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Thioflavin T as an Efficient G-Quadruplex Inducer for the Highly Sensitive Detection of Thrombin Using a New Föster Resonance Energy Transfer System

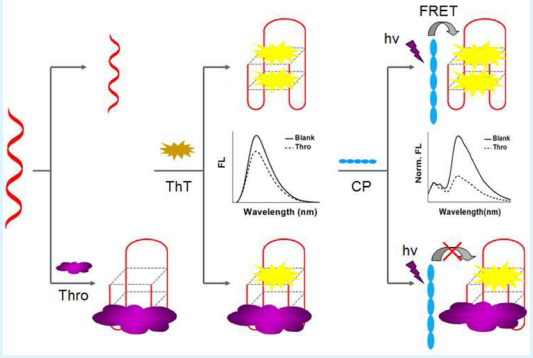
Xingfen Liu,*[,†,∇] Xiaoxiao Hua,[†,∇] Quli Fan,[†] Jie Chao,[†] Shao Su,[†] Yan-Qin Huang,[†] Lianhui Wang,[†] and Wei Huang*[,†,‡]

[†]Key Laboratory for Organic Electronics & Information Displays and Institute of Advanced Materials, Jiangsu National Synergetic Innovation Center for Advanced Materials, Nanjing University of Posts and Telecommunications, Nanjing 210023, China

[‡]Key Laboratory of Flexible Electronics & Institute of Advanced Materials, Jiangsu National Synergetic Innovation Center for Advanced Materials, Nanjing Tech University (NanjingTech), Nanjing 211816, China

Supporting Information

ABSTRACT: We report a new Föster resonance energy transfer (FRET) system that uses a special dye, thioflavin T (ThT), as an energy acceptor and a water-soluble conjugated polymer (CP) with high fluorescence as an energy donor. A simple, label-free, and sensitive strategy for the detection of thrombin in buffer and in diluted serum was designed based on this new system using ThT as an efficient inducer of the G-quadruplex. The difference between the blank and the positive samples was amplified due to distinctive FRET signals because thrombin has little effect on the intercalation of ThT into the G-quadruplex. In the absence of the target, ThT induces the aptamer to form a G-quadruplex and intercalates into it with strong fluorescence. The electrostatic attractions between the negatively charged G-quadruplex and positively charged CP allow a short donor–acceptor distance, resulting in a high FRET signal. However, in the presence of the target, the aptamer forms a G-quadruplex–thrombin complex first, followed by the intercalation of ThT into the G-quadruplex. A long distance exists between the donor and acceptor due to the strong steric hindrance from the large-sized thrombin, which leads to a low FRET signal. Compared with previously reported strategies based on the FRET between the CP and dye, our strategy is label-free, and the sensitivity was improved by an order of magnitude. Our strategy also shows the advantages of being simple, rapid (about 50 min), sensitive, label-free, and low-cost in comparison to strategies based on the FRET between quantum dots and dyes.



KEYWORDS: *G-quadruplex, thioflavin T, conjugated polymer, Föster resonance energy transfer, FRET, thrombin*

INTRODUCTION

The simple, rapid, sensitive, and low-cost detection of proteins plays an important role in biological analysis and medical diagnosis because many diseases show abnormal proteins or protein levels.[1] Antibody-based immunological methods including enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and fluorescence immunoassay are powerful tools to detect protein specifically.[2−4] However, these methods are confronted with some challenges, such as tedious protein modification, multiple washing steps, long assay time, and insufficient sensitivity.[5] Recently, as a alternative to the antibody-based assays, biosensors based on specific aptamer-protein recognition have attracted much attention.[6] Aptamers are specific DNA or RNA strands that show robust binding affinity for various chemical or biological targets.[7] Compared with antibodies, aptamers show the advantages of having small size and simple synthesis as well as easy labeling and good stability because they are specific nucleic acids and can be obtained by an in vitro evolution process called systematic evolution of ligands by exponential enrichment (SELEX).[8] So far, aptamers have been widely employed as excellent biorecognition elements in the design of various high-performance sensors (i.e., electrochemical, fluorometric, colorimetric, electrochemilluminscence, surface-enhanced Raman scattering, and surface plasmon resonance biosensors)[9−18] for application in bioanalysis, diagnostics, therapeutics, drug development, environmental and food analysis, and biocomputing.[19−23]

