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Structure-Switching Signaling Aptamers

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Abstract: Aptamers are single-stranded nucleic acids with defined tertiary structures for selective binding to target molecules. Aptamers are also able to bind a complementary DNA sequence to form a duplex structure. In this report, we describe a strategy for designing aptamer-based fluorescent reporters that function by switching structures from DNA/DNA duplex to DNA/target complex. The duplex is formed between a fluorophore-labeled DNA aptamer and a small oligonucleotide modified with a quenching moiety (denoted QDNA). When the target is absent, the aptamer binds to QDNA, bringing the fluorophore and the quencher into close proximity for maximum fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex. The switch of the binding partners for the aptamer occurs in conjunction with the generation of a strong fluorescence signal owing to the dissociation of QDNA. Herein, we report on the preparation of several structure-switching reporters from two existing DNA aptamers. Our design strategy is easy to generalize for any aptamer without prior knowledge of its secondary or tertiary structure, and should be suited for the development of aptamer-based reporters for real-time sensing applications.

Introduction

Aptamers are single-stranded nucleic acids isolated from random-sequence nucleic acid libraries by "in vitro selection".[1,2] To date, numerous aptamers have been created for a broad range of targets, including metal ions, small organic compounds, metabolites, and proteins.[3,4] The tight-binding capabilities of both DNA and RNA aptamers have been demonstrated in numerous cases including a 2′-aminopyrimidine-containing RNA aptamer for vascular permeability factor/vascular endothelial growth factor with a K_d of 0.14 nM,[5] a 2′-fluoro-modified RNA aptamer for the human keratinocyte growth factor with K_d of 0.3 pM,[6] and a DNA aptamer for platelet-derived growth factor-AB with subnanomolar affinity.[7] Aptamers can also be made to possess a high binding specificity, exemplified by an anti-theophyllin RNA aptamer[8] that displays a >10 000-fold discrimination against caffeine (which differs theophyllin by a methyl group) and an anti-L-arginine RNA aptamer that exhibits a 12 000-fold affinity reduction toward D-arginine.[9] The target versatility, the high binding affinity and specificity, along with the simplicity of in vitro selection, make aptamers attractive as molecular tools for bioanalytical applications. In such cases, it is advantageous if aptamers are able to report on target presence by real-time fluorescence signaling without a need for complex separation steps.

Standard DNA and RNA molecules do not contain intrinsically fluorescent groups. To make aptamers fluoresce, it is necessary to modify aptamers with extrinsic fluorophores. Considerable research activities aiming at designing real-time signaling aptamers have been reported recently. One strategy is to covalently attach a fluorophore at a location of an aptamer that will undergo a target-induced conformational change.[10,11] Such reporters can be created either by rational design if tertiary structure information is available[10] or by in vitro selection using a fluorophore-labeled library.[11] A critical assumption in this approach is that the conformational change might substantially alter the electronic environment of the attached fluorophore to cause a significant change in its fluorescence property. Because of the difficulty in precisely predicting (1) whether the attachment site will undergo a significant conformational change upon target binding and (2) whether such a change could indeed alter the fluorescence property of the attached fluorophore, many

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rationally designed constructs[10] or selected aptamers[11] may have to be tested before a desirable signaling aptamer can be obtained. Therefore, this strategy is not easy to generalize. Furthermore, the known signaling aptamers made by this approach usually exhibit fairly small fluorescence enhancements upon target binding (typically below 2-fold at saturating target concentrations[10,11]) and consequently, their detection sensitivity is relatively low.

