Supporting Information

Aptamer Degradation Inhibiting Combined with DNAzyme Cascade-Based Signal Amplification for Colorimetric Detection of Protein

Jishan Li, Yuhua Jia, Jing Zheng, Wenwan Zhong, Guoli Shen, Ronghua Yang and Weihong Tan

Reagents and Materials. The oligonucleotides used in this work (Table S1) were synthesized from Takara Biotechnology Co. Ltd. (Dalian, China). Recombinant human PDGF-BB and PDGF-AB were purchased from Bowling Vaccine & Pharmaceutical Inc. (San Francisco, CA). Bovine serum albumin (BSA), human serum albumin (HSA), human immunoglobulin G (IgG), thrombin, bovine serum were obtained from Dingguo Biotechnology Co. Ltd. (Beijing, China). *Escherichia coli* Exonuclease I (Exo I) was obtained from New England Biolabs (Ipswich, MA). SYBR Gold (supplied as 10,000×concentrate in DMSO) gel stain for polyacrylamide gel electrophoresis (PAGE) was obtained from Invitrogen (Carlsbad, CA). 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS), Polyacrylic acid (PAA), diethylene glycol (DEG), and mercaptoacetic acid (MAA) were purchased from Sigma-Aldrich Chemical Co. H_2O_2, hemin, tris(hydroxymethyl) aminomethane (Tris), HEPES, Lead acetate ($PbAc_2$), reagents for making the stopping solution and denatured gel, and all other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (electrical resistance of >18.3 MΩ) (Billerica, MA).

Synthesis of PbS Nanocrystals (NCs). To synthesize PbS NCs, first we prepared solution A by heating the mixture of 0.5 mmol $PbAc_2$ and 288 mg PAA in

10 mL DEG at 240 °C, until it turned transparent. At the same time, 4 mmol sulfur powders and 288 mg PAA were dissolved in 15 mL DEG at 240 °C, producing a bright yellow and transparent solution (solution B). Then solution A was quickly injected to solution B with a glass syringe, which caused the temperature drop to 226 °C. After 2 min reaction, the flask was cooled down to room temperature, and black PbS NCs were separated from the solution with ethanol washing and centrifugation. Finally, these PbS NCs were dispersed in 5 mL water for storage. The transmission electron microscopic (TEM) image and the selected-area electron diffraction (SAED) of the prepared NCs were shown as Figure S1 and the diameter of the synthesized PbS NCs is ca 10 nm.

Gel Electrophoresis Assay of Exo I-catalyzed Degradation. Gel electrophoresis was conducted using the 15% polyacrylamide gel. The sample with loading buffer was denatured in boiling water for 1 min and stored on ice before loading. Twenty μL of each sample was loaded into the individual wells, and the gel was run at 200 V for 1 hrs. After 10-min gel staining by using SYBR Gold, the gel image was carried out with a ChemiDoc XRS+ Imaging System (Bio-RAD).

Colorimetric/absorbance Measurement. For the colorimetric measurement, the ABTS/H_2O_2 substrate (1 mM and 2 mM, respectively) was added to the reaction solution to yield a total volume of 300 μL. The curves of absorbance at 405 nm vs. time were obtained using a Hitachi U-4100 UV/Vis spectrophotometer (Kyoto, Japan).

Measurement of the Target Proteins. For detection of target proteins, 50 μL A17E5 (300 nM) in Tris-HCl buffer (pH 7.4, 20 mM K^+, 1 mM Mg^{2+}, 140 mM Na^+) was first incubated with PDGF-BB samples for 30 min under room temperature. And then one 30-min enzymatic digestion process was carried out by the addition of 10 U Exo I and followed another 30-min enzymatic digestion process by the addition of Random DNA (the final concentration is 300 nM). After terminating the exonuclease degradation reaction through a 15-min denaturing process at 85 ^{0}C, the hairpin-structured substrates (the final concentration of G17S8 is 200 nM), PbS NCs

(the final concentration of Pb element is 20 μM) and hemin (the final concentration is 1μM) was added and this mixture was incubated for 1 h under room-temperature. Finally, the absorbance measurement was carried out by addition of ABTS (The final concentration is 1 mM) and H_2O_2 (The final concentration is 2 mM). After the test, the added PbS NCs can be reclaimed via a centrifugation process under 14000g for 20 min.

Cleavage Reactions Using Pb^{2+} or PbS NCs as Cofactor. In general, the 17S and 17E were denatured in the incubation buffer (50 mM HEPES (pH 7.0) with 50 mM $NaNO_3$) at 95 °C for 2 min and then cooled down to room temperature. After 30 min, the mixture was put on ice (0-4 °C) and sat for another 30 min (hybridization solution). Then the cofactor solution was added to initiate the cleavage reaction. The reaction took place on ice and was terminated by adding the equal volume stopping solution. The incubation buffer was treated with the Chelex beads to remove the divalent metal ion impurities before usage. The stopping buffer contained 50mM EDTA, 8M urea, 90mM Tris, 90mM boric acid, 0.05% xylene cyanol and 0.05% bromophenol blue. For rate constant measurement, 60 μL of the 17S (1 μM) and 17E (1.5 μM) hybridization solution was prepared. After removing 5 μL to be the blank reaction, 55 μL metal-containing solution (metal concentration equal to 71.5 μM) was added to cleave the substrate. At various time points of 0.5, 1, 2, 3, 4, 5, 7, 10, 15 min, 10 μL reaction mixture was taken out to mix with 10 μL stop solution. For affinity measurement, 5 μL of the 17S (1 μM) and 17E (1.5 μM) hybridization solution was incubated with 5 μL Pb^{2+} or 10-nm PbS NCs solution for 3 min before 10 μL stop solution was added. The total Pb concentrations were 0.0715, 0.143, 0.715, 1.43, 3.575, 7.15, 14.3, 71.5, 143, and 357.5 μM. The Pb concentration in stock nanomaterials solution was measured by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES). Gel electrophoresis was conducted using the 20% polyacrylamide gel. The sample was denatured in boiling water for 1 min and stored on ice before loading. Twenty μL of each sample was loaded into the individual wells, and the gel was run at 200 V for 2 hrs. The gel image was scanned with a Typhoon

