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RESEARCH PAPER

An aptamer assay using rolling circle amplification coupled with thrombin catalysis for protein detection

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Abstract We describe a sensitive aptamer-based sandwich assay for protein detection on microplate by using rolling circle amplification (RCA) coupled with thrombin catalysis. This assay takes advantage of RCA generating long DNA oligonucleotides with repeat thrombin-binding aptamer sequence, specific aptamer affinity binding to achieve multiple thrombin labeling, and enzyme activity of thrombin for signal generation. Protein target is specifically captured by antibody-coated microplate. Then, an oligonucleotide containing an aptamer for protein and a primer sequence is added to form a typical sandwich structure. Following a template encoded with complementary sequence of aptamer for thrombin, RCA reaction extends the primer sequence into a long oligonucleotide. Many thrombin molecules bind with the RCA product. Thrombin catalyzes the conversion of its chromogenic or fluorogenic peptide substrates into detectable products for final quantification of protein targets. We applied this strategy to the detection of a model protein target, platelet-derived growth factor-BB (PDGF-BB). Due to double signal amplifications from RCA and thrombin catalysis, this assay enabled the detection of PDGF-BB as low as 3.1 pM when a fluorogenic peptide substrate was used. This assay provides a new way for signal generation in RCA-involved assay through direct thrombin labeling, circumventing time-consuming preparation of enzyme-conjugate and affinity probes. This method has promise for a variety of analytical applications.

Keywords Aptamer · Thrombin · Rolling circle amplification · Multiple enzyme labeling · Signal amplification · Protein detection

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Introduction

Aptamers originate from a nucleic acid library through in vitro SELEX process (selective evolution of ligands by exponential enrichment), showing high specificity and affinity to targets [1–3]. As nucleic acid affinity reagents, aptamers possess many advantages in analytical applications and biosensing, such as easy chemical synthesis with low-cost, high-stability, being easy-to-stock, and amplifiable property [4–6]. Numerous assays have been developed for protein detections by using aptamers since the emergence of aptamers [4–9]. The amplifiable property of aptamers enables amplification of nucleic acid sequence to achieve sensitive detection of protein targets in aptamer-based assays by converting protein detection to detection of nucleic acids [4, 10, 11]. Among these assays, polymerase chain reaction (PCR) and rolling circle amplification (RCA) are two popular amplification techniques to enhance the sensitivity of aptamer-based assays [10–12].

RCA is one simple amplification strategy conducted in isothermal condition [10–13]. In a typical RCA reaction, a primer hybridizes with a circle template, which is extended around the template with DNA polymerase. RCA produces a

long, single-strand DNA molecule complementary to the circle template. Hence, hundreds to thousands of repeat units are generated from each template. Through a rational design, the obtained long oligonucleotide can combine with versatile reporters (e.g., fluorescence dyes, electrochemical tag, nanoparticles, enzymes) to generate signals, allowing for signal amplification [10–13]. RCA shows advantages over PCR in simplicity and no need to change temperature [10–13]. RCA strategy has been applied in a variety of formats by combining different detection technology, such as colorimetry, fluorescence, electrochemistry, chemiluminescence, diffractometry, etc. [10–12, 14–20]. In the RCA-based aptamer assay, primer sequence can be easily conjugated with aptamer sequence through chemical synthesis [10–17]. By designing the templates, the DNA products of RCA can be readily customized to contain DNA aptamer, DNAzymes, or other specific DNA sequences, making RCA flexible for many applications [12, 14, 16, 21].

Thrombin-binding aptamers are widely used for detection of thrombin, an important enzyme molecule in blood [22–24]. The binding of aptamer to thrombin does not inhibit the activity of thrombin cleaving small peptide substrate because the aptamer binding site is distinct from the active site of thrombin [25, 26]. Taking advantage of aptamer binding and thrombin catalysis, sensitive assays for thrombin have been developed through affinity capture of thrombin by aptamer and the enzyme activity of captured thrombin in cleaving small peptide substrates to generate detectable products [27–30].

