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Target-Induced Conjunction of Split Aptamer Fragments and Assembly with a Water-Soluble Conjugated Polymer for Improved Protein Detection

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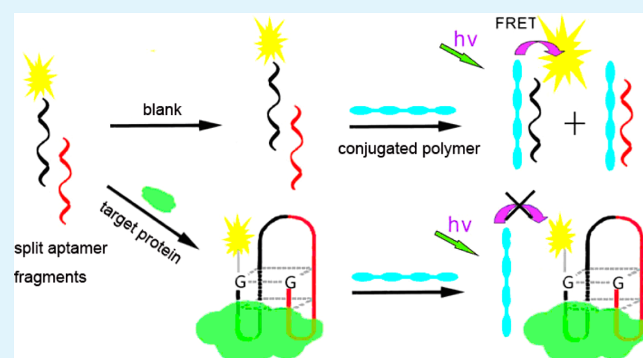
Supporting Information



ABSTRACT: Rapid and sensitive detection of proteins is crucial to biomedical research as well as clinical diagnosis. However, so far, most detection methods rely on antibody-based assays and are usually laborious and time-consuming, with poor sensitivity. Herein, we developed a simple and sensitive fluorescence-based strategy for protein detection by using split aptamer fragments and a water-soluble polycationic polymer (poly{[9,9-bis(6′-(*N*,*N*,*N*-diethylmethylammonium)-hexyl)-2,7-fluorenylene ethynylene]-alt-*co*-[2,5-bis(3′-(*N*,*N*,*N*-diethylmethylammonium)-1′-oxapropyl)-1,4-phenylene] tetraiodide} (PFEP)). The thrombin-binding DNA aptamer was split into two fragments for target recognition. The PFEP with high fluorescence emission was used as energy donor to amplify the signal of dye-labeled DNA probe. In the absence of target, three DNA/PFEP complexes were formed via strong electrostatic interactions, resulting in efficient Föster resonance energy transfer (FRET) between two fluorophores. While the presence of target induces a conjunction of two split aptamer fragments to form G-quadruplex, and subsequent assemble with PFEP leading to the formation of G-quadruplex/thrombin/PFEP complex. The distance between the PFEP and dye increased due to protein's large size, leading to a remarkable decrease of the FRET signal. Compared with the intact aptamer, the use of shorter split aptamer fragments increases the possibility of forming G-quadruplex upon target. Thus, the rate of change of FRET signal before and after the addition of target improved significantly and a higher sensitivity (limit of detection (LOD) = 2 nM) was obtained. This strategy is superior in that it is rapid, has low cost and homogeneous detection, and does not need heating to avoid an unfavorable secondary structure of DNA probe. With further efforts, this method could be extended to a universal way for simple and sensitive detection of a variety of biomolecules.

KEYWORDS: *water-soluble conjugated polymer, aptamer, protein detection, Föster resonance energy transfer (FRET), G-quadruplex*

INTRODUCTION

Methods that allow rapid, simple, sensitive, selective, and cost-effective detection of proteins play important roles in medical diagnosis, prevention and treatment.[1] In general, antibody-based assay is a versatile and powerful tool for specific protein detection.[2] However, it is faced with considerable challenges, such as insufficient sensitivity, long assay time and the involvement of multiple washing steps. As a complement to the antibody-based assay, biosensors based on aptamer-protein recognition have gained increasing attention recently.[3,4]

Aptamers are specific DNA or RNA strands that could recognize various chemical or biological target molecules ranging from metal ions, organic small molecules to proteins, virus, bacterium and even intact tumor cells with high affinity and specificity. Aptamers are obtained by an in vitro evolution process called SELEX (systematic evolution of ligands by exponential enrichment),[5−7] and have many advantages, such as high specificity, simple synthesis, relatively easy labelling, relatively small size, non-immunogenic nature, and good stability, when compared with antibodies.[8] These amazing advantages have attracted much attention in bioanalysis, environmental and food analysis, diagnostics, therapeutics, drug development, and biocomputing.[9−17] In particular, aptamers are extensively used as bio-recognition elements in electrochemical, fluorometric, colorimetric, chemiluminscence, field-effect transistors, and SPR biosensors.[18−30] To enhance

