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ORIGINAL PAPER

Determination of the platelet-derived growth factor BB by a competitive thrombin-linked aptamer-based Fluorometric assay

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Abstract The authors describe a competitive aptamer based assay for detection of the platelet-derived growth factor BB (PDGF-BB; used as a model protein). The assay is making use of thrombin (a serine protease) as an enzyme label for reporting signals. It is taking advantage of a highly selective aptamer and of the fairly specific enzymatic activity of thrombin in terms of cleaving artificial fluorogenic peptide substrates. In a first step, the surface of wells of microplates is coated with PDGF-BB. On addition of a sample containing PDGF-BB, free and bound PDGF-BB compete with each other for binding to a DNA probe that consists of an aptamer sequence for PDGF-BB and a 29-mer aptamer sequence for thrombin. After washing, thrombin is added and will attach to the DNA probe that bound to the PDGF-BB on the microplates. Following addition of a fluorogenic peptide substrate, the bound thrombin will catalyze the cleavage of the substrate to generate a fluorescent product whose fluorescence intensity is measured at excitation/emission wavelengths of 370/440 nm. Fluorescence intensity decreases with increasing PDGF-BB concentration in the sample because less thrombin will bind to the PDGF-BB coated surface of the microplate. Under optimal conditions, PDGF-BB can be quantified in the 0.125 to 3 nM concentration range. This assay was successfully applied to the determination of PDGF-BB in spiked 100-fold diluted human serum.

Keywords Aptasensor · Fluorescence · Enzyme label · Microplate assay · Microtiter plate · Fluorogenic enzyme substrate · Serine protease · DNA probe · Affinity binding · Dual functional probe · Displacement · Indirect analysis

Introduction

Aptamers have been involved in both diagnostic and therapeutic applications as a rival of antibodies. They are generated via systematic evolution of ligands by exponential enrichment (SELEX) technique [1, 2]. They possess several advantages over antibodies, such as simple synthesis, easy labeling, good stability, long shelf life, wide range of targets, and high binding affinity and selectivity. Therefore, various aptamer-based assays with detection formats including fluorescence, electrochemistry, chemiluminescence and colorimetry have been developed [3–5].

Thrombin is an important serine protease in blood [6]. It is also recognized as a biomarker for several diseases because thrombin is a critical mediator of coagulation, inflammation and angiogenesis [6]. Harnessing the analytical feature of aptamers, many sensitive and selective assays for thrombin have been developed by using aptamers for thrombin [6–8]. The widely used aptamers for thrombin include the 15-mer oligonucleotide (5′-GGT TGG TGT GGT TGG-3′) [9] and the 29-mer oligonucleotide (5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′) [10]. The 29-mer aptamer binds to thrombin with a higher affinity ($K_d \approx 0.5$ nM) than the 15-mer

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oligonucleotide ($K_d \approx 100$ nM). Taking advantage of affinity capture and enzyme activity of thrombin in catalyzing cleavage of small peptide substrates, we developed aptamer capture-based assays for thrombin detection [11, 12]. Using thrombin as an enzyme label, recently we have reported a thrombin-linked aptamer assay (TLAA) for non-thrombin protein detection in a sandwich format [13], which relies on thrombin-binding aptamer, enzyme activity of thrombin, and affinity aptamer for protein targets. In the TLAA strategy, the target protein is sandwiched by the antibody on solid surface and a DNA probe that consists of an aptamer sequence for protein target and an aptamer sequence for thrombin. Then, thrombin binds to the sandwich complex through aptamer affinity binding. The attached thrombin catalyzes the cleavage of peptide substrate to detectable product to achieve the detection of target protein. This strategy converts protein target detection into the measurement of thrombin. This approach shows an interesting analytical application of thrombin and thrombin-binding aptamers to non-thrombin target analysis. However, this sandwich assay needs to use a pair of affinity ligands, one antibody and one aptamer. The success relies on the availability of the aptamer or antibody that binds to two distinct regions of the target.

