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Aptamer and rolling circle amplification-involved sandwich assay for platelet-derived growth factor-BB with absorbance analysis†

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This paper described an aptamer-based absorbance assay combining rolling circle amplification (RCA) for platelet-derived growth factor-BB (PDGF-BB). PDGF-BB was specifically captured by an antibody on a microplate, and then bound with an anti-PDGF-BB aptamer connected with a primer sequence, forming a sandwich complex. The primer sequence was extended by RCA reaction to generate a long single-stranded DNA with repeated copies. The amplified DNA product was hybridized with many biotinylated short DNA probes, and then labeled with streptavidin–horseradish peroxidase conjugate (SA–HRP). The HRP catalyzed the conversion of a substrate to a colored product, which was measured by simple absorbance analysis to achieve the detection of PDGF-BB. By involving RCA and HRP catalysis amplification, sensitive detection of PDGF-BB with a good selectivity was obtained; the detection limit reached 3.1 pM.

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Introduction

Aptamers are short, single-stranded DNA or RNA oligonucleotides that are selected from random sequence libraries through *in vitro* systematic evolution of ligands by exponential enrichment (SELEX) and can bind to targets with high affinity and selectivity.^{1–5} The targets of aptamers range from amino acids, drugs, metal ions, peptides to proteins, and even entire cells.^{1–5} Aptamers show advantages in good stability, simple production and synthesis, easy storage, labeling and modification.^{1–5} Aptamer-based assays and biosensors for proteins have attracted wide and increasing interests.^{4–7}

Platelet-derived growth factor (PDGF) is an important growth factor protein, which can be assembled in at least three isoforms, PDGF-AA, PDGF-AB and PDGF-BB. PDGF-BB has a direct relationship with cell transformation and tumour growth and progression.^{8–10} Normally, it is expressed at undetectable or low levels, but it is overexpressed in malignant tumour cells. Therefore, sensitive detection of PDGF-BB is quite important for effective diagnosis and prognosis of many diseases.^{11,12} Aptamers against PDGF-BB have been selected, and they show high binding affinity to PDGF-BB with a dissociation constant (K_d) of about 0.1 nM.¹³ The discovery of the anti-PDGF-BB

aptamers allows the development of aptamer-based assays for PDGF-BB, including affinity capillary electrophoresis,¹⁴ fluorescence assay,^{15,16} electrochemical assay^{17,18} and gold-nanoparticles based assay.¹⁹ To improve detection sensitivity, amplification technology strategies can be applied using an enhanced nanoparticles method and an enzyme amplification method.^{3–5,7,20–22}

Rolling circle amplification (RCA) is a simple and effective signal enhancement technology.^{23,24} In RCA, a circular template hybridizes to primer, and then DNA or RNA polymerase is utilized to produce a long single-stranded DNA or RNA molecule consisting of tandem-repeat complementary sequences of the circular template. The generated and amplified long oligonucleotides can then be detected in a variety of methods using dye-labeled nucleotides or hybridization of fluorophore-labeled or enzyme labeled complementary oligonucleotide probes, which can be measured through electrochemical analysis or optical analysis.^{23,24} Due to signal amplification, a high sensitivity can be obtained by RCA. Compared with polymerase chain reaction (PCR), RCA shows advantages, such as no requirement for expensive and large-scale thermal cycling equipment, suitability for chip-based detection, potential of high-throughput analysis, ability for direct quantification without reference to multiple standard samples.^{23,24} RCA based assays for PDGF-BB using aptamers have been reported in different formats with electrochemical, fluorescence, chemiluminescence, or other detection techniques.^{24–30} In the sandwich formats, a pair of affinity ligands (*e.g.* two aptamers, or one aptamer and one antibody) are needed, while the strategy relying on a binding-induced aptamer switch needs only one aptamer.^{24–30}

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Herein, we described an aptamer-based assay for PDGF-BB detection by combining RCA reaction, enzyme labeling, and simple absorbance detection of enzymatic reaction product. In this strategy, PDGF-BB was captured by an antibody on a microplate, and then the PDGF-BB was bound with an aptamer that contained a primer sequence to form a sandwich complex, followed by a RCA reaction. The generated RCA product was labeled with HRP enzyme, and then HRP converted a substrate to a colored product. The absorbance analysis of the product enabled the detection of PDGF-BB at pM level with high selectivity.