Fluorescence image and the intensity of the cy3 labeled substrate was calculated according to the gray scale of the image. Cleavage yield was defined as the ratio of the amount of the cleaved substrate divided by the total amount of the cleaved substrate and the uncleaved substrate.

Optimization of recognition probe and signal probe. To optimize stem-length of the aptamer probe, aptamer sequences with different stem-length at the 3′-terminus were first treated by Exo I before or after they were incubated with PDGF-BB, then gel electrophoresis was conducted. Briefly, 20 μL 300 nM of AS3, AS4, AS5 or AS6 in Tris-HCl buffer (pH = 7.4, 20 mM K^+, 1 mM Mg^{2+} ,140 mM Na^+) with or without 150 nM of PDGF-BB was first incubated for 1 h under room temperature. Then 4 U Exo I was added into the reaction mixture and incubated for 1 h under 35 ^{0}C. Finally, the exonuclease degradation reaction was terminated through a 15-min denaturing process at 85 ^{0}C and the obtained reaction mixtures were analyzed by gel electrophoresis. As shown in Figure S3, the stem length of 5-bases pair is the best. That is to say, for the aptamer probe with 5-bases stem structure, it will be degraded in the absence of target protein, while the Exo I-catalyzed degradation can be prevented when the aptamer sequence is bound to its protein target.

The carefully designed signal probe has a feasible hairpin-structure (Figure S5) and the thermodynamic parameters of the probe were calculated using bioinformatics software (http://mfold.rna.albany.edu/).[1]

Comparison of Substrate Cleavage Catalyzed by Pb^{2+} and PbS NCs.

The cleavage rates of Pb^{2+} and PbS NCs were compared in the present study. The 8-17 DNAzyme was annealed with the substrate before the addition of the metal-containing solutions. One micromolar Cy3-labelled 17S and 1.5 μM 17E were heated to 95 °C for 2 minutes and hydribized in two 30-min incubation steps, one at room temperature and the other at 4 °C. Afterwards, the solution containing either the free Pb^{2+} or the PbS nanocrystals (NCs) was added to initiate the catalytic reaction. All solutions contained the same Pb concentration of 71.5 μM, and the cleavage reaction took place at 4 °C with a reaction volume of 10 μL. After terminating the

catalytic reaction with a stopping solution at various times, the cleaved product was separated from the full-length substrate by denaturing gel electrophoresis (Figure S7A), and calculated the yield ($\%P_t$) using the band density resulted from the Cy3 emission. The nanostructures did not seem to interfere with the separation probably because they could not enter the gel. The plots of $\%P_t$ vs. reaction time t (Figure S7B) were fit to the following equation

$$\%P_t = \%P_0 + \%P_\infty (1-e^{-kt}) \quad (1)$$

This equation describes the single-turnover cleavage reaction, in which $\%P_0$ and $\%P_\infty$ is the yield at t = 0 and at the endpoint of the reaction ($t = \infty$), respectively, and k is the observed reaction rate.[2,3] Although figure S7B shows that the cleavage by both the PbS NCs and free Pb^{2+} reached completion with the same final yield above 65% at last time, the 10-nm PbS NCs showed a faster cleaving rate during the first 5 minutes. The reaction rates (k_{obs}) from curve fitting were listed in Table S2. The 10-nm NCs led to a cleavage rate 3 times higher than that of the free Pb^{2+} with the same total amount of lead.

The cleavage was not due to the leaked Pb ions from the PbS NCs because the supernatant of NCs solutions after filtration with the Amicon YM-30 filter could not cleave any substrate beyond the background level. The adsorption was disrupted by 0.1% SDS, indicating that electrostatic interaction between the surface cation and the negatively charged SDS or DNA might be dominant driving force for the interaction. Therefore, direct coordination of the DNAzyme with the surface Pb cation may lead to the cofactor activity of the PbS NCs. The affinity of PbS to DNAzyme was quantitatively measured through examining the change of k_{obs} with Pb concentration. The plots of k_{obs} vs. Pb concentration (Figure S7C) followed the equation shown below:

$$k_{obs} = k_{max} \cdot [Pb^{2+}] / (K_{d,app} + [Pb^{2+}]) \quad (2)$$

where $K_{d,app}$ is the apparent dissociation constant. The k_{obs} values were approximated by the linear cleavage rate within the first 3 minutes of reaction. The K_d values are listed in Table S2. Pb ions of the PbS NCs have an affinity more than 10 times higher than that of the free Pb^{2+}. Within each NC, about 11% Pb atoms are present at the

surface, taking into account of its molecular weight, density, and the cubic structure.[2] Since only the surface Pb ions could interact with the adsorbed DNAzyme, the K_d calculation should involve only the surface Pb, which then actually possessed a K_d 100 times lower than the free lead ions. The high affinity of surface cations to DNAzyme may ultimately lead to the cofactor activity of NCs.

