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# Molecular Aptamers for Real-Time Protein–Protein Interaction Study

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**Abstract:** Protein–protein interactions play critical roles in cellular functions, but current techniques for real-time study of these interactions are limited. We report the real-time monitoring of protein–protein interactions without labeling either of the two interacting proteins; this procedure poses minimum effects on the binding properties of the proteins. Our strategy uses a protein/aptamer complex to probe the interactions in a competitive assay where the binding of an aptamer to its target protein is altered by a second protein that interacts with the target protein. Two signal transduction strategies, fluorescence resonance energy transfer (FRET) and fluorescence anisotropy, have been designed to study the interactions of human α-thrombin with different proteins by using two aptamers specific for two binding sites on α-thrombin. Our method has been shown to be simple and effective, does not require labeling of proteins, makes use of easily obtainable aptamers, provides detailed protein–protein interaction information and has excellent sensitivity for protein detection and protein–protein interaction studies. The FRET and the fluorescent anisotropy approaches complement each other in providing insight into the kinetics, mechanisms, binding sites and binding dynamics of the interacting proteins.

**Keywords:** aptamers · fluorescence resonance energy transfer · proteins · structure–activity relationships

The functions of living cells are mostly executed and regulated by proteins. In order to understand how cells fulfill their functions and how they react to changes in the environments, it is necessary to gain insight into how proteins interact with each other under different conditions. It is well known that the functions of proteins in biological systems are highly dependent on their tertiary structures. As a result, chemical modifications to proteins such as dye labeling may cause a reduction or even a loss of protein activities by either directly blocking the active binding sites or affecting the three-dimensional folding of the proteins. Therefore, it is highly desirable to avoid any modifications of proteins when monitoring protein–protein interactions in order to obtain the most "true-to-life" information. However, many commonly used techniques based on molecular separation such as gel electrophoresis and capillary electrophoresis (CE)[1] lack the ability of real-time analysis in homogeneous solutions. A more recent development in protein–protein interactions is the yeast two-hybrid system that was first reported in 1989.[2] Based on transcription activated in yeast nuclei by protein–protein interactions, this method has been widely used to study protein functions, and recently adapted to map protein interactions on a proteome-wide scale.[3,4] However, it can not be done in real-time and involves labor-intensive procedures to fuse the two proteins into a DNA-binding domain and an activation domain. Another technique capable of protein–protein interaction monitoring is based on fluorescence resonance energy transfer (FRET), where two interacting proteins have to be dye-labeled for the energy transfer to take place.

A new strategy for real-time protein interaction study, which requires no protein-labeling, can be developed directly based on aptamers. Aptamers are nucleic acids that may be selected by using a systematic evolution of ligands via an exponential enrichment (SELEX) process.[5,6] Compared with antibodies, aptamers can have similar affinity to their protein targets but are usually much smaller and much easier to reproduce. Quite tolerant to external environment changes and internal modifications, aptamers can be conveniently labeled for various applications. Molecular beacon aptamers combining the superior specificity of aptamers for proteins and the excellent signal transduction mechanism of molecular beacons have been developed for analyte detection without labeling the target.[7,8] Despite being excellent molecular probes for proteins,[7–11] aptamers have not been

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used extensively to study the real-time interactions between their target proteins and other proteins.

Our new strategy directly uses protein-binding aptamers for label-free protein–protein interactions. Two signal transduction strategies, FRET and fluorescence anisotropy, are used to monitor the binding events between the aptamer-binding protein, the "bait protein", and a second protein, the "prey protein". As illustrated in Figure 1a, an aptamer can be labeled with a fluorophore and a quencher to have internal FRET. Binding of the aptamer to the bait protein causes a quenched fluorescence, while the subsequent binding of the prey protein to the bait protein may either displace the aptamer and result in a restoration of fluorescence (sequential incubation) or inhibit the binding of the aptamer and prevent quenching (co-incubation). Many aptamers can be easily labeled with a fluorophore and a quencher to form intramolecular FRET. Folded conformations of many aptamers have been shown to be stabilized by binding to their target molecules.[12–15] In an alternative approach, FRET can be formed using added bases even if the original sequence of the aptamer lacks the necessary conformational changes accompanying the binding to the target molecules.[9]

In the other approach, shown in Figure 1b, an aptamer is labeled with only one fluorophore and the fluorescence anisotropy of the aptamer or the aptamer complex is monitored in real-time. Binding of the aptamer to a much larger bait protein will result in increased fluorescence anisotropy. Further change in the anisotropy of the aptamer-bait complex can be triggered by the interaction between the bait and prey proteins. Co-binding of aptamer and a protein on the same target protein is also possible to be monitored in this way.

The aptamer based assays were applied to study interactions between human α-thrombin and related proteins. Two aptamers binding to two different sites on thrombin were modified to monitor thrombin–protein interactions in real-time. Binding site information as well as binding kinetics could be revealed by combination of FRET and anisotropy based assays.

