

Protein–Protein Interaction Studies Based on Molecular Aptamers by Affinity Capillary Electrophoresis

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Protein–DNA/protein–protein interactions play critical roles in many biological processes. We report here the investigation of protein–protein interactions using molecular aptamers with affinity capillary electrophoresis (ACE). A human α -thrombin binding aptamer was labeled with 6-carboxyfluorescein and exploited as a selective fluorescent probe for studying thrombin–protein interactions using capillary electrophoresis with laser-induced fluorescence. A 15-mer binding DNA aptamer can be separated into two peaks in CE that correspond to the linear aptamer (L-Apt) and the thrombin-binding G-quadruplex structure in the presence of K^+ or Ba^{2+} . In a bare capillary, the peak area of G-quadruplex aptamer (G-Apt) was found to decrease with the addition of thrombin while that of L-Apt remained unchanged. Even though the peak of the G-Apt/thrombin binding complex is broad due to a weaker binding affinity between aptamer and thrombin, we were still able to quantify the thrombin and anti-thrombin proteins (human anti-thrombin III, AT III) based on the peak areas of free G-Apt. The detection limits of thrombin and AT III were 9.8 and 2.1 nM, respectively. The aptamer-based competitive ACE assay has also been applied to quantify thrombin–anti-thrombin III interaction and to monitor this reaction in real time. The addition of poly(ethylene glycol) to the sample matrix stabilized the complex of the G-Apt*thrombin. This assay can be used to study the interactions between thrombin and proteins that do not disrupt G-Apt binding property at Exosite I site of the thrombin. Our aptamer-based ACE assay can be an effective approach for studying protein–protein interactions and for analyzing binding site and binding constant information in protein–protein and protein–DNA interaction studies.

Protein–DNA interaction is very important in DNA replication, recombination, and gene expression.^{1–3} Electrophoresis has been an established method for studying such interactions. Tradition-

ally, the mobility shift gel electrophoresis has been used to study DNA–protein interactions. Recently, the capillary electrophoresis mobility shift assay and affinity capillary electrophoresis (ACE) have been developed to explore protein and DNA interactions.^{4–7} ACE refers to a collection of techniques in which high-affinity binding is used in conjunction with capillary electrophoresis (CE) separation to determine analytes. When used with fluorescent labels and laser-induced fluorescence detection (LIF), this immunoassay technique has demonstrated a potential for high mass sensitivity, rapid separations, simultaneous determination of multiple analytes, and compatibility with automation.^{8–10}

Both competitive and noncompetitive immunoassays have been employed in ACE.^{11–13} In a competitive immunoassay, antigen (Ag) is mixed with a fluorophore-labeled antigen (Ag^*) and a limiting concentration of antibody (Ab). CE-LIF analysis yields two zones that correspond to the Ag^* and the Ab– Ag^* complex. The relative intensities of the Ag^* and Ab– Ag^* allow the quantitation of the original concentration of Ag. In the noncompetitive assay, a dye-labeled Ab (Ab^*) is mixed with Ag. The Ag can be quantified by determining Ag– Ab^* after CE separation. Even though noncompetitive assays offer several advantages over competitive assays, including a larger dynamic range, detection limits more independent of the binding constants, and the ability to discriminate among cross-reactive species, they have been developed rather slowly. The main reasons behind this include that it is not always easy to homogeneously label antibodies with fluorophores, and sometimes it is difficult to separate Ab^* from an Ab^* –Ag complex due to a small electrophoretic mobility difference between Ab^* and Ab^* –Ag. However, the use of antibody fragments along with

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selective labeling procedures may offer homogeneously labeled affinity probes for CE assays.^{13–15} Another possible way to overcome the disadvantages of the noncompetitive assay is to develop alternative types of ligands that not only possess the high binding strength to Ag but also are easy to label and separate.

In this work, we have chosen molecular aptamers for the investigation of protein–protein interactions. A human α -thrombin binding aptamer has been used to explore the binding of thrombin and anti-thrombin proteins. Aptamers are nucleic acid oligonucleotides that can be selected using a systematic evolution of ligands via an exponential enrichment (SELEX) process.^{16–18} Aptamers can be synthesized for a broad range of targets with high affinity and selectivity, including small (dyes, amino acids, nucleotides, drugs) and large (proteins, peptides) targets.^{19–23} Aptamers have been successfully used in many bioanalytical techniques such as flow cytometry,^{24,25} biosensors,^{26–28} ELISA-type assays,²⁹ capillary electrochromatography, and affinity chromatography.^{30,31} Aptamers, especially DNA aptamers, possess several advantages in ACE applications.^{32–34} In particular, aptamers are inexpensively synthesized, once selected, in an automated format, easily labeled with fluorophores, and can be stored for a long period of time without significantly affecting their biological properties. Moreover, an aptamer can be selected with desired affinity toward its target, providing tunability in molecular interaction studies, which is unobtainable with traditional protein–protein binding. Like monoclonal antibodies, DNA aptamers can be designed or selected for different domains (epitopes) of many proteins. However, unlike antibodies, the creation of aptamers does not require immunization and their target domains are not limited by the potential lack of

immunogenicity of a domain. On the other hand, aptamers have predictable behavior in electrophoresis as a result of their uniform charge-to-size ratios. The most important advantage is the aptamer's relatively low molecular mass, enabling taking simple steps for the separation of the complex from the aptamer.

Kennedy and coauthors have successfully applied DNA aptamers to the quantitative analysis of two proteins (IgE, thrombin) in CE.³² In their experiments, the fluorophore-labeled aptamer and protein mixtures were injected and separated by CE. The peak areas of free and protein-bound aptamer were used for the quantification of the proteins. In this way, the detection limits of IgE and thrombin were 46 pM and 40 nM, respectively. The authors pointed out that the binding constant between aptamer and thrombin was significantly weaker than that of aptamer and IgE, resulting in the higher detection limit of thrombin relative to that obtained for IgE. It was presumably believed that lower binding affinity caused a significant loss of the complex of protein–aptamer by dissociation during the electrophoresis process. Unstable aptamer–protein complexes may completely dissociate during the separation, leading to a very broad peak or no peak corresponding to the complex, which in turn makes it difficult to use aptamers for the quantitative analyses of proteins. In a more recent work, they further investigated the electrophoresis conditions required to successfully detect aptamer–ligand complexes.³³ In their report, the tris(hydroxyamino)methane–glycine–potassium buffer at pH 8.4, minimal column length, and a high electric field were required for the successful detection of aptamer–protein complexes. They concluded that these results showed potential for aptamer-based ACE. In another study, Krylov and coauthors proposed a new method that allows for the use of low-affinity aptamers as affinity probes in the quantitative analyses of proteins.³⁴ The method is based on the nonequilibrium capillary electrophoresis of the equilibrium mixture, which allows for the accurate quantitative analysis of proteins even when the aptamer–protein complexes may completely decay during the separation.³⁵

