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ORIGINAL ARTICLE

Highly sensitive chemiluminescence technology for protein detection using aptamer-based rolling circle amplification platform

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Abstract A robust, selective and highly sensitive chemiluminescent (CL) platform for protein assay was presented in this paper. This novel CL approach utilized rolling circle amplification (RCA) as a signal enhancement technique and the 96-well plate as the immobilization and separation carrier. Typically, the antibody immobilized on the surface of 96-well plate was sandwiched with the protein target and the aptamer–primer sequence. This aptamer–primer sequence was then employed as the primer of RCA. Based on this design, a number of the biotinylated probes and streptavidin–horseradish peroxidase (SA–HRP) were captured on the plate, and the CL signal was amplified. In summary, our results demonstrated a robust biosensor with a detection limit of 10 fM that is easy to be established and utilized, and devoid of light source. Therefore, this new technique will broaden the perspective for future development of DNA-based biosensors for the detection of other protein biomarkers related to clinical diseases, by taking advantages of high sensitivity and selectivity.

1. Introduction

Detection of protein biomarkers in clinical samples is of great importance for effective diagnosis and prognosis of many diseases [1]. In general, antibody-based immunoassay systems are versatile and powerful tools for various molecular analyses [2]. Enzyme-linked immunosorbent assay (ELISA) is considered as a standard method to detect protein biomarkers [3]. However, the concentrations of many important biological markers in clinical examples for cancer, infectious diseases, or biochemical processes are too low to be detected using conventional immunoassays [4]. Therefore, several innovative approaches have been developed to improve the sensitivity by combining antibody-based molecular recognition with various

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amplification detection techniques, such as metal ion deposition [5], enzyme amplification [6], bio-barcode detection [7,8], proximity ligation [9,10], metal nanoparticles [11] and quantum dots [12].

Among them, molecular probes based on nucleic acid platforms are emerging. One of the representative examples is the use of aptamer for protein analysis [13–16]. Aptamers are short synthetic nucleotide sequences (DNA or RNA) that have been selected in *in vitro* selection experiments *via* the systematic evolution of ligands by exponential enrichment (SELEX) process [17]. As a novel recognition element, aptamers possess significant advantages including simple synthesis, easy labeling, good stability and design flexibility [18,19]. In addition, aptamer-based assay is easy to combine with other amplification methods, such as gold nanoparticles, polymerase chain reaction (PCR) and rolling circle amplification (RCA) [20–28]. For example, Csordas et al. [22] reported a micromagnetic aptamer PCR (MAP) detection system, which integrated high-gradient magnetic field sample preparation in a microfluidic device with aptamer-based real-time PCR readout, to achieve highly sensitive and quantitative detection of protein targets directly from complex samples. RCA has also been proven to enhance signals for detecting a variety of analytes without thermal cycling. Additionally this RCA process is of linear kinetic amplification model and the RCA product was a single stranded DNA sequence consisting of tandem repeats of the complement of the circular template. Therefore, the RCA amplification strategy has been used to various detection protocols, including optical diffraction [23], fluorescent [24–26] and electrochemical [27,28] ones.

Chemiluminescence (CL) is considered one of the most suited optical detection techniques for developing miniaturized and highly sensitive analytical devices [29]. Even though the quantum efficiency of CL reactions is usually low (in the order of 0.01 or less), the use of enzyme labels such as horseradish peroxidase (HRP) or alkaline phosphatase (ALP), ensures signal amplification and high sensitivity [30,31]. Because of the wide dynamic range of the CL measurements (up to 6 orders of magnitude), the target analyte can be detected in a broad concentration range, from femtomolar to millimolar levels, without the need of sample dilution. Finally, the absence of an excitation light source as in fluorescence measurements makes CL detection less sensitive to interferences attributed to sample components (the only background signal derives from the instrumental noise) and allows a wide range of applications in different fields such as environmental chemistries [32], molecular biology [33,34], pathogenic bacteria [35], clinical diagnosis [36] and cell sensors [37], including several research works in our group [38–41].

Herein, we present an example of the aptamer-based RCA assay by coupling of CL detection for the ultrasensitive protein assay. Platelet-derived growth factor B-chain (PDGF-BB), an important protein for cell transformation and tumor growth and progression, was selected as the model protein.