## Results and Discussion

**FRET signaling aptamer for protein binding**: Human α-thrombin and its aptamers were used as a model system to demonstrate the capability of aptamers to probe protein–protein interactions. α-Thrombin has two positively charged sites termed Exosite I and II on the opposite sides of the protein.[16] Exosite I was found to bind to fibrinogen[17] and hirudin[18] while Exosite II binds to heparin. 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 which was reported to bind to the fibrinogen-binding site of α-thrombin,[19] namely Exosite I. The other DNA aptamer, with a 27 mer backbone length, was determined to bind to the Exosite II of α-thrombin.[12] Both aptamers were found to adopt a G-quartet structure when bound to α-thrombin. A 15 mer Exosite I binding aptamer (15Ap, Table 1) and a 27 mer Exosite II binding aptamer (27Ap, Table 1) with similar thrombin-binding affinity were chosen to study the interactions of α-thrombin with other proteins.

We previously reported a molecular beacon aptamer for α-thrombin detection based on the 15Ap.[8] Here a slightly modified aptamer (FQ-15Ap, Table 1) has been used that incorporates a 6-carboxyfluorescein (6-FAM) at the 5′ end of the DNA as the donor and a Dabcyl at the 3′ end as the quencher. The quenching of 6-FAM emission is caused by energy transfer between 6-FAM and Dabcyl in the protein-binding induced G-quartet structure where the two labels

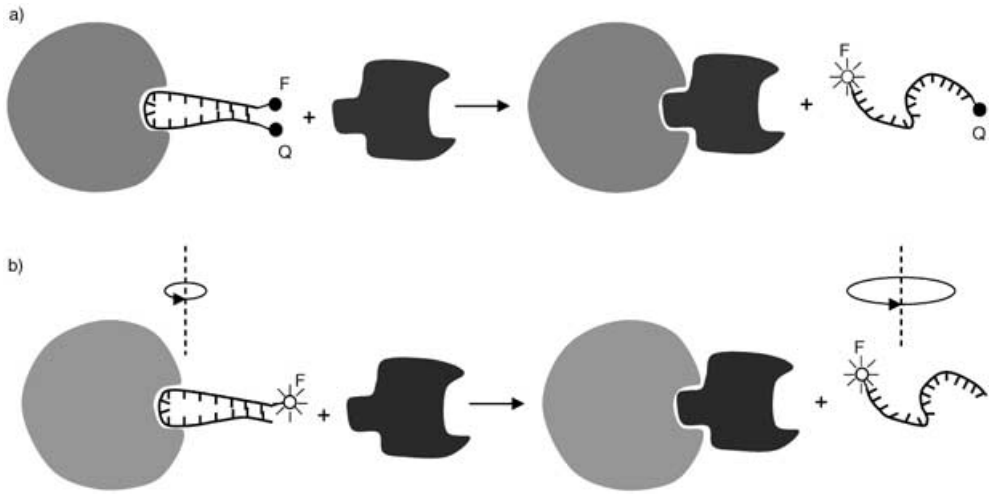


Figure 1. Dye-labeled protein-binding DNA aptamers reporting protein–protein interactions. a) Dual-labeled aptamer with a fluorophore and a quencher. The folded form of the aptamer results in a quenched fluorescence when it binds to the bait protein. The bait–prey protein interaction causes release of the aptamer from the bait protein, leading to a restored fluorescence. b) Single-labeled aptamer. When bound to the much larger bait protein, the aptamer displays slow rotational diffusion. The interaction between bait and prey proteins displaces the aptamer. The unbound aptamer has much faster rotational diffusion. The change in the rotation rate is reported by fluorescence anisotropy of the dye molecule.

  

Table 1. Sequences of the fluorophore-labeled aptamers used in the paper.

| Oligo name | Oligo sequence |
|---|---|
| FQ-15Ap | 5′-(6-FAM)-GGT TGG TGT GGT TGG-(Dabcyl)-3′ |
| T-15Ap | 5′-GGT TGG TGT GGT TGG-(TAMRA)-3′ |
| FQ-27Ap | 5′-(6-FAM)-ACC CGT GGT AGG GTA GGA TGG GGT GGT-(Dabcyl)-3′ |
| T-27Ap | 5′-ACC CGT GGT AGG GTA GGA TGG GGT GGT–(TAMRA)-3′ |
| F-15Ap | 5′-GGT TGG TGT GGT TGG-(6-FAM)-3′ |

