Electronic Supplementary Material (ESI) for Analyst.

A label-free and sensitive fluorescence protein assay was developed on the basis of aptamer exonuclease protection and exonuclease III-assisted recycling amplification

Yanqin Lv,[a] Qingwang Xue,[a]* Xiaohong Gu,[b] Shuqiu Zhang,[b] Jifeng Liu[a]*

[a] *Department of Chemistry, Liaocheng University, Liaocheng, 252059, Shandong, China.*

[b]*Shandong Provincial Key Lab of Test Technology on Food Quality and Safety, Shandong Academy of Agricultural Sciences, Jinan, 250100, China.*

Corresponding author:

Tel: +86-635-8239001; fax: +86-635-8239001. Email: xueqingwang1983@163.com;

liujifeng111@gmail.com

Experimental

Table S1. DNA sequences used in this work

Name	Sequences (5'-3') *
AS-4	5’- TAA TAG GGT TGA ATA AGC ACT GAG GT ***A GAG*** *GGT TGG TGT GGT TGG* -3’
AS-5	5’- TAA TAG GGT TGA ATA AGC ACT GAG GT ***GA GAG*** *GGT TGG TGT GGT TGG* -3’
AS-6	5’- TAA TAG GGT TGA ATA AGC ACT GAG GT ***GGA GAG*** *GGT TGG TGT GGT TGG* -3’
Aptamer probe (AS-5)	5’- TAA TAG GGT TGA ATA AGC ACT GAG GT ***GA GAG*** *GGT TGG TGT GGT TGG* -3’
hairpin DNA	5’-**GGG TTG GGC GGG ATG GG**T TAC CTC AGT GCT TAT TCA ACC CTA TTA-3’

The underlined letters of aptamer probe are the recognition sequences for the underlined letters of hairpin DNA. The italic letters of **AS-4**, **AS-5** and **AS-6** are the aptamer sequences of human thrombin. The bold letters of hairpin DNA is a G-quadruplex sequence, which is caged by the hairpin stem region.

Sequence Optimization of Aptamer Probe

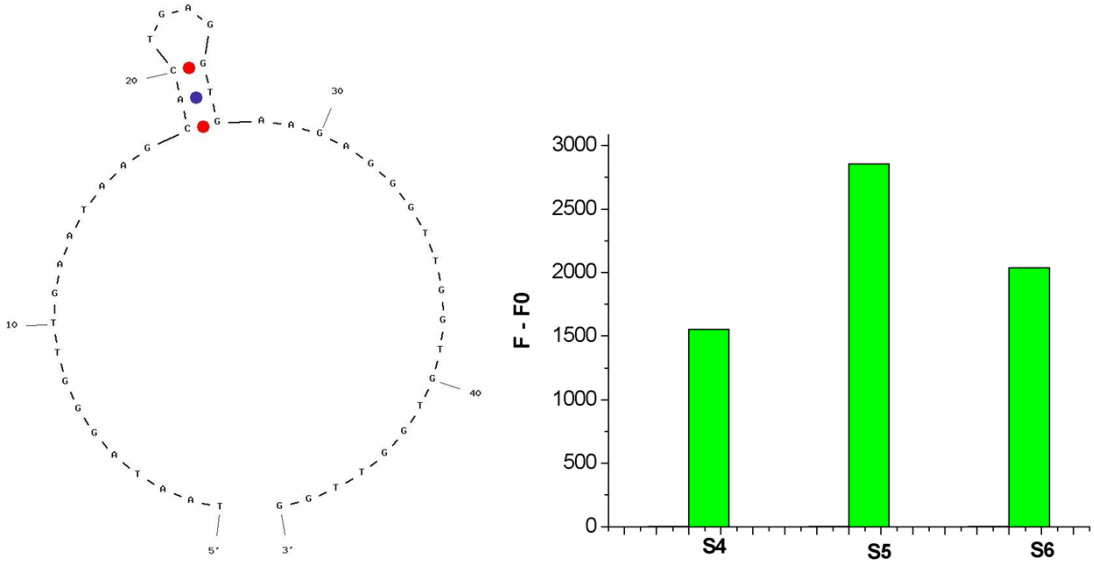


Fig. S1 Sequence optimization of aptamer probe and structure of aptamer probe. The concentration of thrombin is 1.0 nM.