Conjugated polymer (CP) is a new class of fluorescent material characterized by a delocalized electronic structure that allows for efficient electronic coupling and, hence, fast intrachain and interchain energy transfer.[24] The CP also shows a strong absorption and an emission in the ultraviolet–visible (UV–vis) light range due to this unique structure. These

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advantages make CPs an ideal energy donor for a highly efficient Föster resonance energy transfer (FRET) to achieve amplified fluorescence signals and improved detection sensitivities.[25−36] In a typical FRET detection strategy, CP is used as an energy donor, and a dye label on the terminal of an oligonucleotide (or a peptide) acts as an energy acceptor. A pair of fluorophores are in close proximity due to the strong electrostatic attraction between the positively charged CP and the negatively charged DNA, thus leading to a highly efficient FRET. However, the fluorescent labeling of biomolecules with organic dye is laborious, time-consuming, and often expensive. What is more, this process may decrease the quantum yield of fluorophores due to the quenching of specific nucleotides and amino acids, such as guanine and tryptophan.[37−39] In general, only one dye molecule is labeled on one terminal of a single-stranded DNA (ssDNA), and so the fluorescence intensity depends on the concentration of the DNA, which may limit its application when the concentration of DNA is extremely low. As a solution to these problems, DNA intercalating dye was introduced to these FRET strategies to design label-free, low-cost, and time-saving biosensors.[40−47]

A DNA intercalating agent, such as SYBR Green, ethidium bromide (EB), and thiazole orange (TO), is a kind of organic dye that shows a higher affinity for double-stranded DNA (dsDNA) than for ssDNA. These dyes emit strong fluorescence when they intercalate into the groove of duplex but have very weak fluorescence when they exist in random states.[48−50] DNA intercalating agents have been used as probes for various DNA structures in addition to DNA-directed therapeutics.[51] Owing to the fact that more than one dye molecule can intercalate into the grooves of a DNA duplex,[52,53] the sensitivity of the sensor would be remarkably improved by using an intercalating dye and a label-free DNA probe. Studies from our group and others have provided excellent demonstrations on this possibility.[45,54−56] Currently, CP-based FRET strategies have mainly focused on duplex intercalating dyes (i. e., EB, TO, and SYBR); however, a study on the FRET between a G-quadruplex specific intercalating dye and a conjugated polymer has not been reported.

Thioflavin T (ThT), a benzothiazole dye that exhibits enhanced fluorescence upon binding to misfolded protein aggregates (called amyloids), is commonly used in the diagnosis of amyloid fibrils.[57,58] More recently, new research demonstrated that ThT can induce G-rich DNA to fold into the G-quadruplex and produce a much higher fluorescence enhancement (>1700-fold) compared with that of the enhancement (<250-fold) observed in other single- and double-strand DNA forms.[59] At present, the G-quadruplex-specific ThT has attracted increasing interest, and it has been used as a light-up fluorescence probe for the sensing of the G-quadruplex, biothiols, Hg^{2+}, K^{+}, liver-related short gene, and the structure change of the i-motif.[60−65] Inspired by these studies, we constructed a new FRET system using CP as an donor and ThT as an acceptor, and we designed a simple, label-free, and sensitive biosensor for the detection of thrombin in buffer and in diluted serum. Improved sensitivity for thrombin detection was obtained in comparison with that of two recent strategies based on the FRET from the CP to the dye.[34,66] Our strategy also shows the merits of being simpler, faster, and label-free in comparison with the reported assays based on another FRET system, in which quantum dots (QDs) were used as the donor, and a dye was used as the acceptor.[67−69]