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the specificity, sandwich-type assays were developed by using one or two different aptamers.[20,31] However, the sandwich configuration can be realized only when the target molecule has two binding sites to interact with two intact aptamers simultaneously,[32] which is not suitable to those targets with only one aptamer or one binding site. To overcome this obstacle, split aptamer fragments are designed to provide more possibilities for the specific biosensing.[33−38] Compared with intact aptamers, split ones are shorter and have less secondary structure that is unfavorable for target binding. Importantly, the ligand-binding abilities of aptamers are almost not perturbed, although they are split into two or more fragments.[27,39−41]

Conjugated polymers (CPs) are characterized by a delocalized electronic structure that exhibits efficient coupling between optoelectronic segments. This unique structure allows for effective electronic coupling and, therefore, fast intrachain and interchain energy transfer.[42] It is also responsible for a strong absorption and emission in the ultraviolet−visible light (UV-vis) range. These advantages make CPs ideal fluorescent materials for chemical and biological sensing. Assemblies of DNA and CPs have been widely used as optical platforms for the detection of various targets.[43−47] For example, Leclerc's group reported several optical biosensors based on target-induced conformational change of aptamers and water-soluble conjugated polythiophene derivatives.[44] Bazan, Wang, and Liu's groups reported sensitive optical biosensors based on the FRET from highly fluorescent CPs to dye-labeled DNA/PNA probes.[43,45,47] Now, research on the FRET between CPs and dye is primarily focused on the detection of DNA and small molecules such as ATP/K^+. Effect of macromolecules on the FRET process is seldom reported.

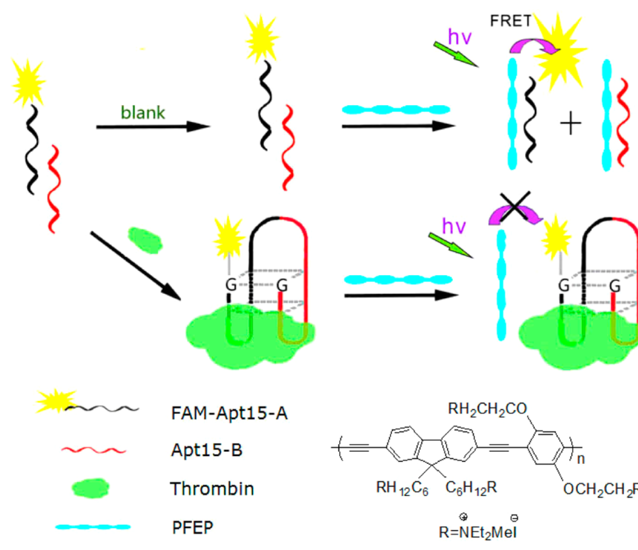
Thrombin is a major biomarker for cardiovascular disease, because it is integral to several coagulant effects, including conversion of fibrinogen to fibrin, cleavage of coagulation factors, and promotion of platelet activation.[48] Therefore, detection of the thrombin level is important for clinical diagnosis. In this work, an improved optical strategy is designed for thrombin detection, using target-induced conjunction of two split aptamer fragments and assembly with a water-soluble polycationic polymer PFEP. Compared with methods using intact aptamer, this strategy exhibits an improved signal-to-background ratio and a higher sensitivity. Furthermore, it is more rapid and simpler than a recently reported strategy based on the FRET between CP and dye-labeled intact aptamer.[49]

■ EXPERIMENTAL SECTION

Materials and Chemicals. Poly{[9,9-bis(6′-(*N*,*N*,*N*-diethylmethylammonium)hexyl)-2,7-fluorenylene ethynylene]-alt-*co*-[2,5-bis(3′-(*N*,*N*,*N*-diethylmethylammonium)-1′-oxapropyl)-1,4-phenylene] tetraiodide} (PFEP) (see Scheme 1) was synthesized according to the previously reported method.[50] The concentration of PFEP refers to the concentration of the repeat unit (RU), which was calculated according to the molecular weight and monomer weights of its neutral polymer. The molecular weight and polydispersity of its neutral polymer are 12 600 and 1.28, respectively.