2. Materials and methods

2.1. Materials and reagents

All chemicals were of analytical grade and were used as received. The DNA-BIND 96-well plate (Costar, 6573) had an *N*-oxysuccinimide surface and was obtained from Corning Inc. (NY, USA). The PDGF-BB proteins and goat anti-human PDGF-BB antibody were purchased from R&D Systems (Minneapolis, MN, USA). The PDGF proteins were reconstituted in 4 mM HCl with 0.2% BSA prior to use. Phi 29 reagent set was purchased from Epicenter (Madison, WI, USA). *Escherichia coli* DNA ligase was obtained from Takara Biotechnology Co., Ltd. (Dalian, China). Streptavidin–horseradish peroxidase (SA–HRP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Sino-American Biotechnology Co. and other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HRP substrate kits were purchased from Millipore Corporation (Billerica, MA, USA). Oligonucleotides were obtained from Invitrogen Biotechnology Co., Ltd. (Shanghai, China), including the following sequences (Table 1).

2.2. Buffers

All of the solutions were prepared with water from a Millipore system (Millipore XQ, MA, USA). Binding buffer (BB) was 0.5 M Na_2HPO_4–NaH_2PO_4, pH 8.5. PBS consisted of 2.7 mM KCl, 1 mM $MgCl_2$, pH 7.4. PBST was PBS containing 0.05% Tween 20. Blocking buffer was PBS containing 5% BSA. Ligation buffer (LB) consisted of 30 mM Tris–HCl, pH 8.0, 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1.2 mM EDTA and 0.1 mM b-NAD. RCA reaction buffer was 40 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$ and 4 mM DTT.

2.3. Apparatus

CL measurement was carried out using a PC-controlled Fluoroskan Ascent FL (Thermo Electron Corporation).

2.4. Sandwich assay with RCA or without RCA

In a typical experiment, goat anti-human PDGF-BB antibody was diluted to 0.75 μg/mL in BB and added into the wells in a 96-well plate (100 μL each well). The plate was incubated for 1 h with gentle mixing at 37 °C. Excess PDGF-BB antibody was removed by decanting the supernatant. The plate was washed and blocked with blocking buffer (150 μL per sample) for 60 min at 37 °C. After decantation of blocking buffer, series dilutions of PDGF-BB were prepared in 100 μL PBS

Table 1 DNA sequences used in this work.

Name	Sequence
Aptamer–primer	5′-TAC TCA GGG CAC TGC AAG CAA TTG TGG TCC CAA TGG GCT GAG TAT TTT TTT TGT CCG TGC TAG AAG GAA ACA GTT AC-3′
Padlock probe	5′-phosphate-TAG CAC GGA CAT ATA TGA TGG ACC GCA GTA TGA GTA TCT CCT ATC ACT ACT AAG TGG AAG AAA TGT AAC TGT TTC CTT C-3′
Biotinylated probe	5′-biotin-GTT TCC TTC TAG CAC-3′

and added into the above wells and incubated for another 40 min with gentle mixing at 37 °C. After washing, 100 μL of 7.5 pmol aptamer-primer complexes in PBS and 5 pmol padlock were added into the wells, which was incubated for 60 min at 37 °C. And then the padlock probe was circularized via ligation by 1 U of *E. coli* DNA ligase in LB (100 μL each well) for 60 min at 37 °C to form the circular template for RCA. The complex was incubated with 100 μL of 40 U phi29 DNA polymerase and 100 μM dNTPs in RCA reaction buffer for 75 min at 37 °C. After a rinsing step, 100 μL of 7.5 pmol biotinylated probe (at the 5′ end) was applied to and hybridized with the resulted RCA products, which then captured the SA–HRP (1:7500 dilution, 100 μL per well). Finally, the plate was washed three times with 150 μL of PBST. The CL signals on the surface of each conjugates were detected directly with 100 μL of commercial CL HRP substrate.

In the assay without RCA, after the binding of PDGF-BB onto the surface of wells, 7.5 pmol aptamer–primer complexes were added to react with the immobilized PDGF-BB. After the plate was washed three times with PBST (150 μL each time), 7.5 pmol of biotinylated probe in 100 μL PBS and 1:7500 dilution of SA–HRP were added and captured on the surface of the wells sequentially (each for 30 min at 37 °C). Finally, the plate was washed three times with 150 μL of PBST. The CL signals were also detected as described above.