Inspired by the previous work [14, 16, 27–30], here, we demonstrated an aptamer assay for protein detection through RCA coupled with thrombin catalysis by using RCA reaction to produce an oligonucleotide binding with multiple thrombin molecules and using thrombin to generate signals. Figure 1 shows the principle of the strategy for protein detection in a sandwich format on the microplate. The protein target is captured by antibody-coated microplate, and then it is bound with an oligonucleotide containing the aptamer for protein target and a primer sequence for RCA reaction. Complementary sequence of the aptamer for thrombin is encoded in the RCA template. The template hybridizes with primer and is circularized by ligase. Following the circular template, RCA reaction extends the primer into a long oligonucleotide that contains many aptamer sequence units for thrombin. The generated DNA product binds with many thrombin molecules, so multiple thrombin molecules are attached on the sandwich complex. As an enzyme molecule, thrombin catalyzes the cleavage of chromogenic or fluorogenic small peptide substrates into detectable products, to achieve final detection of protein targets by absorbance analysis or fluorescence analysis. This strategy provides a new way for signal generation in RCA-involved assay by using thrombin as multiple enzyme labels through direct thrombin-aptamer binding. It does not need tedious and time-consuming process to prepare enzyme-conjugate and affinity probes for enzyme labeling in RCA. Double signal amplification can be achieved in this assay through RCA and thrombin catalysis.

To show the proof of principle of the demonstrated strategy, here, we developed an assay for a model protein target, platelet-derived growth factor-BB (PDGF-BB), which is a potential cancer marker and is known to be related to tumor growth, progression, and transformation [31, 32]. Sensitive detection of PDGF-BB with our assay using absorbance analysis or fluorescence analysis was achieved. As low as 3.1 pM PDGF-BB could be detected in our assay using fluorogenic substrate. The demonstrated strategy shows promise in a variety of analytical applications. RCA-combined thrombin labeling can be applied to other versatile RCA assay formats.

Materials and methods

Reagents and apparatus

The DNA-BIND microplates were obtained from the Corning Inc. (NY, USA). Recombinant human PDGF-BB, PDGF-AB, and PDGF-AA were purchased from Invitrogen. Anti-PDGF-BB antibody was obtained from R&D system (Minneapolis, MN, USA). 10× phi 29 DNA polymerase reaction buffer (RCA buffer) was bought from New England Biolabs (Beijing, China). The phi 29 DNA polymerase and dNTPs were provided by the Epicenter (Madison, WI, USA). The *Escherichia coli* DNA ligase was purchased from Takara Biotechnology Co. Ltd. (Dalian, China). Bovine serum albumin (BSA), hemoglobin (Hb), human immunoglobulin G (IgG), lysozyme (Lys), and human serum sample were obtained from Sigma. Human α-thrombin was ordered from Haematologic Technologies Inc. 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 purchased from Sigma. Ultrapure water was obtained through a Purelab Ultra Elga Labwater system for solution preparation. A microplate reader (Varioskan Flash, Thermo Fisher Scientific, Inc.) was used to record the absorbance and fluorescence signals. Oligonucleotides were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China), and sequences were shown in Table 1.

The following buffers were used. Coupling buffer contained 50 mM Na_2HPO_4 (pH 8.5) solution. Blocking buffer contained 50 mM Na_2HPO_4 and 10 mM Tris–HCl (pH 8.5). PBS buffer (pH 7.5) was composed of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4. SSC buffer (pH 7.2) consisted of 0.3 M NaCl and 0.03 M sodium citrate ($Na_3C_6H_5O_7$). Ligation buffer consisted of 30 mM Tris–HCl