EXPERIMENTAL SECTION

Materials and Chemicals. Conjugated polymer is a polyfluorene derivative doped with benzothiazole (named ThNI), and it was synthesized according to the literature.[70] The synthesis process and optical properties of ThNI have been reported in our recent study.[71] The concentration of ThNI refers to the concentration of the repeated unit (RU), which was calculated on the basis of the molecular weight and monomer weights of its neutral polymer. The molecular weight and polydispersity of its neutral polymer are 319, 900, and 1.1, respectively.[70]

All oligonucleotides used were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Concentrations of the oligonucleotides were determined by measuring their absorbance (also referred to as optical density) at 260 nm. Thioflavin T (3,6-dimethyl-2-(4-dimethylaminophenyl)benzothiazolium cation) was obtained from Sigma-Aldrich and used without further purification. Immunoglobin G was purchased from Shanghai Linc-Bio Science Co., LTD (Shanghai, China). Thrombin was purchased from Sigma-Aldrich. Lysozyme was purchased from Bio Basic Inc. (Toronto, Canada). Bovine serum albumin was purchased from Hangzhou Haoxin Biotech Co., LTD (Hangzhou, China). All other chemicals were obtained from Sigma-Aldrich, Acros, or Alfa and used as received. All solutions were prepared with Milli-Q water (18.2 MΩ·cm).

Experimental Procedures. In all experiments, the aptamer was prepared with 50 mM Tris−HCl buffer (pH 8.0). For the feasibility experiments based on the FRET between the CP and ThT, 16 μL of the 10 μM aptamer was incubated with 8 μL of the 1 μM thrombin or Lys in a certain volume of buffer solution (5 mM Tris−HCl, pH 7.2) at 37 °C for 40 min. The total reaction volume is 50 μL. After that, 4 μL of the 100 μM ThT was added and incubated for 3 min at room temperature. At last, 6 μL of the 10 μM CP solution and a certain volume of buffer solution (5 mM Tris−HCl, pH 7.2) were added to a final volume of 400 μL. The fluorescence of the mixture was detected at the excitation wavelength of 380 nm.

For the control experiment using ThT only, there is little difference from the above procedure. In detail, after the reaction of aptamer and thrombin, ThT was added and incubated for 3 min, and then a certain volume of buffer solution (5 mM Tris-HCl, pH 7.2) was added to a final volume of 400 μL. The fluorescence intensity was measured at an excitation wavelength of 425 nm.

For the specificity assays, three negative proteins (immunoglobin G, bovine serum albumin, and lysozyme) at the same concentration of thrombin were detected, respectively. After that, thrombin in an artificial mixture of several proteins was detected to investigate the selectivity of this strategy.

For the quantitative detection of thrombin in a buffer, 8 μL of thrombin at different concentrations (0.001, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 μM) was incubated with aptamer at 37 °C for 40 min. The other parts of the procedure are the same as the above assays.

For the thrombin detection in diluted serum, fetal bovine serum was diluted 10-fold with buffer (5 mM Tris−HCl, pH 7.2). After that, thrombin of different concentrations was reacted with aptamer in 10% fetal bovine serum at 37 °C for 40 min. Other conditions for this assay were the same as in the above assays.

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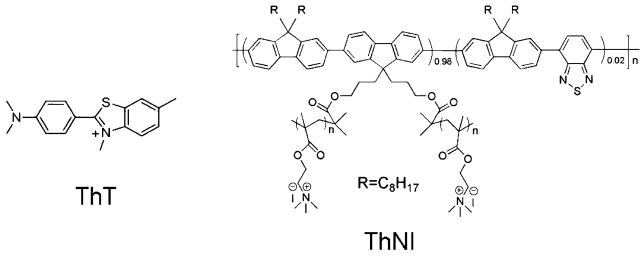
Methods. The absorbance of oligonucleotides was measured using a UV−vis Lambda35 spectrophotometer (PerkinElmer). All fluorescence spectra and FRET measurements were measured at room temperature in a 600 μL quartz cuvette using a PerkinElmer LS-55 fluorometer.