3. Results and discussion

3.1. Design of the aptamer-based assay

A "sandwich-type" detection strategy was employed with HRP as the detection tag in our design (Fig. 1), which involved the sequential immobilization of capture antibody, PDGF-BB and aptamer–primer onto the 96-well plate surface in a classic sandwich assay format. The aptamer–primer had two functional units. The aptamer part was used to recognize and bind PDGF-BB, and the primer part at the 3′-terminus was employed as a universal primer sequence to initiate a linear RCA reaction in the presence of Phi 29 polymerase and dNTPs. Both the 3′- and 5′-terminus of a padlock probe could hybridize to the 3′-terminus of the aptamer–primer. Ligation of the padlock probe resulted in a circular template for RCA, which then produced single-stranded tandem repeated copies of the circular template. The tandem repeated copies in the RCA product were complementary to the biotinylated probe. Thus, the RCA product could hybridize a number of biotinylated probes, which then captured SA–HRP. After the CL substrates were added, the CL signal was recorded immediately with the CL measurement. In contrast, for the assay without RCA, the biotinylated probes directly hybridized the aptamer–primer, which had been immobilized on the surface of the 96-well plate as the formation of antibody–antigen–aptamer complex, and then SA–HRP reacted with the captured biotinylated probes. Finally, the CL signal was also obtained by the reaction with CL substrates and recorded by the CL measurement. All experiments were performed at least triplicate.

3.2. Optimization of reaction parameters

Several parameters were optimized systematically for the highly sensitive approach of protein assay, including the amounts of capture antibody, aptamer–primer, padlock, ligase, polymerase, dNTPs, biotinylated probe and SA–HRP.

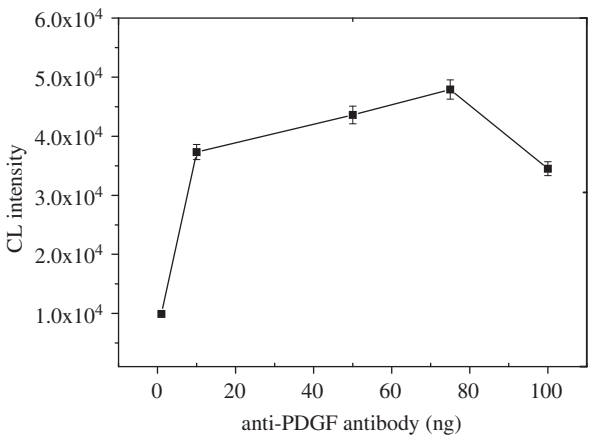

Figure 2 CL intensity *vs.* the capture antibody amount. Experimental conditions: 5.0 pmol of aptamer–primer, 2.1 pmol of padlock, 6 U of ligase, 25 U of polymerase, 100 μM of dNTPs, 2.5 pmol of biotinylated probe, 1:5000 dilution of SA–HRP and 10 pM PDGF-BB. The detection procedure was carried out as described in the Experimental Section 2.4.

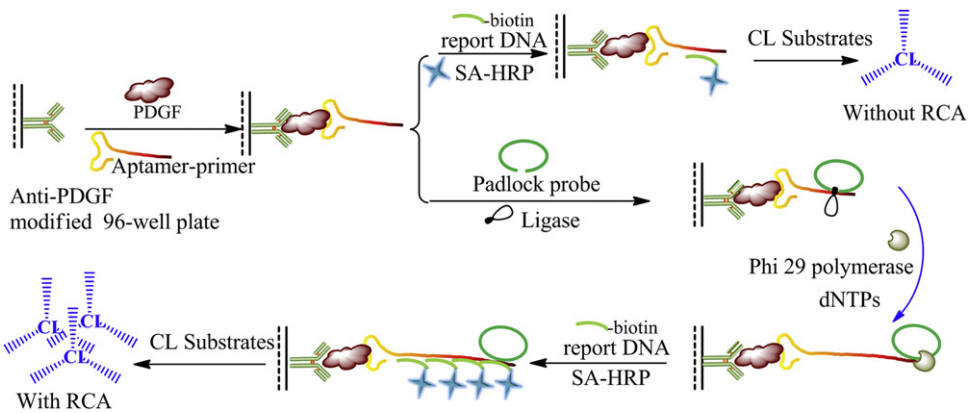

Figure 1 Schematic representation of aptameric system for the detection of PDGF-BB with or without RCA.