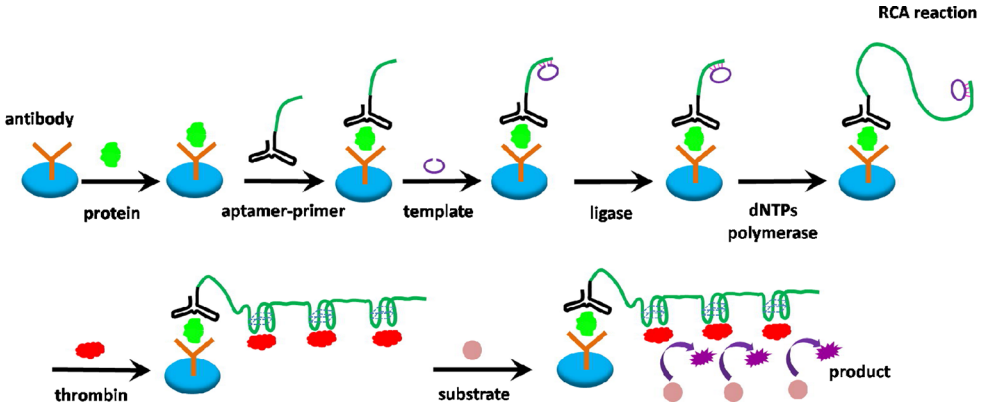

Fig. 1 Schematic of the aptamer assay for protein detection using RCA coupled with thrombin catalysis. Protein target is captured by antibody coated on microplate, and then it is bound with the oligonucleotide (aptamer-primer) containing an aptamer for protein target and a primer sequence to form a sandwich complex. The template encoded with a complementary sequence of the aptamer for thrombin hybridizes with the primer, and it is circularized by ligase. Following the circular template, RCA reaction extends the primer sequence into a long single-stranded DNA sequence for thrombin. The generated long DNA binds with many thrombin molecules, achieving multiple thrombin labeling in sandwich complex. Thrombin catalyzes the cleavage of small peptide substrates into detectable product

(pH 7.5), 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 mM nicotinamide adenine dinucleotide (NAD), 1.2 mM EDTA, and 0.05 mg/mL BSA. Thrombin-binding buffer contained 20 mM Tris–HCl (pH 7.5), 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Thrombin catalysis buffer contained 50 mM Tris–HCl (pH 8.5) and 1 M NaCl. Five types of buffers were used for washing, including buffer A (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween20), buffer B (PBS buffer with 0.1 % Tween 20), buffer C (PBS buffer with 1 mM $MgCl_2$ and 0.1 % Tween 20), buffer D (SSC buffer with 0.05 % Tween 20), and buffer E (thrombin-binding buffer with 0.1 % Tween20).

Table 1 Sequence information for aptamer and used oligonucleotides

Name	Sequence
Apt29	5 -AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3
Apt15	5 -GGT TGG TGT GGT TGG-3
PD44-primer	5 -TAC TCA GGG CAC TGC AAG CAA TTG TGG TCC CAA TGG GCT GAG TA TTTTTTTTTTT *TTG TCC GTG CTA GAA GGA AAC AGT TAC*-3
Template-Apt29	5 -TAG CAC GGA CAA AAAAAAAAAAAAA *AGT CAC CCC AAC CTG CCC TAC CAC GGA CT* AAAAAAAAAAAAAA GTA ACT GTT TCC TTC-3
Template-Apt15	5 -TAG CAC GGA CAA AAAAAAAAAAAAA *CCA ACC ACA CCA ACC* AAAAAAAAAAAAAAA GTA ACT GTT TCC TTC-3

For PD44-primer, the underlined portion is the aptamer sequence of PDGF-BB (PD44) and the italicized portion is the primer sequence. For Template-Apt29, the italicized portion is the complementary sequence of Apt29. For Template-Apt15, the italicized portion is the complementary sequence of Apt15. The underlined portions in Template-Apt15 and Template-Apt29 are the sequences binding to primer

Assay procedure

Antibody was conjugated on the surface of DNA-BIND microplates by the following procedure. The anti-PDGF-BB antibody was diluted to 2 μg/mL in coupling buffer and added into wells of a clear microplate (for absorbance detection) or a black microplate (for fluorescence detection) (100 μL per well). The microplate was incubated for 1 h to conjugate the antibody on the surface of wells of microplate. The excess of PDGF-BB antibody was removed by decanting the supernatant. The wells were washed three times with buffer A and blocked with blocking buffer containing 10 mg/mL BSA (200 μL per well) for 1 h. After decantation of blocking buffer, the wells were washed once with buffer B.