All oligonucleotides employed were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, PRC). The concentrations of the oligonucleotides were determined by measuring their absorbance (also referred to as optical density) at 260 nm. Thrombin (Thro) was purchased from Sigma−Aldrich. Immunoglobin G (IgG) was purchased from Linc-bio Science Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Hangzhou Haoxin Biotech Co., Ltd. Lysozyme (Lys) was purchased from Bio Basic, Inc. Other chemicals were purchased from Sigma−Aldrich, Acros, and Alfa and were used as received. All solutions were prepared with Milli-Q water (18.2 MΩ.cm) from a Millipore system.

Scheme 1. Scheme of Thrombin Detection Based on Split Aptamer Fragments and PFEP, and Chemical Structure of PFEP



Experimental Procedures. For feasibility experiments using split aptamer fragments and intact aptamer, oligonucleotides were prepared with a concentration of 10 μM in tris-HCl buffer (10 mM tris-HCl, 100 mM NaCl, pH 8.0). Apt15-A and Apt15-B of the same volume (2.5 μL) were incubated with thrombin (10 μM, 1 μL) and tris-HCl buffer (total reaction volume is 50 μL) at 37 °C for 30 min. Then, PFEP ([RU] = 50 μM, 2.5 μL) and a certain volume of buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 10.6) were added to a final total volume of 500 μL. After incubation for 5 min at room temperature, the fluorescence of the mixture was detected at an excitation of 404 nm. Control experiments with intact Apt15 were done under the identical conditions.

To study the effect of concentration ratio of PFEP to Apt15-A/Apt15-B on the performance of the biosensor, [PFEP] was fixed as above; the ratios of [PFEP]:[Apt15-A/Apt15-B] were designed to be 5:1, 12.5:1, and 25:1, and the concentrations of Apt15-A/Apt15-B were modified according to these ratios. The fluorescence of the final mixture was detected in buffer of pH 7.4 (20 mM PBS, 140 mM NaCl).

Buffer solutions of a specific pH used in the assays for optimizing reaction condition are shown in Table S1 in the Supporting Information.

Three negative proteins (BSA, IgG, and Lys) were used to replace thrombin to study the specificity of the strategy. Thrombin in a mixture of several proteins was used to investigate the sensor's selectivity in a complex system. Aliquots of various concentrations of thrombin were prepared from stock solution of 10 μM to study the sensitivity of the thrombin sensor.

Methods. The absorbances of oligonucleotides were measured using a UV-vis spectrophotometer (Model 3150, Shimadzu, Japan). All fluorescence and FRET measurements were obtained at room temperature in a 600 μL quartz cuvette using a fluorometer (Model RF-5301, Shimadzu, Japan).

■ RESULTS AND DISCUSSION

A schematic description of thrombin detection based on split aptamer fragments and PFEP, as well as the chemical structure of PFEP, are shown in Scheme 1. The 15-mer thrombin-binding aptamer (Apt15, 5′-GGTTGGTGTGGTTGG-3′) was split into two fragments according to the work reported by Chen et al.[41] and Plaxco et al.[51] One of the split aptamer

fragments was labeled with fluorescein (FAM) and called Apt15-A (5′-FAM-GGTTGGTG-3′) and the other fragment was called Apt15-B (5′-TGGTTGG-3′). In the absence of target protein, the two fragments were independent and both could bind with PFEP via strong electrostatic interactions resulting in three types of complexes, including Apt15-A/PFEP, Apt15-B/PFEP, and Apt15-A/PFEP/Apt15-B. The binding of Apt15-A with PFEP leads to a highly efficient FRET from the PFEP to the FAM labeled on the Apt15-A. However, in the presence of thrombin, a target-induced G-quadruplex and subsequent G-quadruplex/thrombin/PFEP complex were formed with two split aptamer fragments, thrombin and positively charged PFEP. Because of the strong steric hindrance from large-sized target protein, the distance between the PFEP and FAM increased; therefore, the FRET signal decreased.