In this work, we have investigated the effects of an electrophoresis buffer containing different metal ions on the conformation of the aptamers. Furthermore, we found that adding an appropriate concentration of poly(ethylene glycol) (PEG) to the aptamer and thrombin mixture might stabilize the complex of aptamer–thrombin. Finally, we further studied the protein–protein interactions of thrombin and anti-thrombin proteins using molecular aptamers. These studies should provide useful information in using molecular aptamers for protein–protein interactions studies.

EXPERIMENTAL SECTION

Chemicals and Buffers. The 6-carboxyfluorescein (6-FAM) was labeled at the 5' end of aptamer (5'-FAM/GGT TGG TGT GGT TGG-3') obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Thrombin (MW ~36 700), human anti-thrombin III (AT III, MW ~58 000), and a monoclonal antibody anti-human thrombin (AHT, MW ~150 000) were obtained from Haematologic Technologies Inc. (Essex Junction, VT). A sulfated hirudin fragment 54–65, Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO₃H)-Leu-Gln (HirF, MW 1500), fluorescein, PEG (MW 8000), and poly(*N*-vinyl-2-pyrrolidone) (PVP, 1 300 000) were from Sigma-Aldrich, Inc. (St. Louis, MO). The electrophoresis buffers consisted of 25

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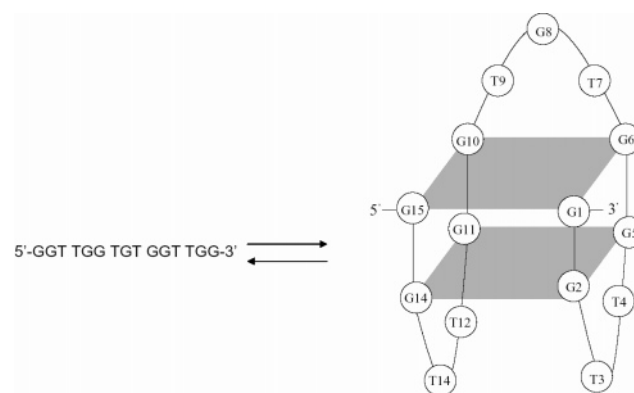
mM tris(hydroxyamino)methane (Tris), 192 mM glycine, and 0–10 mM KCl, LiCl, MgCl₂, or BaCl₂ at pH 8.4 were used for separating aptamer. The electrophoresis buffer, 10 mM Tris-HCl pH 8.4 and 15 mM KCl, was for quantifying thrombin and studying protein–protein interactions. All solutions were made in the electrophoresis buffer including the stock solutions of protein, aptamer, PEG, and fluorescein (internal standard). All reagents for the buffers were obtained from Fisher Scientific Co. L.L.C. (Pittsburgh, PA).

Apparatus. The basic design of the separation system has been previously described.³⁶ Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL) was used to drive electrophoresis. The entire detection system was enclosed in a black box with an HV interlock. The high-voltage end of the separation system was put in a laboratory-made plexiglass box for safety. A 2.5-mW Ar ion laser with 488-nm output (Spectra Physics, Mountain View, CA) was used for excitation. The emission was collected with a 20× objective (numeric aperture 0.25). One 520-nm interference filter was used to block scattered light before the emitted light reached the photomultiplier tube (Hamamatsu R928, Hamamatsu Photonics K.K., Hamamatsu, Japan). The amplified currents were transferred directly through a 50-kΩ resistor to a 24-bit A/D interface at 10 Hz (AT-MIO-16, National Instruments, Austin, TX) and stored in a personal computer. Capillaries (Polymicro Technologies, Phoenix, AZ), 50-μm i.d. and 365-μm o.d., were used for electrophoresis separations with or without PVP coating.

Separation of Aptamer. Samples were prepared in a separation buffer and consisted of 200 nM aptamer and 10 nM fluorescein (internal standard). They were injected into the capillary (total length, 35 cm; effective length, 10 cm) hydrodynamically ($\Delta h = 5$ cm) for 10 s. Separation buffers were constituted of 25 mM Tris, 192 mM glycine, and 0–10 mM KCl at pH 8.4. The electrophoresis separation was carried out with an electric field of 285 V/cm.

Aptamer-Based ACE. In thrombin quantification, the aptamer was mixed with thrombin and fluorescein (internal standard) in the electrophoresis separation buffer (10 mM Tris-HCl pH 8.4 and 15 mM KCl) and incubated for 60 min at room temperature. The final concentrations of aptamer, fluorescein, and thrombin were 200 nM, 10 nM, and 0–1.0 μM, respectively. The resulting samples injected into the capillary (total length, 50 cm; effective length, 25 cm) hydrodynamically ($\Delta h = 10$ cm) for 10 s. The electrophoresis separation was carried out with an electric field of 350 V/cm. For the quantification of anti-thrombin proteins (AT III, HirF, AHT), aptamers were mixed with thrombin in the electrophoresis separation buffer (10 mM Tris-HCl pH 8.4 and 15 mM KCl) and incubated for 60 min at room temperature. The desired concentrations of anti-thrombin proteins were mixed with aptamer–thrombin complex solutions and incubated for another 60 min. Fluorescein was added to the resulting samples as an internal standard to 10 nM. The final concentrations of aptamer, thrombin, and anti-thrombin proteins were 200 nM, 200 nM, and 0–10.0 μM, respectively. The samples were injected into the capillary (total length, 40 cm; effective length, 25 cm) hydrodynamically ($\Delta h = 10$ cm) for 10 s. The electrophoresis separation was carried out with an electric field of 500 V/cm. At the end of

Scheme 1. Linear and G-Quadruplex Structures of the Thrombin Binding DNA Aptamer^a



^aShaded squares represent quinine quartets.

each run, the capillary was rinsed with 0.5 N NaOH for 10 min to remove the protein adsorbed on the capillary.