Competition assay and displacement assay have the advantages of needing only one affinity ligand probe (i.e. single-site binding) and the reduction in the time required for the assay (only one capture incubation step). Competitive aptamer based assays have a wide application in chemical and biochemical analysis. Taking advantage of the affinity of aptamer to target, Baldrich et al. reported two displacement assays for the detection of thrombin [14]. In the first assay, biotinylated aptamer for thrombin is immobilized on streptavidin-coated plates, and enzyme-labeled thrombin is added and incubated. Then, unmodified thrombin is subsequently added to displace the enzyme-labeled thrombin. In the second assay, thrombin is immobilized on microtiter plates, and biotinylated aptamer is incubated, and then free thrombin is subsequently added to displace the aptamer from the complex. In the second assay, the detection of thrombin is achieved with addition of streptavidin-labeled horseradish peroxidase (HRP). Hansen et al. reported an electrochemical displacement aptamer assay for thrombin and lysozyme [15]. They immobilized the thiolated aptamers of thrombin and lysozyme onto the gold substrate simultaneously, followed with the binding of the CdS quantum-dot labeled with thrombin and the PbS quantum dot labeled with lysozyme. As the sample containing detection targets are added, the quantum-dot labeled proteins are displaced by the targets, and can be measured through electrochemical stripping detection. Wang et al. described a competition between immobilized tetracycline-BSA and free tetracycline for the binding to biotinylated aptamer, which was conjugated to the streptavidin-HRP to achieve the detection of tetracycline [16]. Cao et al. reported a competitive electrochemical assay for thrombin that based upon the competitive binding of thrombin and thrombin-gold nanoparticle-glucose oxidase bioconjugate with the aptamer on the electrode [17].

Here we described a competitive format of thrombin-linked aptamer assay (TLAA) for the detection of platelet-derived growth factor BB (PDGF-BB), an important protein related with cell transformation and tumor growth and progression [18–20]. PDGF-BB was used here as a model protein to show the proof of concept of the competitive TLAA and to further expand the application of TLAA. The PDGF-BB was conjugated on the surface of microplates. Free PDGF-BB in solution competed with the PDGF-BB coated on the microplates to the binding of a DNA probe that contains a PDGF-BB aptamer sequence and a thrombin aptamer sequence. The DNA probe attached to the coated PDGF-BB then bound with thrombin through affinity interaction between aptamer and thrombin. Thrombin catalyzed the cleavage of a fluorogenic peptide substrate to a fluorescent product. The more free PDGF-BB was present in sample solution, the less DNA probe was attached onto PDGF-BB on the plates, and less thrombin was labeled on the microplate, causing decrease of fluorescence signals. This competitive TLAA enabled detection of PDGF-BB at 0.125 nM. This assay was also successfully applied to PDGF-BB analysis in complex sample matrix (e.g. diluted serum).

Experimental

Reagents and materials

The high-binding black 96-well NUNC Maxisorp plates (USA, http://www.thermofisher.com) were used. Recombinant human PDGF-BB, PDGF-AB and PDGF-AA were purchased from Invitrogen (USA, http://www.thermofisher.com). Bovine serum albumin (BSA), human immunoglobulin G (IgG) and lysozyme (Lys) were obtained from Sigma (USA, http://www.sigmaaldrich.com). Human α-thrombin was bought from Haematologic Technologies Inc. (Essex Junction, VT) (http://www.haemtech.com). The fluorogenic peptide substrate of thrombin, N-p-tosyl-Gly-Pro-Arg-7-amido-4-methylcoumarin hydrochloride, was purchased from Sigma (USA, http://www.sigmaaldrich.com). The ultrapure water was obtained through a Purelab Ultra Elga Labwater system. A microplate reader (Varioskan Flash, Thermo Fisher Scientific, Inc) was used to record the fluorescence signals.

