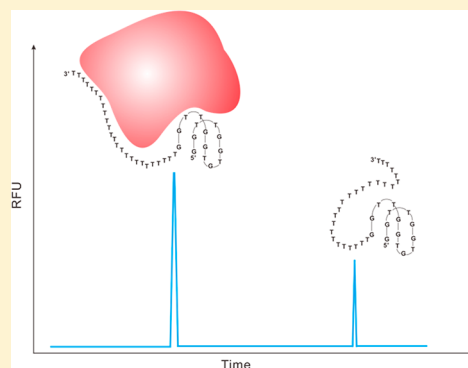


Enhancing the Affinity of Anti-Human α -Thrombin 15-mer DNA Aptamer and Anti-Immunoglobulin E Aptamer by PolyT ExtensionYunlong Bai,^{†,‡} Yapiiao Li,^{†,§} Dapeng Zhang,^{†,§} Hailin Wang,^{†,§} and Qiang Zhao^{*,†,§}[†]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China[‡]Institute of Environmental Science, Shanxi University, Taiyuan 030006, China[§]University of Chinese Academy of Sciences, Beijing 100049, China

Supporting Information

ABSTRACT: Aptamer affinity capillary electrophoresis–laser-induced fluorescence (CE-LIF) for protein detection takes advantage of aptamers for their ease of synthesis and labeling, small size, and having many negative charges. Its success relies on the high binding affinity of aptamers. One 15-mer DNA aptamer (5′-GGT TGG TGT GGT TGG-3′, Apt15) shows desirable specificity for human α -thrombin, an important enzyme with multiple functions in blood. However, Apt15 has weak binding affinity, and the use of Apt15 in affinity CE-LIF analysis remains challenging. Here we reported that extension of Apt15 at the 3′-end with a polyT tail having length of 18 T or longer significantly enhanced its affinity and enabled a well-isolated and stable peak for thrombin–aptamer complex in affinity CE. It was likely that the improvement of binding affinity resulted from double binding, an additional interaction of the polyT tail with thrombin in addition to the Apt15 section binding to thrombin. With dye-labeled Apt15 having a T₂₅ tail, we achieved detection of thrombin at concentrations as low as 0.1 nM by affinity CE-LIF. This aptamer probe specifically bound to human α -thrombin, showing negligible affinity for human β - and γ -thrombin, which are proteolyzed derivatives of human α -thrombin and share similar structure. This strategy of adding a polyT extension also enhanced the binding affinity of anti-immunoglobulin E aptamer in CE-LIF analysis, showing that the affinity enhancement approach is not limited to the thrombin-binding aptamer and has potential for more applications in bioanalysis.



Aptamer affinity capillary electrophoresis coupled with laser-induced fluorescence (CE-LIF) is a powerful technique in target detection and molecular interaction studies, having strengths of rapid analysis, low sample consumption, efficient separation, and high sensitivity.^{1–4} The unique features of aptamer probes, such as having many negative charges, small size, and easy labeling with fluorophore, make aptamers suitable for affinity CE-LIF analysis of proteins based on binding-induced change of electrophoretic mobility of aptamer.^{5–17} Aptamer affinity CE-LIF has been applied to detection of a variety of proteins and study of protein–aptamer interactions.^{5–16} Aptamers with high binding affinity are favorable for aptamer affinity CE-LIF analysis of protein and many other applications.

Thrombin is an important protease in blood with multiple functions in many physiological processes, and it is also a potential biomarker for some diseases.^{18–22} Aptamer-based assays for thrombin have attracted increasing attention.^{17,23,24} Among the frequently used aptamers for thrombin,^{25,26} a 29-nucleotide aptamer (5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′, denoted as Apt29 here) binds to the heparin-binding site of human α -thrombin with a dissociation constant (K_d) around 0.5 nM.²⁶ One 15-mer DNA aptamer (5′-GGT TGG TGT GGT TGG-3′, denoted as Apt15 here)

binds to the fibrinogen-binding site of human α -thrombin with a K_d around 100 nM.^{25–28} Though Apt15 has weak binding affinity, it is more desirable than Apt29 for selective detection of human α -thrombin because Apt15 specifically binds to human α -thrombin and shows negligible binding to human β - and γ -thrombin which are proteolyzed derivatives of human α -thrombin.^{27,29,30} In contrast, Apt29 does not distinguish between human α -, β -, and γ -thrombin.^{26,27,30,31}

Application of Apt15 for thrombin detection in aptamer affinity CE-LIF is quite challenging because the weak affinity of Apt15 makes the complex unstable and causes fast dissociation of bound aptamer during CE separation.^{5,7,8} Many efforts have been attempted for detection of thrombin with an Apt15 probe in affinity CE-LIF, by reducing the capillary length or increasing electric field to accelerate the separation in a suitable buffer.^{5,7,8} However, baseline separation of the complex from the unbound Apt15 probe was not possible⁵ or was difficult to achieve^{7,8} in CE-LIF analysis, and a large amount of aptamer probe was

Received: June 15, 2017

Accepted: August 1, 2017

Published: August 1, 2017



required. The obtained detection limit fell in the range from about 10 to 100 nM.^{5,7,8}

Here we report a polyT extension strategy to significantly enhance affinity of Apt15 and achieve a stable and well-separated aptamer–thrombin complex in affinity CE-LIF analysis. Attaching a polyT tail having a length of at least 18 T nucleotides on the 3′-end of Apt15 greatly improved the binding affinity of the aptamer. It was likely that an additional interaction between polyT tail and human α -thrombin occurred when the Apt15 section bound to thrombin, causing enhancement of binding affinity of aptamer due to these double interactions. The use of Apt15 with a T₂₅ tail enabled CE-LIF detection of human α -thrombin at concentrations as low as 0.1 nM. Apt15 with a T₂₅ tail shared the binding site of Apt15 on human α -thrombin and still maintained the capability to distinguish human α -thrombin from human β - and γ -thrombin. In addition, by attaching a polyT tail on the 5′-end of tetramethylrhodamine (TMR)-labeled aptamer for immunoglobulin E (IgE), enhanced binding affinity of aptamer was also achieved in CE-LIF analysis. The strategy of enhancing the binding affinity of an aptamer through adding a polyT extension may be applicable for other aptamers of protein targets. This affinity enhancement approach shows potential for other applications in biosensing and bioanalysis.

EXPERIMENTAL SECTION

Reagents. Lysozyme, platelet-derived growth factor BB (PDGF-BB), hemoglobin (Hb), and human immunoglobulin G (IgG) were ordered from Sigma (St. Louis, MO). Human IgE was purchased from Athens Research & Technology (Athens, GA). Human α -, β -, and γ -thrombin were obtained from Haematologic Technologies Inc. (Essex Junction, VT). Human serum was purchased from Zhongke Chenyu Biotechnology (Beijing, China). All unlabeled and TMR-labeled DNA oligonucleotides were synthesized and purified by Sangon Biotech (Shanghai, China). The sequences of DNA oligonucleotides are listed in Table S1 in Supporting Information. TG buffer (25 mM Tris and 192 mM glycine, pH 8.3) was prepared with chemicals of analytical pure grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and filtered with 0.22 μ m filter (Millipore, Bedford, MA). All solutions were prepared with water (18.2 M Ω) from Elga Labwater system (Purcell Ultra Genetic).

Apparatus. A laboratory-built CE-LIF system was used.^{9,16} A fused-silica capillary (Yongnian Company, Hebei, China; 365 μ m o.d., 25 μ m i.d.) with total length of 40 cm was employed, and the effective length was 34 cm. TMR was excited by a helium–neon laser (543.5 nm) (Melles Griot, Irvine, CA), and the fluorescence was filtered through a filter (model FF01-575/15-25, Rochester, NY) and collected by a photomultiplier tube (PMT). The signal from the PMT was analyzed by a HW-2000 chromatographic workstation (Qianpu Software, Shanghai, China).