PEG-Assisted Aptamer-Based ACE. To study the effect of PEG, aptamers were mixed with thrombin in 10 mM Tris-HCl pH 8.4, 15 mM KCl, and 0–10% PEG and incubated for 60 min at room temperature. The final concentrations of aptamer and thrombin were 200 nM. The electrophoresis buffer consisted of 10 mM Tris-HCl pH 8.4 and 15 mM KCl. The resulting samples were injected into the PVP-coated capillary (total length, 15 cm; effective length, 5 cm) hydrodynamically ($\Delta h = 1.5$ cm) for 20 s. The electrophoresis separation was carried out with an electric field of 666 V/cm. Similarly, to quantify thrombin and study the interactions of thrombin and anti-thrombin proteins (AT III, AHT, HirF), the desired concentrations of thrombin and anti-thrombin proteins were mixed with aptamer and aptamer–thrombin complex solutions, respectively. The resulting samples were injected and separated in a PVP-coated capillary. The electrophoresis buffer consisted of 10 mM Tris-HCl pH 8.4 and 15 mM KCl, and the separation was carried out with an electric field of 666 V/cm. At the end of each run, the capillary was rinsed with 5% PVP for 10 min.

RESULTS AND DISCUSSION

Conformation of Aptamer. It has been reported that a 15-mer thrombin binding aptamer adopts an intramolecular G-quadruplex structure (Scheme 1) in the presence of K⁺.^{37–40} Its affinity for thrombin has been associated with the inhibition of thrombin-catalyzed fibrin clot formation.^{41,42} Studies with circular dichroism, temperature-dependent UV spectroscopy, differential scanning calorimetry, isothermal titration calorimetry, capillary electrophoresis, NMR, and mass spectrometry have revealed intramolecular G-quadruplex structures of the 15-mer aptamer in the presence of various metal ions.^{37–40,43–45} The G-quadruplex

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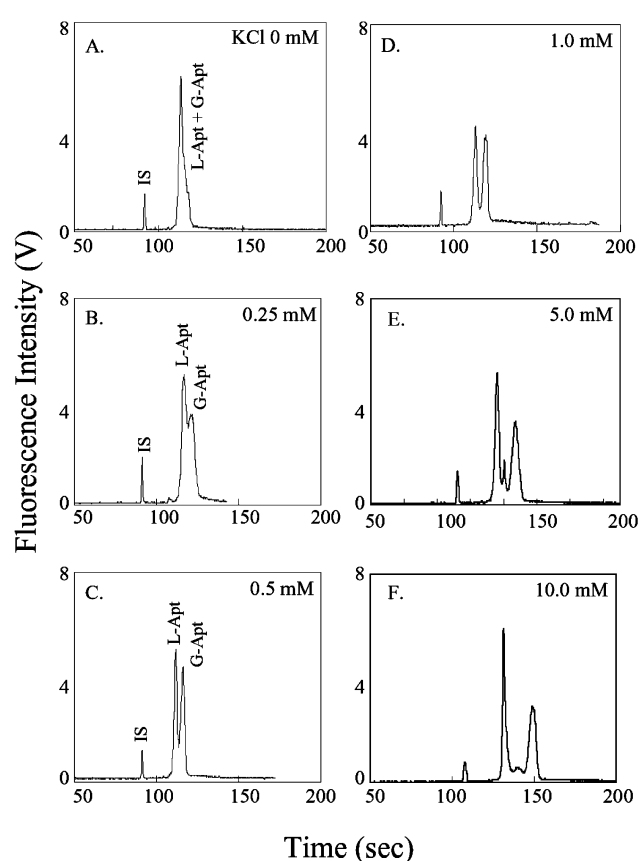


Figure 1. Capillary electrophoresis traces of thrombin binding aptamer in the presence of 25 mM Tris, 192 mM glycine, and various concentrations of KCl. Separation buffer was composed of 25 mM Tris, 192 mM glycine and 0 (A), 0.25 (B), 0.5 (C), 1.0 (D), 5.0 (E), and 10.0 mM (F) KCl at pH 8.4. Samples were prepared in a separation buffer and contained a final concentration of 200 nM aptamer and 10 nM fluorescein (internal standard). The samples were injected into the capillary (total length, 35 cm; effective length, 10 cm) hydrodynamically ($\Delta h = 5$ cm) for 10 s, and an electric field of 285 V/cm was applied to drive the separation.

aptamer (G-Apt) is stable both kinetically and thermodynamically because of the tight association of cations with quinone residues.^{46–48} The stability of the G-Apt is a very important factor in the detection of thrombin in ACE because thrombin will only bind to the G-quartet form of the aptamer.^{42,49} Here, we evaluated the stability of the G-Apt in the presence of several metal ions (K^+ , Li^+ , Ba^{2+} , Mg^{2+}). Figure 1 shows the electropherograms for the aptamer with increasing concentrations of KCl. The aptamer adopted a primarily linear form in the absence of any metal ions. However, we suspect that there might have been an unstable G-Apt based on the tailing of the aptamer peak. The aptamer was separated into two peaks when K^+ was present. The first and second peaks corresponded to the linear aptamer (L-Apt) and the G-Apt, respectively. The peak area ratio of the G-Apt to L-Apt form

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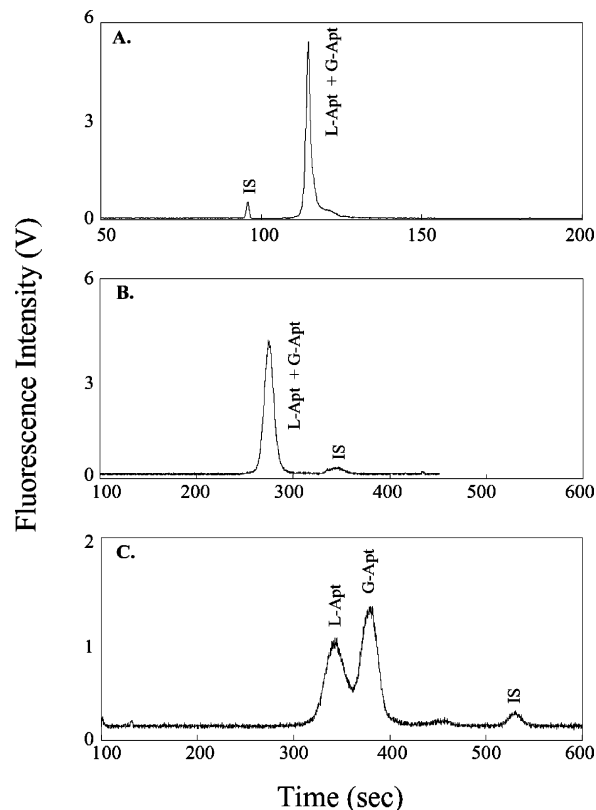