are in close proximity. When excess α-thrombin was added to an FQ-15Ap solution at room temperature, the fluorescence of 6-FAM dropped about 55 percent (Figure 2). It is known that high metal ion concentrations, especially the presence of $K^+$, can promote the formation of G-quartets.[20,21] It is thus expected that there will be less fluorescence change induced by G-quartet formation when thrombin is added to such high salt concentrations, which could reduce the detection capability of the protein–protein assay. However, by using a buffer without any metal ions was found to inhibit protein–protein interactions. By keeping a 50 mM NaCl concentration in the buffer, we were able to sustain the protein activities and get relatively high fluorescence quenching induced by protein binding to the aptamer. When α-thrombin was added into a control 15 mer aptamer that was labeled only with 6-FAM, no significant fluorescence change was observed (Figure 2); this indicates that the fluorescence decrease in the FQ-15Ap-thrombin binding experiment was due to the binding-induced conformational change of the aptamer rather than a direct quenching of the dye 6-FAM by α-thrombin. We did not observe the quenching i) under conditions where thrombin would not bind the aptamer beacon, and ii) with a scrambled aptamer beacon to which thrombin does not bind. This result was consistent with reported molecular beacon aptamer study.[8]

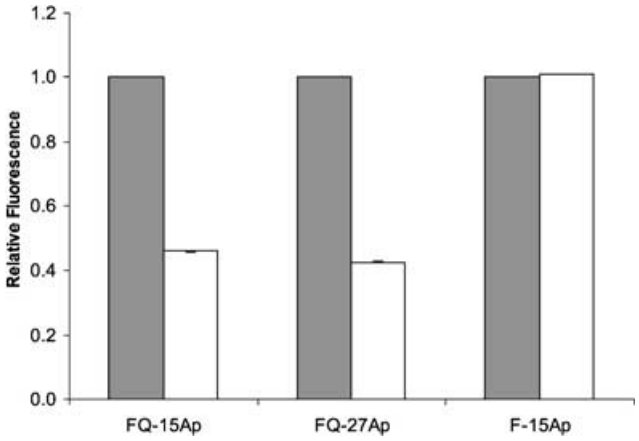


Figure 2. Human α-thrombin binding induced relative fluorescence change of dual-labeled aptamers. 6-FAM florescence intensities of 100 nM FQ-15Ap, FQ-27Ap and F-15Ap were recorded before (gray bar) and after (white bar) the addition of 500 nM α-thrombin.

The sequence of the Exosite II-binding 27 mer aptamer was adopted from a previous report.[16] This aptamer was also labeled with 6-FAM and Dabcyl similar to FQ-15Ap. With the addition of α-thrombin, FQ-27Ap displayed decreased 6-FAM fluorescence (Figure 2) because 6-FAM and Dabcyl at the two ends of the aptamer were brought closer in the quadruplex structure.

**Dual-labeled aptamers for thrombin-protein binding study**: The 1:1 molar ratio FQ-15Ap (or FQ-27Ap)/α-thrombin solution (bait solution) was used to identify the interactions of α-thrombin with other proteins. When a second protein (prey protein) binds to the same site of α-thrombin as the FQ-15Ap, the aptamer is expected to be displaced and the freed aptamer will shift back to a more relaxed conformation, resulting in restored 6-FAM fluorescence. A sulfated fragment of hirudin that contained the C-terminal 13-residue[18] (HirF) instead of hirudin was used for binding α-thrombin. The addition of HirF to the FQ-15Ap bait solution caused a sharp fluorescence increase (Figure 3a), which was expected since both HirF and FQ-15Ap bound to the same site of α-thrombin. Control experiments showed that there was no fluorescence change when HirF was added to a FQ-15Ap in the absence of thrombin (data not shown); this indicates that there was no direct interaction between the aptamer and HirF. The time course results showed that this competitive binding reaction was fast as the aptamer departed within seconds after HirF was added to the aptamer–thrombin complex solution.

Several other proteins were also investigated for interactions with α-thrombin by using the FQ-15Ap bait solution. The addition of an antibody, anti-human thrombin (AHT), caused no significant change in the fluorescence of 6-FAM (Figure 3a). While this result indicates that AHT does not compete with the aptamer for the Exosite I of α-thrombin, we can not exclude the possibility that AHT still binds to α-thrombin but at a different site of α-thrombin. More experiments were done to address this issue (results are presented later in this paper). A serine protease inhibitor antithrombin III (AT3) was also tested in the bait solution. A slow-signal increasing trend was observed for AT3 (Figure 3a). Addition of excess AT3 further increased the 6-FAM fluorescence, but the fluorescence intensity never exceeded that of the FQ-15Ap solution in the absence of α-thrombin (data not shown), meaning that the signal change was not due to direct interaction between the aptamer and AT3. This result could be explained in that the binding of AT3 to α-thrombin may have caused a conformational change in α-thrombin that rendered the binding with the aptamer at Exosite I unstable.[22] It has been reported that the interaction of AT3 with the active site of serine proteinases is a multi-step, covalent bond forming process,[23] which should be the reason for the slow reaction rate observed here.