Experimental

Reagents and materials

The DNA BIND 96-well clear plate (Costar) was purchased from Corning Inc. (NY, USA). The mouse antibody for PDGF-BB was obtained from R&D systems (Minneapolis, MN, USA). Streptavidin-horseradish peroxidase (SA-HRP) was purchased from Westang Bio-Tech (Shanghai, China). Ready-to-use 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was offered by Thermo Scientific. $10\times$ phi29 DNA polymerase reaction buffer was bought from New England Biolabs (Beijing, China). The phi29 DNA polymerase and dNTPs were provided by Epicenter (Madison, WI, USA). The *E. coli* DNA ligase was purchased from Takara Biotechnology Co., LTD (Dalian, China). Bovine serum albumin (BSA), hemoglobin (Hb), human immunoglobulin G (IgG) and lysozyme were obtained from Sigma. Human α -thrombin was bought from Haematologic Technologies Inc.

The DNA oligos were synthesized by Sangon Biotech Co., LTD (Shanghai, China) and the sequences of DNA oligos used in the study were as follows: aptamer-primer: 5'-TACTCAGGCAC TGCAAGCAATTGTGGTCCCAATGGGCTGAGTATTTTGTCCG TGCTAGAAGGAAACAGTTAC-3' (the italicized part was the aptamer sequence and the bold-faced part was the primer sequence); template: 5'-phosphate-TAGCACGGACATATATGA TGGTACCGCAGTATGAGTATCTCTATCACTACTAAGTGGAAT GTAAGTGTTCCTTC-3'; biotinylated probe: 5'-biotin-GTTTCC TTCTAGCAC-3'.

The following buffers were used in the experiment. Coupling buffer was 50 mM Na_2HPO_4 (pH 8.5). Blocking buffer contained 50 mM Na_2HPO_4 and 10 mM Tris-HCl (pH 8.5). Ligation buffer consisted of 30 mM Tris-HCl (pH 7.5), 4 mM MgCl_2 , 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM nicotinamide adenine dinucleotide (NAD), 1.2 mM EDTA and 0.05 mg mL^{-1} BSA. PBS buffer solution (pH 7.3) 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 ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$). Three washing buffers were used in the study, including washing buffer A (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.05% Tween 20), washing buffer B (PBS, pH 7.3, 0.1% Tween 20), and washing buffer C (SSC buffer with 0.05% Tween 20, pH 7.2).

Detection of PDGF-BB

First, the anti-PDGF-BB antibody ($2\text{ }\mu\text{g mL}^{-1}$) in coupling buffer was added into the 96-well microplate (100 μL per well) and incubated for 1 h. Then, the plate was washed three times with

washing buffer A and blocked with blocking buffer containing 10 mg mL^{-1} BSA (200 μL each well) for 1 h. After removing the blocking buffer and washing the plate once with washing buffer B, 100 μL of PDGF-BB in PBS containing 2 mg mL^{-1} BSA was added into each well. After 1 h incubation, the solution was removed, and the wells were rinsed three times with washing buffer B. After that, 100 μL of 100 nM aptamer-primer prepared in PBS containing 1 mM MgCl_2 (heated at $85\text{ }^\circ\text{C}$ for 3 min, and then cooled at room temperature) was applied and incubated for 30 min. After discarding the solution and washing the plate three times with washing buffer B, 100 μL of DNA template (56 nM) in SSC buffer containing 0.05% Tween 20 was added into the plate. After 1 h incubation, the plate was rinsed three times with washing buffer C, and then 100 μL of 0.02 U μL^{-1} *E. coli* DNA ligase in ligation buffer was added into the plate and incubated for 1 h. Then, 100 μL of RCA reaction buffer containing 0.1 mM dNTPs, 0.4 U μL^{-1} phi29 DNA polymerase and 0.1 mg mL^{-1} BSA was directly applied in the wells and incubated for 1 h. After rinsing three times with washing buffer C, 100 μL of biotinylated probe (0.36 μM) diluted in SSC buffer containing 0.05% Tween 20 was added in the wells. After incubation for 30 min and washing the plate three times with washing buffer C, 100 μL of 80-fold diluted SA-HRP was added into the wells and incubated for 30 min. Then, the plate was washed four times with washing buffer B, and 100 μL of TMB substrate solution was added into the wells. After incubation for 15 min, the enzyme reaction was stopped by adding 100 μL of phosphoric acid solution (1.46 M). Finally, the absorbance was measured at the wavelength of 450 nm by a plate reader (Variskan Flash, Thermo Fisher Scientific, Inc.). The plate was incubated at $37\text{ }^\circ\text{C}$ in all experiments.