It is reported that the electrostatic interactions between CP and oligonucleotides with different conformations (ssDNA, dsDNA, G-quadruplex) are quite different, which can be used to discriminate DNA hybridization, SNP, cleavage, and target binding.[52,53] To test the feasibility of our strategy, split aptamer fragments Apt15-A and Apt15-B were used for thrombin detection and the intact aptamer Apt15 was used as a control. Firstly, the UV-vis absorption and fluorescence emission spectra of the donor (PFEP) and acceptor (FAM-Apt15-A) were recorded (see Figure S1 in the Supporting Information). As we have reported,[54] the fluorescence spectra of PFEP and the UV-vis absorption spectra of FAM overlapped well and an efficient FRET could occur between them. Thus, strong FRET signals should be observed when PFEP was mixed with Apt15, Apt15-A, or Apt15-A/Apt15-B, and weakened FRET signals would be obtained due to the formation of G-quadruplex/thrombin in the presence of a target. Figure 1 shows the results of a feasibility study. As might be expected, both split Apt15-A/Apt15-B and intact Apt15 could bind with PFEP and highly efficient FRET ($I_{525\ nm}/I_{440\ nm}$ = 6.77 for the former and $I_{530\ nm}/I_{440\ nm}$ = 6.72 for the latter) occurred simultaneously. However, when the thrombin is present, weakened FRET signals were observed for split aptamer fragments ($I_{525\ nm}/I_{440\ nm}$ = 2.6) and intact aptamer ($I_{530\ nm}/I_{440\ nm}$ = 4.84). It is worth noting that a more remarkable decrease of FRET signal was observed for split Apt15-A/Apt15-B than for intact Apt15. It implied that both intact aptamer and split aptamer fragments could form target-induced G-quadruplex via intramolecular hydrogen-bonding interactions and a subsequent G-quadruplex/thrombin/PFEP complex formed via electrostatic interactions. As the distance between the PFEP and dye increased due to strong steric hindrance from the large-sized target protein, efficient FRET was blocked and decreased FRET signals were observed. It is reported that the binding of thrombin on the aptamer can limit the electronic transfer from the electroactive polythiophene to the electrode,[55] the blocking capacities of the protein is consistent with the results here. In addition, compared to the emission spectra employing the intact aptamer, a blue shift of 4 nm was observed when using split aptamer fragments. We propose that the complexes of Apt15/PFEP and Apt15/thrombin/PFEP are slightly bigger than Apt15-A/PFEP/Apt15-B and Apt15-A/thrombin/Apt15-B/PFEP complexes, because of fewer bases in split aptamer fragments.

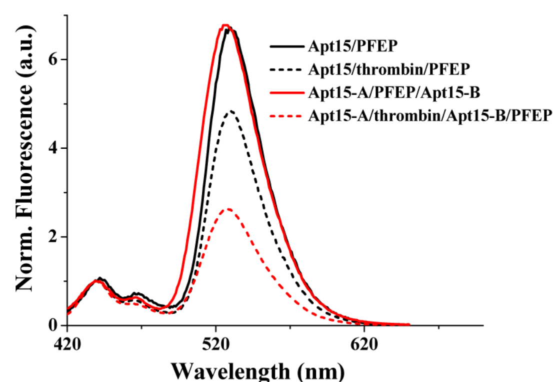


Figure 1. Normalized fluorescence of thrombin detection with intact Apt15 (black curves) and split Apt15-A/Apt15-B (red curves).