A series of 100 μL PDGF-BB in PBS buffer containing 1 mM $MgCl_2$ and 4 mg/mL BSA were added into the above-mentioned wells and incubated for 30 min. PDGF-BB was captured on the antibody-coated microplates. After washing with buffer C for three times, 100 μL of 200 nM oligonucleotide probe (named PD44-primer) in PBS containing 1 mM $MgCl_2$ was added to the wells and incubated for 30 min (before addition, the PD44-primer was heated at 85 °C for 3 min and cooled to room temperature). After the wells were rinsed with buffer C for three times, 100 μL of 50 nM template in SSC buffer containing 0.05 % Tween 20 was added into the wells, and incubated for 1 h to hybridize with the PD44-primer. The excess template was removed by washing with buffer D for three times. And then, the template was circularized via ligation by ligase (0.02 U/μL) in ligation buffer (100 μL each well) for 1 h.

RCA reaction was conducted for 1 h in 100 μL of RCA buffer containing 0.4 mM dNTPs, 0.05 U/μL phi29 DNA polymerase, and 0.1 mg/mL BSA. The wells were washed three times with buffer D. One hundred microliters of 20 nM

thrombin in thrombin-binding buffer was added and incubated for 30 min. After washing the wells with buffer E for three times, thrombin catalyzed cleavage of the chromogenic substrate (0.375 mM) or fluorogenic substrate (0.059 mM) in 100 μL of thrombin catalysis buffer for 1 h. Finally, the generated product was measured by a plate reader. For assay using chromogenic substrate, the absorbance was measured at the wavelength of 405 nm. For assay using fluorogenic substrate, the fluorescence was measured at 440 nm with the excitation of 370 nm. The incubation temperature was 37 °C in all steps of experiments.

Result and discussion

Design of the RCA-based aptamer assay coupled with thrombin catalysis for PDGF-BB

Figure 1 shows the schematic diagram of the aptamer assay for protein detection using RCA coupled with thrombin catalysis for PDGF-BB detection. Anti-PDGF-BB antibody was coated on the surface of wells of microplate through covalent conjugation. PDGF-BB was captured by the coated antibody on microplate. Next, the oligonucleotide probe (PD44-primer) containing an aptamer sequence (PD44: 5 -TAC TCA GGG CAC TGC AAG CAA TTG TGG TCC CAA TGG GCT GAG TA-3 [33]) for PDGF-BB and a primer sequence for RCA was added and bound with the captured PDGF-BB, forming a sandwich complex. The template encoded with complementary sequence of the aptamer for thrombin was added and hybridized to the primer and this template was circularized by ligase through ligation reaction. Subsequently, in the presence of phi29 DNA polymerase and dNTPs, following the circular template, RCA reaction extended the primer into a long single-stranded DNA containing many copies of aptamer sequences for thrombin. Many thrombin molecules were attached onto the RCA product. Finally, the thrombin catalyzed the cleavage of chromogenic substrate or fluorogenic substrate into detectable products, allowing for the quantification of PDGF-BB.