3.2.1. Effect of the capture antibody amount

The amount of capture antibody on the wells played an important role in the performance of the immunoassay. Several amounts of anti-PDGF-BB antibody were investigated as shown in Fig. 2. Elevated CL intensity was observed with increasing amount of the capture antibody, and peaked at 75 ng of capture antibody. Further increase in the amount of capture antibody to 100 ng resulted in a decrease of the CL intensity. This was attributed to steric and electrostatic hindrances, arising from more tightly packed capture antibody on the surface of wells, limiting access to the surface-bound capture antibody by PDGF-BB antigen. Thus, 75 ng capture antibody was selected for subsequent experiments.

3.2.2. Effects of the amounts of the aptamer–primer and padlock

The effects of aptamer–primer and padlock amounts were subsequently examined and optimized. CL intensity increased in the range of 0.1–7.5 pmol of aptamer–primer and then decreased slightly (Fig. 3A). CL intensity increased with increasing amount of padlock sequences over the range of 0.1–5.0 pmol, and then decreased (Fig. 3B). Thus, 7.5 pmol of aptamer–primer and 5.0 pmol of padlock sequences were selected in further studies.

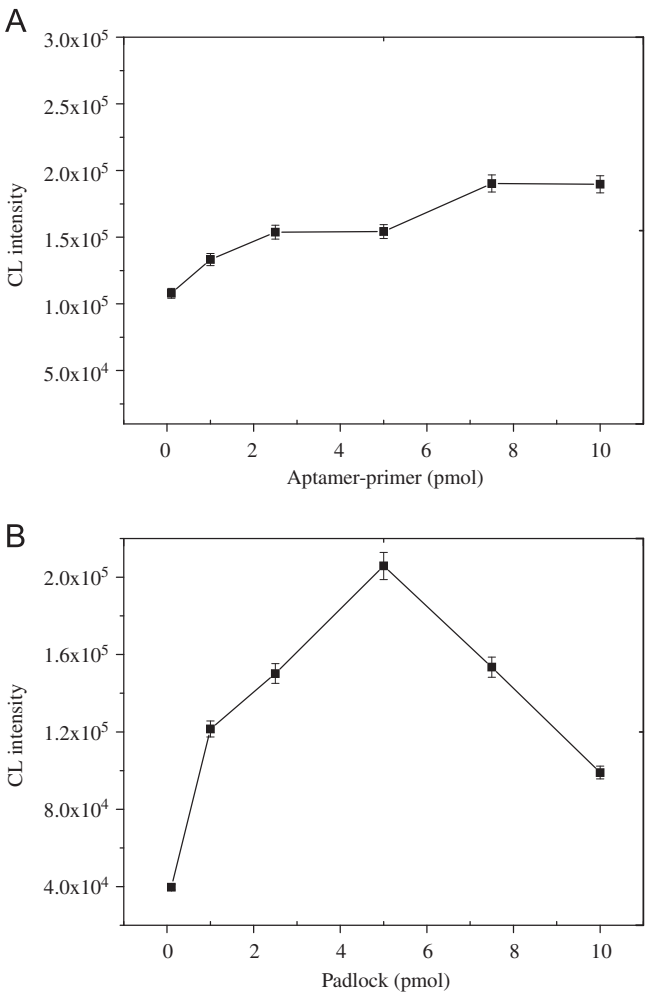

Figure 3 CL intensity *vs*. the amounts of aptamer–primer (A) and padlock (B). Experimental conditions: 75 ng of PDGF-BB antibody, 6 U of ligase, 25 U of polymerase, 100 μM of dNTPs, 2.5 pmol of biotinylated probe, 1:5000 dilution of SA–HRP and 10 pM PDGF-BB. A, 2.1 pmol of padlock; B, 7.5 pmol of aptamer–primer. The detection procedure was carried out as described in the Experimental Section 2.4.

