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Fluorescence Anisotropy Analysis for Mapping Aptamer−Protein Interaction at the Single Nucleotide Level

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Supporting Information

ABSTRACT: Structural characterization of aptamer−protein interactions is challenging and limited despite the tremendous applications of aptamers. Here we for the first time report a fluorescence anisotropy (FA) approach for mapping the interaction of an aptamer and its protein target at the single nucleotide level. Nine fluorescently labeled aptamers, each conjugated to a single tetramethylrhodamine at a specified nucleotide in the aptamer, were used to study their interactions with thrombin. Simultaneous monitoring of both fluorescence anisotropy changes and electrophoretic mobility shifts upon binding of the fluorescently modified aptamer to the protein provides unique information on the specific nucleotide site of binding. T25, T20, T7 and the 3′-end were identified as the close contact sites, and T3, C15T, and the 5′-end were identified as the sites distant from the binding. This approach is highly sensitive and does not require cross-linking reactions. Studies of aptamer−protein interactions using this technique are potentially useful for design, evolution, and modification of functional aptamers for a range of bioanalytical, diagnostic, and therapeutic applications.

Nucleic acid aptamers have shown great promise in biotechnology, medical diagnostics, and therapeutics.[1] Diverse applications in affinity recognition, biosensing, ultrasensitive detection, and separation have mainly taken advantage of the high affinity and specificity of the selected aptamers.[2−5] Further applications of aptamers could greatly benefit from the structural knowledge of how aptamers specifically bind to their targets. Classic structural approaches such as X-ray crystallography and NMR are very time-consuming.[6] An indirect approach based on photocross-linking analysis is useful, but it may only identify a few point-contact sites because of the inefficient cross-linking reactions. Cross-linkings occur only between the photoreactive nucleotide analogues and 4 electron-rich amino acid residues among the 20 amino acid residues.[7] X-ray crystallography analysis has shown multiple close contacts in protein−DNA binding.[8] Currently, there is very limited structural determination of aptamer−protein complexes.[8]

Here we, for the first time, propose a fluorescence polarization or anisotropy (FA) approach for sensitive characterization of site proximity in the architecture of aptamer−protein complexes at the single nucleotide level. This approach takes advantage of changes in FA of a fluorophore-labeled aptamer upon its binding to its target protein. Because FA is dependent on the molecular volume and the local movement of the fluorescent molecule, close contact between two interacting molecules near the fluorophore will result in slower movement and increases in FA values. The close contact of the labeled nucleotides in an aptamer to the associated protein can be derived from the increased FA response. Experimentally, the labeled unbound aptamer and aptamer−protein complex are separated by capillary electrophoresis, and FA is used as a measure of fluorophore local movement of the separated species. The anisotropy changes (Δr) between the unbound aptamer and the aptamer−protein complex may reflect relative orientation of the aptamer in the aptamer−protein complex. This novel approach builds on our recent DNA wrapping study showing that wrapping of fluorescently labeled DNA damage probes to the DNA repair proteins significantly increases FA responses.[9]

In the aptamer−protein system, anisotropy is a complex function of multiple parameters, and no explicit relation between Δr and "what causes" Δr has yet been derived. Δr can be caused by two important factors: (i) the closeness of the labeled fluorophore in the aptamer to the associated protein and (ii) structural changes of the aptamer near the labeled fluorophore upon binding. For the proposed method to be valid (informative of the closeness), the structural change contribution to Δr should be much smaller than the contribution of the closeness. There are previous data that indicate that Δr is less than a certain value upon structural changes of the aptamer caused by small ligand binding.[10] However, no one has ever measured the anisotropy change caused by the structural changes upon protein binding. Accordingly, one of the principal goals of this work is to demonstrate that closeness can induce much greater Δr than the structural changes.

To demonstrate the proof-of-principle, we chose to study the interaction of human α-thrombin and aptamer TA29. TA29 may bind human α-thrombin in a quadruplex/duplex structure[11] (Supporting Information [SI], Scheme S1). A series of singly labeled aptamers (including mutants) were synthesized with the same sequence as TA29 or with a single nucleotide substitution (SI, Table S1). These aptamers were labeled with a single fluorescent dye, tetramethylrhodamine (TMR), either at the 5′- or 3′-end or internally at thymidines through a flexible linker (SI, Scheme S2). The labeling did not occupy any hydrogen-bonding position nor generate a steric barrier for base stacking

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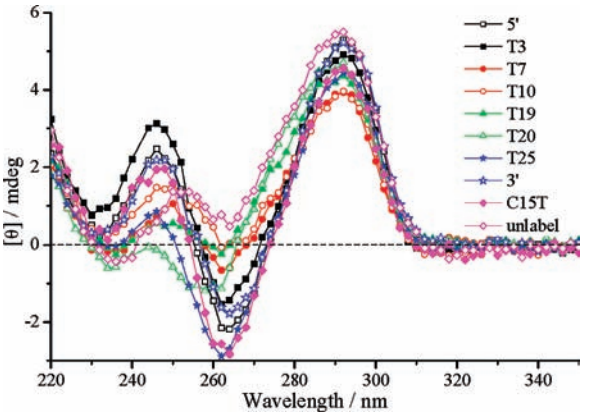

Figure 1. CD spectra of 10 μM TA29 aptamers in 10 mM Tris-HCl, pH 7.5, 150 mM KCl at room temperature. Each aptamer was labeled with a single TMR dye at the indicated nucleotide position.