Other studies have focused on designing molecular beacon-based signaling aptamers (denoted "aptamer beacons")[12−16] through the adaptation of the molecular beacon concept originally designed for the detection of nucleic acid targets by nucleic acid hybridization.[17] Yamamoto et al. reported the first aptamer beacon designed from an RNA aptamer that interacts with the Tat protein of HIV.[13] These researchers split the aptamer into two molecules, one of which was formulated into a hairpin-shaped beacon molecule (after the addition of a few nucleotides to tie the two ends of the RNA into a hairpin structure and the attachment of a fluorophore at one end of the RNA and a quencher at the other end). In the absence of Tat, the two RNA molecules exist independently; the beacon half of the aptamer adopts the hairpin structure, emitting a low level of fluorescence. When Tat is introduced, the beacon changes its structure in order to engage the other half of the aptamer for binding to Tat; the disruption of the hairpin structure causes physical separation of the fluorophore-quencher pair, resulting in a fluorescence enhancement. The successful design of the above signaling aptamer is achieved because the original RNA aptamer has a unique secondary structure that contains a long stretch of paired nucleotides to permit the splitting of the aptamer into two molecules. Therefore, it can be difficult to use the same strategy for other aptamers that lack such a secondary structure feature. An alternative molecular beacon strategy has been reported by Hamaguchi et al.[12] in which they place an intact aptamer as the loop segment of a molecular beacon. However, this strategy is difficult to generalize as well, particularly for large aptamers and the aptamers in which the two ends of the aptamer sequence do not move away from each other after target binding (e.g., the anti-ATP DNA aptamer[18,19]). Moreover, tying the two ends of an aptamer into a hairpin structure could significantly alter the correct tertiary folding of the aptamer and consequently, such a modified aptamer may lose its binding ability. For example, only one of the three anti-thrombin aptamer beacons designed by Hamaguchi et al.[12] based on a known anti-thrombin DNA aptamer[20] was able to retain the thrombin-binding ability, whereas the other two failed to interact with thrombin completely.[12] Considering that aptamers have variable sizes and different kinds of secondary structures and that many aptamers may not have an easily determined secondary structure, there is an obvious need to establish a signaling-aptamer designing strategy that is easy to generalize and has little restrictions on the size and secondary structure of aptamers.

Herein, we describe a simple and general approach for preparing solution-based signaling aptamers that function by a coupled structure-switching/fluorescence-dequenching mechanism. Our strategy exploits the unique ability of each DNA aptamer to adopt two distinct structures—a DNA duplex with a complementary DNA sequence, and a tertiary complex with the target for which the aptamer is created. Our signaling aptamers take advantage of target-induced switching between a DNA/DNA duplex and a DNA/target complex. Generation of a signal upon formation of the DNA/target complex is obtained by using a fluorophore-labeled DNA aptamer and a small complementary oligonucleotide that is covalently modified with a quencher (denoted QDNA). In the absence of the target, the aptamer naturally binds to the QDNA, bringing the fluorophore and the quencher into close proximity for highly efficient fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex rather than the aptamer-QDNA duplex, triggering the release of the QDNA from the fluorophore-labeled aptamer. The dissociation of the QDNA is accompanied by the increase of fluorescence intensity because of fluorescence dequenching. On the basis of this strategy, we have successfully engineered several fluorescent reporters from two existing DNA aptamers, one that is specific for ATP and the other that binds thrombin.

Experimental Section

DNA Oligonucleotides and Chemical Reagents. Standard and modified DNA oligonucleotides were all prepared by automated DNA synthesis using cyanoethylphosphoramidite chemistry (Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University). 5′-Fluorescein and 3′-DABCYL (4-(4-dimethylaminophenylazo)benzoic acid) moieties were introduced using 5′-fluorescein phosphoramidite and 3′-DABCYL-derivatized controlled pore glass (CPG) (Glen Research, Sterling, Virginia) and were purified by reverse phase HPLC. HPLC separation was performed on a Beckman-Coulter HPLC System Gold with a 168 Diode Array detector. The HPLC column was an Agilent Zorbax ODS C18 Column, with dimensions of 4.5 × 250 mm and a 5 μm bead diameter. A two-solvent system was used for the purification of all DNA species, with solvent A being 0.1 M triethylammonium acetate (TEAA, pH 6.5) and solvent B being 100% acetonitrile. The best separation results were achieved by a nonlinear elution gradient (10% B for 10 min, 10%B to 40%B over 65 min) at a flow rate of 0.5 mL/min. The main peak was found to have very strong absorption at both 260 and 491 nm. The DNA within 2/3 of the peak-width was collected and dried under vacuum. Unmodified DNA oligonucleotides were purified by 10% preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE), followed by elution and ethanol precipitation. Purified oligonucleotides were dissolved in water and their concentrations were determined spectroscopically. Human factor Xα and human factor IXα were purchased from Haematologic Technologies (Essex Jct., VT). Human thrombin, bovine serum albumin (BSA), adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), deoxyadenosine 5′-triphosphate (dATP), uridine 5′-triphosphate (UTP), guanosine 5′-triphosphate (GTP), cytosine 5′-triphosphate (CTP),