Capillary Electrophoresis–Laser-Induced Fluorescence Analysis. TMR-labeled aptamer probes were incubated with thrombin in sample buffer containing 10 mM Tris-HCl (pH 7.5) and 5 mM KCl, and TMR dye (internal standard, IS) was also added in the sample buffer to correct possible variance of migration time in CE-LIF analysis. In calculation of peak area, the internal standard was not used. For analysis of IgE, sample buffer contained 10 mM Tris-HCl (pH 7.5) and 1 mM MgCl₂, and no TMR dye was added because the migration positions of TMR dye and IgE–aptamer complex overlapped.

After incubation for 1 h in ice (incubation temperature and time were optimized), the sample was injected by applying a positive voltage of 15 kV for 5 s. CE separation was conducted in TG buffer by applying a voltage of 20 kV at 25 °C. In experiments, before each injection of sample, the capillary was rinsed with 20 mM NaOH for 3 min, water for 2 min, and TG buffer for 2 min.

RESULTS AND DISCUSSION

Isolation of Thrombin–Aptamer Complex in Affinity Capillary Electrophoresis–Laser-Induced Fluorescence.

Figure 1A shows electropherograms of CE-LIF analysis of human α -thrombin with 5′-TMR-Apt15. In the absence of thrombin, the peak of 5′-TMR-Apt15 appeared at about 2.6 min, and the internal standard of TMR dye migrated at about 1.8 min. We did not find the peak for human α -thrombin–5′-TMR-Apt15 complex in CE-LIF analysis when 10 nM 5′-TMR-Apt15 was incubated with human α -thrombin in sample buffer, though the aptamer peak area decreased in the presence of human α -thrombin.⁸ At high concentrations of thrombin (higher than 20 nM), the baseline increased slightly after the aptamer peak migrated. Even when the concentrations of human α -thrombin increased to 200 nM, no complex peak appeared at early migration time. A stable thrombin–Apt15 complex was not achieved in CE separation due to weak affinity of Apt15, in agreement with the previous report.^{5,7}

We conjugated a series of polyT tails, having different lengths ranging from 10 to 35 T, at the 3′-end of Apt15 that had TMR labeling at the 5′-end (Table S1), and then we tested these aptamer probes in CE-LIF analysis of human α -thrombin (Figure 1B). When the attached polyT had a length of 18 T, a remarkable and well-isolated peak for human α -thrombin–aptamer probe complex began to appear in CE-LIF analysis (Figure 1B). It shows that a polyT tail with a minimum length of 18 T is required to achieve a stable thrombin–aptamer probe complex in CE separation. The peak areas of complexes linearly increased with further increase in length of polyT attached to Apt15 in the range from 18 to 25 T. The peak areas of complexes obtained with 5′-TMR-Apt15-T₂₅, 5′-TMR-Apt15-T₃₀, and 5′-TMR-Apt15-T₃₅ are close.

According to the previously reported method (shown in Supporting Information),^{9,16,32} the apparent dissociation constants (K_d) of TMR-labeled Apt15 having polyT tail with length ranging from 18 to 35 T were estimated and are summarized in Figure 1C: 13 nM for 5′-TMR-Apt15-T₁₈, 0.6 nM for 5′-TMR-Apt15-T₂₄, 0.6 nM for 5′-TMR-Apt15-T₂₅, 0.7 nM for 5′-TMR-Apt15-T₃₀, and 0.7 nM for 5′-TMR-Apt15-T₃₅. The results show that Apt15 derivatives with polyT tails longer than 18 T have high binding affinity for human α -thrombin. Among these tested aptamer probes, 5′-TMR-Apt15-T₂₅ is preferred in CE-LIF analysis because it enables a large peak area for the complex, and the peak of the complex is well isolated from that of the unbound aptamer probe.

In contrast, no complex peak was observed in CE-LIF analysis when TMR-labeled T₂₅ oligonucleotide probe (5′-TMR-T₂₅) was incubated with human α -thrombin (Figure S1). The peak of 5′-TMR-T₂₅ did not significantly change with increasing thrombin, showing that T₂₅ did not bind to human α -thrombin.

The use of 5′-TMR-Apt15 having different lengths of polyA tail on the 3′-end (Table S1) did not allow formation of a complex peak during CE-LIF analysis of human α -thrombin (10 nM). The result shows that the enhancement of binding

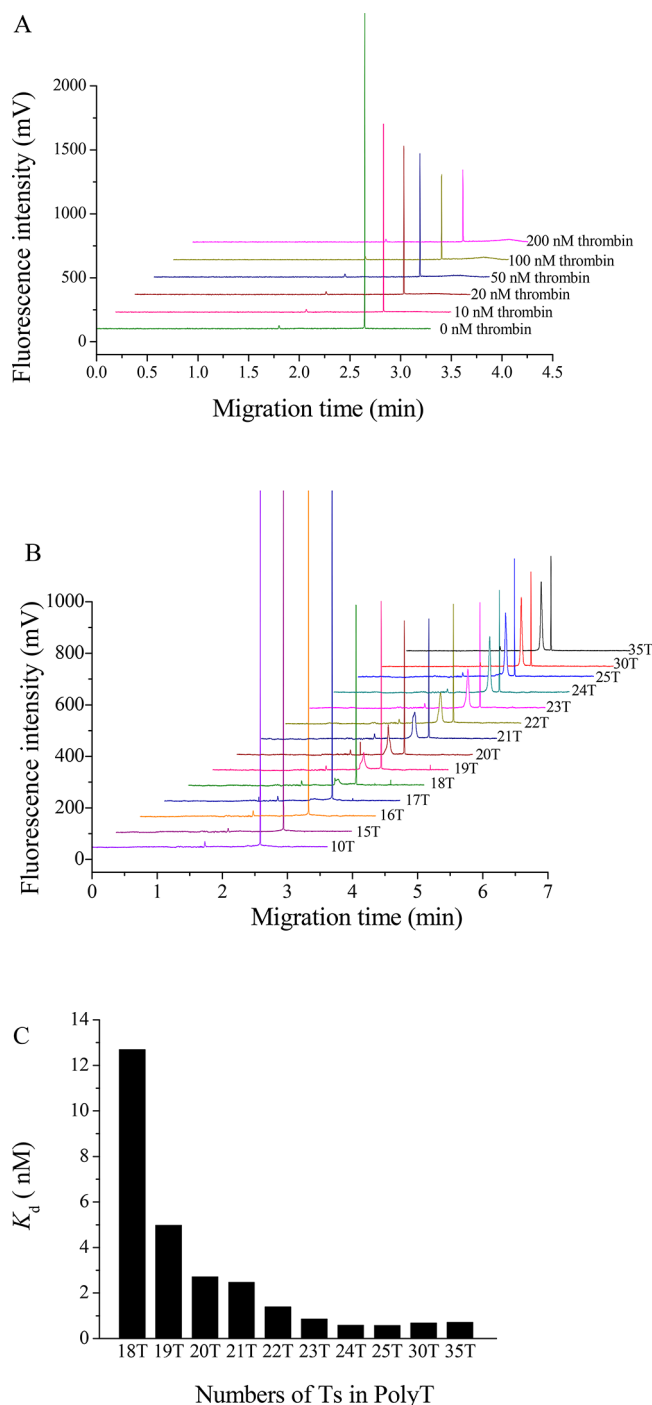


Figure 1. (A) Electropherograms of 5'-TMR-Apt15 (10 nM) in the presence of varying concentrations of human α -thrombin in sample buffer. (B) Electropherograms of aptamer probes (10 nM) with varying lengths of polyT tail at the 3'-end of Apt15 in the presence of human α -thrombin (10 nM) in sample buffer. Length of the polyT tail is indicated for each trace. (C) Apparent dissociation constant (K_d) versus length of polyT attached on Apt15, ranging from 18 to 35 T.

affinity of Apt15 with a polyT tail is related to the types of nucleotide in the extension. The added polyA may interact with the T bases of Apt15, possibly affecting the binding affinity of Apt15. When the added 25-nucleotide tail on the 3'-end contained a random T and G sequence (5'-TMR-Apt15-(TG)₂₅ in Table S1), a complex peak was also observed during CE-LIF analysis of human α -thrombin (10 nM) (Figure S2),

showing that the added random T and G sequence also enhanced the binding affinity of aptamer.