Figure 2. Capillary electrophoresis traces of thrombin binding aptamer in the presence of 25 mM Tris, 192 mM glycine, and various other metal ions. Separation buffer were composed of 25 mM Tris, 192 mM glycine, and 10 mM LiCl (A), 2.5 mM $MgCl_2$ (B), and 2.5 mM $BaCl_2$ (C) at pH 8.4. Samples prepared in a separation buffer and contained a final concentration of 200 nM aptamer and 10 nM fluorescein (internal standard). Other experimental conditions same as in Figure 1.

increased with the increased concentration of K^+ . This result agrees with other reports that the stability of the G-Apt is dependent upon the concentration of K^+ .^{39,40,45} Furthermore, the increase in the K^+ concentration was found to improve the resolution between the L-Apt and G-Apt peaks. On the other hand, Figure 2 shows that other metal ions, such as Li^+ , Mg^{2+} , and Ba^{2+} , were not capable of stabilizing the G-Apt or effectively separating the G-Apt from the L-Apt. It is reported that cations with an ionic radius in the range of 1.3–1.5 Å fit well within the two G-quartets of the complex.³⁹ Our results were in agreement with the reports that K^+ , Rb^+ , NH_4^+ , Sr^{2+} , and Ba^{2+} are able to form stable intramolecular cation–Apt complexes, while Li^+ , Na^+ , Cs^+ , Mg^{2+} , and Ca^{2+} only form weak complexes. Among the four metal ions (K^+ , Li^+ , Mg^{2+} , Ba^{2+}) chosen in our experiments, the aptamer displayed a stable G-Apt peak with K^+ or Ba^{2+} present. As shown in Figure 2, as little as 2.5 mM Ba^{2+} could effectively stabilize the peak of the G-Apt. However, similar to Mg^{2+} , Ba^{2+} associates with high affinity to the phosphate backbone of oligonucleotides and decreases the mobility of the aptamer. This was demonstrated by the change in the aptamer peak position relative to the fluorescein peak. On the other hand, the decrease of the mobility of fluorescein indicated a severely reduced electroosmotic flow, which led to peak broadening, increased separation time, and decreased resolution. For this reason, Ba^{2+} was certainly not suitable for the study of protein–DNA and protein–protein

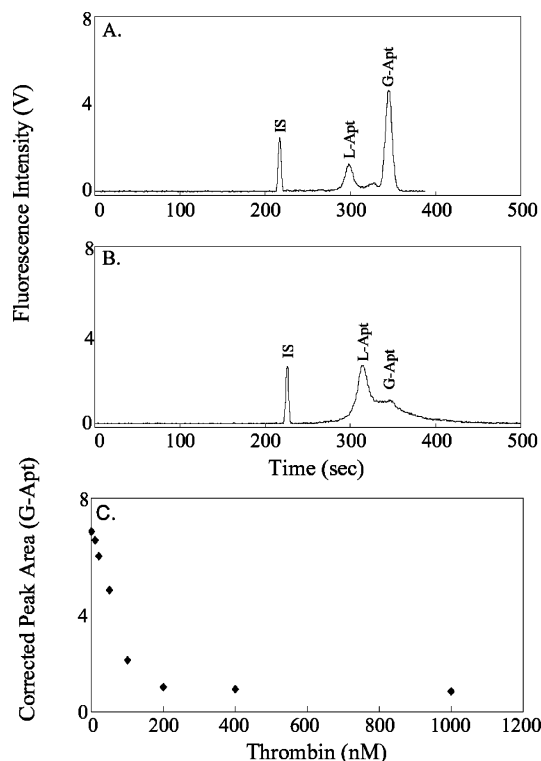


Figure 3. Determination of thrombin using aptamer-based ACE. Electropherograms obtained for 200 nM aptamer with 0 (A) and 200 nM thrombin (B). Calibration curve constructed using samples containing 200 nM aptamer with various concentrations of thrombin (C). The electrophoresis separation buffer was 10 mM Tris-HCl and 15 mM KCl at pH 8.4. Samples prepared in separation buffer and consisted of a final concentration of 200 nM aptamer, 10 nM fluorescein (internal standard), and 0–1.0 μ M thrombin. The samples were injected into the capillary (total length, 50 cm; effective length, 25 cm) hydrodynamically ($\Delta h = 10$ cm) for 10 s, and an electric field of 350 V/cm was applied to drive the separation. Peak areas were corrected for variations in injection volume by dividing by the area of the internal standard peak.

interactions in ACE. Thus, KCl was chosen to be added to sample matrix and electrophoresis buffer in further experiments.

Quantification of Thrombin. As mentioned before, literature reports had pointed out that the unstable aptamer–protein complex would undergo dissociation in the electrophoresis process.^{32–34} This caused complex peak broadening or disappearance unless the hydrodynamic flow was used to force the complex through the capillary.³² Although the flow-assisted method allows quantification of the aptamer–protein complex, it is inconvenient and may eliminate some of the advantages of CE. A relatively long capillary (effective length 25 cm) was used in our experiments. The advantage of a long capillary was that there was less interference with the peak of the G-Apt by the aptamer–thrombin peak (Apt*thrombin) because the complex of aptamer–thrombin would almost completely decay in a longer capillary, which is good for detection of thrombin based solely on changes in aptamer peak.

Panels A and B of Figure 3 compare the electropherograms obtained for 200 nM aptamer without and with 200 nM thrombin in the sample. Fluorescein was used as an internal standard (IS) to correct variations in the injection volume. The electrophoresis buffer was further optimized from before (25 mM Tris, 192 mM

glycine, and 10 mM KCl at pH 8.4, Figure 1F) to 10 mM Tris-HCl and 15 mM KCl at pH 8.4. In Figure 3A, the peak area ratio (G-Apt/L-Apt) was calculated to be 2.97, which was significantly higher than that in Figure 1F (1.09). The electropherogram with thrombin (Figure 3B) shows a large decrease in the free G-Apt peak, which can be attributed to the binding of the G-Apt to the thrombin. However, no changes to the L-Apt peak were observed, which agreed with the reports that only G-Apt binds to thrombin.^{42,49} A calibration curve was constructed (Figure 3C) based on the peak area of the free G-Apt as a function of thrombin concentration. The curve is linear up to 200 nM thrombin, and a 1:1 binding ratio between the aptamer and thrombin is clearly demonstrated. The limit of detection (LOD), which is calculated as the concentration yielding a signal change 3 times the peak-to-peak noise, was 9.8 nM, which is lower than previous reported 40 nM.³² We suspect that the main reason is that the quantification in the literature did not differentiate between G-Apt and L-Apt, while we focused on the peak area change of the G-Apt, which might have resulted in a higher level of sensitivity.