Two thrombin-binding DNA aptamers have been widely used. One has 15 bases with a lower binding affinity (denoted as Apt15, K_d ~100 nM), and the other is a 29-mer aptamer with high binding affinity (denoted as Apt29, K_d ~0.5 nM) (sequence information shown in Table 1) [22–24]. We tried two kinds of templates to generate the 15-mer thrombin-binding aptamer sequence and the 29-mer DNA aptamer, respectively. The template encoded the complementary sequence of Apt15 could be used to generate repeat sequences of Apt15 in RCA product, and it was denoted as Template-Apt15. The other template, called Template-Apt29, could be used to produce tandem Apt29 sequences in the RCA product, as the template contained the complementary sequence of Apt29. We first tested the feasibility of the assay using chromogenic substrate, N-p-tosyl-Gly-Pro-Arg-p-nitroanilide. The labeled thrombin could cleave the chromogenic substrate into p-nitroaniline, and the generated p-nitroaniline was measured at 405 nm [30]. As Fig. 2 shows, we observed great signal increase in the presence of PDGF-BB over the signal from blank sample when either of the templates was used in the assay, showing our strategy is feasible for the detection of PDGF-BB. When Template-Apt29 was used, the obtained signal increase over the blank was much higher than that from the assay using Template-Apt15. The enhanced signal can be attributed to the higher binding affinity of Apt29 than Apt15. Thus, Template-Apt29 was used in our assay.

Optimization of experimental conditions

We further investigated experimental conditions of assays for the detection of PDGF-BB, including the amount of primer, template, ligase, polymerase, dNTPs, and thrombin. We measured the absorbance signal from the blank sample and the absorbance signal from PDGF-BB (0.25 or 0.5 nM). We subtracted the absorbance signal of the blank from the absorbance signal of the PDGF-BB sample to get the net increase of absorbance signal (ΔAbs). The effects of experimental conditions were evaluated by measuring the change of ΔAbs.

The obtained ΔAbs increased with increasing concentration of PD44-primer (see Electronic Supplementary Material (ESM) Fig. S1) A high ΔAbs value was obtained when the concentration was higher than 100 nM. Though 400 nM PD44-primer could further increase the signal, 200 nM PD44-primer was selected for subsequent experiments because a low blank signal was observed at this concentration. Furthermore, the obtained ΔAbs increased as the concentration of ligase increased from 0.005 to 0.02 U/μL, and then ΔAbs slightly decreased when the concentration of ligase further increased (see ESM Fig. S2). The results show the use of ligase at the tested concentration range can allow effective ligation of the template to form a circular template. Thus,

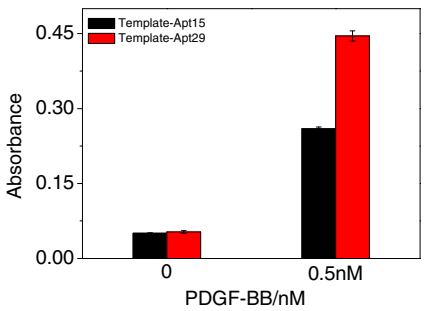

Fig. 2 The absorbance signal obtained in the assay using different templates (Template-Apt15 and Template-Apt29). Experimental conditions: 200 nM PD44-primer, 50 nM template, 0.02 U/μL ligase, 0.1 U/μL polymerase, 0.2 mM dNTPs, 10 nM thrombin

0.02 U/μL of ligase was selected due to the maximum ΔAbs was observed at this concentration.

We also tested the influence of template concentration on the obtained ΔAbs. The maximum ΔAbs was obtained with template at 50 nM, so 50 nM template was preferred (see ESM Fig. S3). The amount of dNTPs had a large effect on the obtained ΔAbs. A high ΔAbs was obtained when the concentration of dNTPs ranged from 0.4 to 1.6 mM (Fig. 3). The further increase of dNTPs caused decrease of ΔAbs, and this phenomenon is consistent with the previous report [20] The high concentrations of dNTPs may cause the generated products wrapped with other, which affects the formation of right conformation of aptamer of thrombin and the affinity binding of thrombin. However, the exact reason for the signal decrease caused by higher concentrations of dNTPs is not known yet. dNTPs at 0.4 mM was applied in the assay due to a high signal was obtained at this concentration.