The key point of our proposed strategy was that, when the thrombin is recognized, residually free aptamer sequences are completely degraded by Exo I. The exonuclease I (Exo I) can efficiently catalyze degradation of free single stranded DNA probes in the 3' to 5' direction. Therefore, the stem-length and structure of the aptamer probe was optimized in order to make sure that the aptamer bound preferentially to the target protein while residually free aptamer sequences are completely degraded by Exo I. Aptamer probes with different stem-length between the aptamer sequence (GGT TGG TGT GGT TGG) at the 3' -terminus and DNAzyme sequence (TAA TAG GGT TGA ATA AGC ACT GAGGT) at the 5'-terminus were used to investigate for electing a proper probe (**Fig. S1)**. Based on the fluorescence change of the systems with or without 1.0 nM human thrombin, the signal-to-background ratio level (the positive signal (F) deducts the negative signal (F0). The negative signal (F0) means the buffer sample including all assay reagents excluding the target protein) was used to evaluate the aptamer probe performance; the highest signal-to-background ratio was observed for AS-5. For this group, the AS-5 has the less bases hybridization structure and therefore showed a rather high degradation rate and high ability of interaction with target. While the AS-4 probe and the AS-6 probe have the more bases hybridization structure and therefore showed a rather low degradation rate and low ability of interaction with target. Especially, for the AS-4 probe, the AS-4 probe has the most bases hybridization structure. Therefore the AS-4 probe showed a low signal-to-background ratio. Thus, the AS-5 probe was chosen as the optimum aptamer probe in the experiments.

Optimization of the aptamer oligonucleotide probe concentration

The concentration of aptamer probe plays an important role in the sensing process. To guarantee to all protein molecules be recognized and detected, an appropriate concentration of aptamer oligonucleotide probe is expected to yield the optimization signal. The effect of the concentration of aptamer probe on the fluorescent signal was shown in **Fig. S2**. The fluorescence intensity increased with the increase in the concentration of aptamer probe. When the concentration of aptamer probe reached 100 nM, the maximum fluorescence intensity was achieved. Thereafter, the fluorescence response exhibited a gradual decrease with a further increase in the concentration of aptamer probe. This was probably because a large excess of aptamer probe disturbed the binding between the target protein and its aptamer, and increased the background response with the extension of time due to inactivation of exonuclease I. Thus, 100 nM was selected as the optimum concentration in the experiment due to its best signal-to-noise level.

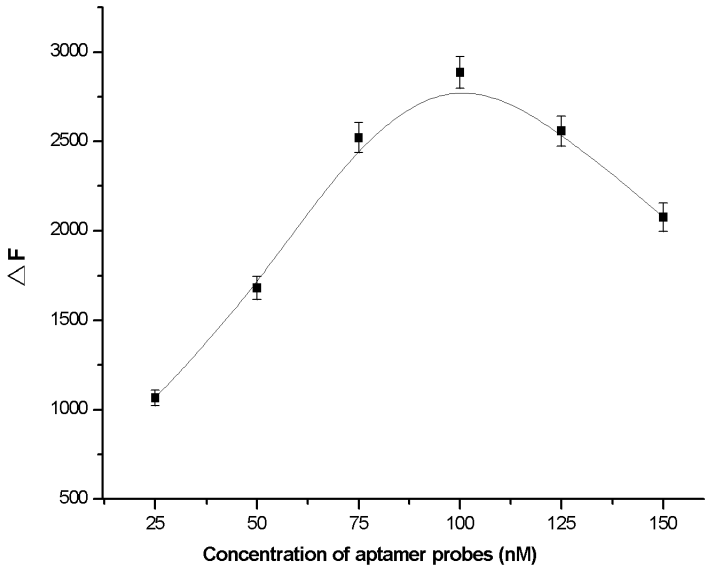


Fig. S2 Effect of the aptamer concentration on the fluorescent signal intensity. The concentration of thrombin is 1.0 nM.