Effect of surface coating on the cleavage reaction of PbS NCs was also investigated. The PbS NCs were coated with PAA molecules (average Mw ~ 1,800). The carboxyl groups on PAA act like bidentate ligands and coordinately bind to the NC surface cations.[4,5] The DNAzyme may need to compete with the PAA molecules for the coordination sites on NC surface, which would be difficult if PAA molecules are crosslinked or conjugated with larger biomolecules. To prove this hypothesis, we coupled the 10-nm PbS with human IgG (Mw ~ 150 kDa), one random 40-nt ssDNA (Mw ~ 13 kDa) that had no enzymatic activity, and the 8-17 enzyme strand (17E), respectively, and tested their capability in cleaving the 17S substrate. The unconjugated PbS NCs and NCs coupeld with IgG (PbS-IgG) or random DNA (PbS-DNA) were added to the annealed 17E-17S solution (17E:17S ratio was 1.25:1 with 1 μM Cy3-17S) at 4 °C to perform the typical cleavage reaction for 10 minutes. The final Pb concentration in these solutions was 71.5 μM. However, no cleavage by the PbS-IgG and PbS-DNA was observed (Figure S7D), indicating that after conjugation the PbS NCs exhibited no catalytic effect on the 8-17 DNAzyme. Crosslinking the large biomolecules with PAA, which gives more complete surface coverage, seems to block the interaction between the DNAzyme and the NC surface, inhibiting the cleavage reaction.

Effect of Random DNA on the Sensing System. To investigate the effect of random DNA on the aptamer-protein binding interaction, 20 μL 300 nM of A17E5 in Tris-HCl buffer (pH = 7.4, 20 mM K^+, 1 mM Mg^{2+} ,140 mM Na^+) was first incubated with or without 150 nM of PDGF-BB for 30 min under room temperature. And then one 30-min enzymatic digestion process was carried out by the addition of 4 U Exo I and followed another 30-min enzymatic digestion process by the addition of 0 or 300

nM Random DNA. Finally, the exonuclease degradation reaction was terminated through a 15-min denaturing process at 85 ^{0}C and the obtained reaction mixtures were analyzed by gel electrophoresis. As shown in Figure S8A, clear A17E5 band (lane 3) can still be observed when the random DNA is contained, while no significant band can be observed if the target protein PDGF-BB is not presented (lane 4), indicating that the addition of random DNA sequence has no effect on the aptamer-protein binding interaction and the digestion reaction of Exo I.

To investigate the effect of random DNA on the signal probe of G17S8, the following experiments were carried out. Briefly, the 300 μL solution of '**(a)** 200 nM G17S8 + PbS NCs' (Pb element concentration is 20 μM), '**(b)** 200 nM G17S8 + PbS NCs + 300 nM R1', '**(c)** 200 nM G17S8 + PbS NCs + 300 nM R1 + 50 nM A17E5', or '**(d)** 200 nM G17S8 + PbS NCs + 50 nM A17E5' was first incubated for 30 min, then hemin was added (the final concentration is 1 μM) and the mixture was incubated for 30 min under room-temperature to form the HRP-mimicking DNAzyme. Finally, the absorbance measurement was carried out by addition of ABTS and H_2O_2. As shown in Figure S8B, the solutions of (a) and (b) have the same absorbance, and there are not significant difference between solution (c) and (d), indicating that the random DNA has no effect on the signal probe of G17S8 and also no effect on the cleavage reaction of substrates.

To verify whether the introduction of random DNA can reduce background signal, several experiments were carried out. **Experiment (a)**: The solution of '300 μL 200 nM G17S8 + PbS NCs (Pb element concentration is 20 μM)' was first incubated with 1 μM hemin for 1 h under room temperature, then absorbance measurement was carried out by addition of ABTS and H_2O_2. **Experiment (b)**: The solution of '300 μL 200 nM G17S8 + PbS NCs + 50 nM A17E5' was first incubated with 1 μM hemin for 1 h under room temperature, then absorbance measurement was carried out by addition of ABTS and H_2O_2. **Experiment (c)**: 10 U Exo I was added into the 50 μL 300 nM A17E5 solution and incubated for 1 h under 35 ^{0}C, then the exonuclease degradation reaction was terminated through a 15-min denaturing process at 85 ^{0}C and the solution was diluted to 300 μL using Tris-HCl buffer. Finally,

'G17S8 (the final concentration is 200 nM) + PbS NCs + hemin (the final concentration is 1 μM)' was added and this mixture was incubated for 1 h under room-temperature, then absorbance measurement was carried out by addition of ABTS and H_2O_2. **Experiment (d)**: 10 U Exo I was added into the 50 μL 300 nM A17E5 solution and incubated for 30 min under 35 ^{0}C, then another 30-min enzymatic digestion process was conducted by the addition of Random DNA (the final concentration is 300 nM). After terminating the exonuclease degradation reaction through a 15-min denaturing process at 85 ^{0}C, the solution was diluted to 300 μL using Tris-HCl buffer. Finally, 'G17S8 (the final concentration is 200 nM) + PbS NCs + hemin (the final concentration is 1 μM)' was added and this mixture was incubated for 1 h under room-temperature, then absorbance measurement was carried out by addition of ABTS and H_2O_2. As shown in Figure S8C, the absorbance curve for the random DNA-introduced solution (curve d) is closed to the background curve (curve a), indicating that the addition of random DNA can move the equilibrium of enzymatic digestion reaction toward to the positive direction, causing further degradation of the free A17E5 sequences, thereby reducing the background signal resulted from those free sequence-triggered substrate cleavage.