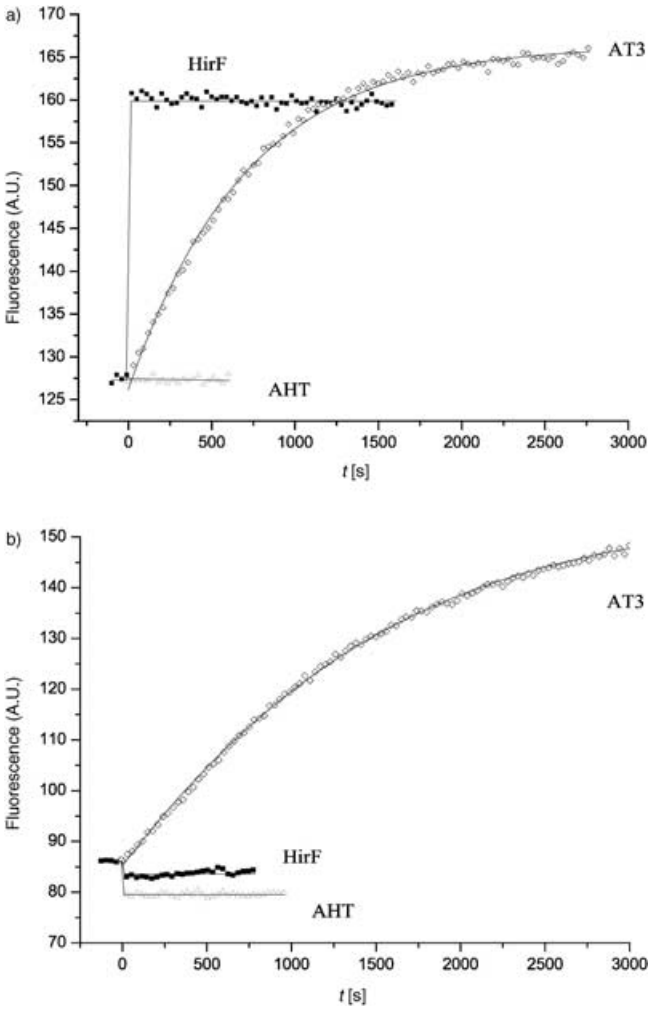


Figure 3. Dual-labeled aptamers for α-thrombin/protein interactions. a) In a solution of mixed 100 nM FQ-15Ap and 100 nM α-thrombin, 200 nM AT3 (◇), 500 nM HirF (■) or 300 nM AHT (△) was added at 0 s and fluorescence of 6-FAM was continuously monitored. b) In a solution of mixed 100 nM FQ-27Ap and 100 nM α-thrombin, 300 nM AT3 (◇), 500 nM HirF (■) or 300 nM AHT (△) was added at 0 s and fluorescence of 6-FAM was continuously monitored.

Bovine serum albumin (BSA) was used as a control protein for interaction with α-thrombin. No fluorescence change was observed for BSA (data not shown). Another set of control experiments were conducted by adding the prey proteins to be tested to an FQ-15Ap buffer solution without α-thrombin. None of the proteins affected fluorescence of the aptamer (data not shown), meaning they did not interact with either the aptamer or the fluorophore.

We found that different thrombin to aptamer ratios resulted in different sensitivity of the assay for protein–protein interactions. For example, for a thrombin to aptamer ratio of 2:1, it took more prey protein to cause similar quantity of signal change compared to a 1:1 ratio. For that reason, 1:1 ratio of thrombin to aptamer was used in all our experiments.

Different proteins were investigated in a FQ-27Ap/α-thrombin bait solution in a similar way as in the FQ-15Ap based assay. The results for HirF and AHT showed slightly decreased signals instead of any increase (Figure 3b), indicating no displacement of FQ-27Ap took place. By contrast, antithrombin III still displayed a gradual increase in 6-FAM fluorescence, meaning that, contrary to a previous report,[22] binding between thrombin and the serpin antithrombin III can also destabilize binding at Exosite II. Again, the slow interaction between AT3 and thrombin caused rather gradual displacement of FQ-27Ap.

**Fluorescence anisotropy (FA) based aptamer probes for protein interactions**: To address some of the unresolved problems in FRET experiments such as how AT3 binds to α-thrombin and what happens between AHT and α-thrombin, we developed a complementary strategy based on fluorescence anisotropy. Fluorescence anisotropy is widely used for studying the interactions of biomolecules due to its capability of sensing changes in molecular size or molecular weight.[24] We labeled the thrombin aptamers with only one TAMRA dye at the 3′ end to create anisotropy aptamer probes, the 15 mer T-15Ap and the 27 mer T-27Ap (Table 1).

The T-15Ap was first investigated for its ability to probe protein interactions. When a 1:1 molar ratio of T-15Ap and α-thrombin was mixed together, the anisotropy of T-15Ap increased more than 30% (data not shown). This bait solution was then tested with different prey proteins (Figure 4a). The anisotropy dropped within seconds upon addition of HirF to the bait solution and remained almost constant after that. This result correlates well with the result from the FRET-based experiment and may be explained as a quick displacement of the aptamer by HirF at the Exosite I binding site of α-thrombin. The anisotropy decreased as a result of the increased concentration of unbound aptamer which had a much lower molecular weight than that of the aptamer–protein complex. The reaction was rapid, indicating noncovalent bonds were most likely involved in the binding between HirF and α-thrombin.