The amount of polymerase also had significant influence on the obtained ΔAbs. (Fig. S4 in ESM) When the concentration of polymerase was lower than 0.05 U/μL, the obtained ΔAbs was low. When the concentration of polymerase increased from 0.05 to 0.4 U/μL, the obtained ΔAbs decreased. A similar phenomenon was also observed in a previous study [20]. The real reason for the decrease of signals caused by high concentration of polymerase is still not known; 0.05 U/μL of polymerase was applied in the assay. The amount of thrombin was also optimized (Fig. 4). The use of thrombin at 5 nM gave low ΔAbs, while the ΔAbs sharply increased when thrombin at 10 nM was used; 20 nM of thrombin enabled the assay to give the maximum ΔAbs. Further increase of thrombin cause ΔAbs decreased. We do not know what caused the signal decreasing when thrombin at concentrations higher than 20 nM was applied; 20 nM thrombin was finally used in the assay.

In addition, we also did a control experiment without using RCA. In the control experiment, after sandwiching the PDGF-BB with the capture antibody coated on microplate and PD44-primer, we added thrombin into the wells. After incubation

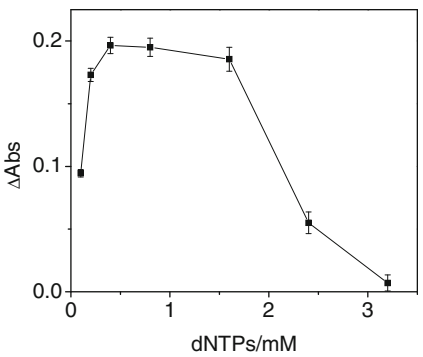

Fig. 3 The effect of dNTPs concentration on net increase of absorbance signal (ΔAbs) obtained from PDGF-BB sample. Experimental conditions: 0.25 nM PDGF-BB, 200 nM PD44-primer, 25 nM template, 0.02 U/μL ligase, 0.1 U/μL polymerase, 10 nM thrombin

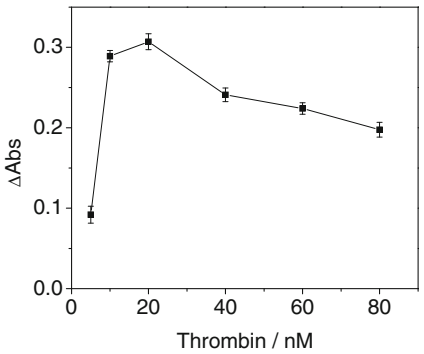

Fig. 4 The effect of thrombin concentration on net increase of absorbance signal (ΔAbs) obtained from PDGF-BB sample. Experimental conditions: 0.25 nM PDGF-BB, 200 nM PD44-primer, 50 nM template, 0.02 U/μL ligase, 0.4 mM dNTPs, and 0.05 U/μL polymerase

and washing, the chromogenic substrate of thrombin was added and was incubated. In this control experiment, the obtained absorbance signal was close to that from the blank sample. The result shows that the thrombin could not bind with oligonucleotide probe PD44-primer in the sandwich complex because PD44-primer did not contain the thrombin-binding aptamer sequence.

Detection of PDGF-BB

Under the optimized conditions, our assay allowed successful detection of varying concentrations of PDGF-BB. Figure 5a shows that ΔAbs increased with the increasing PDGF-BB

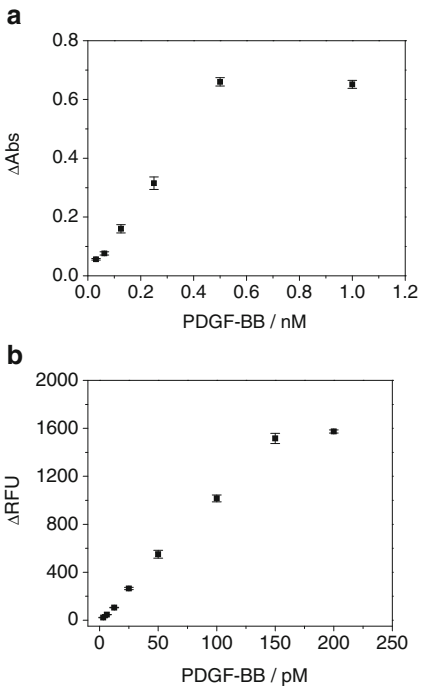

Fig. 5 **a** Detection of PDGF-BB by the assay using chromogenic substrate. **b** Detection of PDGF-BB by the assay using fluorogenic substrate