RESULTS AND DISCUSSION

The conjugated polymer used in this study was a cationic polyfluorene derivative doped with ThNI. It was synthesized according to the literature,[70] and the synthesis process and characterization data were reported in our recent study.[71] The chemical structures of ThNI and the fluorescent dye ThT are shown in Scheme 1.

Scheme 1. Chemical Structures of ThT and CP (ThNI)

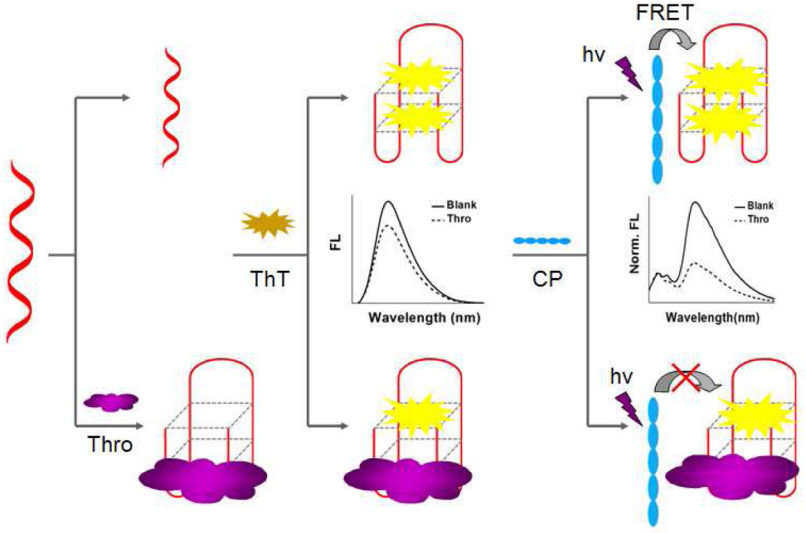


Scheme 2 describes the strategy of the ThT-induced G-quadruplex folding and further signal amplification via a highly efficient FRET from the CP to ThT for the detection of thrombin. The DNA probe is label-free, and it is the 29-mer aptamer of thrombin with the sequence 5′-AGTCCGTGG-TAGGGCAGGTTGGGGTGACT-3′. For the strategy using ThT only, ThT induced the G-rich DNA probe to fold into a G-quadruplex and then intercalated into it with high fluorescence in the absence of thrombin. However, the presence of thrombin still led to high fluorescence due to the weak hindrance from the G-quadruplex−thrombin complex because ThT is a small molecule that combines on the G-quartet, whereas thrombin is a macromolecule, and the 29-mer aptamer binds its heparin-binding domain.[53,59,72,73] Thus, it is impossible to distinguish the blank from the positive samples using ThT only.

Significantly, when the conjugated polymer was introduced to the above system, the difference between the blank and positive samples is amplified remarkably due to the distinguishable FRET signals. A highly efficient FRET occurs in the absence of thrombin because the electrostatic attraction between the negatively charged G-quadruplex and the positively charged CP allowed for a short distance between the donor and acceptor. In comparison, a low-efficiency FRET occurs in the presence of thrombin due to a long distance between the donor and acceptor caused by the strong steric hindrance from the large-sized protein.