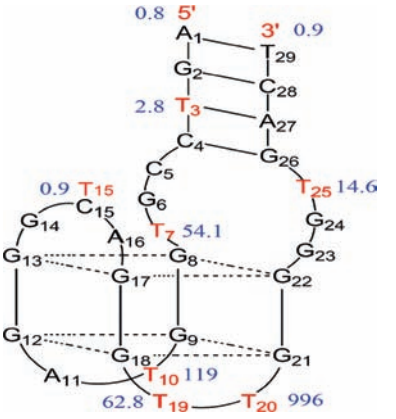

Figure 2. Measured dissociation constants (K_d, nM) for the complexes between thrombin and the modified aptamers. Each aptamer was labeled with a single TMR dye at the specified nucleotide site. K_d values were determined by the CE mobility shift assay.

(SI, Scheme S2). The synthesized aptamers were tested for their ability to form secondary conformation and to bind with human α-thrombin.

The secondary structure of the unlabeled and labeled TA29 (labeled at 5′, T3, T7, T10, C15T, T19, T20, T25, or 3′) was investigated by circular dichroism (CD) analysis. Similar to the unlabeled TA29, all the labeled TA29 aptamers (without mutation) displayed a peak at 292 nm and a valley at 263 nm (Figure 1), showing typical CD characteristics of an antiparallel G-quadruplex conformation.[12] In contrast, only three of nine mutated TA29 aptamers formed the antiparallel G-quadruplex conformation (SI, Figure S1). It is evident that all the labeled and unmutated TA29 aptamers preserved their ability to form proper secondary structure. This is consistent with the fact that the chosen labeling does not reduce the capacity of hydrogen bonding and base stacking for the labeled nucleotides.

The interaction of human α-thrombin and TA29 was measured by a capillary electrophoresis (CE) mobility shift assay with laser-induced fluorescence polarization (LIFP) detection.[9,13] Benefiting from highly efficient CE separation, the unbound TA29 can be well resolved from the complex of thrombin−TA29 (SI, Figure S2), enabling the simultaneous measurement of the FA values of the bound and unbound TA29 by online coupled LIFP detection. Although performed in the same set of CE experiments, the dissociation constants (K_d) were measured by the CE mobility shift assay, independently of the FA measurement. The K_d values are indicative of the binding strength, and FA values (r) from the fluorophores labeled at different sites suggest the nature of contact (distant or close).

The K_d values and FA values were mapped in Figures 2 and 3 according to the secondary structure of TA29 and summarized in Tables S2 and S3 in the SI, respectively. These aptamers can be classified into three groups according to the magnitude of the measured affinity. The first, with negligible perturbation, includes the 3′-, 5′-, and C15T-labeled aptamers, showing a high affinity ($K_d \approx 0.8-0.9$ nM); the second includes T3- and T25-labeled aptamers with a K_d of 2.8−14.6 nM, indicating a moderate affinity perturbation; and the last includes T7-, T10-, T19-, and T20-labeled aptamers with a K_d of 54.1−996 nM, displaying a high affinity perturbation.

To examine whether the fluorescent labeling of the aptamer alters the site of the aptamer binding to its target, we performed a series of competitive binding experiments using both the