The AT3 curve showed a different decreasing trend with time. It was rather slow and gradual, similar to the FRET-based result. Even though the FRET assay clearly illustrated that the aptamer was displaced, it did not provide much information about how this displacement took place. There could be several pathways that the AT3/α-thrombin interaction might have taken. One of them is that the AT3 molecules would quickly bind to the active site of α-thrombin, and a slow conformational change of α-thrombin induced by AT3 binding then caused FQ-15Ap to leave Exosite I. In another pathway, AT3 would slowly attack thrombin while such interaction would force the aptamer to leave thrombin at the same time. The FRET-based method could not differentiate between these two mechanisms. On the other hand, using fluorescence anisotropy, if the AT3/α-thrombin interaction underwent the first pathway, the increased molecular weight through the binding of AT3 to α-thrombin/aptamer complex in the first step would introduce an initial anisotropy increase. Then, the anisotropy would slowly decrease

  

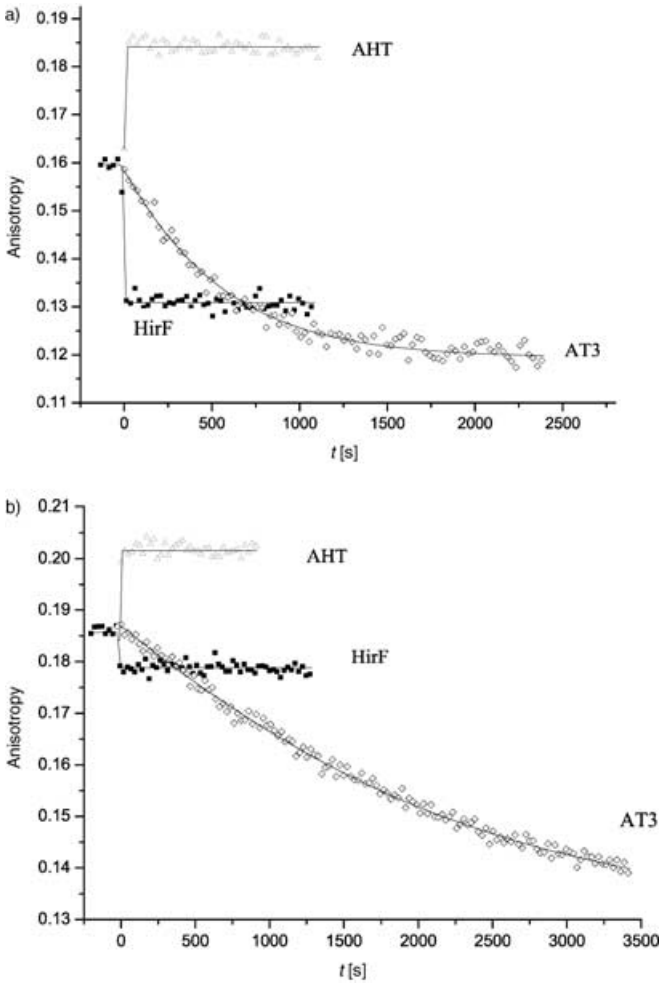


Figure 4. TAMRA-labeled aptamers for α-thrombin/protein interactions based on fluorescence anisotropy. a) In a solution of mixed 100 nM FQ-15Ap and 100 nM α-thrombin, 200 nM AT3 (◇), 500 nM HirF (■) or 300 nM AHT (△) was added at 0 s and anisotropy of TAMRA was recorded in real-time. b) Same experiments as in a) using the T-27Ap aptamer. 200 nM AT3 (◇), 500 nM HirF (■) or 300 nM AHT (△) was added to the aptamer/α-thrombin mixture solution at 0 s.

from that point on as the T-15Ap slowly became unbound. However, the real-time anisotropy detection of the AT3/α-thrombin interaction (Figure 4a) demonstrated no such initial anisotropy jump. The anisotropy experiments support the second pathway better as the mechanism for this protein–protein interaction. The anisotropy approach is shown here to be able to provide insight into the kinetics and mechanisms of the targeted interactions, which will be highly useful in understanding proteins' functions. It is our belief that site-directed aptamers enable real-time, sensitive studies on protein–protein interaction.

In contrast, AHT caused an immediate anisotropy increase of T-15Ap when added to the aptamer/α-thrombin bait solution (Figure 4a). This anisotropy increase suggested a binding event between AHT and α-thrombin. Furthermore, it is clear that the binding happened at a different site than Exosite I, which added extra weight to the aptamer/α-thrombin complex. This result correlated with the FRET assay which showed the aptamer was not displaced by AHT. The binding of AHT and α-thrombin was further confirmed using gel electrophoresis (Figure 5). One advantage of the anisotropy-based method over the FRET-based method and many other techniques might be that it can differentiate interactions at different binding sites.