The attachment position of the polyT tail on Apt15 had great influence on the binding affinity of aptamer probe and the CE-LIF separation of human α -thrombin. We conjugated a T₂₅ tail on the 5'-end of Apt15 (T₂₅-Apt15) and attached a TMR label on either the 5'- or 3'-end (Table S1). We then tested these aptamer probes (5'-TMR-T₂₅-Apt15 and T₂₅-Apt15-3'-TMR) in CE-LIF analysis for human α -thrombin (Figure 2). For 5'-

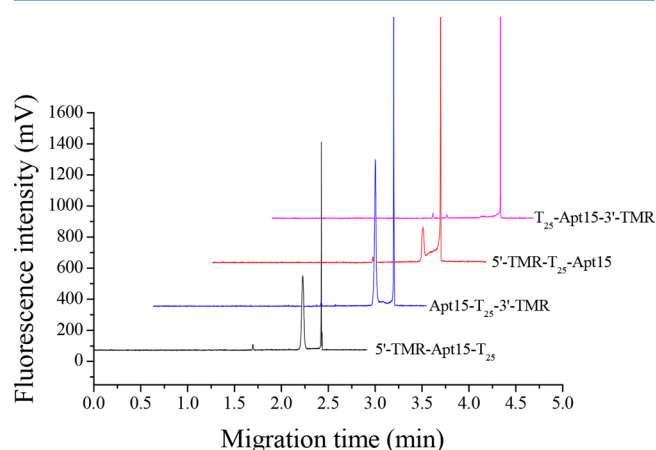


Figure 2. Performance of Apt15 having a T₂₅ tail attached at the 3'- or 5'-end in CE-LIF analysis of thrombin. Aptamer probe (10 nM) was incubated with human α -thrombin (10 nM) in sample buffer.

TMR-T₂₅-Apt15, we observed a peak for human α -thrombin–aptamer probe complex in CE-LIF analysis, but the peak for the complex was not well isolated from that for unbound aptamer probe (Figure 2). The peak area of 5'-TMR-T₂₅-Apt15–thrombin complex is smaller than that obtained for 5'-TMR-Apt15-T₂₅–thrombin complex. For T₂₅-Apt15-3'-TMR, the peak for human α -thrombin–aptamer probe complex is very small. The apparent K_d values of T₂₅-Apt15-3'-TMR and 5'-TMR-T₂₅-Apt15 were estimated as 228 nM and 8.8 nM, respectively, according to the previously reported method.^{9,16} This shows that adding T₂₅ on the 3'-end of Apt15 enables much better improvement of binding affinity of Apt15.

When Apt15-T₂₅-3'-TMR (with TMR labeling on 3'-end) was used, the peak area for thrombin–aptamer complex was higher. Aptamer probe Apt15-T₂₅-3'-TMR exhibited a higher fluorescence intensity, about 2 times that of 5'-TMR-Apt15-T₂₅. For 5'-TMR-Apt15-T₂₅, TMR was conjugated on the 5'-end, closer to the G bases of Apt15, and fluorescence quenching possibly occurred.^{33,34} For Apt15-T₂₅-3'-TMR, such quenching was reduced as TMR was far from the G bases in the aptamer sequence. The apparent K_d of Apt15-T₂₅-3'-TMR was estimated to be about 3.2 nM.⁹ Both 5'-TMR-Apt15-T₂₅ and Apt15-T₂₅-3'-TMR can be used as aptamer probes for detection of human α -thrombin in CE-LIF analysis.

Binding Behavior of Aptamer Probe on Thrombin. To explore the binding behavior of 5'-TMR-Apt15-T₂₅ on human α -thrombin, we performed competition experiments in CE-LIF analysis by using unlabeled oligonucleotide probes to displace 5'-TMR-Apt15-T₂₅. We incubated human α -thrombin (10 nM) with 5'-TMR-Apt15-T₂₅ (10 nM) and varying concentrations of unlabeled Apt15, Apt15-T₂₅, or T₂₅ in sample buffer and conducted CE-LIF analysis (Figure 3). As Figure 3A shows, upon addition of Apt15, the peak for 5'-TMR-Apt15-T₂₅–

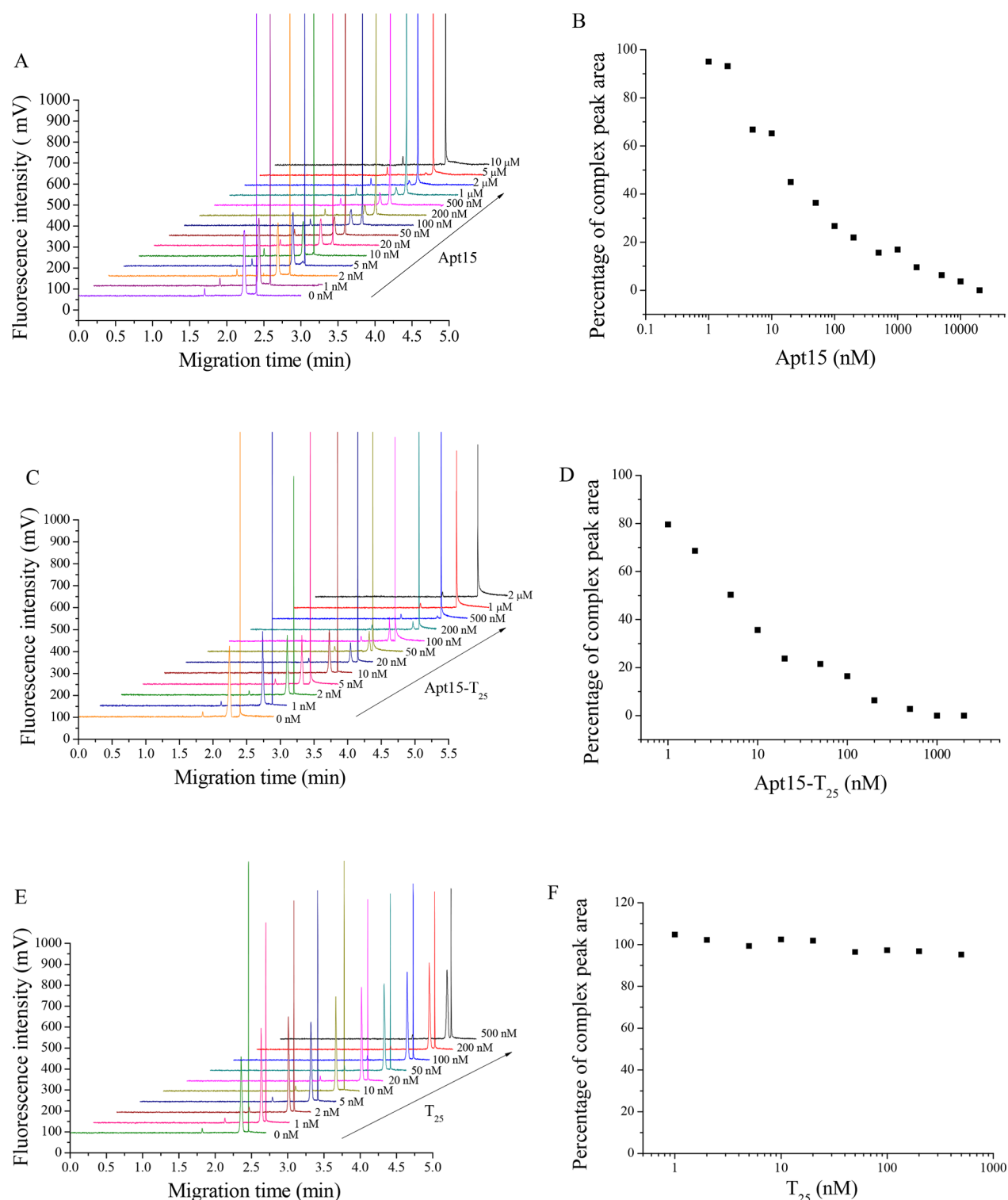


Figure 3. Competition CE-LIF experiments with (A, B) unlabeled Apt15, (C, D) unlabeled Apt15-T₂₅, or (E, F) unlabeled T₂₅. 5'-TMR-Apt15-T₂₅ (10 nM), human α -thrombin (10 nM), and varying concentrations of unlabeled (A) Apt15, (C) Apt15-T₂₅, or (E) T₂₅ were incubated in sample buffer. Relationships between the concentration of unlabeled DNA—(B) Apt15, (D) Apt15-T₂₅, or (F) T₂₅—and the percentage of complex peak area in the presence of unlabeled DNA relative to complex peak area in the absence of unlabeled DNA are also shown.

