ligands to human α -thrombin. *Nucleic Acids Res* 22:2619–2626
  21. Janke R, Genzel Y, Wahl A, Reichl U (2010) Measurement of key metabolic enzyme activities in mammalian cells using rapid and sensitive microplate-based assays. *Biotechnol Bioeng* 107:566–581
  22. Ihara M, Suzuki T, Kobayashi N, Goto J, Ueda H (2009) Open-sandwich enzyme immunoassay for one-step noncompetitive detection of corticosteroid 11-deoxycortisol. *Anal Chem* 81:8298–8304
  23. Edwards KA, Wang Y, Baeumner AJ (2010) Aptamer sandwich assays: human alpha-thrombin detection using liposome enhancement. *Anal Bioanal Chem* 398:2645–2654
  24. Edwards KA, Baeumner AJ (2010) Aptamer sandwich assays: label-free and fluorescence investigations of heterogeneous binding events. *Anal Bioanal Chem* 398:2635–2644
  25. Baldrich E, Acero JL, Reekmans G, Laureyn W, O'Sullivan CK (2005) Displacement enzyme linked aptamer assay. *Anal Chem* 77:4774–4784
  26. He P, Shen L, Cao Y, Li D (2007) Ultrasensitive electrochemical detection of proteins by amplification of aptamer-nanoparticle bio bar codes. *Anal Chem* 79:8024–8029
  27. Müller J, Becher T, Braunstein J, Berdel P, Gravius S, Rohrbach F, Oldenburg J, Mayer G, Potzsch B (2011) Profiling of active thrombin in human blood by supramolecular complexes. *Angew Chem Int Ed* 50:6075–6078
  28. Shimada J, Maruyama T, Kitaoka M, Kamiya N, Goto M (2012) Microplate assay for aptamer-based thrombin detection using a DNA-enzyme conjugate based on histidine-tag chemistry. *Anal Biochem* 421:541–546
  29. Jiang YX, Fang XH, Bai CL (2004) Signaling aptamer/protein binding by a molecular light switch complex. *Anal Chem* 76:5230–5235
  30. Li Y, Lee HJ, Corn RM (2007) Detection of protein biomarkers using RNA aptamer microarrays and enzymatically amplified surface plasmon resonance imaging. *Anal Chem* 79:1082–1088
  31. Chen Y, Nakamoto K, Niwa O, Corn RM (2012) On-chip synthesis of RNA aptamer microarrays for multiplexed protein biosensing with SPR imaging measurements. *Langmuir* 28:8281–8285
  32. Hao L, Zhao Q (2016) Using fluoro modified RNA aptamers as affinity ligands on magnetic beads for sensitive thrombin detection through affinity capture and thrombin catalysis. *Anal Methods* 8:510–516