References

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[2] D. Faulhammer and M. Famulok, *Angew. Chem. Int. Ed.* 1997, **35**, 2837-2841.
[3] R. P. G. Cruz, J. B. Withers and Y. Li, *Chem. Biol.* 2004, **11**, 57-67.
[4] T. K. Stage-Zimmermann and O. C. Uhlenbeck, *RNA* 1998, **4**, 875-889.
[5] A. K. Brown, J. Li, C. M. B. Pavot and Y. Lu, *Biochem.*, 2003, **42**, 7152-7161.

Table S1. DNA sequences used in this work.

Name	Sequences (5'-3') *
A17E5	CATCTCTTCTCCGAGCCGGTCGAAATAGTTAGTACAGGCTACGGCACGTAGAGCATCACCATGAT CCTGT
G17S8	CCCGCCCTTGAGACTAACTAT**rA**GGAAGAGATG**GGGTAGGGCGGGTTGGGT**
Random DNA	CCTGACTTTTATGCC
AC5	CATCTCTTCCTATAGTTAGTACAGGCTACGGCACGTAGAGCATCACCATGATCCTGT
AS3	AAA **AGG** CTACGGCACGTAGAGCATCACCATGAT **CCT**
AS4	AAA **CAGG** CTACGGCACGTAGAGCATCACCATGAT **CCTG**
AS5	AAA **ACAGG** CTACGGCACGTAGAGCATCACCATGAT **CCTGT**
AS6	AAA **CACAGG** CTACGGCACGTAGAGCATCACCATGAT **CCTGTG**
17S	ACT CAC TAT **rA** G GAA GAG ATG-Cy3
17E	CAT CTC TTC TCC GAG CCG GTC GAA ATA GTG AGT

*Notes: The green bases in A17E5 is the DNAzyme (17E) which can hybridized with its substrate (17S, the orange bases in G17S8) and trigger cleavage of the substrate, the purple bases is the aptamer of PDGF-BB. The blue boldface in G17S8 is the G-rich DNA sequence which can form the HRP-mimicking DNAzyme together with hemin and the underlined bases can form stem-structure of the molecular probe. 'rA' in G17S8 and 17S indicates the RNA base. The green bases in AC5 are perfectly matched with the orange bases of G17S8. The boldfaces in AS3, AS4, AS5 and AS6 are the complementary bases.

Table S2. Summary of the reaction rate constants and dissociation coefficients obtained from fitting the curves shown in Figure 1 with equation (1) and (2), respectively. The standard deviations were obtained with three replicates.

Type	k_{obs} **(min^{-1})**	$K_{d,app}$ **(μM)**
Pb^{2+}	0.73 ± 0.04	54.4 ± 31.7
10nm-PbS NC	2.11 ± 0.07	4.7 ± 1.8

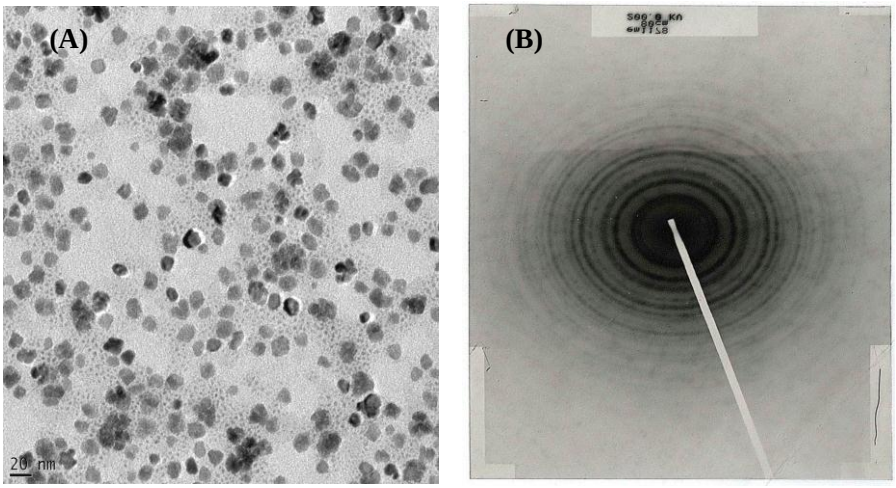


Figure S1. Transmission electron microscopic (TEM) image (A) and selected-area electron diffraction (SAED) (B) of the synthesized PbS NCs.