thrombin complex decreased. The presence of 20 nM unlabeled Apt15 caused about 50% decrease in complex peak area (Figure 3B). Addition of 20 μ M unlabeled Apt15 caused complete disappearance of the complex peak. The result indicates that Apt15 competes with 5'-TMR-Apt15-T₂₅ for binding to human α -thrombin in CE-LIF analysis. It has been known that Apt15

binds to the fibrinogen-binding site of human α -thrombin.^{26,28} This suggests that the Apt15 section of 5'-TMR-Apt15-T₂₅ possibly still binds to the fibrinogen-binding site of human α -thrombin.

Unlabeled Apt15-T₂₅ also competed with 5'-TMR-Apt15-T₂₅ for binding to human α -thrombin (Figure 3C,D). With

increasing unlabeled Apt15-T₂₅, the peak for 5'-TMR-Apt15-T₂₅-human α -thrombin complex significantly decreased. The presence of 5 nM unlabeled Apt15-T₂₅ caused about 50% decrease in complex peak area (Figure 3D). The complex peak completely disappeared when 1 μ M unlabeled Apt15-T₂₅ was present in sample buffer. This result shows that 5'-TMR-Apt15-T₂₅ and Apt15-T₂₅ have the same binding site on thrombin. It also proves that Apt15-T₂₅ has higher binding affinity for thrombin than Apt15.

In contrast, when unlabeled T₂₅ was present, the peak for 5'-TMR-Apt15-T₂₅-thrombin complex did not show remarkable change (Figure 3E,F). This shows that T₂₅ does not displace the bound Apt15-T₂₅ from thrombin and further confirms that T₂₅ does not bind to thrombin, as Figure S1 suggests.

The enhanced binding affinity of aptamer Apt15 having a polyT tail is probably caused by an additional interaction between the polyT tail and human α -thrombin when the Apt15 section binds to human α -thrombin. The double binding of polyT tail and the Apt15 section to thrombin significantly strengthens the interaction between human α -thrombin and aptamer. The additional interaction between polyT and thrombin requires polyT of a suitable length (as long as 18 T) (Figure 1C). The polyT tail with length shorter than 18 T perhaps is not long enough to reach the binding site on thrombin or cannot have a strong interaction with thrombin. However, the details of how the polyT tail interacts with human α -thrombin, and which part of thrombin binds the polyT tail, remain unknown.

Capillary Electrophoresis–Laser-Induced Fluorescence Detection of Human α -Thrombin. We successfully detected varying concentrations of human α -thrombin by using 5'-TMR-Apt15-T₂₅ probe (10 nM; Figure 4) or Apt15-T₂₅-3'-TMR (Figure S3) in CE-LIF analysis. With increasing thrombin, the complex peak increased gradually, and the peak for unbound aptamer probe decreased (Figure 4). Use of 5'-TMR-Apt15-T₂₅ probe allowed detection of human α -thrombin at concentrations as low as 0.1 nM. The peak area for the complex shows a linear relationship with the concentration of human α -thrombin ranging from 0.1 to 10 nM ($y = 64\,112x$, $R^2 = 0.997$). In CE-LIF analysis, Apt15-T₂₅-3'-TMR also enabled the detection of human α -thrombin at 0.1 nM (Figure S3) with a linear detection range from 0.1 to 10 nM ($y = 129\,415x$, $R^2 = 0.990$), and the sensitivity is higher due to the high fluorescence intensity of Apt15-T₂₅-3'-TMR probe.

The detection limit for human α -thrombin in this work is much lower than those previously reported for CE-LIF detection of thrombin by use of dye-labeled Apt15, more than 100-fold reduction.^{5,7,8} Table S2 summarizes the comparison of our method and previously reported methods for thrombin detection by aptamer-based affinity CE-LIF with respect to type of aptamer, detection limit, detection range, total length of capillary, and effective length of capillary. The Apt15-T₂₅ probe shows advantages over Apt15 in CE-LIF analysis of thrombin, and the peak for Apt15-T₂₅ probe–thrombin complex is more stable. Compared with the work where Apt29 was used as affinity ligand in CE-LIF analysis,^{9–11} the sensitivity obtained by use of Apt15-T₂₅ is comparable to that obtained by use of Apt29. Clearly, dye-labeled Apt15-T₂₅ probe enables a higher sensitivity for thrombin detection in CE-LIF than Apt15 probe.^{5,7,8}

Selectivity of Apt15-T₂₅ Probe in Capillary Electrophoresis–Laser-Induced Fluorescence Analysis. We examined the selectivity of CE-LIF analysis of human α -

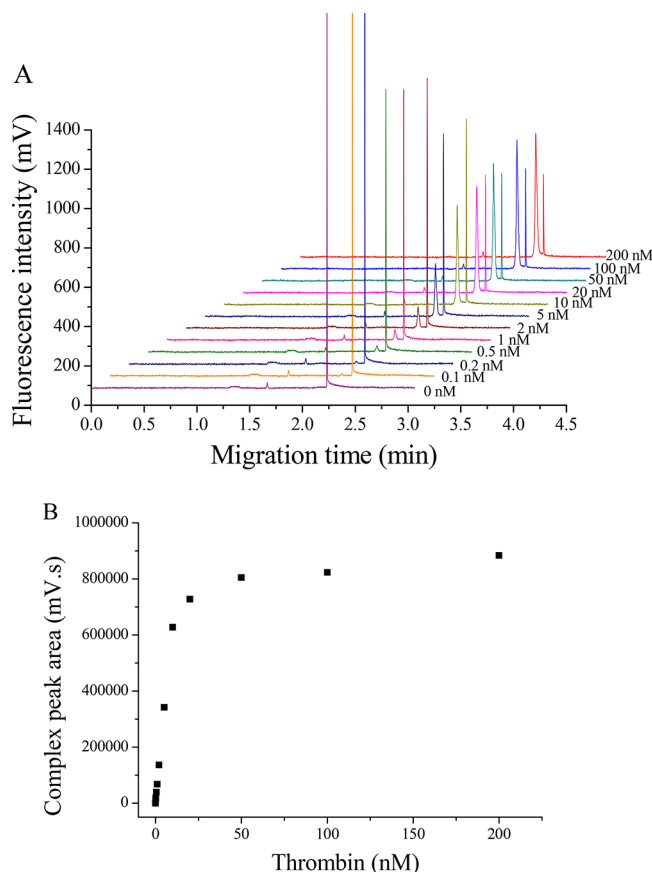


Figure 4. (A) Electropherograms of CE-LIF detection of varying concentrations of human α -thrombin by use of 5'-TMR-Apt15-T₂₅ (10 nM). (B) Relationship between peak area for complex and concentration of thrombin.

thrombin using 5'-TMR-Apt15-T₂₅ as aptamer probe by testing other proteins (10 nM) including human β -thrombin, human γ -thrombin, human prothrombin, hemoglobin (Hb), human immunoglobulin G (IgG), lysozyme, and platelet-derived growth factor BB (PDGF-BB). When 5'-TMR-Apt15-T₂₅ was used, no complex peak was observed for the tested proteins (Figure S4). These tested proteins did not show interference with human α -thrombin detection. This shows that 5'-TMR-Apt15-T₂₅ can discriminate human α -thrombin from human β - and γ -thrombin and does not bind to β - and γ -thrombin, which are autoproteolyzed derivative of human α -thrombin.^{29,30} It has been known that γ -thrombin contains the heparin-binding site instead of the fibrinogen-binding site,^{27,29,30} so this indicates that 5'-TMR-Apt15-T₂₅ does not bind to the heparin-binding site of α -thrombin. In contrast, we also applied Apt29 with TMR labeling on the 5'-end in CE-LIF analysis, and in this case human α -, β -, and γ -thrombin gave similar peaks for thrombin–Apt29 complexes (data not shown). This confirms that Apt29 does not have the capability to distinguish the differences between human α -, β -, and γ -thrombin,^{27,31} even though Apt29 has high binding affinity in CE-LIF analysis. Therefore, the aptamer probe Apt15-T₂₅ shows better performance for specific detection of human α -thrombin in aptamer affinity CE-LIF analysis, having an advantage over the Apt29 probe in selectivity.