concentration. A good linear relationship between the ΔAbs and PDGF-BB concentration was obtained in the range from 0.031 to 0.5 nM ($y = 1.31x$, $R^2 = 0.998$). The detection limit was 0.031 nM, according to signal increase higher than three times of the standard deviation of the blank. When PDGF-BB concentration is higher than 0.5 nM, the saturated signals were observed, which can be attributed to that the chromogenic substrate was totally converted into product by thrombin. The limited antibody on the surface of microplate can also cause the saturated signals.

To evaluate the efficiency of RCA reaction, we also did a control experiment, in which only one thrombin molecule was labeled on the sandwich complex by using a DNA probe (5 - TAC TCA GGG CAC TGC AAG CAA TTG TGG TCC CAA TGG GCT GAG TA TTTTTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3) containing the aptamer for PDGF-BB (PD44) and the aptamer for thrombin (Apt29) to replace PD44-primer. When PDGF-BB was captured by antibody on the microplate, then the DNA probe was added to form a sandwich complex. After that, single thrombin bound with the probe through binding between thrombin and Apt29. Thrombin then catalyzed the hydrolysis of its chromogenic substrate for signal generation. In this control experiment, except without using RCA reaction steps, other experimental conditions were the same as that used in the RCA assays. In this control experiment, PDGF-BB ranging from 0.5 to 32 nM was detected with a linear relationship between the ΔAbs and PDGF-BB concentration ($y = 0.021x$, $R^2 = 0.997$) in the assay using single thrombin labeling (Fig. S5 in ESM). The slope of the linear fitting equations corresponding to multiple thrombin labeling obtained by RCA is 62-fold of the slope of the linear fitting equation corresponding to single thrombin labeling. The result shows RCA caused 62-fold signal amplification by multiple thrombin labeling. More than 62 repeat aptamer sequences for thrombin binding may be generated through RCA. The RCA amplification efficiency is close to the reported value at similar experimental conditions [14].

To further improve the sensitivity of the assay, we used fluorogenic substrate instead of chromogenic substrate in the assay. In this case, the labeled thrombin catalyzed the cleavage of the fluorogenic substrate, N-p-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin, into a fluorescent product. The product was measured at emission wavelength of 440 nm with an excitation at 370 nm. Figure 5b shows the detection of PDGF-BB with the assay using fluorogenic substrate. Fluorescence intensity was shown with relative fluorescence unit (RFU). Net fluorescence intensity increase (ΔRFU) was obtained by subtracting the obtained fluorescence intensity of blank sample from the obtained fluorescence signal of PDGF-BB sample. Due to the sensitive fluorescence measurement, the detection limit of the assay was further put down to 3.1 pM, a good linear relationship between ΔRFU and concentration of PDGF-BB in the range from 3.1 to 150 pM was obtained ($y = 10.2x$, $R^2 = 0.998$). When PDGF-BB with concentrations higher than 150 pM, the obtained signals were not further significantly increased because the used fluorogenic substrate was all converted into the fluorescent product at this condition. The increase of fluorogenic substrate may extend the upper detection range. The obtained sensitivity of our assay is higher or comparable to some previously reported aptamer-based assays for PDGF-BB [17, 18, 34–39]. Although the sensitivity of our assay was lower than that obtained from assays using multiple signal amplification or highly sensitive analysis approach [14, 16, 20], our assay provides a simple way to generate enhanced signals from RCA by introducing many thrombin molecules as enzyme labels. Through direct binding with enzyme molecule of thrombin, our assay greatly reduces the tedious and time-consuming steps to prepare enzyme-conjugates and affinity probes for enzyme labeling in RCA.