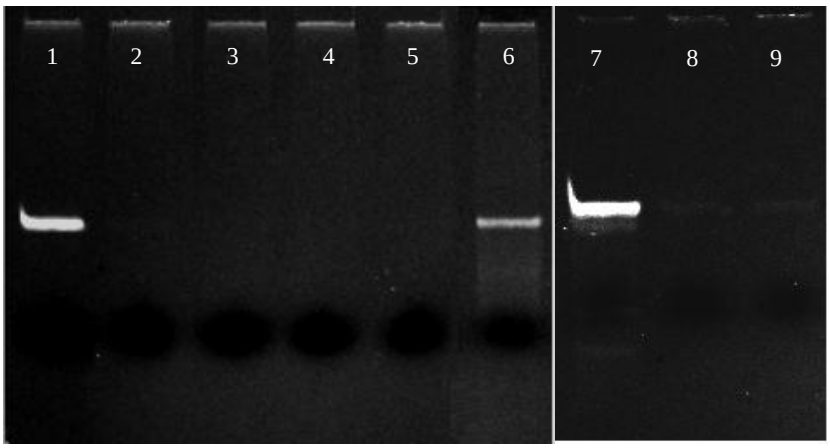


Figure S2. Gel images for analysis of Exo I-catalyzed degradation under the presence of different proteins. Lane 1and 7, A17E5; lane 2 and 8, A17E5 + Exo I; lane 3, A17E5 + 600 nM BSA + Exo I; lane 4, A17E5 + 600 nM human IgG + Exo I ; lane 5, A17E5 + 600 nM HSA + Exo I; lane 6, A17E5 + 150 nM PDGF-BB + Exo I; lane 9, A17E5 + 600 nM PDGF-AB + Exo I. Experiment process: 20 μL 300 nM of A17E5 in Tris-HCl buffer (pH = 7.4, 20 mM K^+, 1 mM Mg^{2+} ,140 mM Na^+) with or without different proteins was first incubated for 1 h under room temperature. Then 4 U Exo I was added into the reaction mixture and incubated for 1 h under 35 ^{0}C. Afterwards, the exonuclease degradation reaction was terminated through a 15-min denaturing process at 85 ^{0}C and the obtained reaction mixtures were analyzed by gel electrophoresis.

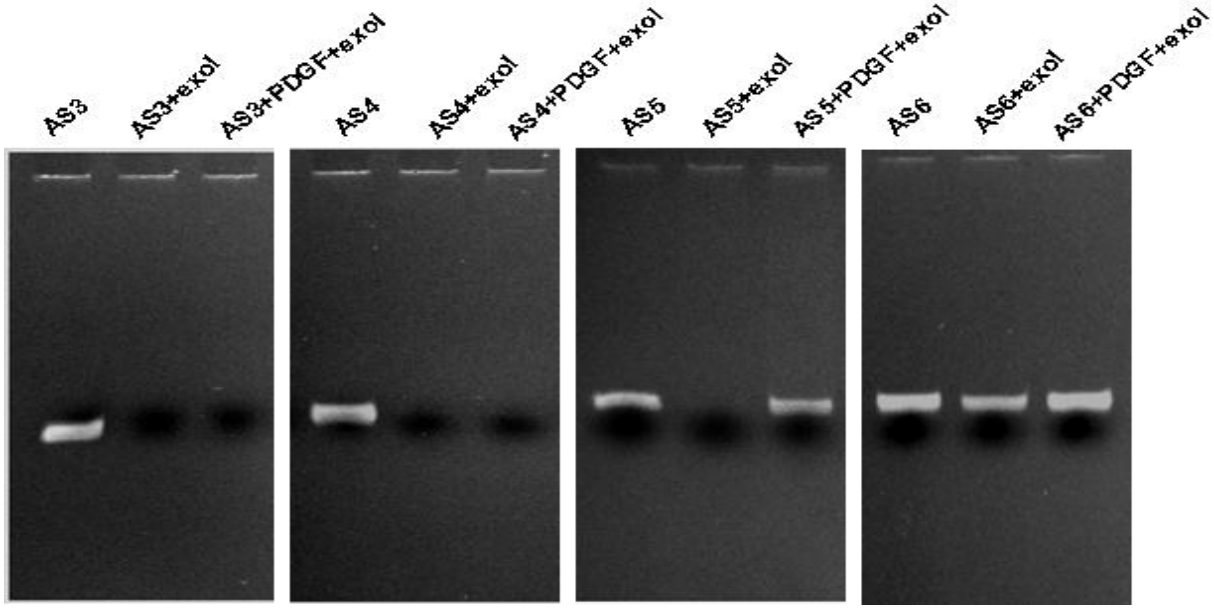


Figure S3. Gel images for optimization of aptamer probe. The experiment process is described as the experimental part.