Results and discussion

Strategy of PDGF-BB detection using aptamers and RCA with absorbance analysis

Scheme 1 shows the schematic diagram of the aptamer and RCA-involved sandwich assay for PDGF-BB detection by absorbance analysis. Antibody is coated on a microplate, and PDGF-BB is specifically captured from the sample mixture by the coated antibody. Then, an aptamer-primer that contains an aptamer sequence against PDGF-BB and a primer sequence is added to bind with the PDGF-BB, and a sandwich complex is formed. The primer part of the sandwich complex can hybridize with a DNA template, and the template is ligated to form a circular template. Following the circular template, the RCA reaction produces a long DNA molecule with tandem repeated copies of complementary sequence of template in the presence of dNTPs and polymerase. The generated DNA product hybridizes with many biotinylated DNA probes, and these attached probes then bind with streptavidin-labeled HRP (SA-HRP) through the interaction between biotin and streptavidin, thus the extended DNA oligo is labeled with many HRP molecules. HRP converts the substrate of TMB to a colored product. The product becomes yellow when an acid stopping solution is added, and it can be measured by recording the absorbance at

Scheme 1 The schematic diagram of aptamer and RCA-involved absorbance assay for PDGF-BB.

450 nm. The detection of PDGF-BB is finally achieved by the absorbance analysis.

Optimization of experimental conditions

First, we tested whether the strategy was feasible for PDGF-BB detection. As shown in Fig. 1, a high absorbance signal was observed in the presence of PDGF-BB, while the blank sample gave only a small absorbance signal, showing that PDGF-BB could be detected in the demonstrated strategy.

We then investigated the effects of the reaction reagents on assay performance, including aptamer-primer, template, ligase, polymerase, dNTPs, biotinylated DNA probe, and SA-HRP. As the concentration of the aptamer-primer increased from 12.5 nM to 100 nM, the absorbance change obtained by PDGF-BB at 50 pM increased, and then the absorbance change decreased when the concentration of aptamer further increased (Fig. 2). Thus, 100 nM of aptamer-primer was selected for the following studies. The effect of the concentration of the template on the absorbance change was also tested in the range from 7 nM to 112 nM. The absorbance change increased with increasing template in the range from 7 nM to 56 nM, and

Fig. 1 Testing the feasibility of PDGF-BB detection. Experimental condition: aptamer-primer, 100 nM; template, 56 nM; ligase, 0.02 U μL^{-1} ; dNTPs, 0.1 mM; polymerase, 0.4 U μL^{-1} ; biotinylated probe, 0.36 μM ; SA-HRP, 80-fold diluted.

Fig. 2 Effect of the aptamer-primer on signal change obtained by PDGF-BB. Experimental condition: PDGF-BB, 50 pM; template, 56 nM; ligase, 0.02 U μL^{-1} ; dNTPs, 0.1 mM; polymerase, 0.4 U μL^{-1} ; biotinylated probe, 0.36 μM ; SA-HRP, 80-fold diluted.

reached a maximum value when 56 nM of template was applied (Fig. S1†). Therefore, 56 nM of template was employed for the further experiments. No large influence was observed in the obtained absorbance change when the ligase concentration varied from 0.0025 to 0.04 U μL^{-1} . Ligase at a concentration of 0.02 U μL^{-1} was chosen for the subsequent experiments.

Phi29 DNA polymerase and dNTPs are two important factors for extending the primer, and their amount significantly affected the obtained absorbance change. The absorbance change was large when polymerase at 0.4 U μL^{-1} was applied (Fig. 3). When the concentration of polymerase was higher than 0.4 U μL^{-1} , the obtained absorbance change by PDGF-BB decreased. The use of 0.1 mM dNTPs could give a large absorbance change, and dNTPs with concentration higher than 0.1 mM caused an eliminated absorbance change. Therefore, 0.4 U μL^{-1} polymerase and 0.1 mM dNTPs were applied in this work.