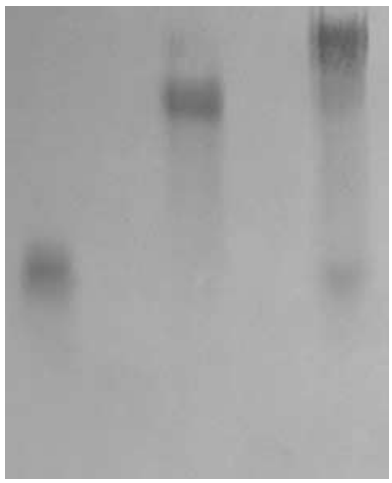

Figure 5. Binding between α-thrombin and anti-human thrombin (AHT) confirmed by gel electrophoresis on a 7.5% native Tris-HCl gel. Left lane contained 50 pmole of α-thrombin. Middle lane had 32 pmole of AHT. Right lane had mixture of 32 pmole of AHT and 50 pmole of α-thrombin.

Bait solutions containing T-27Ap and α-thrombin were also used to probe protein–protein interactions at the Exosite II of α-thrombin (Figure 4b). HirF caused a slightly lower anisotropy change even though it binds to Exosite I. Considering HirF is a rather small molecule ($M_W$ ~ 1.5 kDa), the small anisotropy decrease was likely caused by HirF displacing T-27Ap. However, this decrease was much smaller compared with that of T-15Ap. AT3 displayed a gradually decreasing anisotropy as it slowly replaced T-27Ap. In contrast, AHT induced an instant anisotropy increase similar to what was found with T-15Ap, suggesting that AHT does not affect binding at Exosite II and probably binds to a third site of α-thrombin other than Exosite I and II.

**Quick evaluation of binding constants of protein–protein interactions**: By using the aptamer/thrombin system with known thermodynamic properties, it is possible to obtain the dissociation constant ($K_d$) of the protein–protein binding reactions by doing one single fluorescence measurement in our competitive assay. In a competitive assay, such as the HirF/thrombin interaction detection described in this work, the interaction of aptamer and its target protein is a known system. The addition of the prey protein may shift the equilibrium of the aptamer/bait protein binding reaction and cause a signal change. Based on the known aptamer/α-

thrombin interaction and equilibrium conditions, theoretically it was possible to calculate the $K_d$ of α-thrombin/prey protein binding reaction using a single signal change that occurred when the prey protein was added to the aptamer/α-thrombin complex solution.

Assume $C_A$ molar of the total T-15Ap aptamer concentration and $C_T$ molar of the total α-thrombin concentration, when $C_P$ molar of prey protein is present in the mixture, it will displace T-15Ap and result in a decreased anisotropy value of $r_{new}$. $r_{new}$ can be represented using the following equation:

$$r_A \cdot x + r_{AT} \cdot (1-x) = r_{new}$$

where $r_A$ and $r_{AT}$ are anisotropies of the two fluorescent species in the solution, T-15Ap and T-15Ap/α-thrombin complex, respectively, and × is fraction of the unbound T-15Ap aptamer. Since $r_A$ and $r_{AT}$ are known properties of the aptamer/α-thrombin system and $r_{new}$ is the measured new anisotropy, it is easy to find out that:

$$x = \frac{r_{new} - r_{AT}}{r_A - r_{AT}}$$

Then the concentrations of unbound and bound T-15Ap are:

$$[\text{T-15Ap}] = C_A \cdot x \quad [\text{T-15Ap/α-thrombin}] = C_A \cdot (1-x)$$

Because the dissociation constant of aptamer/α-thrombin reaction ($K_{d/AT}$) is already known, then:

$$[\text{α-thrombin}] = \frac{K_{d/AT} \cdot [\text{T-15Ap/α-thrombin}]}{[\text{T-15Ap}]}$$

Since $C_T$ = [α-thrombin] + [T-15Ap/α-thrombin] + [prey/α-thrombin], [prey/α-thrombin] = $C_T$ − [α-thrombin] − [T-15Ap/α-thrombin];

similarly, $C_P$ = [prey/α-thrombin] + [prey protein], so [prey protein] = $C_P$ − [prey/α-thrombin];

finally, the dissociation constant of α-thrombin/prey protein binding reaction ($K_{d/TP}$) is given by the following Equation:

$$K_{d/TP} = \frac{[\text{prey protein}] \cdot [\text{α-thrombin}]}{[\text{prey/α-thrombin}]}$$

Using a simple computer program, it is possible to routinely calculate protein–protein binding affinity by using data obtained from the aptamer-based competitive assay for protein–protein interactions. Despite the quick and easy evaluation of $K_d$ by using the above described method, it is important to note that errors in sample handling and fluorescence signal measurements as well as inaccuracy in the aptamer/thrombin binding constant might lead to a considerable amount of uncertainty in the calculated dissociation constants. In this case, multiple measurements may be required. We also notice that there is no information about the stoichiometry of binding and the potential of "cooperativity" in any of the binding reactions in our estimation. This simple method for evaluating the binding constant can at least be used as a quick estimation in protein–protein interaction studies.