A DNA probe with the sequence, 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′, was synthesized and purified by Sangon Biotech (Shanghai, China, http://www.sangon.com). In the

sequence, the boldface portion was the aptamer for PDGF-BB, and the underlined portion was the aptamer for thrombin [13]. A polyT sequence was used as a linker between these two aptamer sequences.

The following buffers were used in the experiments. Coating buffer consisted of 0.1 M Na_2CO_3 (pH 9.6). Blocking buffer was PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5) with 10 $g{\cdot}L^{-1}$ BSA. Assay buffer consisted of PBS, 1 mM $MgCl_2$, and 4 $g{\cdot}L^{-1}$ BSA. Thrombin catalysis buffer was composed of 50 mM Tris-HCl (pH 8.5) and 1 M NaCl. Two washing buffers were used, including buffer A (PBS and 0.1 % Tween 20), buffer B (PBS, 1 mM $MgCl_2$ and 0.1 % Tween 20).

Detection of PDGF-BB

First, PDGF-BB coated microplate was prepared by the following procedure. 100 μL of PDGF-BB (15.5 nM) in coating buffer was added to the wells of microplate, and incubated overnight at 4 °C. The wells were washed three times with 150 μL of washing buffer A. 200 μL of blocking buffer was added and incubated for 1 h at 37 °C to block nonspecific binding sites of wells. After that, the wells were washed once with washing buffer A. Subsequently, 100 μL of sample solution containing various concentrations of PDGF-BB and DNA probe (0.035 nM) in assay buffer was added to the wells, and incubated for 30 min at 37 °C. After washing with buffer B, 100 μL of thrombin (10 nM) in assay buffer was added, and incubated for 30 min at 37 °C. The wells were then rinsed with buffer B for three times. 100 μL of thrombin catalysis buffer containing fluorogenic peptide substrate (0.06 mM) was added and incubated for 2 h at 37 °C. Finally, the fluorescence was measured immediately by the microplate reader with excitation and emission at 370 nm and 440 nm, respectively.

Results and discussion

Detection principle of the competitive TLAA for PDGF-BB

Figure 1 shows the principle of TLAA in a competitive format (competitive TLAA) for the detection of PDGF-BB. First, PDGF-BB coated microplate was prepared. Then, the PDGF-BB sample in solution and a DNA probe containing an aptamer for PDGF-BB and a 29-mer aptamer for thrombin were introduced into the PDGF-BB coated microplate. After washing, thrombin was added and bound with the DNA probe that attached to the PDGF-BB coated on microplate through the specific aptamer-thrombin interaction. Finally, thrombin catalyzed the cleavage of a fluorogenic peptide substrate into a detectable fluorescent product, generating fluorescence signal. The presence of PDGF-BB in sample solution causes decrease of fluorescence signal as the free PDGF-BB competed with the coated PDGF-BB on microplate for binding with the DNA probe. Thus, the detection of PDGF-BB in sample solution was achieved.

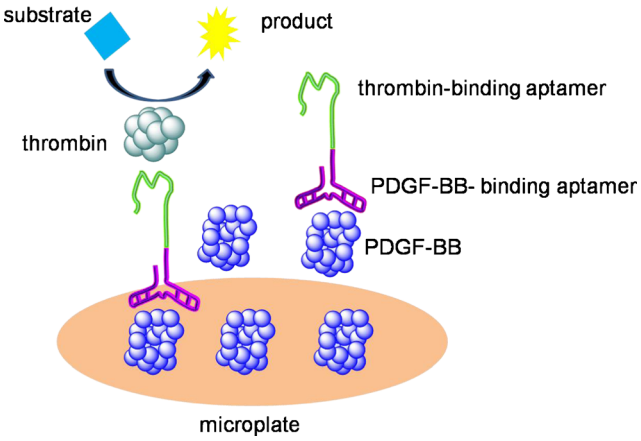

Fig. 1 Schemes of the competitive thrombin-linked aptamer assay (TLAA) for detection of PDGF-BB. Free PDGF-BB in solution and the PDGF-BB coated on the microplate competitively bind with a DNA probe containing the aptamer for PDGF-BB and the aptamer for thrombin. Thrombin binds with the DNA probe attached on the PDGF-BB coated on microplate and catalyzes the cleavage of fluorogenic peptide substrate to fluorescent product, generating signals. The increase of free PDGF-BB in solution leads to a decreased fluorescence signal