Next, we further tested the possibility of detection of human α -thrombin in a complex sample matrix. Thrombin spiked in a 50-fold diluted human serum sample was successfully detected

with 5'-TMR-Apt15-T₂₅ in CE-LIF analysis (Figure S5). The detection limit for human α -thrombin was about 0.2 nM in 50-fold diluted human serum sample. The linear detection range was from 0.2 to 10 nM ($y = 38\,614x$, $R^2 = 0.977$). This shows that 5'-TMR-Apt15-T₂₅ is capable of detecting α -thrombin in a complex sample matrix. Apt15-T₂₅-3'-TMR also enabled the detection of α -thrombin spiked in 50-fold diluted serum sample. Thrombin spiked in 20-fold diluted serum sample could also be detected; however, the recovery rate was lower than in the 50-fold diluted serum sample. Some components in serum sample may cause nonspecific interactions and influence the binding between thrombin and aptamer, so more dilution of serum sample is needed for analysis.

Enhancing Binding Affinity of Anti-IgE Aptamer by Attaching PolyT Tail. To test whether this strategy of enhancing aptamer affinity by attaching polyT on the end of the aptamer can be applied to aptamers for other protein targets, we also tried the aptamer for IgE (denoted as AptIgE, Table S3).³⁵ PolyT having different lengths (10, 20, 30, and 40 T) was attached on the 5'-end of anti-IgE aptamer probe that had TMR labeling at the 3'-end (Table S3). When the TMR-labeled aptamer, AptIgE-3'-TMR (10 nM), was applied, a small complex peak was observed in CE-LIF analysis of IgE (10 nM; Figure 5A). When anti-IgE aptamers having polyT tails on the 5'-end (T₁₀-AptIgE-3'-TMR, T₂₀-AptIgE-3'-TMR, T₃₀-AptIgE-3'-TMR, and T₄₀-AptIgE-3'-TMR) were used in CE-LIF analysis, larger complex peaks were obtained (Figure 5A). We calculated the apparent K_d values of the tested aptamer probes.^{9,16} (Figure 5B). The apparent K_d of AptIgE-3'-TMR was about 50 nM, while the K_d of aptamer with polyT tail on the 5'-end was lower. The K_d of T₄₀-AptIgE-3'-TMR was about 15 nM, indicating the binding affinity of aptamer was improved about 3-fold by attaching T₄₀ to the 5'-end of anti-IgE aptamer. This result suggests that enhancement of binding affinity by attaching polyT on the end of the aptamer is not limited to the anti-thrombin aptamer Apt15 but also is applicable for aptamers of other protein targets.

CONCLUSION

In summary, we reported a strategy to enhance the binding affinity of aptamers through introduction of a polyT extension on the aptamer. The extension of 15-mer DNA aptamer on the 3'-end with a polyT tail greatly improved the binding affinity and facilitated formation of a stable human α -thrombin-aptamer probe complex in aptamer affinity CE-LIF analysis. The enhancement effect on binding affinity depended on the length and attachment position of the polyT tail. The polyT tail possibly had an additional interaction on thrombin when the Apt15 section bound with thrombin, significantly tightening the affinity binding. By using Apt15 with a T₂₅ tail conjugated on the 3'-end, we achieved sensitive detection of human α -thrombin with a detection limit of 0.1 nM. The Apt15 probe with polyT tail shared the binding site of Apt15 on human α -thrombin and had the capability to distinguish human α -thrombin from human β - and γ -thrombin. The strategy of enhancing binding affinity of aptamer through adding a suitable length of polyT on the end of the aptamer also improved the affinity of anti-IgE aptamer in CE-LIF analysis, showing the demonstrated approach is not limited to anti-thrombin aptamer. This strategy has potential for more applications in affinity separation, bioanalysis, and biosensing using aptamers.

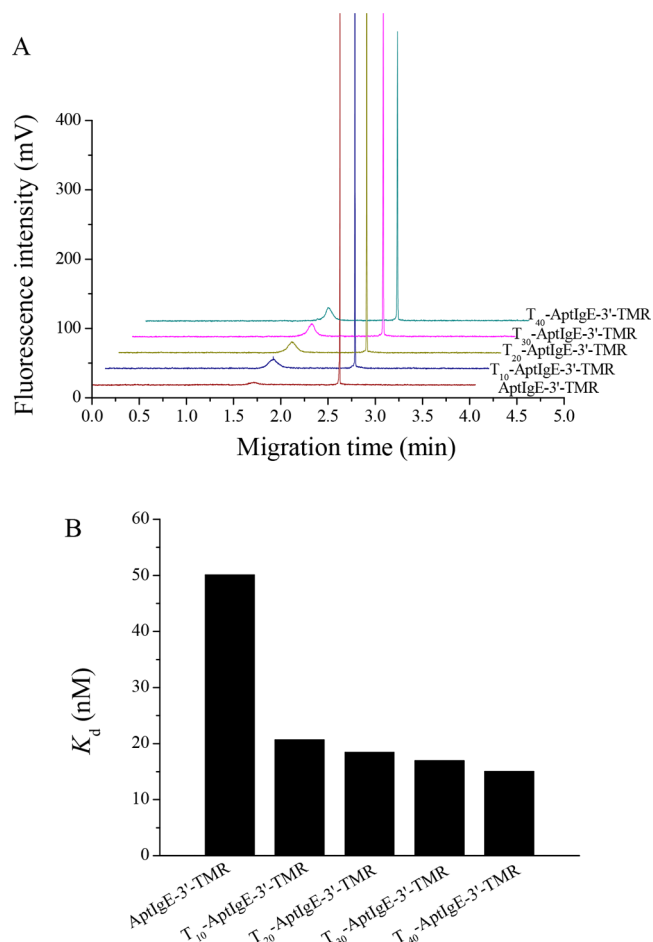


Figure 5. CE-LIF analysis of binding between IgE and anti-IgE aptamer probes (AptIgE) with different lengths of polyT at the 5'-end and TMR labeling at the 3'-end. Length of the polyT tail ranged from 0 to 40 T. (A) Electropherograms of different aptamer probes (10 nM) in the presence of IgE (10 nM) in sample buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM MgCl₂. (B) Apparent dissociation constant (K_d) versus TMR-labeled AptIgE probe having polyT attached on the 5'-end, with the length ranging from 0 to 40 T.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b02313.

Additional text describing calculation of apparent dissociation constants; three tables listing tested DNA oligonucleotides and detection of thrombin; five figures showing electropherograms of 5'-TMR-T₂₅, comparison of aptamers in CE-LIF analysis, detection of human α -thrombin by Apt15-T₂₅-3'-TMR, specificity test, and detection of human α -thrombin in spiked sample (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge support from National Natural Science Foundation of China (Grants 21575153 and 21435008) and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB14030200).