We demonstrated this capability by calculating $K_d$ of α-thrombin/HirF binding reaction using a reported 15 mer aptamer–thrombin $K_{d/AT}$ of 75 nM.[25] With anisotropy of 100 nM free 15 mer aptamer and 100 nM 1:1 aptamer/thrombin mixture to be $0.121 \pm 0.003$ and $0.160 \pm 0.003$, respectively. Three measurements of anisotropy after HirF addition resulted in a calculated $K_d$ of $180 \pm 50$ nM for HirF/thrombin binding, which is in the range comparable to previously reported (150 nM).[12]

## Conclusion

Our results have shown that without any modification to two proteins, their interaction can be monitored in real-time by using aptamer-based assays. The FRET and fluorescence anisotropy approaches used in this paper are found to complement each other. The FRET-based assay relies on direct measurements of sample fluorescence, which makes it highly sensitive and selective. It also has the potential to be easily adapted for binding-site-specific protein interaction screening with high throughput in an array format. Compared with FRET-based approach, fluorescence anisotropy has shown to offer a large amount of information about protein–protein binding that is not readily available by using other techniques including FRET.

Neither approach requires labeling of the interacting target protein or the probe protein, allowing true real-time monitoring of the interactions between the two proteins based on their unaffected biological activities. While each one excels in different aspects of protein interaction study, we found that the combination of the two fluorescence techniques was capable of providing detailed knowledge about the kinetics of the protein–protein binding as well as the mechanism and binding site information of the interactions. This is not possible or at least not easily obtainable with many other current techniques.

The aptamer competitive assay should also hold the potential for studying interactions between proteins and other molecules such as small organic molecules, as well as DNA and RNA. With the development of an automated aptamer selection system and its capability to carry out aptamer selection for multiple targets in parallel,[26] it is expected that aptamers can be rapidly developed for a growing number of proteins. It is thus possible to build a large array of aptamers for protein-drug candidate interactions in large scale drug discovery, or in whole cell protein–protein interactions for disease diagnosis and functional proteomics.

  

## Experimental Section

**Materials**: Dye-labeled aptamers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The sequences of the fluorophore-labeled 15 and 27 mer thrombin–aptamers used in this paper are listed in Table 1. All aptamers were purified with HPLC.

Human α-thrombin ($M_W$ ~36.7 kDa), human antithrombin III (AT3) ($M_W$ ~58 kDa) and a monoclonal antibody anti-human thrombin (AHT) ($M_W$ ~150 kDa) were obtained from Haematologic Technologies Inc. (Essex Junction, VT). Bovine serum albumin (BSA) ($M_W$ ~67 kDa) and a sulfated hirudin fragment 54–65, Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr($SO_3$H)-Leu-Gln (HirF) ($M_W$ ~1.5 kDa), were from Sigma-Aldrich, Inc. (St. Louis, MO). All tests were performed in a 20 mM Tris-HCl buffer with a pH 7.6 that contained 50 mM NaCl and 5% (v/v) glycerol. All reagents for the buffer were obtained from Fisher Scientific (Pittsburgh, PA).

**Fluorescence FRET and anisotropy measurements**: Fluorescence measurements were performed on a Fluorolog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ). For FRET-based assays, the fluorescence of 6-FAM was monitored with an excitation wavelength of 488 nm and an emission wavelength of 515 nm. For anisotropy-based experiments, the fluorescence of TAMRA was monitored with 555 nm as the excitation and 580 nm as the emission wavelength. Slit widths were varied to yield the best signals. All measurements were carried out in a 100 μL cuvette. In the aptamer/thrombin binding experiments, a very small volume of α-thrombin at a high concentration was added to an aptamer solution in the cuvette to make a molar ratio of aptamer and thrombin 1:1, and the fluorescence signals were recorded before and after the addition. For protein–protein binding reaction, an aptamer/thrombin mixture at 1:1 molar ratio was placed in the cuvette, and small volumes of the second protein solution at high concentrations were added to the mixture to make the desired prey protein concentrations. All dilution effects caused by the addition of samples to the original solutions were corrected during data analysis.