Optimization of experimental conditions

Figure 2 shows the obtained typical fluorescence signal in the absence of or in the presence of PDGF-BB sample in competitive TLAA. In the absence of PDGF-BB sample (blank), a strong fluorescence signal was observed, indicating the DNA probe was attached to the PDGF-BB that was coated on the

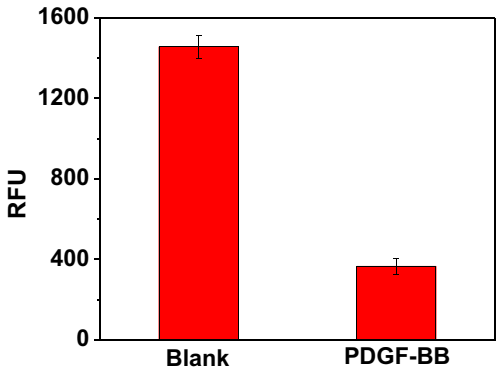

Fig. 2 Testing the feasibility of competitive TLAA for PDGF-BB detection. Blank sample and 3 nM PDGF-BB were analyzed (relative fluorescence unit, RFU) Experimental conditions: 100 μL of 15.5 nM PDGF-BB was used to prepare PDGF-BB coated microplate. 0.035 nM DNA probe was applied in competition process. Thrombin at 10 nM was used in thrombin-binding process

microplates. In contrast, a greatly decreased signal was observed when 3 nM PDGF-BB in sample solution was applied, suggesting that most of DNA probe bound to target PDGF-BB in solution instead of the coated PDGF-BB on microplate. Thus, the result shows that competitive TLAA is feasible for detection of PDGF-BB.

We then optimized the following parameters in experiments: (a) concentration of PDGF-BB in coating buffer for preparation of PDGF-BB coated microplate; (b) concentration of DNA probe; (c) concentration of thrombin. First, the concentration of PDGF-BB for the preparation of PDGF-BB coated microplate was optimized. More PDGF-BB was coated on the surface of wells of the microplate with increase of PDGF-BB concentration, giving higher fluorescence signal of the blank sample in TLAA in the obtained PDGF-BB coated microplate. (shown in Fig. S1 in Supplementary Material). We chose PDGF-BB at 15.5 nM in the coating buffer for preparation of the PDGF-BB coated microplate as a large signal was obtained at this condition. The efficiency of the competition process depends on the amount of the DNA probe used. The decrease of fluorescence signal caused by the added PDGF-BB in solution, ΔRFU, was obtained by subtracting the blank signal from the signal caused by added PDGF-BB. As shown in Fig. S2, the maximum absolute ΔRFU was obtained when 0.035 nM DNA probe was applied. We used 0.035 nM DNA probe in the assay. The concentration of thrombin also has great effect on the obtained absolute ΔRFU (shown in Fig. S3). 10 nM of thrombin was used in the competitive TLAA as the maximum absolute ΔRFU was obtained at this condition.