At first, to investigate the possibility of FRET from the CP and ThT, we recorded the UV−vis absorption and fluorescence emission spectra of the ThNI and ThT. As shown in Figure 1A, ThNI has a characteristic emission peak at 420 nm and a weak shoulder peak at 441 nm. ThT has an absorption peak at 412 nm. The well-overlapping spectra provided a possibility of producing an efficient FRET process as long as they are in a close proximity. Next, the necessity for the use of conjugated polymers and the feasibility for the detection of thrombin via FRET between ThNI and ThT were investigated by comparing the response of the blank to that of thrombin or lysozyme before and after the addition of ThNI. Figure 1B shows the results for the detection of proteins using ThT only. The blank and lysozyme show higher fluorescence signals than did thrombin because ThT can induce the aptamer to fold into a G-quadruplex, followed by its intercalation into the G-quartets. In comparison, the fluorescence response of thrombin is slightly smaller (approximately 20%) than that of the blank and negative protein because the combination of aptamer and thrombin hindered the ThT from intercalating into the G-quartet. Surprisingly, when ThNI was introduced as an energy donor for FRET, the fluorescence signal of the blank, target protein, and negative protein is quite different. We observed an increased fluorescence intensity of ThNI at 419 nm and a decreased fluorescence intensity of ThT at 490 nm in comparison to the spectrum of the blank (Figure S1 in Supporting Information). It demonstrated that a highly efficient

Scheme 2. Descriptions of the ThT-Induced G-Quadruplex and Further Amplification Detection via the FRET from the CP to ThT



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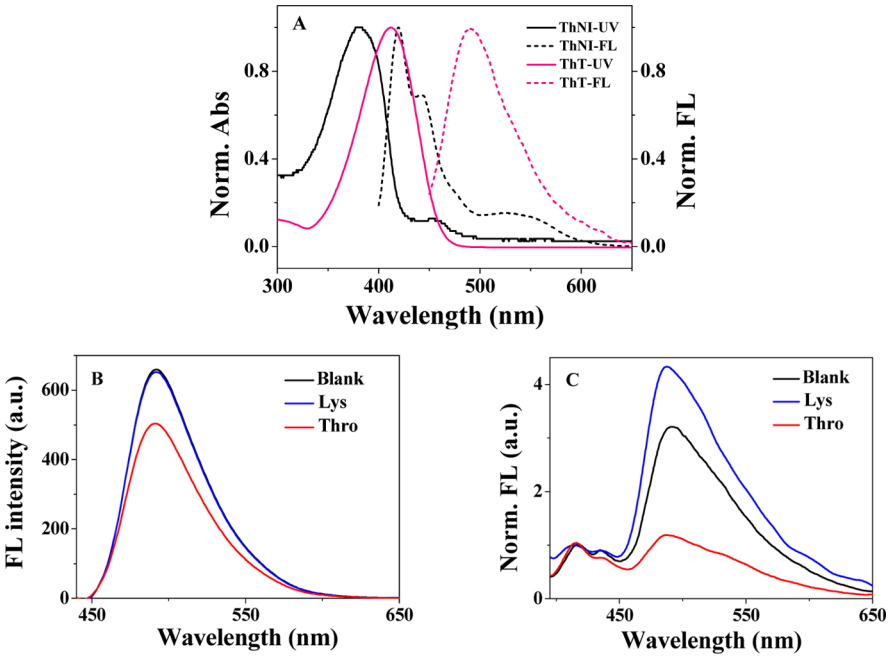

Figure 1. Normalized UV−vis absorption spectra and fluorescence spectra of donor (ThNI) and acceptor (ThT) (A). Fluorescence spectra of thrombin and lysozyme (both at 20 nM) detection without ThNI (B) and with ThNI (C).

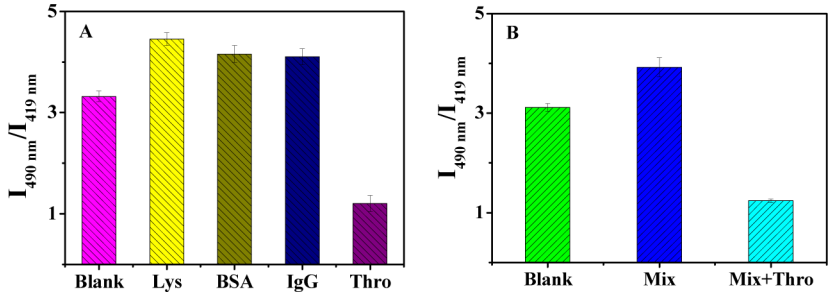

Figure 2. (A) Response of $I_{490\ nm}/I_{419\ nm}$ to different proteins (each at 20 nM). (B) Response of $I_{490\ nm}/I_{419\ nm}$ to Mix (a mixture of IgG, BSA, and Lys, each at 20 nM) and thrombin (20 nM) in Mix.