Researchers have demonstrated that the space charge density around the DNA controls the efficiency of FRET from the CP to the fluorescein.[45] The form of CP/G-quadruplex pair increases the FRET efficiency, because of stronger electrostatic interactions between the more-condensed G-quadruplex and the CP, compared to the CP/ssDNA pair when K^+ is bound.[45] However, we observed converse results: the FRET signals of thrombin/G-quadruplex/PFEP decreased, compared to the aptamer/PFEP complexes. The reason might be that the size of thrombin is so large that it causes strong steric hindrance, leading to low-efficiency FRET from the PFEP to the FAM. The results are consistent with a recent report for protein discrimination using conjugated oligoelectrolytes/ssDNA aggregates.[56] It implied that the steric hindrance of protein plays a more important role than the space charge density of DNA in the FRET from PFEP to dye-labeled aptamer.

Considering the fact that the biosensor works in a "turn-off" mode, and FRET signals of the blanks may be different when the concentration of aptamer is changed or the assays were performed in solutions with different pHs, a percentage of change in FRET signals was used to evaluate the sensor's performance.

$$\Delta R\ (\%) = \frac{R_0 - R}{R_0} \times 100$$

R_0 and R are the ratios of acceptor/donor fluorescence intensity ($I_{530\ nm}/I_{440\ nm}$ or $I_{525\ nm}/I_{440\ nm}$) in the absence and presence of target protein, respectively. We found that ΔR is much higher when using split aptamer fragments (ΔR = 61%) than using the intact aptamer (ΔR = 28%). It can be attributed to the following two factors:

(i) *The difference in the electrostatic force between PFEP and oligonucleotides of different length.* The long intact aptamer has stronger electrostatic interactions and short split fragments have weaker interactions with PFEP. Thus, it is easier to pull the oligonucleotide away from the PFEP and form a thrombin-induced G-quadruplex in the assay using split aptamer fragments.

(ii) From the perspective of molecular thermal motion, the long intact aptamer moves slower and has smaller collision probability with thrombin than split aptamer fragments. Therefore, it is easier for split aptamer fragments to form G-quadruplex in the presence of thrombin, although, in theory, the probability of forming G-quadruplex with two split fragments is less than that with one intact aptamer.

Taken together, we propose that more G-quadruplex/thrombin/PFEP complexes may be formed in the system