Analytical performance of the competitive TLAA

Figure 3 shows the change in ΔRFU as a function of the concentration of PDGF-BB sample. With the increase of PDGF-BB in sample, the absolute ΔRFU increased. Fluorescence decrease is linear in the tested concentration range from 0.125 nM to 3 nM ($y = -379.4x$, $R^2 = 0.993$, where y represented the ΔRFU, x represented the protein concentration). The detection limit was 0.125 nM. The sensitivity was compared with that from recently reported aptamer based assays for PDGF-BB (shown in Table 1) [21–35]. The sensitivity of our assay is comparable to that reported in some methods [21–27, 32, 34]. The use of amplification strategy through rolling circle amplification (RCA) or highly sensitive techniques can provide extremely high sensitivity for detection of PDGF-BB [28–31, 33, 35], better than our results. Compared with the sandwich TLAA assay [13], the competitive TLAA has lower sensitivity. Although many sensitive aptamer-based assay have been reported for the detection of PDGF-BB [28–31, 33, 35], here we demonstrate the competitive TLAA for protein detection is feasible by using the detection of PDGF-BB as an example. Our established method can be applied for the detection of PDGF-BB with the requirement of moderate sensitivity. Our entire assay procedure only requires one affinity ligand probe. Other detection methods for thrombin can be used in our assay formats though we applied fluorescence detection here [6].

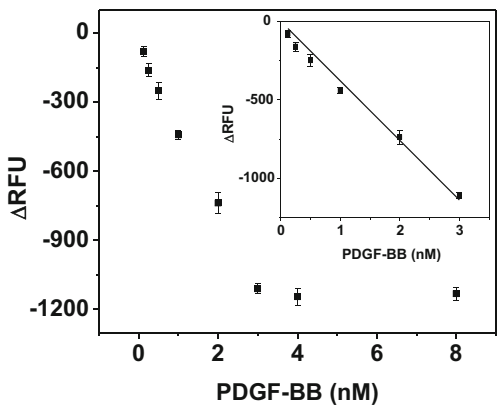

Fig. 3 The detection of PDGF-BB by the competitive TLAA in assay buffer. The inset shows the linear fitting curve for linear detection range

Specificity of the assay

To evaluate the specificity of the competitive TLAA for PDGF-BB detection, we chose some other proteins including human immunoglobulin G (IgG), thrombin, and lysozyme (Lys). As shown in Fig. 4, the tested thrombin (20 nM), IgG (100 nM), and lysozyme (100 nM) did not cause remarkable decrease of fluorescence signal. The tested proteins did not interfere with the detection of PDGF-BB. This can be attributed to the inherent specific binding between the aptamer and its target protein.

In addition, A PDGF dimer composed of two different types of monomers (A and B chains) occurs in three variants: PDGF-BB, PDGF-AB and PDGF-AA. The ΔRFU for 2 nM PDGF-BB was about two times higher than that for 2 nM PDGF-AB, and a negligible ΔRFU was obtained in the presence of 2 nM of PDGF-AA. The results can be explained by that the aptamer used here binds to these variants with different affinities [36, 37]. The signal caused by PDGF-AA was low because the aptamer did not bind to PDGF-A chain. PDGF-AB consists of both A and B chains, and the PDGF-B chain can bind to the aptamer. One PDGF-AB can only bind to one aptamer for PDGF-BB, while PDGF-BB can bind with two aptamers and has higher binding affinity [37]. Therefore, PDGF-AB can cause some signal change, but the signal change is smaller than that caused by PDGF-BB. The phenomenon is consistent with the previous reports [34, 35, 38]. The result clearly demonstrates that the competitive TLAA has a good selectivity for discrimination of PDGF-BB from other proteins.