Optimization of the exonuclease I

In the assay, to realize the separation-free procedure, we exploit exonuclease I to degrade all unbound protein aptamer probes. Exonuclease I catalyzes the removal of nucleotides from single-stranded DNA in the 3' to 5' direction. Without a protein target, the aptamer probe would be degraded to 5'-terminal dinucleotides with the release of deoxyribonucleoside 5'-monophosphates from the 3'-terminal of the single-stranded DNA chains. In the assay, the oligonucleotides that bind thrombin are protected, whereas free single-strands aptamer probes are degraded by exonuclease I. To guarantee to completely degrade all free single-strands aptamer probes, we investigate the concentration of exonuclease I.

In this experiment, the time of exonuclease digestion is pivotal to quantify analyte protein because exonuclease I will drive the equilibrium between the bound and unbound aptamer toward the unbound aptamer. Within limited time, there is very little effect of exonuclease on the moving of this equilibrium. After the same time of digestion, the concentrations of free aptamer without binding thrombin in each sample are extremely low and almost the same. The proportions of bound thrombin to total thrombin in each sample are almostly unvaried during the limited time. Therefore, we investigate the cleavage time of exonuclease I.

The effect of the exonuclease I concentration and the cleavage time on the fluorescent signal was shown in **Fig. S3**. It shows that 20 units exonuclease I was selected as the optimum concentration for degrading the free protein aptamer probes.

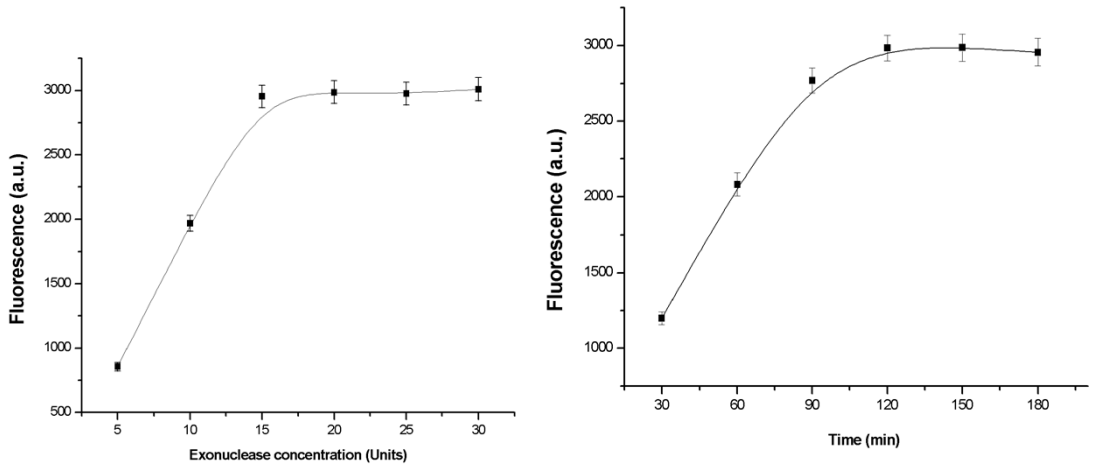


Fig. S3 Effect of the exonuclease I concentration and the cleavage time on the fluorescent signal intensity. The concentration of thrombin is 1.0 nM.