■ REFERENCES

- (1) Hu, S.; Dovichi, N. J. *Anal. Chem.* **2002**, *74*, 2833–2850.
- (2) Harstad, R. K.; Johnson, A. C.; Weisenberger, M. M.; Bowser, M. T. *Anal. Chem.* **2016**, *88*, 299–319.
- (3) Gayton-Ely, M.; Pappas, T. J.; Holland, L. A. *Anal. Bioanal. Chem.* **2005**, *382*, 570–580.
- (4) Albishri, H. M.; Deeb, S. E.; AlGarabli, N.; AlAstal, R.; Alhazmi, H. A.; Nachbar, M.; El-Hady, D. A.; Watzig, H. *Bioanalysis* **2014**, *6*, 3369–3392.
- (5) German, I.; Buchanan, D. D.; Kennedy, R. T. *Anal. Chem.* **1998**, *70*, 4540–4545.
- (6) Pavski, V.; Le, X. C. *Anal. Chem.* **2001**, *73*, 6070–6076.
- (7) Buchanan, D. D.; Jameson, E. E.; Perlette, J.; Malik, A.; Kennedy, R. T. *Electrophoresis* **2003**, *24*, 1375–1382.
- (8) Huang, C. C.; Cao, Z. H.; Chang, H. T.; Tan, W. H. *Anal. Chem.* **2004**, *76*, 6973–6981.
- (9) Song, M. Y.; Zhang, Y. X.; Li, T.; Wang, Z. X.; Yin, J. F.; Wang, H. L. *J. Chromatogr. A* **2009**, *1216*, 873–878.
- (10) Li, Y. L.; Guo, L.; Zhang, F. C.; Zhang, Z. Y.; Tang, J. J.; Xie, J. W. *Electrophoresis* **2008**, *29*, 2570–2577.
- (11) Zhang, H.; Li, X. F.; Le, X. C. *J. Am. Chem. Soc.* **2008**, *130*, 34–35.
- (12) Shen, R.; Guo, L.; Zhang, Z.; Meng, Q.; Xie, J. *J. Chromatogr. A* **2010**, *1217*, 5635–5641.
- (13) Zhang, H.; Li, X.-F.; Le, X. C. *Anal. Chem.* **2009**, *81*, 7795–7800.
- (14) Zhang, H.; Wang, Z.; Li, X. F.; Le, X. C. *Angew. Chem., Int. Ed.* **2006**, *45*, 1576–1580.
- (15) Wang, H.; Lu, M.; Le, X. C. *Anal. Chem.* **2005**, *77*, 4985–4990.
- (16) Zhang, D. P.; Lu, M. L.; Wang, H. L. *J. Am. Chem. Soc.* **2011**, *133*, 9188–9191.
- (17) Li, F.; Zhang, H.; Wang, Z.; Newbigging, A. M.; Reid, M. S.; Li, X. F.; Le, X. C. *Anal. Chem.* **2015**, *87*, 274–292.
- (18) Davie, E. W.; Kulman, J. D. *Semin. Thromb. Hemostasis* **2006**, *32* (Suppl 1), 3–15.
- (19) Crawley, J. T.; Zanardelli, S.; Chion, C. K.; Lane, D. A. *J. Thromb. Haemostasis* **2007**, *5* (Suppl 1), 95–101.
- (20) Franchini, M.; Mannucci, P. M. *Semin. Thromb. Hemostasis* **2012**, *38*, 95–101.
- (21) Lawson, J. H. *Semin. Thromb. Hemostasis* **2006**, *32* (Suppl1), 98–110.
- (22) Maragoudakis, M. E.; Tsopanoglou, N. E. *Thrombin: Physiology and Disease*; Springer Science: New York, 2009.
- (23) Liu, J.; Cao, Z.; Lu, Y. *Chem. Rev.* **2009**, *109*, 1948–1998.
- (24) Deng, B.; Lin, Y.; Wang, C.; Li, F.; Wang, Z.; Zhang, H.; Li, X. F.; Le, X. C. *Anal. Chim. Acta* **2014**, *837*, 1–15.
- (25) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564–566.
- (26) Tasset, D. M.; Kubik, M. F.; Steiner, W. *J. Mol. Biol.* **1997**, *272*, 688–698.
- (27) Paborsky, L. R.; McCurdy, S. N.; Griffin, L. C.; Toole, J. J.; Leung, L. L. *K. J. Biol. Chem.* **1993**, *268*, 20808–20811.
- (28) Krauss, I. R.; Merlino, A.; Randazzo, A.; Novellino, E.; Mazzarella, L.; Sica, F. *Nucleic Acids Res.* **2012**, *40*, 8119–8128.
- (29) Soslau, G.; Goldenberg, S. J.; Class, R.; Jameson, B. *Platelets* **2004**, *15*, 155–166.
- (30) Lundblad, R. L.; Nesheim, M. E.; Straight, D. L.; Sailor, S.; Bowie, J.; Jenzano, J. W.; Roberts, J. D.; Mann, K. G. *J. Biol. Chem.* **1984**, *259*, 6991–6995.
- (31) Zhao, Q.; Wang, X. *Biosens. Bioelectron.* **2012**, *34*, 232–237.
- (32) Berezovski, M.; Nutiu, R.; Li, Y.; Krylov, S. N. *Anal. Chem.* **2003**, *75*, 1382–1386.
- (33) Torimura, M.; Kurata, S.; Yamada, K.; Yokomaku, T.; Kamagata, Y.; Kanagawa, T.; Kurane, R. *Anal. Sci.* **2001**, *17*, 155–160.
- (34) Zhang, D.; Zhao, Q.; Zhao, B.; Wang, H. *Anal. Chem.* **2012**, *84*, 3070–3074.
- (35) Wiegand, T. W.; Williams, P. B.; Dreskin, S. C.; Jouvin, M.; Kinet, J.; Tasset, D. *J. Immunol.* **1996**, *157*, 221–230.

Supporting Information

Enhancing the Affinity of Anti-Human α -Thrombin 15-mer DNA Aptamer and Anti-Immunoglobulin E Aptamer by PolyT Extension

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Calculation of Apparent Dissociation Constants in CE-LIF Analysis

The apparent dissociation constant (K_d) was estimated by the formula below.^{9,16}

$$K_d = \frac{[\text{Protein}](1 + A1/(A2 + A3)) - [\text{Aptamer}]}{1 + (A2 + A3)/A1}$$

A1 is the peak area of unbound aptamer. A2 represents the peak area of aptamer-protein complex. A3 is the dissociated part from the complex during CE separation. [Protein] and [Aptamer] are the total concentrations of protein and aptamer in the sample buffer, respectively. A detailed procedure was described in previous report.^{9,16} We calculated the A1, A2, and A3 by measuring the corresponding area in the chromatographic software. We drew the baseline of the electropherogram curve, and the corresponding dissociation part between the complex peak and unbound aptamer probe peak could be observed. The area of the dissociation part was then measured.

Table S1. List of tested DNA oligonucleotides for the study of thrombin-aptamer binding

Name	Sequence
5'-TMR-Apt15	5'- TMR -GGT TGG TGT GGT TGG -3'
5'-TMR-Apt15-T ₁₀	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT T-3'
5'-TMR-Apt15-T ₁₅	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT-3'
5'-TMR-Apt15-T ₁₆	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT T-3'
5'-TMR-Apt15-T ₁₇	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TT-3'
5'-TMR-Apt15-T ₁₈	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT-3'
5'-TMR-Apt15-T ₁₉	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT T-3'
5'-TMR-Apt15-T ₂₀	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TT-3'
5'-TMR-Apt15-T ₂₁	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT-3'
5'-TMR-Apt15-T ₂₂	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT T-3'
5'-TMR-Apt15-T ₂₃	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TT-3'
5'-TMR-Apt15-T ₂₄	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TT-3'
5'-TMR-Apt15-T ₂₅	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TTT T-3'
5'-TMR-Apt15-T ₃₀	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3'
5'-TMR-Apt15-T ₃₅	5'- TMR -GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT-3'
5'-TMR-Apt15-A ₁₀	5'- TMR -GGT TGG TGT GGT TGG AAA AAA AAA A-3'
5'-TMR-Apt15-A ₁₅	5'- TMR -GGT TGG TGT GGT TGG AAA AAA AAA AAA AAA-3'
5'-TMR-Apt15-A ₂₀	5'- TMR -GGT TGG TGT GGT TGG AAA AAA AAA AAA AAA AAA AA-3'
5'-TMR-Apt15-A ₂₅	5'- TMR -GGT TGG TGT GGT TGG AAA AAA AAA AAA AAA