FRET occurs in the absence of the target or in the presence of the negative protein due to the strong electrostatic interactions between the G-quadruplex−ThT complex and CP. The normalized fluorescence spectra in Figure 1C shows that the blank produced a high FRET signal ($I_{490\ nm}/I_{419\ nm}$ = 3.2), and the lysozyme produced a higher signal ($I_{490\ nm}/I_{419\ nm}$ = 4.3) than did the blank. However, the thrombin gave a much lower signal ($I_{490\ nm}/I_{419\ nm}$ = 1.17). A higher signal of lysozyme over the blank may be attributed to the amphipathicity of protein, which may enhance the electrostatic interactions between the G-quadruplex−ThT complex and CP because it cannot combine aptamer. However, when the aptamer combines with thrombin, the distance between the ThT and ThNI increased, owing to a strong steric hindrance from large-sized thrombin, resulting in a remarkably decreased FRET signal.

To study the specificity and selectivity of this strategy, we detected three nontarget proteins (immunoglobin G (Ig G), bovine serum albumin (BSA), and lysozyme (Lys)). In addition, thrombin in an artificial complicated system was detected using this strategy. As illustrated in Figure 2A, three control proteins generated even higher FRET signals ($I_{490\ nm}/I_{419\ nm}$ is 4.10, 4.15, and 4.45 for IgG, BSA, and Lys, respectively) than did the blank because they cannot combine aptamers. Thus, the ThT-induced G-quadruplex formed in the system and the ThT molecules intercalated into the G-quartet. Strong electrostatic interactions occurred between the negatively charged G-quadruplex−ThT and the highly positively charged CP because the ThT molecule only has limited positive charges.[74] Owing to the existence of amphipathic protein, the electrostatic interactions between the ThNI and oligonucleotide were enhanced, which resulted in increased FRET signals. However, a remarkably decreased FRET signal ($I_{490\ nm}/I_{419\ nm}$ = 1.2) was observed upon thrombin (20 nM) due to the specific combination of aptamer and thrombin as well as the strong steric hindrance from the large-sized thrombin, which prevent the ThT and ThNI from approaching each other. Furthermore, thrombin (20 nM) in an artificial complex system was detected to evaluate the selectivity of our biosensor. The results in Figure 2B demonstrate that the $I_{490\ nm}/I_{419\ nm}$ for Mix (a mixed protein of the above three control proteins, each at 20 nM) is 3.92, which is slightly larger than that of the blank ($I_{490\ nm}/I_{419\ nm}$ = 3.12). However, the FRET signal for thrombin in Mix decreased to 1.24. Thus, this biosensor shows superior selectivity for thrombin over other proteins because of the high specificity and affinity between the