Specificity test

We tested a few non-target proteins in place of PDGF-BB with the same experimental procedures to assess the specificity of our assay using fluorogenic substrate for PDGF-BB detection. As shown in Fig. 6, the addition of the high concentrations of the non-target proteins (Hb, IgG, Lys at 100 nM and thrombin at 20 nM) did not cause interference with detection of PDGF-BB. In addition, we also tested two common PDGF variants (PDGF-AA and PDGF-AB). Compared with the blank signal, PDGF-AB (50 pM) could cause a slight signal increase, but much lower than that from PDGF-BB (50 pM). The signal from PDGF-AA (50 pM) was also close to the blank signal. The results could be explained by that the aptamer was originally selected to bind to the B chain. PDGF-AB consists of both A and B chains meaning the PDGF-AB can also bind to the aptamer with lower affinity, while PDGF-AA does not bind with aptamer [38, 39]. The results show that our assay

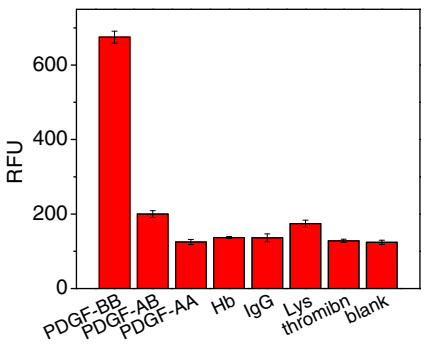

Fig. 6 Specificity test for detection of PDGF-BB by the assay using fluorogenic substrate; 50 pM PDGF-BB, 50 pM PDGF-AB, 50 pM PDGF-AA, 100 nM Hb, 100 nM IgG, 100 nM Lys, and 20 nM thrombin were tested

has a good selectivity for discrimination of PDGF-BB from other proteins. The result for specificity test of the assay using chromogenic substrate was shown in Fig. S5 in ESM, and PDGF-BB could also be selectively tested.

We further assessed the performance of the assay for the detection of PDGF-BB in a complex sample matrix. PDGF-BB spiked in the 100-fold diluted human serum was analyzed. As shown in Fig. S6A in ESM, PDGF-BB in the range of 0.031–0.5 nM in the 100-fold diluted serum sample could still be detected ($y = 1.35x$, $R^2 = 0.993$) when the assay using chromogenic substrate was applied. When the assay using fluorogenic substrate was applied, PDGF-BB ranging from 3.1 to 150 pM could be detected in the 100-fold diluted human serum sample ($y = 9.75x$, $R^2 = 0.995$) (Fig. S6B in ESM). The result indicates that our assay can be used for detection of PDGF-BB in complex sample matrix.

Conclusion

We reported an aptamer assay for protein detection by combining rolling circle amplification (RCA) and thrombin catalysis, using the detection of PDGF-BB as an example. In this strategy, we achieved multiple thrombin molecules attached on the target protein by using RCA to generate oligonucleotide containing aptamer sequences for thrombin. The final detection of target was obtained by cleaving small peptide substrates into detectable products with thrombin. The double amplification approaches from RCA and thrombin catalysis improved the sensitivity. With this strategy, we achieved detection of PDGF-BB in a sandwich format on PDGF-BB antibody-coated microplate by using an oligonucleotide containing aptamer for PDGF-BB and a primer of RCA. This method provides a simple way for signal generation in RCA assay with multiple thrombin labels through thrombin-aptamer binding. This strategy is not limited in sandwich assays using aptamer as affinity ligands, other affinity ligands (e.g., antibody) can be used when RCA primer can be labeled on the affinity ligands to generate aptamer sequences for thrombin. It shows promise in a variety of analytical applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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