Determination of Dissociation Constant (K_d). The value of K_d was determined under the conditions of the experiments. The equilibrium concentration of free G-Apt is proportional to the area of the G-Apt peak (A_g^{eq}).³⁴

$$[\text{G-Apt}]_{eq} = cA_g^{eq} \quad (1)$$

where c is a constant. The equilibrium concentration of the complex (G-Apt*thrombin) is equal to

$$[\text{G-Apt*thrombin}]_{eq} = [\text{G-Apt}]_o - [\text{G-Apt}]_{eq} = c(A_g^o - A_g^{eq}) \quad (2)$$

where A_g^o is the peak area of G-Apt without thrombin. The ratio R of the two equilibrium fractions

$$R = [\text{G-Apt}]_{eq}/[\text{G-Apt*thrombin}]_{eq} = A_g^{eq}/(A_g^o - A_g^{eq}) \quad (3)$$

The knowledge of ratio R is sufficient for the determination of K_d :

$$K_d = \{[\text{thrombin}]_o(1 + R) - [\text{G-Apt}]_o\}/(1 + 1/R) \quad (4)$$

where $[\text{thrombin}]_o$ and $[\text{G-Apt}]_o$ are the initial concentrations of thrombin and G-Apt, respectively.

The fluorescence intensities of the aptamer were found to be unchanged on a fluorometer under different concentrations of KCl, indicating the conformational change of the aptamer did not affect the fluorescence intensity of the labeled 6-carboxyfluorescein. As a result, the $[\text{G-Apt}]_o$ could be calculated from Figure 3A by the areas of peak L-Apt (A_l^o) and G-Apt (A_g^o)

$$[\text{G-Apt}]_o = [\text{Apt}]_o A_g^o / (A_l^o + A_g^o) \quad (5)$$

Based on six experiments with different concentrations of thrombin and aptamer, the value of K_d (G-Apt*thrombin) was calculated using eqs 4 and 5 to be 20 nM. This value is smaller

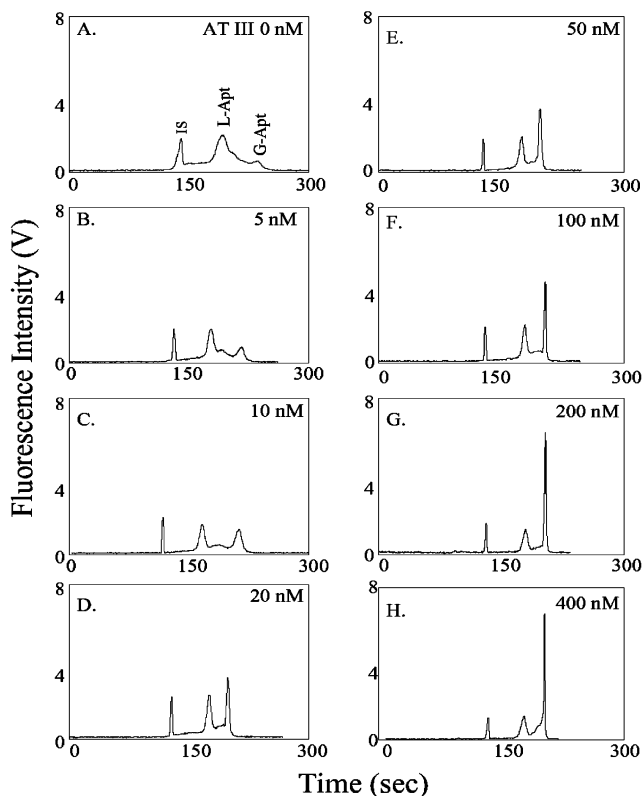


Figure 4. Determination of AT III using aptamer-based ACE. Electropherograms obtained for 200 nM aptamer with 200 nM thrombin and various concentrations of AT III. In electropherograms A–H, AT III concentrations were 0, 5, 10, 20, 50, 100, 200, and 400 nM, respectively. Aptamers were mixed with thrombin and incubated for 60 min at room temperature. The desired concentrations of AT III were mixed with aptamer–thrombin complex solutions. The resulting samples added fluorescein as an internal standard to 10 nM with incubation for another 60 min. Separation was carried out at a constant electric field of 500 V/cm. Other conditions as in Figure 3.

than those obtained by Kennedy et al. (450 nM) and Krylov et al. (240 nM). This can be explained by the fact that the binding affinity measured here is between the G-Apt and thrombin, which is supposed to be stronger than that between thrombin and the overall aptamer (G-Apt + L-Apt).

Competitive Assay. Thrombin has two positive-charged sites termed exosites I and II on the opposite sides of the protein.^{50,51} Exosite I was found to bind to fibrinogen⁵² and hirudin^{53–55} while exosite II binds to AT III⁵⁶ and AHT.⁵⁷ Two different aptamers have been identified that have high affinity and selectivity for α -thrombin. The first one is a 15-mer single-stranded DNA aptamer (in this work), which was reported to bind to the

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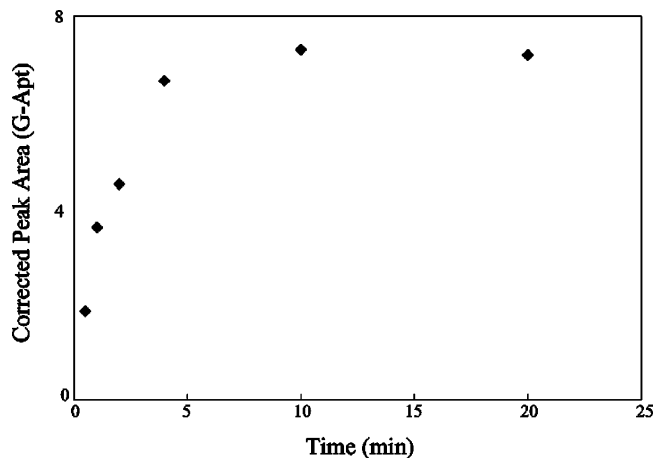