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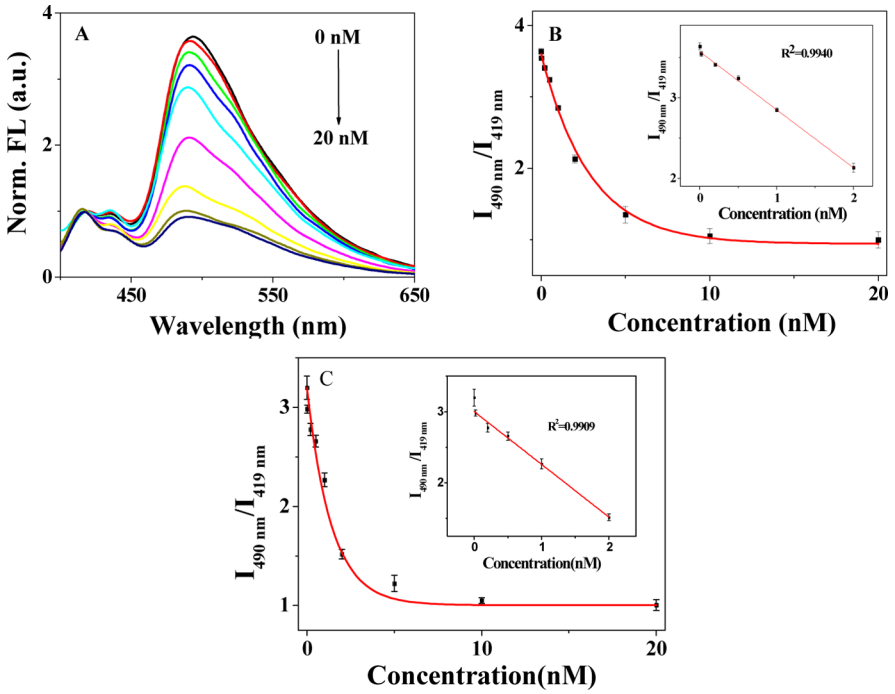

Figure 3. (A) Normalized fluorescence spectra and (B) the relationship between $I_{490\ nm}/I_{419\ nm}$ and thrombin concentrations (0, 0.02, 0.2, 0.5, 1, 2, 5, 10, and 20 nM) in buffer. The inset shows the linear relationship in the range of 0.02−2 nM. (C) Relationship between $I_{490\ nm}/I_{419\ nm}$ and thrombin concentrations. The concentrations are the same as those shown in panel B in 10% serum. The inset shows the linear relationship in the range of 0.02−2 nM.

Table 1. Comparison of Several Fluorometric Sensors for Thrombin Detection Based on the Two FRET Systems, CP-Dye and QDs-Dye

FRET pair	materials	labeling	LOD	time	sensor type	ref
CP-dye	ThNI, ThT, and aptamer[a]	no	0.2 nM	short	homogeneous	this work
CP-dye	PFEP, FAM-Apt15-A, and Apt15-B[b]	yes	2 nM	short	homogeneous	34
CP-dye	PFP-2F, FAM-TBA15, and TBA15-NPs[c]	yes	1.06 nM	long	solid-state	66
CP-dye	PT, NH_2-aptamer-Cy3, and glass slide[d]	yes	62 pM	medium	solid-state	75
QDs-dye	QDs, Cy3-DNA, and SH-aptamer	yes	~22 nM	long	homogeneous	68
QDs-dye	QDs, BOBO-3, and NH_2-aptamer[e]	yes	1 nM	medium	homogeneous	67
QDs-dye	QDs, Atto-647N-DNA, and NH_2-aptamer[f]	yes	0.5 nM	medium	homogeneous	69

[a]ThNI: a polyfluorene derivative doped with benzothiazole. ThT: thioflavin T. [b]PFEP: a cationic conjugated polyfluorene derivate. FAM: fluorescein. Apt15-A: a part of 15-mer antithrombin aptamer. Apt15-B: another part of 15-mer antithrombin aptamer. [c]PFP-2F: a positively charged polyfluorene derivate. TBA15: the 15-mer antithrombin aptamer. NPs: silica nanoparticles. [d]PT: polythiophene. Cy3: cyanine 3. [e]BOBO-3: a dimeric cyanine dye, 1,1′-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3 -thiazole)-2-methylidene]-pyridinium tetraiodide. [f]ATTO-647N: a cationic dye that labels the red spectral region.

aptamer and thrombin (K_d = 0.5 nM for the 29-mer aptamer).[72]