Table 1 An overview of some recently reported aptamer-based assays for PDGF-BB

Methods and materials	Linear range	LOD	Ref.
Colorimetry assay based on silver decahedral nanoparticles	0.177–7.1 nM	177.5 pM	21
Fluorescence assay based on target-induced silver nanoclusters formation	1–50 nM	370 pM	22
Fluorescence resonance energy transfer assay with graphene oxide surface	0.167–1.167 nM	167 pM	23
Electrochemiluminescence assay with magnetic beads solid support	0.1–1000 nM	80 pM	24
Electrochemistry assay with target binding-induced rolling circle amplification	0.084–8.4 nM	63 pM	25
Chemiluminescence assay using gold nanoparticles through hydroxylamine amplification	0.06–6 nM	60 pM	26
Fluorescence polarization assay based on multiple protein-DNA-protein structures	0.1–6 nM	68 pM	27
Chemiluminescence assay based on exonuclease-assisted cascade autocatalytic recycling amplification	1 pM-10 nM	0.68 pM	28
Chemiluminescence assay based on rolling circle amplification and gold nanoparticles	0.2–200 pM	0.06 pM	29
Electrochemiluminescence assay with glucose oxidase modified gold nanoparticles	0.1–500 pM	0.017 pM	30
Electrochemistry assay with catalase functional DNA-platinum nanoparticles dendrimer	0.05 pM-35 nM	0.02 pM	31
Scanometric assay based on aptamer functionalized silver nanoparticles	0.195–12.5 nM	195 pM	32
Electrochemistry assay with hyperbranched rolling circle amplification	5–80 fM	1.6 fM	33
Affinity capillary electrophoresis with laser induced fluorescence detection	0.5–50 nM	50 pM	34
Fluorescence assay based on three-dimensional carbon microarrays	5 pM-100 nM	5 pM	35
Sandwich thrombin-linked aptamer assay	0.016–2 nM	16 pM	13
Fluorescence competitive thrombin-linked aptamer assay	0.125–3 nM	125 pM	This work

To evaluate the applicability of this competitive TLAA for the target analysis in complex sample matrix, we investigated the detection performance of this assay for PDGF-BB in diluted serum samples. Under similar conditions, different concentrations of PDGF-BB spiked in the 100-fold diluted human serum were detected. As shown in Fig. S4, the detection limit was 0.125 nM, and a linear relationship between the ΔRFU and concentration of PDGF-BB was achieved in the range from 0.125 nM to 2 nM ($y = -423.6x$, $R^2 = 0.983$). The results indicate that the assay for detection of PDGF-BB in 100-fold diluted serum sample yields similar assay performance with that obtained in the binding buffer. It shows the assay can be applied in a complex sample matrix.

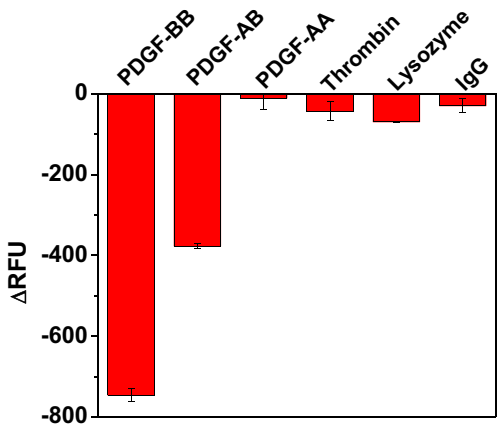

Fig. 4 Specificity test of the competitive TLAA for PDGF-BB detection by assessing PDGF-BB (2 nM) along with other proteins including PDGF-AB (2 nM), PDGF-AA (2 nM), thrombin (20 nM), lysozyme (100 nM), and IgG (100 nM)

Conclusions

In summary, we demonstrated a competitive thrombin-linked aptamer assay for the detection of PDGF-BB using thrombin as an enzyme label. PDGF-BB in solution and PDGF-BB coated on microplate competed for the affinity binding to a DNA probe that contained the aptamer for PDGF-BB and the aptamer for thrombin. Measurement of thrombin bound with the DNA probe allowed for final signal generation. This assay format only requires one affinity ligand for the target instead of two affinity ligands in sandwich assay. The sensitivity of the present assay is not high compared with some sensitive assays for PDGF-BB, further improvement is possible by combining sensitive techniques and amplification methods. The preparation of PDGF-BB coated microplate needs a long time, and improved coating process will help. When the PDGF-BB coated microplate is ready prior to use, the microplate-based assay allows for rapid sample handling and fast analysis, having potential for high throughput analysis. Though PDGF-BB is used as a model protein in our present assay, this assay format shows promise for detection of other proteins with thrombin as a label by using the corresponding aptamers sequence in the DNA probe used, and it can expand the analytical application of thrombin and aptamers.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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