The biotinylated probe had a complementary part to the repeated sequence of the DNA oligo product of the RCA

Fig. 3 Effect of the phi29 DNA polymerase on obtained absorbance change. Experimental condition: PDGF-BB, 50 pM; aptamer-primer, 100 nM; template, 56 nM; ligase, 0.02 U μL^{-1} ; dNTPs, 0.1 mM; biotinylated probe, 0.36 μM ; SA-HRP, 80-fold diluted.

Fig. 4 Detection of PDGF-BB at varying concentrations with aptamer and RCA-involved absorbance assay.

reaction, and it could hybridize with the extended primer. Through the strong interaction between biotin and streptavidin, the SA-HRP could be labeled on the DNA oligo product of the RCA reaction. No remarkable variation in absorbance was caused with the biotinylated probe concentration ranging from 0.045 μM to 0.76 μM . It was likely that the amount of probe was enough when the concentration of probe reached 0.045 μM . Finally, 0.36 μM of biotinylated probe was applied for further studies.

The amount of the conjugate of streptavidin and HRP (SA-HRP) had a great influence on the absorbance (Fig. S2†). The blank and the signal increased simultaneously with the increase in the amount of the SA-HRP. However, the signal to blank ratio reached a maximum value when the 80-fold diluted SA-HRP was applied. Hence, 80-fold diluted SA-HRP was chosen in further experiments.

Detection of the PDGF-BB with RCA

Under the optimized condition, PDGF-BB at varying concentrations was successfully detected (Fig. 4). A good linear relationship was obtained between the absorbance change and the

Fig. 5 Selectivity of the assay for PDGF-BB. The concentration of tested protein: IgG, 6.7 μM ; Hb, 14.7 μM ; lysozyme, 0.69 μM ; thrombin, 10 nM; PDGF-AA and PDGF-AB, 50 pM; PDGF-BB, 50 pM.

concentration of the PDGF-BB in the range from 3.1 pM to 200 pM ($y = 0.0194x - 0.11$, $R^2 = 0.993$), and the detection limit was about 3.1 pM, which was determined by the absorbance change over the blank higher than 3 times the standard deviation of the blank signal. The obtained sensitivity is higher than that of a few aptamer-based assays for PDGF-BB due to the RCA amplification and HRP amplification.^{14,16,31–33} Compared with other RCA-involved and aptamer-based assays for PDGF-BB, the sensitivity of our assay is comparable to or lower than the reported sensitivity (Table S1†).^{26–30}

Selectivity

The selectivity of our assay for PDGF-BB was assessed by testing a few other proteins. As shown in Fig. 5, the absorbance signals for the high concentrations of the tested proteins (IgG, Hb, lysozyme, thrombin) were close to the blank value. PDGF-AA and PDGF-AB did not cause interference for the detection of PDGF-BB. The presence of high concentrations of tested proteins and PDGF-BB did not significantly influence the detection of PDGF-BB when these proteins coexisted with PDGF-BB. The results show that our strategy has good selectivity for the detection of PDGF-BB.

To examine whether this method could be used to detect PDGF-BB in a complex sample matrix, we analyzed PDGF-BB spiked in a diluted serum sample. The detection of PDGF-BB in 1% bovine serum was investigated. The spiked PDGF-BB could be successfully detected in the diluted serum samples (Fig. S3†). The recovery of PDGF-BB in 1% bovine serum ranged from 80% to 88% for the tested PDGF-BB at 12.5 pM, 25 pM, and 50 pM. This result shows the assay can be used to detect PDGF-BB in a complex sample matrix.

Conclusions

In summary, this paper described an aptamer-based absorbance assay for PDGF-BB detection by involving RCA reaction and horseradish peroxidase labeling as a signal enhancement technology. PDGF-BB was captured by antibody coated microplate, and then bound with aptamer-primer. The RCA reaction extended the primer to a long DNA oligo, which was then labeled with HRP. HRP catalyzed the chromogenic substrate to generate a colored product to achieve the final detection of PDGF-BB in absorbance analysis. This assay was sensitive and specific. The detection limit reached 3.1 pM. The strategy could be used to detect PDGF-BB in diluted serum sample.

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