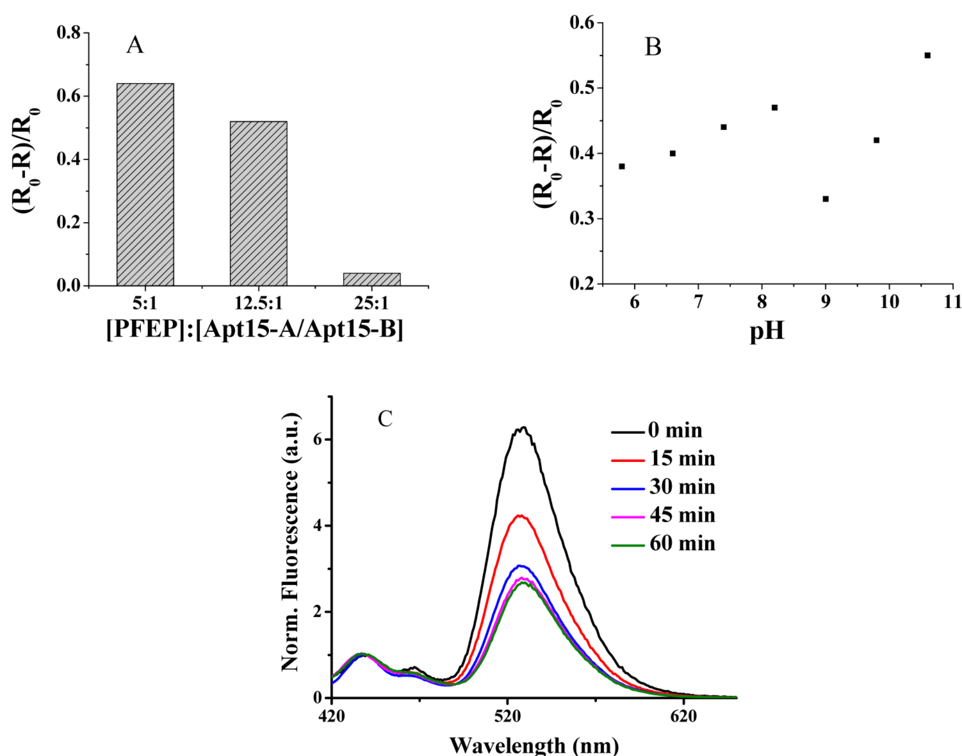


Figure 2. Responses of $(R_0 - R)/R_0$ to (A) different [PFEP]:[Apt15-A/Apt15-B] ratios and (B) different pH values. (C) Normalized fluorescence of thrombin detection at different incubation times.

using split aptamer fragments, since the ligand-binding ability of aptamer is not perturbed when it was split into two or more fragments.

We then tried to optimize the conditions of the assay. Firstly, the concentration ratio of PFEP to split aptamer fragments was designed as 5:1, 12.5:1, and 25:1. Results in Figure 2A indicated that the value of ΔR decreased as the PFEP concentration increased. The reason might be that when the [PFEP]:[Apt15-A/Apt15-B] ratio is 5:1, the number of positive charges in PFEP is comparable to the negative charges in Apt15-A and Apt15-B, almost all the PFEP binding with G-quadruplex/thrombin. In comparison, when the [PFEP]:[Apt15-A/Apt15-B] ratio is 12.5:1 or 25:1, the PFEP is excessive, and a certain amount of self-aggregates of PFEP formed in the system. The formation of aggregates will lead to decreased fluorescence emission due to self-quenching of PFEP and decreased the FRET signal from the PFEP to the dye in the absence of target (blanks in Figure S2 in the Supporting Information). Secondly, the system with split aptamer fragments was tested in solutions of different pHs (5.8, 6.6, 7.4, 8.2, 9.0, and 10.6). As shown in Figure 2B, the highest ΔR value was obtained in a solution with a pH of 10.6. Of note, it changes irregularly in the solutions of other pH values. It might be attributed to a difference in the net charges of G-quadruplex/thrombin complex. Because of the amphiphilicity property of protein and the fact that thrombin has an isoelectric point (pI) at pH 7.1, it brings positive charges at pH < pI, weak electrostatic attraction occurred between G-quadruplex/thrombin and PFEP, thus relatively low FRET signals were observed. However, when the pHs of solutions are higher than 7.1, the thrombin brings negative charges, and the electrostatic attraction between G-quadruplex/thrombin and PFEP becomes stronger. However, the distance between the donor and the acceptor increased, because of the strong steric hindrance from the large-sized protein. In addition, fluorescence emission strength in PFEP is different for different solutions of pH 5.8, 6.6, 7.4, 8.2, 9.0, and 10.6, because of the different aggregation statuses resulting from $\pi-\pi$ stacking interactions (see Figure S3 in the Supporting Information). Therefore, the ΔR value of thrombin detection exhibits an irregular change in the range of 5.8−10.6. Thirdly, the extent of reaction between split aptamer fragments and target was investigated by incubating Apt15-A/Apt15-B and thrombin for 0−60 min at 37 °C. As shown in Figure 2C, with the extension of incubation time, the FRET signal decreased remarkably in 30 min, and decreased very slowly after 30 min. For the above reasons, the concentration ratio (PFEP to Apt15-A/Apt15-B) of 5:1, an incubation time of 30 min, and detection in buffer of pH 10.6 were selected as optimal conditions to perform the assays.