	AAA AAA AAA A-3'
5'-TMR-Apt15-A ₃₀	5'- TMR -GGT TGG TGT GGT TGG AAA AAA AAA AAA AAA AAAA AAAAA AAAAA A-3'
5'-TMR-Apt15-A ₃₅	5'- TMR -GGT TGG TGT GGT TGG AAA AAA AAA AAA AAA AAAA AAAAA AAAAA A-3'
5'-TMR-Apt15-(TG) ₂₅	5'- TMR -GGT TGG TGT GGT TGG TTG TGT GTG TTG GTG GTT TGG GGG G-3'
Apt15-T ₂₅ -3'-TMR	5'-GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TTT T- TMR -3'
5'-TMR-T ₂₅ -Apt15	5'- TMR -TTT TTT TTT TTT TTT TTT TTT TTT TGG TTG GTG TGG TTG G-3'
T ₂₅ -Apt15-3'-TMR	5'-TTT TTT TTT TTT TTT TTT TTT TTT TGG TTG GTG TGG TTG G - TMR -3'
5'-TMR-T ₂₅ Apt15-T ₂₅	5'- TMR -TTT TTT TTT TTT TTT TTT TTT TTT T-3' 5'-GGT TGG TGT GGT TGG TTT TTT TTT TTT TTT TTT TTT TTT T-3'
Apt15 T ₂₅	5'-GGT TGG TGT GGT TGG-3' 5'-TTT TTT TTT TTT TTT TTT TTT TTT T-3'

Table S2 Comparison of CE-LIF detection of thrombin using dye-labeled aptamers

Aptamer	Total Length of capillary	Effective length of capillary	Inner diameter of capillary	Concentration of aptamer probe	Detection limit	Linear range for detection	Reference
Apt15	50 cm	25 cm	50 µm	200 nM	9.8 nM	9.8-200 nM	8
Apt15	15 cm	5 cm	50 µm	200 nM	10.9 nM	10.9-200 nM	8
Apt15	37 cm	7 cm	50 µm	800 nM	100 nM	100-900 nM	7
Apt15	30 cm	7 cm	50 µm	2000 nM	40 nM	NA ^a	5
Apt29	30 cm	23.5 cm	25 µm	10 nM	0.083 nM	0.083-10 nM	9
Apt29	30.2 cm	20 cm	75 µm	200 nM	2 nM	2-400 nM	10
Apt29	30.2 cm	20 cm	75 µm	10 nM	10 nM	10-300 nM	10
Apt29	40 cm	NA	20 µm	100 nM	0.1 nM	1-100 nM	11
Apt15-T ₂₅	40 cm	34 cm	25 µm	10 nM	0.1 nM	0.1-10 nM	This work

^a NA means not available

Table S3. List of tested DNA oligonucleotides for the study of IgE-aptamer binding

Name	Sequence
AptIgE-3'-TMR	5'-GGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCC CCT- TMR -3'
T ₁₀ -AptIgE-3'-TMR	5'-TTTTTTTTTTGGGGCACGTTTATCCGTCCCTCCTAG TGCGTGCCCT- TMR -3'
T ₂₀ -AptIgE-3'-TMR	5'-TTTTTTTTTTTTTTTTTTTTTTGGGGCACGTTTATCCGT CCCTCCTAGTGGCGTGCCCT- TMR -3'
T ₃₀ -AptIgE-3'-TMR	5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGGC GTTTATCCGTCCCTCCTAGTGGCGTGCCCT- TMR -3'
T ₄₀ -AptIgE-3'-TMR	5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTGGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCC CCT- TMR -3'

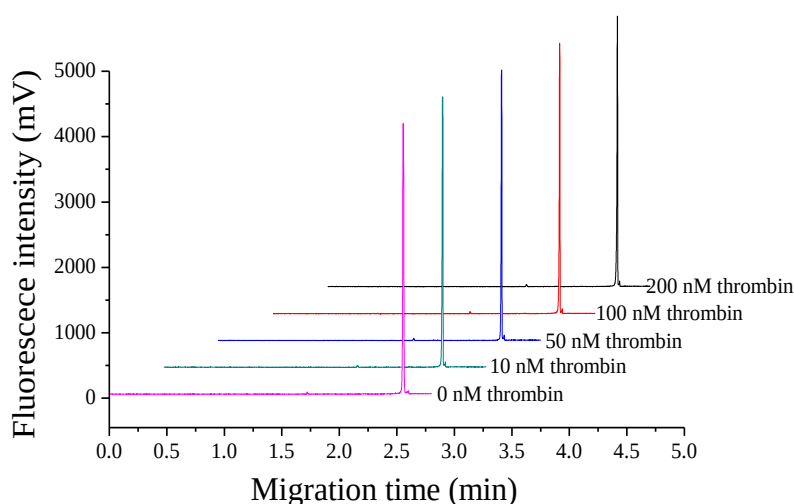


Figure S1. Electropherograms of the 5'-TMR-T₂₅ (10 nM) in the presence of varying concentrations of human α -thrombin.

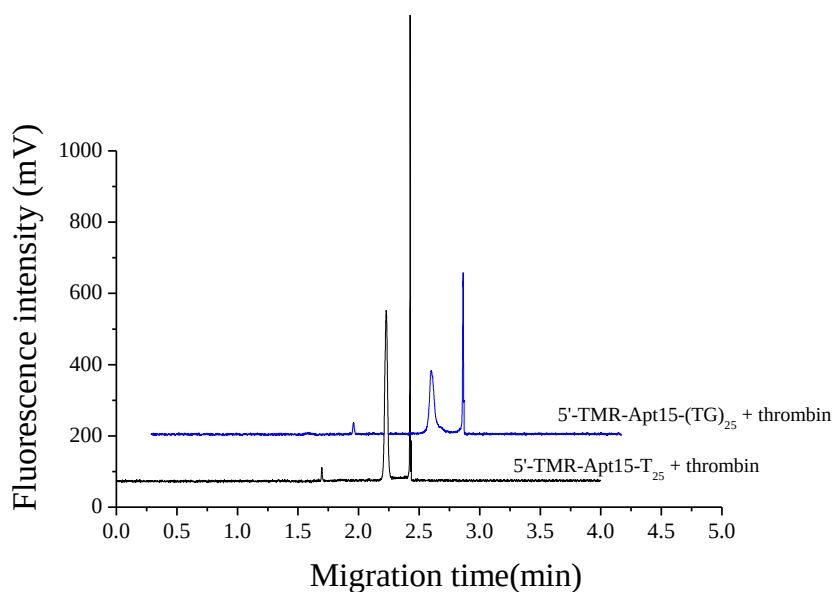


Figure S2. Comparison of 5'-TMR-Apt15-(TG)₂₅ and 5'-TMR-Apt15-T₂₅ in CE-LIF analysis of human α -thrombin. Blue curve shows the electropherogram of the Apt15 attached with a 25-nt tail containing random T and G on 3' terminal (5'-TMR-Apt15-(TG)₂₅, shown in Table S1) in CE-LIF analysis of thrombin. Black curve shows the electropherogram of 5'-TMR-Apt15-T₂₅ in CE-LIF analysis of thrombin. Aptamer probe (10 nM) was incubated with human α -thrombin (10 nM) in sample buffer.