The sensitivity of this strategy was first studied by detecting thrombin at different concentrations (0 to 20 nM) in a buffer solution. As shown in Figure 3A,B, with an increasing concentration of thrombin, the normalized fluorescence intensity of ThT ($I_{490\ nm}/I_{419\ nm}$) gradually decreased. The FRET signal decreased linearly over the thrombin concentration range from 0.02 nM to 2 nM (y = 3.570 − 0.720x, R^2 = 0.9940, inset of Figure 3B). The limit of detection (LOD) is estimated to be 0.2 nM for thrombin in a buffer based on the equation $F - 3\sigma$, where F is the average value of the blank, and σ is the standard deviation of the blank. The sensitivity was improved by one order of magnitude in comparison with that of the recently reported thrombin assays.[34,66]

Furthermore, to evaluate the ability of this sensing system to detect thrombin in real and complicated matrixes, thrombin in 10-fold diluted fetal bovine serum was tested. The fetal bovine serum was diluted with 5 mM Tris−HCl buffer. Using the diluted serum as the reaction medium, the same process for thrombin determination (0, 0.02, 0.2, 0.5, 1, and 2 nM) in aqueous buffer was operated. As shown in Figure 3C, $I_{490\ nm}/I_{419\ nm}$ declined with the increasing concentration of thrombin. Significantly, we also obtained a linear range for thrombin in 10% serum, which was between 0.02 nM and 0.2 nM (y = 3.002−0.742x, R^2 = 0.9909, inset in Figure 3C). Additionally, we observed that the FRET signals were slightly lower than those in aqueous buffer. It may be attributed to the fact that substances in serum can quench the fluorescence of ThT; however, serum cannot quench but slightly enhance the fluorescence of ThNI (Figures S2 and S3 in the Supporting Information). The LOD for thrombin in diluted serum was estimated to be 0.2 nM based on the above $F - 3\sigma$ equation. The same linear range was obtained in the diluted serum

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samples, demonstrating a high robustness of our sensor in a complex biological sample matrix.

Finally, we listed the performance of several fluorometric methods for thrombin detection based on two kinds of FRET systems, CP-dye and QDs-dye. As shown in Table 1, our method showed the advantages of being label-free, sensitive, and time-saving over some recently reported methods. Compared with that of two similar homogeneous strategies based on the CP-dye FRET system, the limit of detection for thrombin was improved by one order of magnitude besides the label-free detection.[34,66] Compared with solid-state sensors based on the CP-dye FRET system, it showed the merits of being simpler, faster, and low-cost because tedious operations (centrifugation, washing, drying, and ultrasonic operations) and a long assay time were required for the solid-state detection.[66,75] In addition, our strategy also shows the merits of being fast, low-cost, label-free, and sensitive compared with the fluorometric methods based on the QDs-dye FRET system because the conjugation of biomolecules on the surface of QDs takes a long time (several hours), and labeled DNA is much more expensive than unlabeled.[67−69] Thus, we provide a simple, rapid, sensitive, and low-cost strategy that has better analytical performance and higher sensitivity to thrombin detection.

CONCLUSIONS

In summary, we demonstrated a homogeneous, label-free, and sensitive strategy for the detection of thrombin. Because the binding of thrombin with the G-quadruplex has little effect on ThT intercalation, the use of ThNI could amplify the difference between the blank and positive samples via efficient FRET. Compared to the results from recently reported strategies based on the FRET between the CP and dye, the sensitivity was improved by one order of magnitude. This is the first time that ThT and CP were employed as a donor−acceptor pair to construct FRET-based sensors, which utilized the specificity of ThT for the G-rich sequence and the amplification ability of CP, thus affording a favorable analytical performance. This work also provides opportunities to develop sensitive, label-free, and homogeneous biosensors for other targets using this CP-ThT system.

ASSOCIATED CONTENT

Supporting Information

Graphs showing the fluorescence spectra of thrombin and lysozyme detection based on the FRET between ThNI and ThT and of the responses of ThNI and ThT to the diluted serum and to serum of different concentrations, respectively. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b03662.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: iamxfliu@njupt.edu.cn.
*E-mail: wei-huang@njtech.edu.cn.

Author Contributions

▽X.L. and X.H. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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