Optimization of the concentration of hairpin DNA probe

In the assay, the protected aptamer probes by target act as catalysator to trigger hybridizing with hairpin DNA probe. Upon interaction with the protected aptamer probes, the DNA probe was opened to yield the fluorescent ZnPPIX/G-quadruplex structure in the presence of Exonuclease III. The fluorescence signals are detected, which can be used as a quantitative measure for the protein concentration. To achieve desirable analytical characteristics, the effect of the concentration of the hairpin DNA probe was investigated. As shown in **Fig. S4**, the fluorescence intensity of the aptasensor increased with increasing the concentration of hairpin DNA probe. The fluorescent intensity response exhibits a rapid increase with an increase in the concentration of hairpin DNA probe and trends to a constant value at 75 nM, indicating the saturation of the bond between aptamer and thrombin. Thus, 75 nM was selected as the optimum concentration in the experiment due to its best signal-to-noise level.

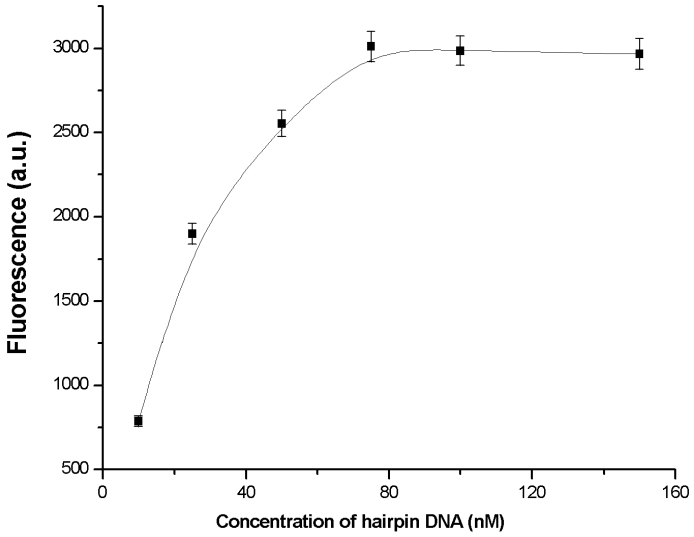


Fig. S4 Effect of the hairpin DNA concentration on the fluorescent signal intensity. The concentration of thrombin is 1.0 nM

Exo III-aided recycling amplication reaction

To achieve desirable analytical characteristics, the Exo III enzyme reaction time was optimized. As shown in **Fig. S5**, when the cleavage time was prolonged, the fluorescence of the final solution increased as well, which indirectly displayed the procedure of cleavage by Exo III. The results showed a linear increase in fluorescence between 0 and 40 min, and then, the intensity continued to increase slowly. Control groups also had a slight intensity increase which might be caused by the contamination of the Exo III enzyme with DNA nucleases. Taking both the effectiveness and the speed into consideration, we chose 45 min as the Exo III enzyme reaction time for our reactions.

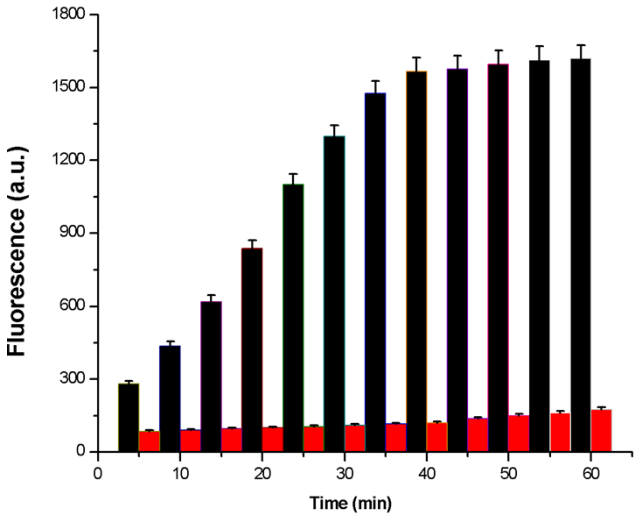
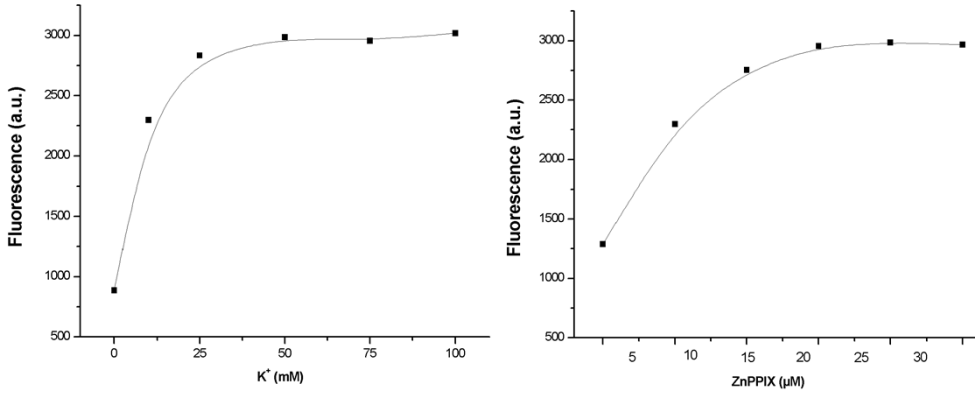