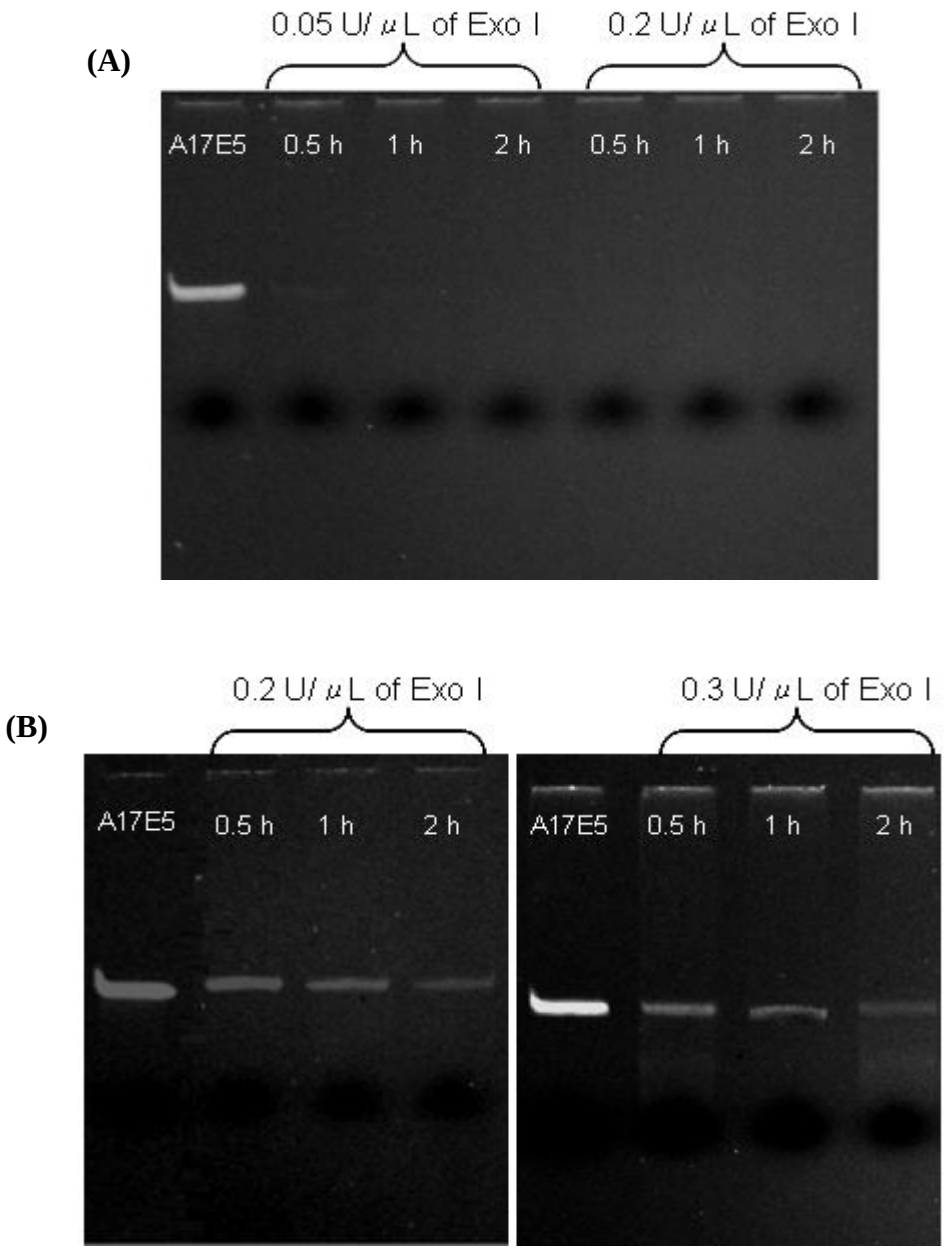
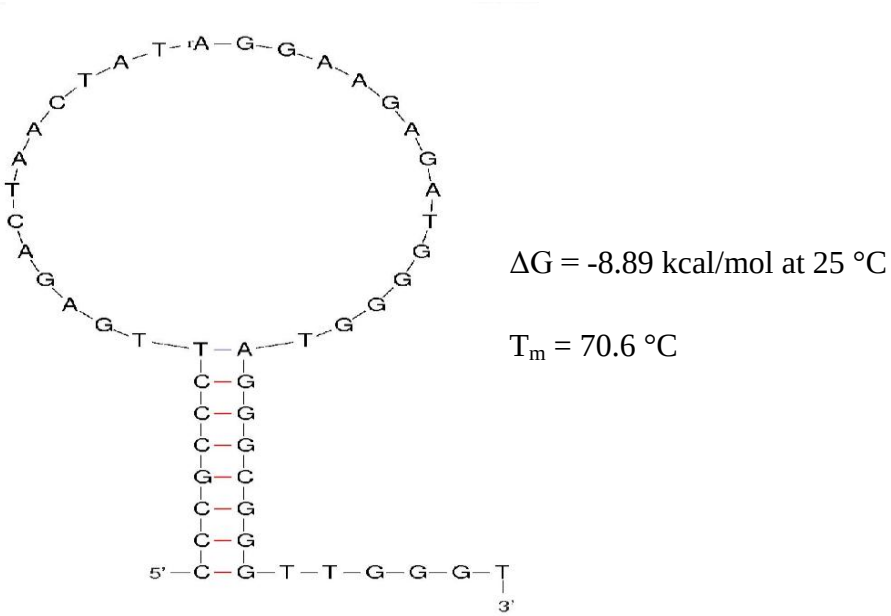


Figure S4. Gel images for optimizing the reaction conditions of Exo I-catalyzed degradation. Gel electrophoresis was carried out according to the experimental part.

(A) 20 μL 300 nM of A17E5 in Tris-HCl buffer (pH = 7.4, 20 mM K^+, 1 mM Mg^{2+} ,140 mM Na^+) was first incubated for 0.5 h, 1 h or 2 h under the presence of 0.05 U/μL or 0.2 U/μL Exo I at room temperature, and then the exonuclease degradation reaction was terminated through a 15-min denaturing process at 85 ^{0}C and the obtained reaction mixtures were analyzed by gel electrophoresis.

(B) 20 μL 300 nM of A17E5 in Tris-HCl buffer was first incubated with 100 nM of PDGF-BB for 30 min under room temperature. And then one 0.5 h, 1 h, or 2 h enzymatic digestion process was carried out under the presence of 0.2 U/μL or 0.3 U/μL Exo I. Finally, the exonuclease degradation reaction was terminated through a 15-min denaturing process at 85 ^{0}C and the obtained reaction mixtures were analyzed by gel electrophoresis.



G17S8

Figure S5. Computed secondary structure of G17S8. The fold was performed under the conditions of Tris-HCl buffer (pH = 7.4, 20 mM K^+, 1 mM Mg^{2+} ,140 mM Na^+) and room temperature (25 ^{0}C) using the Zucker DNA folding program.