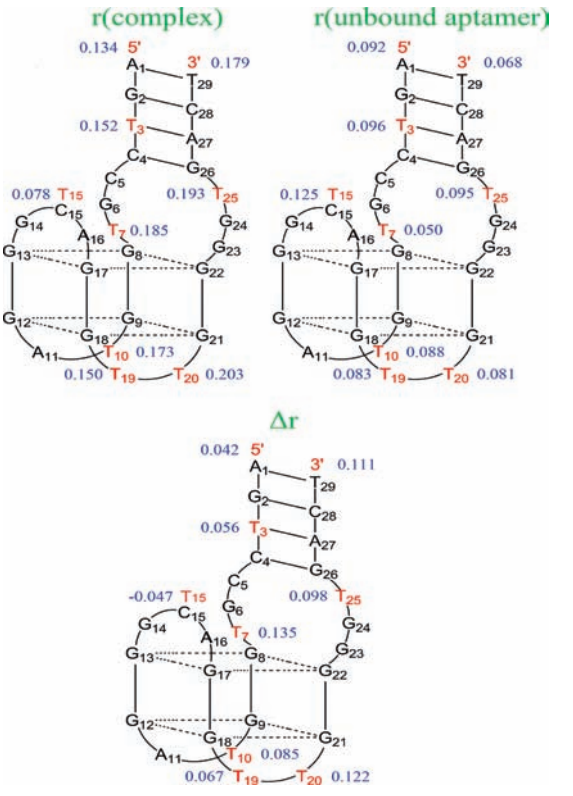

Figure 3. FA response of TMR-labeled thymidine in the aptamer−thrombin complexes (top left), the unbound aptamers (top right), and the anisotropy change after the aptamers bind to thrombin (bottom). C15 was substituted by T15 for testing the FA response of C15. Each aptamer is labeled with a single fluorescent dye at the specified site.

labeled and unlabeled aptamers. The unlabeled TA29 aptamer is believed to specifically bind to the heparin-binding exosite of human α-thrombin.[11a] If the fluorescently labeled TA29 aptamer binds on the same binding sites as the unlabeled aptamer, the labeled TA29 aptamer is expected to be displaced from the thrombin−aptamer complex by the excess unlabeled aptamer. Indeed, the competitive binding assay shows the expected behavior (SI, Figure S3), suggesting that the fluorescent

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labeling of the aptamer did not alter the binding site of the aptamer.

Both the 3′- and 5′-ends are adjacently located in the duplex stem of the thrombin-binding-induced G-quadruplex/duplex structure for TA29 and can be used as a reference for each other. As shown in Figure 3, the anisotropy of the 3′-labeled TA29 greatly increases upon binding to human α-thrombin (Δr = 0.111). However, the binding of 5′-labeled TA29 to α-thrombin just slightly increases the anisotropy (Δr = 0.042). Regarding the same molecular volume of the aptamer−protein complex (MW ≈ 47 kDa) and similar bonding chemistry (covalently bonding to phosphate group), both the binding-induced large increase in anisotropy (Δr = 0.111) and the high anisotropy value of the complex (r = 0.179) suggest that the 3′-end of TA29 aptamer is more closely contacted to the associated protein than the 5′-end.

The close contact of the 3′-end of the aptamer is further supported by the larger increase in FA values of the T25-labeled aptamer (adjacent to the 3′-end) compared to the FA values of the T3-labeled aptamer (adjacent to the 5′-end) when they bind to α-thrombin. The T25-labeled aptamer (adjacent to 3′-end) has a large increase in FA response upon the binding to α-thrombin (Δr = 0.098). In contrast, T3-labeled aptamer (adjacent to 5′-end) only has a moderate FA increase upon α-thrombin binding (Δr = 0.056). These results suggest that 3′-end of the aptamer is closer to the associated protein than the 5′-end.

A previous X-ray crystallography study of the complex of thrombin−15mer aptamer clearly showed that the corresponding site of C15 in the 15mer aptamer was distant from the heparin-binding exosite of thrombin.[8a] Since the model TA29 aptamer possesses the same core sequence as that of the 15mer antithrombin aptamer, we hypothesize that the C15 in the TA29 aptamer is a distant site for the aptamer binding to thrombin. If this hypothesis is correct, the aptamer with fluorescent labeling on the C15 site should generate a smaller FA increase when the aptamer binds to thrombin. To test this hypothesis, we measured the FA value of the modified aptamer and its thrombin complex. The C15 that cannot be commercially labeled was substituted by T15 (C15T). Indeed, the bound C15T-labeled aptamer displays the smallest FA response among all tested aptamers (r = 0.078, Figure 3, top left), supporting our hypothesis. Moreover, the substitution does not change the binding affinity (Figure 2), indicating that C15 is located in a variable region and makes less contribution to the high affinity binding. All these results coincide with previous work,[11] demonstrating the reliability of the FA method for detection of the distant sites in the aptamer−protein architecture.

T19 and T20 are adjacent to each other and are located at the same loop of a core antiparallel G-quadruplex conformation. However, T20-labeled aptamer can induce a much larger FA increase (Δr = 0.122) than T19-labeled aptamer (Δr = 0.067) upon thrombin binding (Figure 3). These results suggest a closer contact of T20 to the associated protein.

The suggested close contact of T20 is further supported by binding affinity analysis. It is evident that the labeling of T20 by TMR can cause the largest affinity decrease (1200 times), indicating the significant contribution of T20 to the high affinity binding. The affinity perturbation should be attributed to incorrect secondary conformation and/or a steric exclusion effect caused by the labeling. Because of the formation of appropriate antiparallel G-quadruplex conformation as shown by CD analysis (Figure 1) and no change in binding sites as shown by a competitive binding assay, the largest decrease in affinity of the T20-labeled TA29 aptamer may mainly arise from the steric exclusion effect. The steric exclusion effect is expected if the site is closely contacted with the bound protein. Therefore, the implicated steric exclusion effect further supports the close contact of T20 to the bound protein.