Fig. S5 Time course of the fluorescence intensity in the presence of 100 pM thrombin or without thrombin (the controls).

Optimization of the concentration of ZnPPIX and K^+

ZnPPIX can bind to the GGGTTGGGCGGGATGGGG G-quadruplex structure. The nature of the G-quadruplex configuration is controlled by adding K^+ ions. In the presence of K^+ ions, the parallel configuration is favored. To achieve the best sensing performance, the concentration of ZnPPIX and K^+ were also optimized. The experimental results indicated that a concentration of 50 mM of K^+ and 25 μM of ZnPPIX could provide maximum S/N ratio for the sensing system (**Fig. S6**).



（A） （B）

Fig. S6 The effect of the concentration of K^+ and ZnPPIX on the fluorescence response of the sensing system. The concentration of thrombin is 1.0 nM.

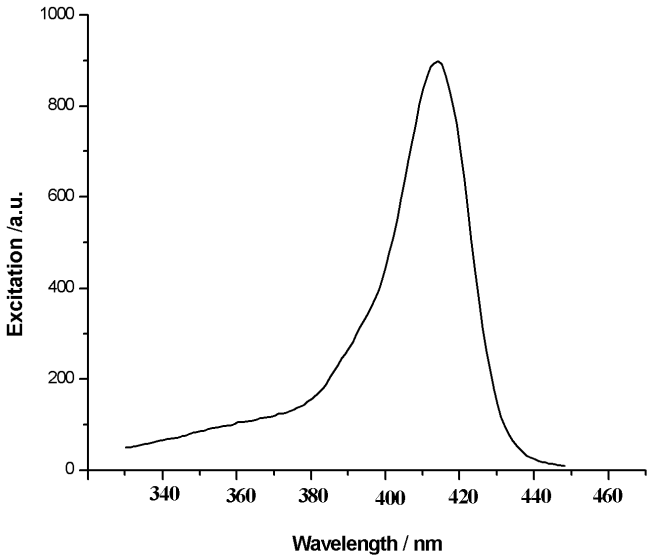


Fig. S7 Excitation spectrum of the ZnPPIX/G-quadruplex complex.

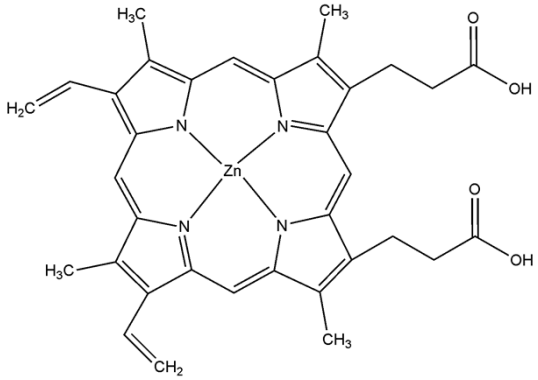


Fig. S8 Molecular structure of ZnPPIX