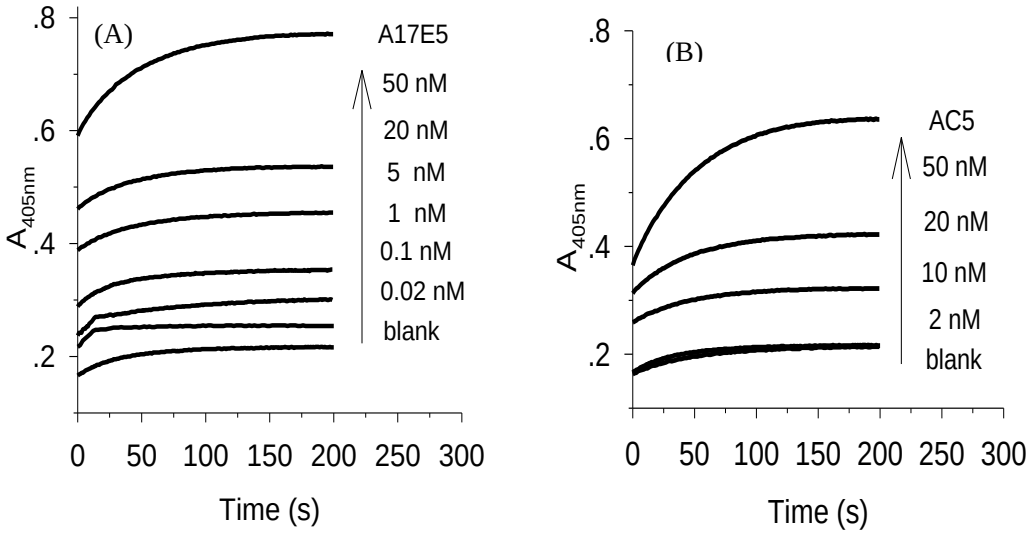


Figure S6. Comparison of the DNAzyme cascade-based sensing system with the traditional hairpin-structured probes. Time-dependent absorbance recording at 405 nm in the DNAzyme-mediated ABTS-H_2O_2 system was carried out when 200 nM G17S8 was incubated with different concentrations of A17E5 (A) and AC5 (B). The reaction solution contains 1 μM hemin and 20 μM Pb element (PbS NCs).