Figure 5. Using thrombin binding aptamer to monitor thrombin–AT III interaction. A solution of mixed aptamer and thrombin incubated in electrophoresis buffer for 60 min. Then AT III were mixed with the aptamer/thrombin complex solution and incubated for another 60 min. The final concentration of aptamer, thrombin, and AT III were 200 nM. After the resulting sample incubation for different times, rapid injection into the capillary and the separation were carried out at a constant electric field of 500 V/cm. Other conditions as in Figure 3.

fibrinogen binding site of α -thrombin, namely, exosite I.⁵⁸ The other DNA aptamer, with a 27-mer backbone length, was determined to bind to the exosite II of α -thrombin.⁵⁹ Both aptamers were found to adopt a G-quartet structure when bound to α -thrombin.^{59,60} We previously used the complex of aptamer–thrombin to probe thrombin–protein interactions in a competitive assay where the binding of the aptamer to thrombin was altered by a second protein that interacts with thrombin. Two signal transduction strategies, fluorescence energy transfer and fluorescence anisotropy, have been designed to study the interactions of thrombin with different proteins using two aptamers specific for two binding sites on α -thrombin.⁶¹ Here, we have further demonstrated the results by aptamer-based ACE. As shown in Figure 4, the increasing concentration of AT III caused an increase in the G-Apt peak area as expected. This result agreed with another report that the binding of AT III to thrombin may cause a conformational change in thrombin that rendered the binding with the aptamer at exosite I unstable.⁵⁶ In Figure 5, the reaction between AT III and thrombin was monitored in real time. This is the first time aptamer-based ACE has been used for real-time protein–protein interactions. The reaction of thrombin and AT III was completed within 10 min, and the result agreed with our previous work.⁶¹ A calibration curve was made based on the peak area of free G-Apt versus AT III concentration (Figure 6). The curve is almost linear up to 200 nM AT III, and a 1:1 binding ratio between the thrombin and AT III was displayed. Based on these data, the LOD was calculated to be 2.1 nM.

The addition of another antibody, 400 nM AHT (2 times the concentration of thrombin), caused no significant change in the peak of the G-Apt (data not show). While this result indicates that

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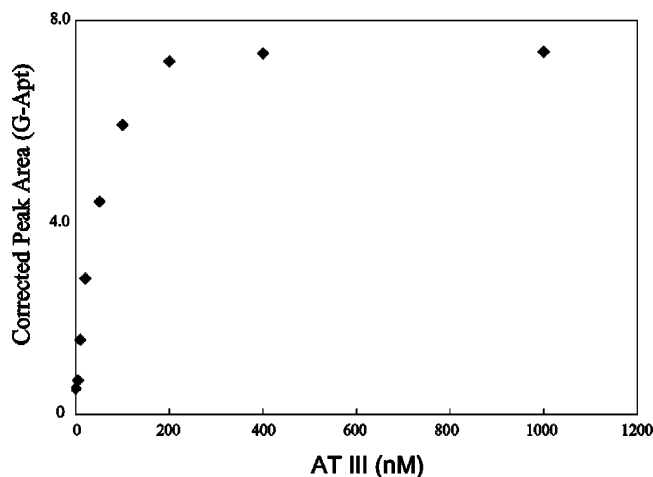


Figure 6. Calibration curve constructed using samples containing 200 nM aptamer, 200 nM thrombin, and various concentrations of AT III (0–1.0 μ M). Peak area of G-Apt was corrected for variations in injection volume by dividing by the area of the internal standard peak. Other conditions as in Figure 3 and Figure 4.

AHT does not compete with the aptamer for the exosite I of thrombin, it cannot exclude the possibility that AHT still binds to thrombin but at a different site of thrombin. More experiments were done to demonstrate this point, and the results are presented later in this paper. A sulfated fragment of hirudin that contained the C-terminal 13-residue (HirF) instead of hirudin was used for studying binding with thrombin. Although the K_d of HirF–thrombin (150 nM) at pH 7.4 is similar to the reported K_d of aptamer–thrombin (200 nM) and both HirF and aptamer bind to the same site (exosite I) of thrombin,⁵⁹ HirF caused no significant change in the peak of the G-Apt even when the added concentration was as high as 10 μ M (50 times the concentration of thrombin). It has been reported that binding of hirudin (65 amino acids) and some derived hirudin fragments to thrombin strongly depends on pH.⁶² The optimum pH for the interaction between hirudin and thrombin was found to be between pH 7.5 and pH 8.0. The K_d value increased at higher pH values, and the plot of $-\log K_d$ against pH displayed an asymptotic slope of -2 in the alkaline pH range. As a result, the sample and electrophoresis buffer at pH 8.4 in our experiments may also cause a much higher K_d between HirF and thrombin than reported in the literature.⁵⁹ On the other hand, the relative lower salt concentration of buffer used in this experiment may also have an impact on the binding affinity of HirF.

Effect of PEG. Unlike AT III, which can displace G-Apt from a G-Apt*thrombin complex and cause changes in the area of the G-Apt peak, the thrombin antibody AHT would not affect G-Apt/thrombin binding. It is necessary to observe the peak of the G-Apt*thrombin complex and analyze its mobility shift to study the interactions between AHT and thrombin. To resolve the problem of complex dissociation during CE, a short PVP-coated capillary (effective length 5 cm) was used to shorten the analysis time and prevent the protein adsorption on the capillary. However, with a total separation time less than 50 s, peak broadening was not improved (Figure 7A). CAE for a gel mobility shift assay,