It is worthy considering that if the Apt15-A individually can form the G-quadruplex on thrombin, that could lead to a decreased FRET signal. We then performed the assay of protein detection using Apt15-A. The results (see Figure S4 in the Supporting Information) showed that the FRET signal from PFEP to Apt15-A is almost the same as that of split Apt15-A/Apt15-B and intact Apt15. We attributed this to the same concentration of donor and acceptor, although the length of oligonucleotide is different. Not surprisingly, thrombin cannot be distinguished from three other control proteins (IgG, BSA, and Lys), because the FRET signals changed only marginally, no matter what protein is added. These results implied that Apt15-A individually cannot be used to detection thrombin because G-quadruplex consists of two G-quartets that containing eight guanosines, it can be formed only with intact aptamer or two split fragments in the presence of thrombin.

In order to investigate the specificity of the protein biosensor, three non-target proteins (IgG, BSA, and Lys) were used as controls. At first, four proteins were tested

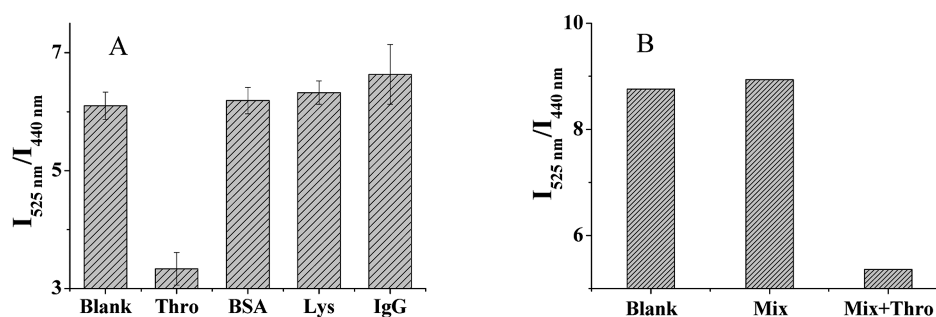


Figure 3. (A) Response of the $I_{525\ nm}/I_{440\ nm}$ ratio to thrombin (20 nM) and three negative proteins. (B) Response of the $I_{525\ nm}/I_{440\ nm}$ to thrombin (10 nM) in a complex system (Mix is a mixture of IgG, BSA, and Lys, each at 10 nM, and the value is an average of two parallel experiments).