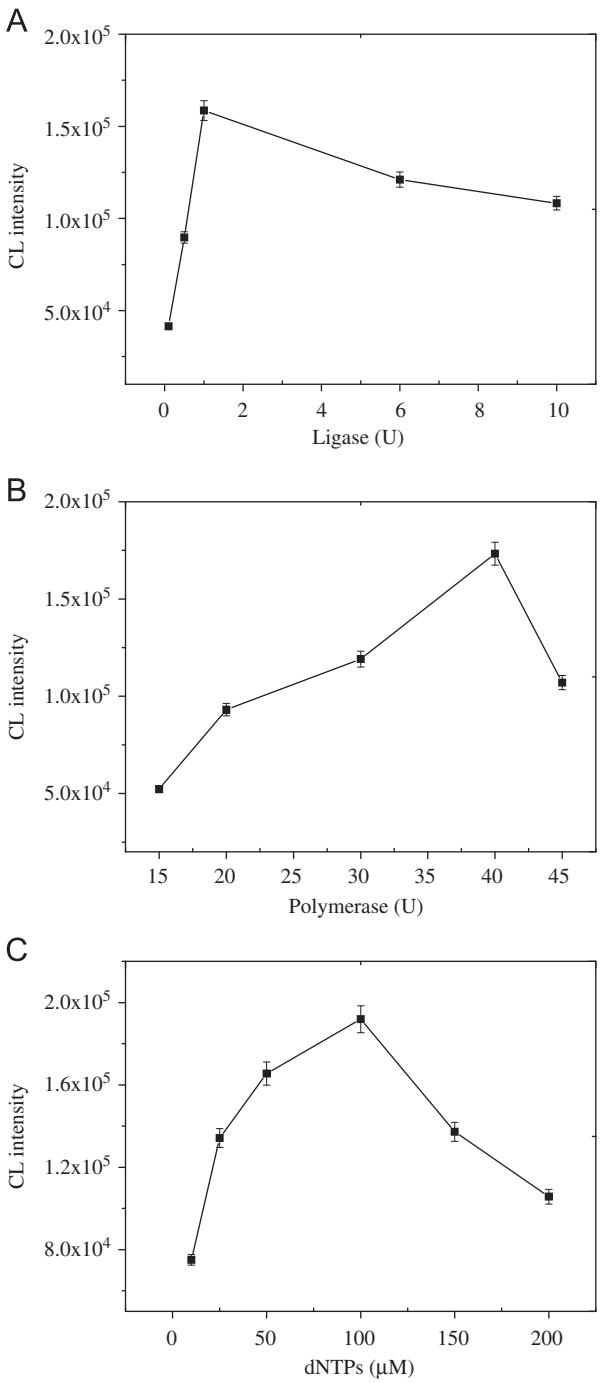

Figure 4 CL intensity *vs*. concentration of RCA reagents. Experimental conditions: 75 ng of PDGF-BB antibody, 7.5 pmol of aptamer–primer, 5.0 pmol of padlock, 2.5 pmol of biotinylated probe, 1:5000 dilution of SA–HRP and 10 pM PDGF-BB. A, 25 U of polymerase, 100 μM of dNTPs; B, 1 U of ligase and 100 μM of dNTPs; C, 1 U of ligase and 40 U of polymerase. The detection procedure was carried out as described in the Experimental Section 2.4.

3.2.3. Dependence of CL intensity on the amount of RCA reagents

The amounts of ligase, polymerase and dNTPs also affected the CL signal in some extent. As shown in Fig. 4, CL intensity started to increase rapidly as the concentration of ligase increased from 0 to 1 U, and reached a maximum with 1 U of ligase. Further increase in the amount of ligase to 10 U caused a decrease in the CL intensity. Thus, 1 U of ligase was selected for subsequent experiments. Similarly, 40 U of polymerase and 100 μM of dNTPs were selected respectively in further studies.

3.2.4. Dependence of CL intensity on the amount of report reagents

The amounts of biotinylated probe and SA–HRP were two vital factors for the amplification of CL signal. Therefore, these parameters were also investigated. As shown in Fig. 5, the range of 0.5–10.0 pmol of biotinylated probe and 1:1000 to 1:15 000 dilution of SA–HRP were studied for the optimal condition. Finally, the maximum CL intensity was obtained by using 7.5 pmol of biotinylated probe and 1:7500 dilution of SA–HRP, which were thus employed for the subsequent experiments.