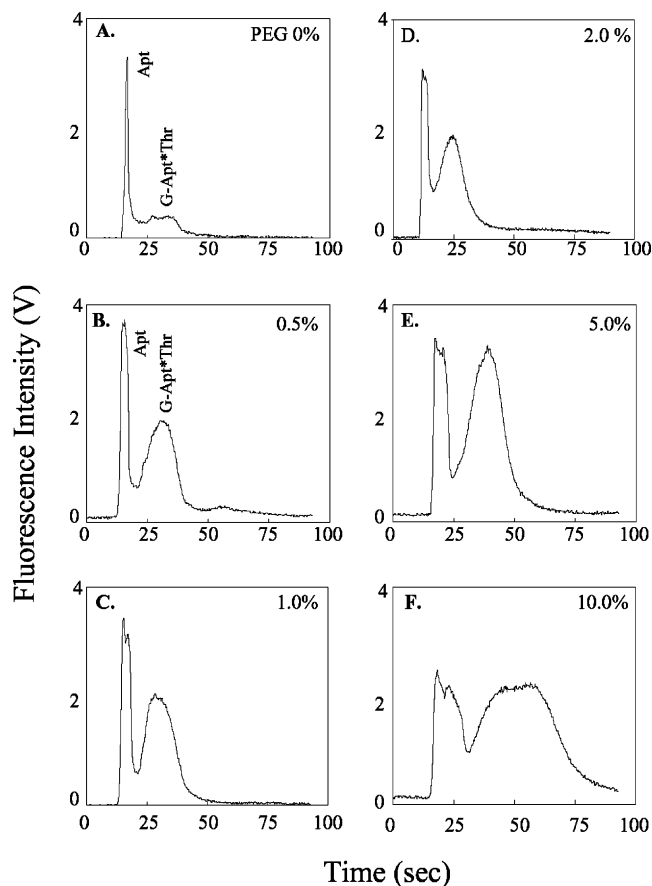


Figure 7. Effect of PEG on the stability of G-Apt*thrombin complex. Aptamers were mixed with thrombin in 10 mM Tris-HCl at pH 8.4, 15 mM KCl, and 0 (A), 0.5 (B), 1.0 (C), 2.0 (D), 5.0 (E), and 10% (F) PEG, incubated for 60 min at room temperature. The final concentrations of aptamer and thrombin were 200 nM. The electrophoresis buffer was 10 mM Tris-HCl and 15 mM KCl at pH 8.4. The resulting samples were injected into the PVP-coated capillary (total length, 15 cm; effective length, 5 cm) hydrodynamically ($\Delta h = 1.5$ cm) for 20 s. The electrophoresis separation was carried out with an electric field of 666 V/cm.

which also involves separation of DNA–protein complexes, has used buffers containing soluble linear polymers.^{63–66} These buffers may aid the separation by interacting with solutes and capillary walls to prevent adsorption. In addition, polymers have previously been used to promote the stabilizing cage effect, which stabilized protein complexes during electrophoresis.^{63–66} Polymer in the region where the complexes have dissociated may retard further separation of the two components and lead to an enhanced probability for a reassociation. Furthermore, the dissociation step may be retarded by the polymer, if the dissociation requires complex–complex interactions.⁶⁷ Finally, polymer may increase local concentrations of analytes that cause lower level of dissociation of complex. Given all the background information, we tried adding PEG to sample matrix. As shown in Figure 7B–F, addition of PEG clearly revealed the peak of the G-Apt*thrombin complex.

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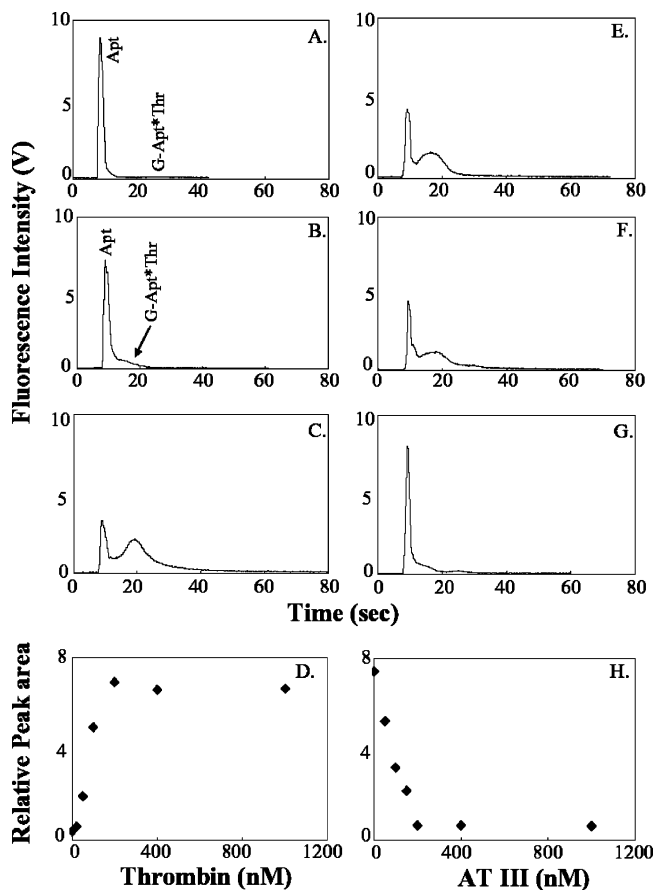


Figure 8. Determination of thrombin and AT III using PEG-containing sample matrix. Each sample contained a final concentration of 200 nM aptamer and 0 (A), 50 (B), and 200 nM (C) thrombin. In (E–G), each sample contained a final concentration of 200 nM aptamer, 200 nM thrombin, and 50 (E), 100 (F) and, 200 nM (G) AT III. (D) and (H) are the calibration curves constructed with various concentrations of thrombin and AT III, respectively. The sample matrix consisted of 10 mM Tris-HCl, 15 mM KCl, and 2% PEG at pH 8.4. The electrophoresis buffer was 10 mM Tris-HCl and 15 mM KCl at pH 8.4. Other conditions as in Figure 4 and Figure 7.