Fluorescence anisotropy was also measured with Fluorolog-3 spectrofluorometer. The measurements were based on the following equation:[24]

$$\text{Anisotropy } r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}}$$

where the subscripts V and H refer to the orientation (vertical or horizontal) of the polarizers for the intensity measurements, with the first subscript indicating the position of the excitation polarizer and the second for the emission polarizer. G is the G factor of the spectrofluorometer, which is calculated as $G = I_{HV}/I_{HH}$. The G factor represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light, and is dependent on the emission wavelength. For a certain dye, the G factor would be measured and used throughout the experiments that used the same dye. Then the spectrofluorometer would keep the excitation polarizer vertical and rotate the emission polarizer from vertical to horizontal position to measure the intensities for anisotropy calculation. For TAMRA, all intensities were measured at an emission wavelength of 580 nm with an excitation wavelength of 555 nm. Time-based anisotropy measurements were carried out by continuously monitoring anisotropy. With an integration time of 1.5 s, each anisotropy measurement would take about 6.1 s.

**Gel electrophoresis**: Gel electrophoresis was performed on a Mini-Protean 3 precast gel system (Bio-Rad Laboratories, Inc., Hercules, CA). Samples loaded on a 7.5% resolving Tris-HCl native gel (Bio-Rad Laboratories, Inc., Hercules, CA) were run at 150 V for 150 min. The gel was then taken out, rinsed with ultra-pure water and stained with Coomassie blue stain reagent (Fisher Scientific, Pittsburgh, PA) for 1 h. A digital camera was used to image the stained gel.

## Acknowledgements

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[1] L. Tao, R. T. Kennedy, *Electrophoresis* **1997**, *18* 112–117.
[2] S. Fields, O. Song, *Nature* **1989**, *340*, 245–246.
[3] P. Uetz, L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson, J. R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, J. M. Rothberg, *Nature* **2000**, *403*, 623–627.
[4] T. Ito, T. Chiba, R. Ozawa, M. Yoshida, M. Hattori, Y. Sakaki, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 4569–4574.
[5] C. Tuerk, L. Gold, *Science* **1990**, *249*, 505–510.
[6] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818–822.
[7] X. Fang, A. Sen, M. Vicens, W. Tan, *ChemBioChem* **2003**, *4*, 829–834; C.-C. Huang, Z. Cao, H.-T. Chang, W. Tan, *Anal. Chem.* **2004**, *76*, 6973–6981.
[8] J. J. Li, X. Fang, W. Tan, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 31–40.
[9] N. Hamaguchi, A. Ellington, M. Stanton, *Anal. Biochem.* **2001**, *294*, 126–131.
[10] L. S. Green, C. Bell, N. Janjic, *Biotechniques* **2001**, *30*, 1094–1096, 1098, 1100.
[11] J. S. Hartig, S. H. Najafi-Shoushtari, I. Grune, A. Yan, A. D. Ellington, M. Famulok, *Nat. Biotechnol.* **2002**, *20*, 717–722.
[12] D. M. Tasset, M. F. Kubik, W. Steiner, *J. Mol. Biol.* **1997**, *272*, 688–698.
[13] L. S. Green, D. Jellinek, R. Jenison, A. Ostman, C. H. Heldin, N. Janjic, *Biochemistry* **1996**, *35*, 14413–14424.
[14] M. N. Stojanovic, P. de Prada, D. W. Landry, *J. Am. Chem. Soc.* **2001**, *123*, 4928–4931.
[15] K. Padmanabhan, K. P. Padmanabhan, J. D. Ferrara, J. E. Sadler, A. Tulinsky, *J. Biol. Chem.* **1993**, *268*, 17651–17654.
[16] M. T. Stubbs, W. Bode, *Thromb. Res.* **1993**, *69*, 1–58.
[17] M. C. Naski, J. W. Fenton, J. M. Maraganore, S. T. Olson, J. A. Shafer, *J. Biol. Chem.* **1990**, *265*, 13484–13489.
[18] T. J. Rydel, A. Tulinsky, W. Bode, R. Huber, *J. Mol. Biol.* **1991**, *221*, 583–601.
[19] L. R. Paborsky, S. N. McCurdy, L. C. Griffin, J. J. Toole, L. L. Leung, *J. Biol. Chem.* **1993**, *268*, 20808–20811.
[20] C. C. Hardin, T. Watson, M. Corregan, C. Bailey, *Biochemistry* **1992**, *31*, 833–841.
[21] H. Ueyama, M. Takagi, S. Takenaka, *J. Am. Chem. Soc.* **2002**, *124*, 14286–14287.
[22] J. C. Fredenburgh, A. R. Stafford, J. I. Weitz, *J. Biol. Chem.* **2001**, *276*, 44828–44834.
[23] S. P. Leytus, S. W. Peltz, W. F. Mangel, *Biochim. Biophys. Acta* **1983**, *742*, 409–418.
[24] W. Tan, K. Wang, T. Drake, *Curr. Opin. Chem. Biol.* **2004**, *8*, 547–553.
[25] R. F. Macaya, J. A. Waldron, B. A. Beutel, H. Gao, M. E. Joesten, M. Yang, R. Patel, A. H. Bertelsen, A. F. Cook, *Biochemistry* **1995**, *34*, 4478–4492.
[26] J. C. Cox, A. D. Ellington, *Bioorg. Med. Chem.* **2001**, *9*, 2525–2531.

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