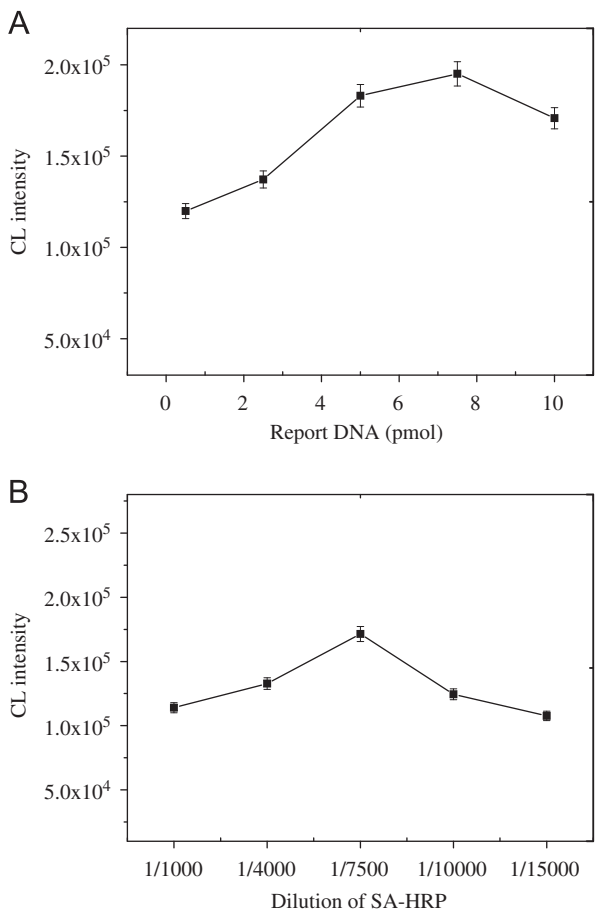

Figure 5 CL intensity *vs*. the concentration of biotinylated probe and SA–HRP. Experimental conditions: 75 ng of PDGF-BB antibody, 7.5 pmol of aptamer–primer, 5.0 pmol of padlock, 1 U of ligase, 40 U of polymerase, 100 μM of dNTPs and 10 pM PDGF-BB. A, 1:5000 dilution of SA–HRP; B, 2.5 pmol of biotinylated probe. The detection procedure was carried out as described in the Experimental Section 2.4.

3.2.5. Analytical performance with or without RCA

Under the above-optimized experimental conditions, the quantitative behavior of the method was assessed by monitoring the dependence of the CL intensity on the concentration of the target PDGF-BB. As shown in Fig. 6, a linear relationship was obtained between the CL intensity and the amount of PDGF-BB in the range of 10 fM–1 nM (lgI = 0.836 lgC + 0.928,

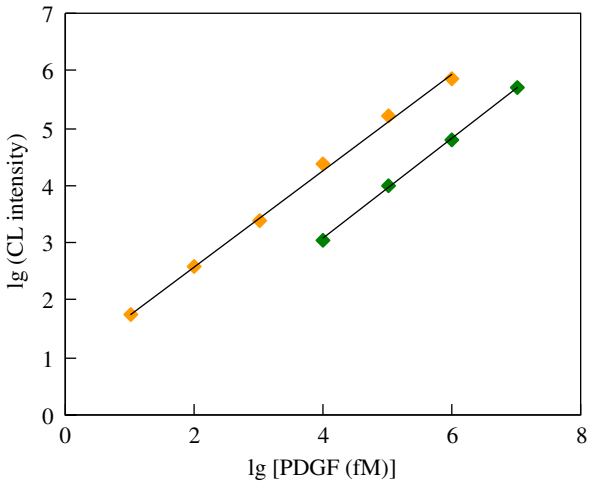

Figure 6 Log–log calibration data for PDGF-BB by using the protocol with RCA (up) and without RCA (down). The detection procedure was carried out as described in the Experimental Section 2.4.

Table 2 Comparison of sensitivity for different aptamer-based assays for PDGF-BB.