Our finding that T20 is closer than T19 to the bound thrombin is consistent with a previously published low-resolution model showing T20 is closer than T19 to the protein.[11] Previous cloning and sequencing of the SELEX-selected aptamers of thrombin showed that the position 19 (A/T) was variable but the position 20 (T) was conserved.[11] This may suggest the importance of T20 and the relatively lower contribution of T19 to the high affinity binding, further supporting our conclusion. The identified distant site of C15T is also consistent with previous X-ray crystallography and photocross-linking analysis.[11]

Similarly, judging from the high-affinity perturbation and the largest increase in anisotropy upon the α-thrombin binding (Figures 2 and 3), T7 is also identified as a close contact site for the binding of the aptamer to α-thrombin.

Taken together, T7, T20, T25, and the 3′-end were identified as the close contact sites, and C15T, T3, and the 5′-end, as three distant sites for the binding of TA29 aptamer to human α-thrombin (SI, Table S4).

Structures of aptamers may change after binding to their target proteins, as in the case of thrombin binding to its aptamer.[11] The wide range of the observed Δr values from a series of labeled nucleotides is consistent with the idea of closeness of the labeled sites and the relative change of aptamer structure at these sites after the aptamer binds to thrombin. The large positive Δr values for the thrombin complex with nucleotides labeled at sites T7, T20, T25, and the 3′-end are consistent with the interpretation of their close contact with thrombin upon binding. The negative Δr value for the thrombin complex of the nucleotide labeled at site C15T also suggests a structural change of the aptamer near this site after the aptamer binds to thrombin. The higher anisotropy value (r = 0.125) of this labeled nucleotide than that of others (r = 0.05−0.09) prior to binding may be due to a structure that restricts the local movement of the G-quadruplex at this site. Upon binding to thrombin, the structural change of the aptamer in the thrombin−aptamer complex results in a substantial increase in anisotropy values for sites T7, T20, T25, and the 3′-end (close contact region) and a decrease in anisotropy values for site C15T.

It may be hypothesized that the observed significant increases in anisotropy after the binding of our designed aptamers to the target protein could be due to the close contact and possible changes in the aptamer structure. To test this hypothesis, we have determined the relative contributions of these two possible factors to the overall changes of the anisotropy values. Our experimental data on anisotropy changes upon conformational change of the atpamers and Peyrin's work[10] indicate that the possible contribution of the structural changes to the increased anisotropy is small, much smaller than the observed anisotropy increase. We elaborate this point further as the following.

First, we have shown that the unbound aptamers when labeled at different sites have similar anisotropy values. This result is consistent with previous reports.[14]

Second, Peyrin et al. showed that structural changes of a Texas Red-labeled aptamer (an anti-L-tyrosinamide 49-mer DNA aptamer) induced by its binding to a small target molecule (L-tyrosinamide) resulted in only a minimum increase in anisotropy

values.[10] In their experiment, the highest increase of anisotropy was only $\Delta r = 0.012$. This is in contrast to the much higher increase in anisotropy ($\Delta r = 0.098-0.135$) that we have observed in our experiment. The highest increase we observed for the close contact site was $\Delta r = 0.135$. This is an order of magnitude higher than the contribution from the structural change (Peyrin's value of 0.012).

Third, we conducted a set of experiments by using thrombin aptamers that are in the G-quadruplex (in 10 mM Tris-HCl, pH 7.5, plus 150 mM KCl) and non-G-quadruplex (in 10 mM Tris-HCl, pH 7.5) conformations. As previously reported, the G-quadruplex structure is the conformation of binding to thrombin, while the non-G-quadruplex structure does not bind to thrombin.[11] We determined the fluorescence anisotropy values of both conformations of nine aptamers each labeled at different sites, and their differences were only 0.006 to −0.029 (SI, Table S5). These results further support that the contribution from the structural changes to the overall anisotropy values is negligible.

Therefore, between the two factors (the close contact and the structural change), the main contribution to the significant increase of Δr is the close contact. The contribution from the possible structural change is much smaller.

In summary, we for the first time have probed the aptamer−protein interaction at single-nucleotide resolution. The combined CE mobility shift and FA approach eliminate any cross-linking reactions and can be used to map the close contacts of multiple nucleotides in the whole aptamer to the associated protein. In addition, multiple distant sites can also be identified. Because of the importance of the multiple close contacts in macromolecular interactions, this technology is potentially useful for artificial evolution, design, and modification of functional aptamers for a range of bioanalytical, diagnostic, and therapeutic applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Experimental details and supporting materials. This material is available free of charge via the Internet at http://pubs.acs.org

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