The L-Apt and G-Apt cannot be separated due to the fact that the experiments were done in a very short time. However, it did not effect the quantification of thrombin and AT III by the complex peak of G-Apt*thrombin. The optimum concentration of PEG was found to be 2.0%. From the changes in the peak profiles with increasing PEG concentrations, we believe that one main reason for peak broadening is that PEG affects the interactions between G-Apt and thrombin and the stability of the complexes. The effect might be greater for the complexes than for the aptamer. It is also possible that, in the presence of high concentrations of PEG, not only has G-Apt a strong interaction with thrombin but other forms of Apt may also interact with thrombin. Viscosity difference between the sample zone and the electrophoresis buffer is not the main reason for the broadening. This has been demonstrated by adding the same concentration of PEG as in the sample to the separation buffer, which caused a little decrease of band broadening of the peak of the G-Apt*thrombin complex. We think it could be that the constant collisions with the PEG polymer during the separation perturb the complex to some extent.⁶⁶

Panels A–C of Figure 8 show the increased peak of G-Apt*thrombin and the decreased peak of the free aptamer with

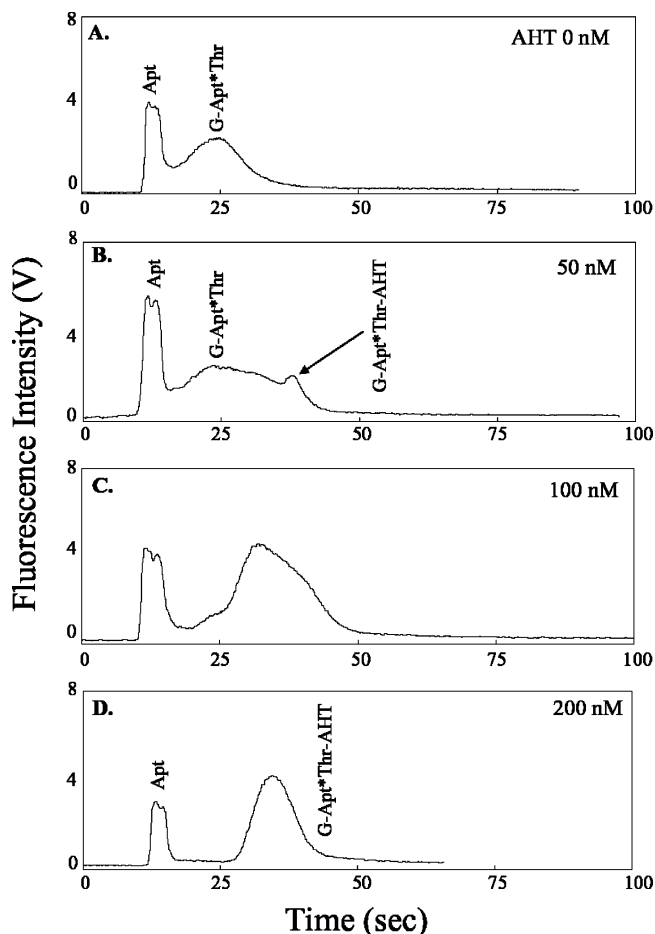


Figure 9. Binding between G-Apt*thrombin and AHT confirmed by capillary electrophoresis. In a solution of mixed aptamer and thrombin incubated in 10 mM Tris-HCl, 15 mM KCl, and 2% PEG at pH 8.4 for 60 min. Then AHT was mixed with the aptamer/thrombin complex solution and incubated for another 60 min. Each sample contained a final concentration of 200 nM aptamer, 200 nM thrombin, and 0 (A), 50 (B), 100 (C), and 200 nM (D) AHT. Other conditions as in Figure 7.

increasing thrombin. The calibration curve in Figure 8D has a linear range up to 200 nM thrombin. The LOD of thrombin is 10.9 nM. In a similar way, AT III was quantified using the G-Apt*thrombin-based competitive assay (Figure 8E–H). The electropherograms with increasing AT III have shown a decreased peak of G-Apt*thrombin and an increased peak of free aptamer. These results once again confirmed the displacement of G-Apt by AT III in CE. The LOD of AT III was estimated at 21.2 nM.

Mobility Shift. The substrate specificity of thrombin is regulated by binding of macromolecular substrates and effectors to exosites I and II.⁵⁵ Exosites I and II have been reported to be linked allosterically, such that binding of a ligand to one exosite results in nearly total loss of affinity for ligands at the alternative exosite, whereas other studies support the independence of the interactions.⁵⁵ Previous results in bare capillaries revealed that AHT had no effect on binding between the aptamer and thrombin. To study the interaction between thrombin and AHT in ACE, it might be helpful to analyze the mobility change of the G-Apt*thrombin complex with the addition of AHT. Using the PEG-assisted ACE, electropherograms were obtained (Figure 9A–D) that clearly show changes in the migration time of the

G-Apt*thrombin complex as AHT concentration was varied. We further optimize the concentration of PEG in this experiment and found 2% PEG was still best. As shown in Figure 9B–D, this mobility shift can be attributed to the increased overall molecular mass of the G-Apt*thrombin*AHT binding complex. Control experiments revealed that no mobility shift was observed when 200 nM aptamer was mixed with only 400 nM AHT. Figure 9B (aptamer 200 nM, thrombin 200 nM, and AHT 50 nM) displays an unresolved small peak (arrow point and the migration time of that peak is equal to that of the second peak in Figure 9D (aptamer 200 nM, thrombin 200 nM, and AHT 200 nM)). This result reveals that only a small fraction of G-Apt*thrombin binds with AHT when the concentration of AHT is lower than G-Apt*thrombin. The highly broadened peaks in Figure 9B,C indicate the shift from the G-Apt*thrombin complex to the G-Apt*thrombin*AHT complex. In addition, the dissociation constant of AHT–thrombin is not very low at 14 nM,⁶⁸ which contributed to the dissociation of G-Apt*thrombin*AHT complex at low AHT concentrations. Even though quantification of AHT is difficult, the interaction between thrombin and AHT, and the fact that AHT and G-Apt bind to different sites of thrombin, have been clearly revealed using this mobility shift assay in ACE.

CONCLUSIONS

In this work, we have demonstrated that the 15-mer thrombin binding DNA aptamer adopts two different forms in the presence

of K^+ or Ba^{2+} and only the G-quadruplex form can bind thrombin to form a complex. Binding between aptamer and proteins is thus highly dependent on the conformation of the molecular aptamers. The presence of thrombin and anti-thrombin III only affected the G-aptamer peak in affinity capillary electrophoresis. The G-aptamer-based CE analysis showed a higher binding affinity between G-aptamer and thrombin. As a result, a better detection limit of thrombin could be achieved. The aptamer-based competitive affinity capillary electrophoresis assay has been also applied to quantify thrombin–anti-thrombin III interaction and to monitor this reaction in real time. We have also shown that a mobility shift-based affinity capillary electrophoresis assay, using poly-(ethylene glycol) in the sample matrix, can be used to study the interactions between thrombin and proteins that do not disrupt G-aptamer binding property at exosite I site of the thrombin. We believe that this aptamer-based affinity capillary electrophoresis assay can be an effective alternative approach for studying protein–protein interactions and for analyzing binding site information and binding constants.

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