Analytical method	Label	Detection limit
Fluorescence	Cy3	0.4 nM [25]
Fluorescence	FAM and DABCYAL	6.8 pM [26]
Fluorescence	Fluorescein	110 pM [42]
Fluorescence	TOTO	0.1 nM [44]
Fluorescence	SYBR-green	0.1 ng/mL [45]
Fluorescence	Dyes	10 ng/μg [46]
Electrochemical	AP	10 fM [27]
Electrochemical	Methylene blue	63 pM [28]
Electrochemical	Fc	1.0 pg/mL [43]
Densitometry	Gold nanoparticles (AuNPs)	83 aM [20]
Diffractometric	Microbeads	10 pM [23]
Photoluminescence	Au-nanodots NPs	10 pM [47]
Colorimetric	AuNPs	35 nM [48]
Capillary Electrophoresis	FAM	50 pM [49]
Luminescence	$[Ru(phen)_2(dppz)]^{2+}$	1.0 nM [50]
Chemiluminescence	HRP	10 fM (this work)

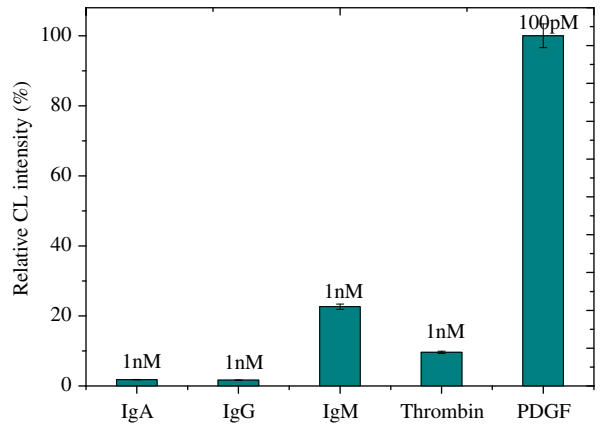

Figure 7 Selectivity of the proposed strategy. Experimental conditions: 75 ng of PDGF-BB antibody, 7.5 pmol of aptamer–primer, 5.0 pmol of padlock, 1 U of ligase, 40 U of polymerase, 100 μM of dNTPs, 7.5 pmol of biotinylated probe, 1:7500 dilution of SA–HRP, 100 pM PDGF-BB and 1 nM of IgA, IgG, IgM and thrombin. The detection procedure was carried out as described in the Experimental Section 2.4.

$R^2=0.997$, where I is the CL intensity and C is the concentration of target PDGF-BB). And the detection limit was estimated at 10 fM. For comparison, the performance of aptamer-based sandwich assay without RCA was also studied. A good linear correlation was presented between the CL intensity and the concentration of PDGF-BB in the range of 10 pM–10 nM ($\lg I=0.879 \lg C-0.446$, $R^2=0.998$). A 1000-fold enhancement of sensitivity was achieved based on RCA amplification. Thus, it was demonstrated that RCA is powerful for signal amplification. Furthermore, our CL protocol allowed detection of PDGF-BB down to fM level and was competitive with other aptamer-base PDGF-BB assays as reported previously (Table 2).

3.2.6. Detection specificity

Sensitivity and specificity were the two critical factors for a successful assay system. In the proposed strategy, the sensitivity of our assay compared favorably with previous efforts. The detection specificity was also determined and evaluated by the aptameric recognition function. As shown in Fig. 7, compared with 100 pM PDGF-BB, the CL intensity from 1 nM of the non-target proteins (IgG, IgA, IgM, interferon and thrombin) was low. It was demonstrated that the CL signal was specifically triggered by the aptamer/target binding.

4. Conclusion

A new CL platform for the determination of protein has been demonstrated by the combination of the aptamer as the identification unit and RCA as the amplification unit. This aptamer-RCA based CL technique has significant advantages, including improved sensitivity and high selectivity. The detection limit of this novel protocol was demonstrated down to 10 fM, which is a 1000-fold improvement compared to non-RCA assay, and also comparable to or even better than what was provided by most previous reports. Furthermore, our protocol might discriminate the target protein from 10-fold concentration of other proteins. In summary, this aptamer-RCA based CL technique possesses great potential to serve as the PDGF-BB diagnostic tool in clinical test by virtue of its sensitivity and selectivity. In addition, this new approach can also be easily extended to the detection of other biomarkers relating to other diseases, such as IgE or thrombin, etc.

Acknowledgments

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