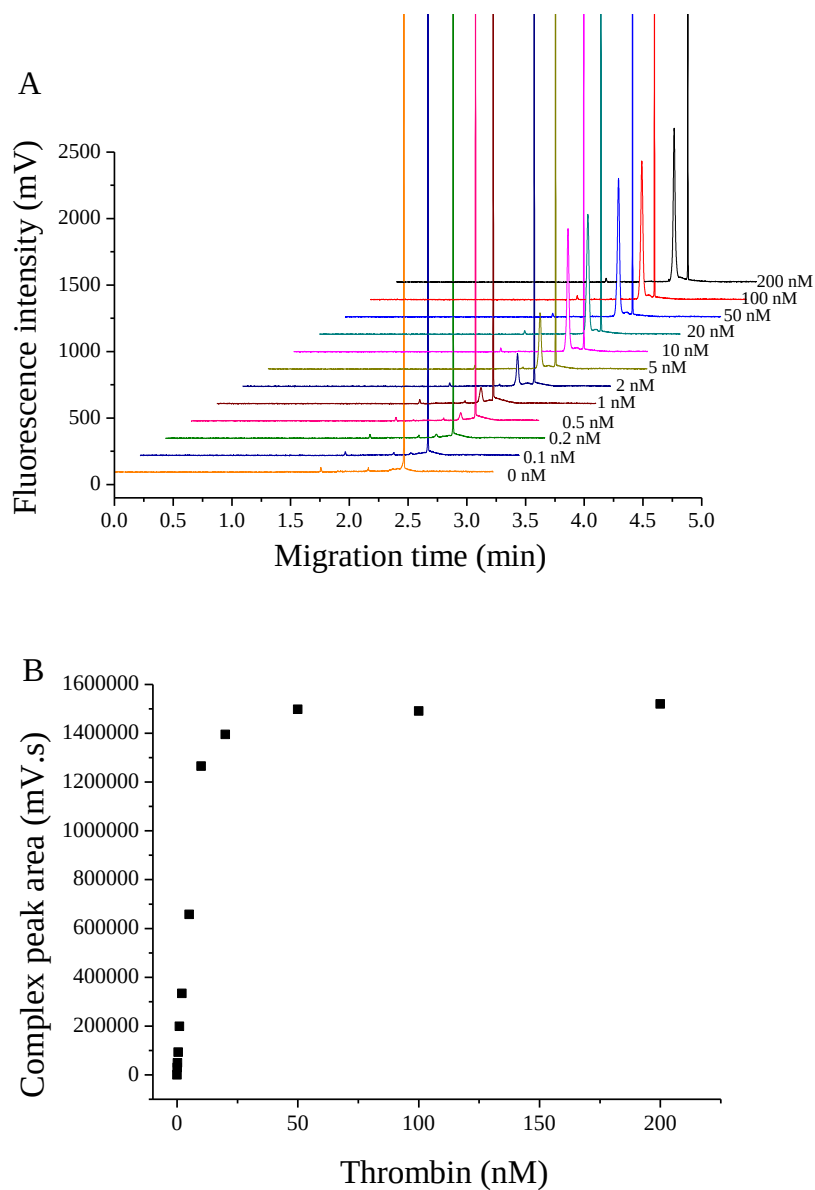


Figure S3. CE-LIF detection of human α -thrombin using Apt15-T₂₅-3'-TMR. (A) Electropherograms of corresponding to Apt15-T₂₅-3'-TMR (10 nM) and increasing concentrations of human α -thrombin. (B) Relationship between the peak area of the complex and concentration of human α -thrombin.

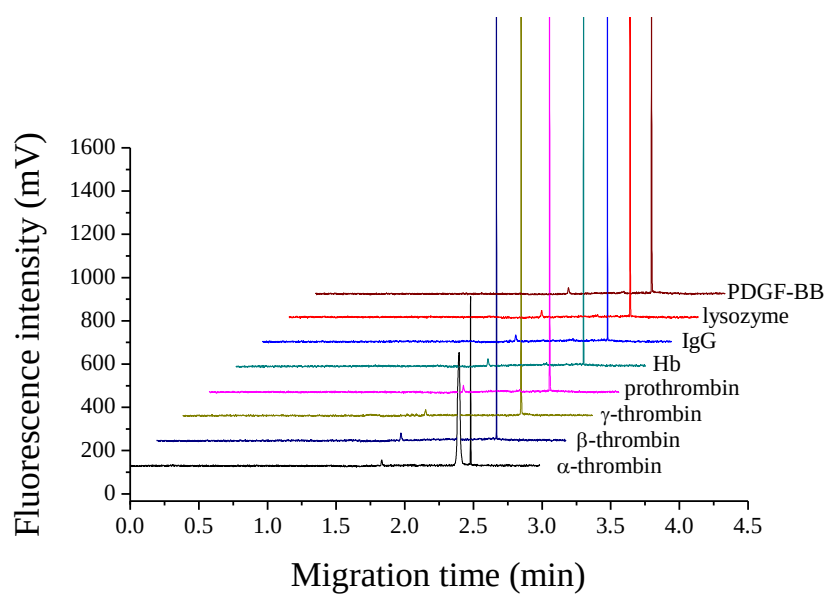
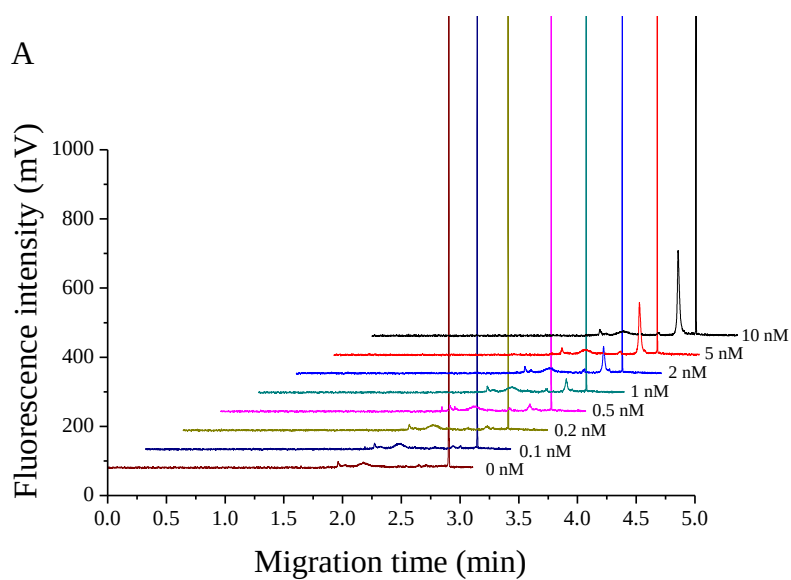


Figure S4. Specificity test of CE-LIF detection of human α -thrombin using 5'-TMR-Apt15-T₂₅. 5'-TMR-Apt15-T₂₅ (10 nM) was incubated with tested proteins (10 nM) for CE-LIF analysis.



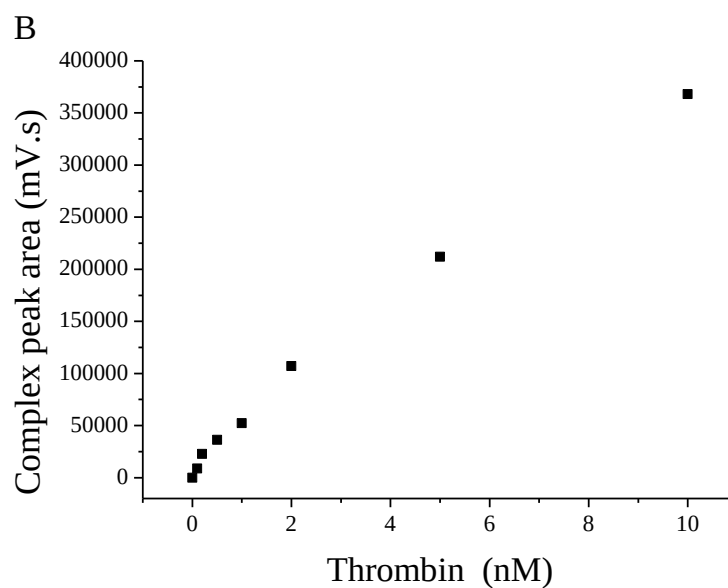


Figure S5. Detection of human α -thrombin spiked in 50-fold diluted serum sample by CE-LIF using 5'-TMR-Apt15-T₂₅. (A) Electropherograms corresponding to Apt15-T₂₅-5'-TMR (10 nM) and increasing concentrations of human α -thrombin spiked in 50-fold diluted serum by sample buffer (B) Relationship between the peak area of the complex peak and the concentration of thrombin.