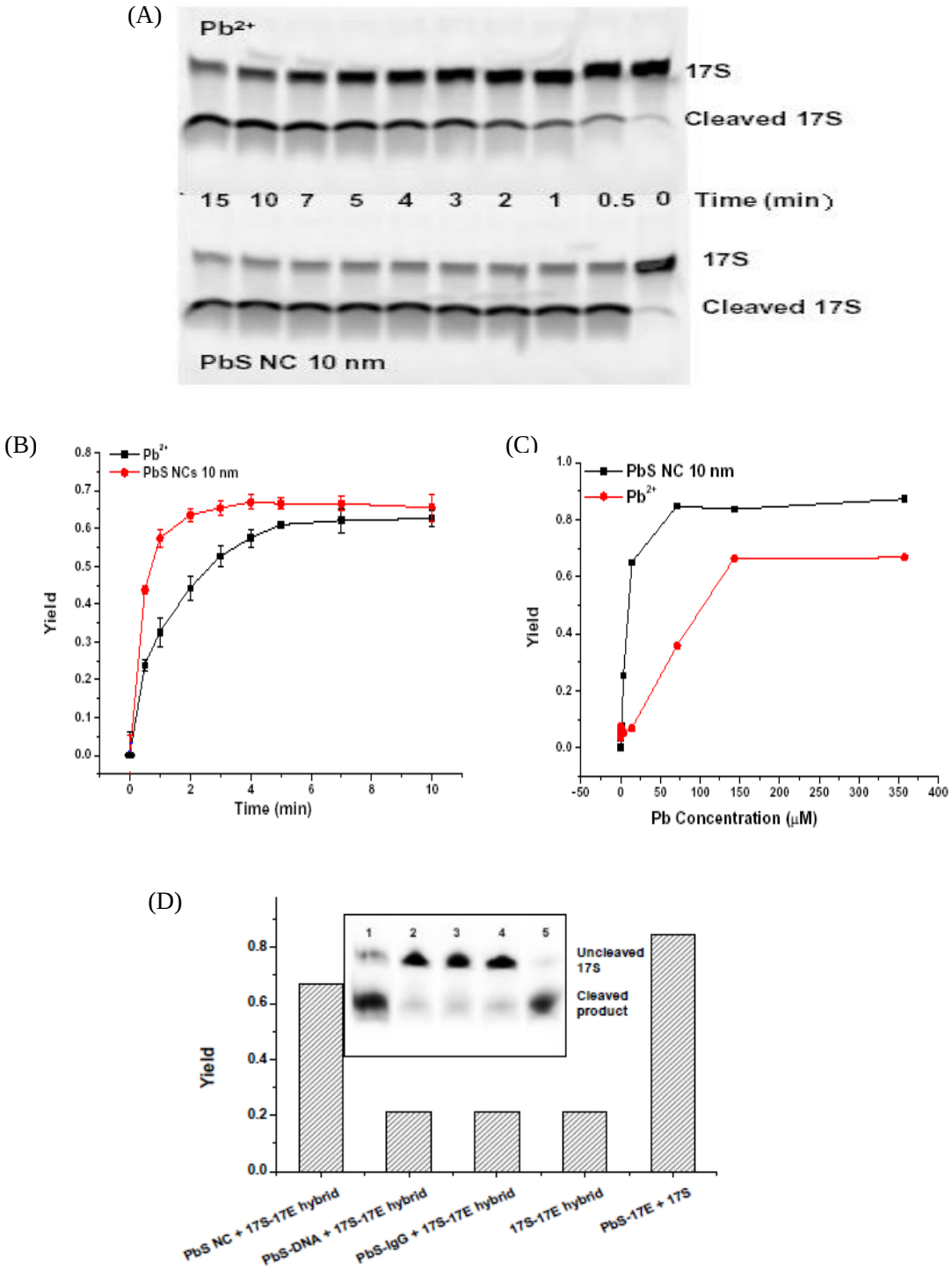


Figure S7. (A) Gel images for analysis of substrate cleavage at the presence of different cofactors. (B) Comparison of substrate cleavage catalyzed by Pb^{2+} and PbS NCs. Error bars were obtained from 3 repeated measurements. (C) Change of cleavage rate with Pb element concentration, the reaction mixture was incubated for 3 min before 10 μL stop solution was added. Only one comparison was shown here, while three data sets were used to calculate the K_d values. (D) Cleavage yields with different types of PbS NCs bioconjugates. The inserted picture was the gel image. The reaction mixture was incubated for 10 min before 10 μL stop solution was added.

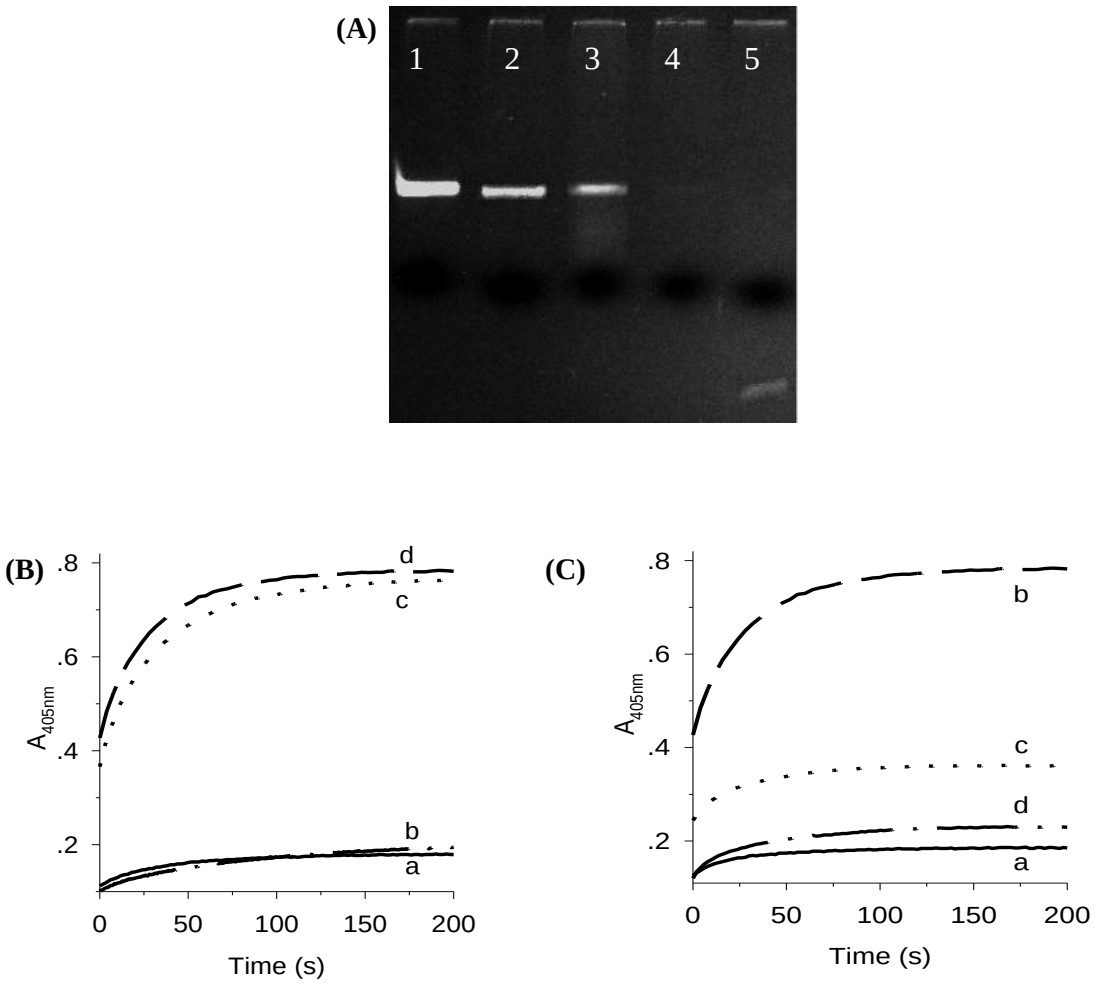


Figure S8. The effect of random DNA on the sensing system. The experiment process is described as the experimental part. (A) Gel images to investigate the effect of random DNA on the aptamer-protein binding interaction. Lane 1, A17E5; lane 2, A17E5 + PDGF-BB + Exo I; lane 3, A17E5 + PDGF-BB + R1 + Exo I; lane 4, A17E5 + R1 + Exo I; lane 5, R1. (B) The effect of random DNA on the signal probe of G17S8. Curve a, G17S8; Curve b, G17S8 + R1; Curve c, G17S8 + A17E5 + R1; Curve d, G17S8 + A17E5. (C) The effect of random DNA on the background signal. Curve a, G17S8; Curve b, G17S8 + A17E5; Curve c, G17S8 + A17E5 + Exo I; Curve d, G17S8 + A17E5 + R1 + Exo I.