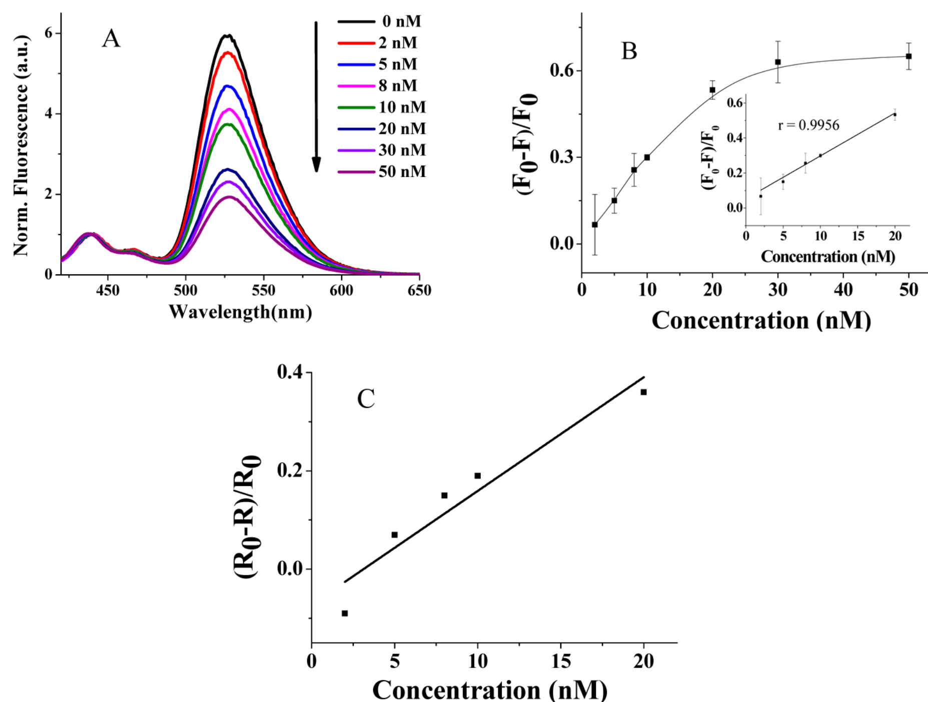


Figure 4. (A) Normalized fluorescence spectra and (B) the change of thrombin detection ($(R_0 - R)/R_0$) at different concentrations (2, 5, 8, 10, 20, 30, 50 nM) using the split aptamer. Inset shows the relationship between the quantity $(R_0 - R)/R_0$ and thrombin concentration in the range of 2−20 nM. (C) Response of $(R_0 - R)/R_0$ for the detection of thrombin (2−20 nM) with intact aptamer (the value is an average of two parallel experiments).

respectively with split aptamer fragments. As illustrated in Figure 3A, compared with the blank ($I_{525\ nm}/I_{440\ nm}$ = 6.1), the addition of thrombin (20 nM) gave a significantly decreased FRET signal ($I_{525\ nm}/I_{440\ nm}$ = 3.3). Notably, the other three proteins produced even higher FRET signals ($I_{525\ nm}/I_{440\ nm}$ = 6.2, 6.3, and 6.6 for BSA, Lys, and IgG, respectively) than the blank, because they cannot form the G-quadruplex with two split aptamer fragments. Because of the amphiphilicity of proteins, the electrostatic interactions between the PFEP and oligonucleotide were enhanced by proteins, thus leading to increased FRET signals. Furthermore, thrombin (10 nM) in a complex system was detected using split aptamer fragments. Results (Figure 3B) demonstrated that the $I_{525\ nm}/I_{440\ nm}$ ratio for Mix (a mixture of the above three control proteins, each at 10 nM) is 9.0, which is slightly higher than that of the blank ($I_{525\ nm}/I_{440\ nm}$ = 8.8). However, the FRET signal for thrombin in Mix is remarkably decreased to 5.4. Thus, this biosensor showed excellent selectivity for thrombin over other proteins, because of high specificity and affinity between Apt15 or Apt15-A/Apt15-B and thrombin.

Furthermore, the split aptamer-based sensing strategy was used to test thrombin with different concentrations from 0 to 50 nM. As shown in Figures 4A and 4B, the FRET signal decreased as the thrombin concentration increased, and a linear relationship (correlation coefficient of r = 0.9956) over the low-concentration range (0−20 nM) was observed. The limit of detection (LOD, defined as three times of the standard deviation of the blank signal) was calculated to be 2 nM. However, when using intact aptamer to detect thrombin in the low-concentration range (0−20 nM), the FRET signal is quite unstable (Figure 4C), and the LOD might be determined to be 10 nM at most. Thus, the sensitivity of this split aptamer-based strategy is comparable with most reported approaches and is superior to the method using the intact aptamer. Compared to a recently reported conjugated polymer amplified fluorescence method,[49] it is more rapid and simpler, in addition to a similar nanomolar detection limit.

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■ CONCLUSIONS

We have developed a novel simple and sensitive fluorescence aptamer sensor for the detection of thrombin. The use of two shorter split aptamer fragments greatly increases the possibility of forming G-quadruplex upon thrombin, leading to an improved signal-to-background ratio and a higher sensitivity. This method has advantages of being sensitive, rapid, homogeneous, and simple, compared with most reported approaches. Furthermore, the use of split aptamer fragments avoids a heating process for removing the secondary structure of the long DNA/RNA probe, so our strategy provides alternative ideas for designing simple and sensitive aptamer biosensors.

■ ASSOCIATED CONTENT

Supporting Information

UV-vis absorption and fluorescence spectra of donor (PFEP) and acceptor (FAM), response of $I_{525\ nm}/I_{440\ nm}$ for protein detection using Apt15-A individual, fluorescence emission intensity of PFEP in solutions of different pHs, and the composition of solutions with different pH values are included in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

∇These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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