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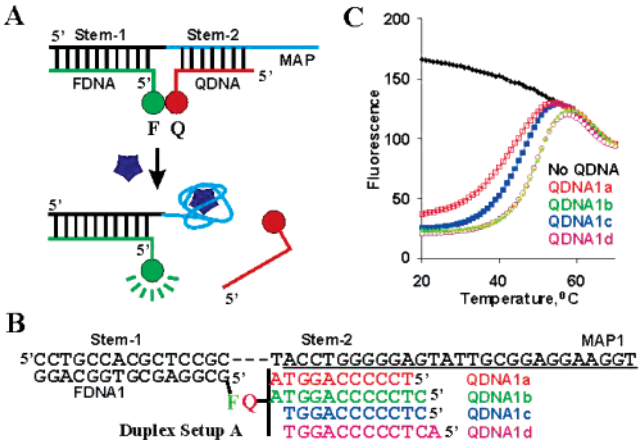


Figure 1. Design of structure-switching signaling aptamers. (A) Working principle. A tripartite, fluorescence-quenching, two-stem duplex assembly can be constructed using a fluorophore (F)-containing FDNA (in green), a quencher (Q)-containing QDNA (in red) and an unmodified DNA molecule (MAP) that contains an FDNA-binding sequence (in black) and a target-binding motif (in light blue). When the target (blue star) is introduced, the duplex structure is transformed into the target-aptamer complex with a concomitant release of QDNA and an enhancement of fluorescence intensity. (B) An ATP-binding aptamer as a model system. The original 27-nt aptamer (underlined) is appended with a sequence at the 5′-end for the formation of the 15-bp stem-1 with FDNA1. Four QDNAs, each containing a DABCYL at the 3′-end, were used to establish a suitable stem-2. (C) Thermal denaturation profiles of DNA solutions containing FDNA1 and MAP1 (filled black diamonds) as well as FDNA1 and MAP1 with one of the following QDNAs: QDNA1a (red open squares), QDNA1b (filled green triangles), QDNA1c (filled blue squares), and QDNA1d (open purple circles). The experiments were carried out as described in the experimental protocol section.

were purchased from Sigma and their solution concentrations were determined by standard spectroscopic methods.

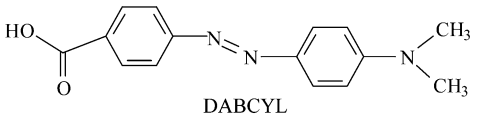
Fluorescence Measurements. The following concentrations of oligonucleotides were used for fluorescence measurements (if not otherwise specified): 40 nM for FDNA1, 80 nM for the aptamers (MAPs) and 120 nM for the quenchers (QDNAs). The ratio of FDNA:MAP:QDNA was set to be 1:2:3 to ensure a low background signal. Under this setting, the vast majority of FDNA molecules would form a duplex structure with MAP and the resulting FDNA-MAP duplexes would also be able to engage a QDNA molecule for fluorescence quenching. DNA solutions also contained 300 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tris·HCl (pH 8.3) for the ATP reporters, and 5 mM KCl, 1 mM $MgCl_2$ and 20 mM Tris·HCl (pH 8.3) for the thrombin reporter. The fluorescence intensities were recorded on a Cary Eclipse Fluorescence Spectrophotometer (Varian) with excitation at 490 nm and emission at 520 nm. To obtain the thermal denaturation profile of a particular reaction mixture, the DNA solution was heated to 90 °C for 5 min, and the temperature was then decreased from 90 °C to 20 °C at a rate of 1 °C/min. The fluorescence intensity was recorded automatically for every 1 °C drop in temperature. Measurements of fluorescence intensities from specific samples are detailed in each figure legend.

Results

Signaling Aptamer for ATP Sensing. A scheme demonstrating the concept of the duplex-to-complex structural transition coupled with fluorescence dequenching is shown in Figure 1A. Three synthetic DNA oligonucleotides are used: the first one is modified with a fluorophore at the 5′-end (denoted FDNA), the second one is labeled with a quencher at the 3′-end (denoted QDNA), and the third DNA molecule (denoted MAP) consists of an aptamer domain and an FDNA-binding motif. The QDNA is complementary to the aptamer in the sequence segment near the FDNA-binding motif. In the absence of the target, the three DNA molecules are expected to assemble into the tripartite duplex structure in which the fluorophore and the quencher are situated in close proximity, leading to efficient fluorescence quenching. Because the aptamer domain has a propensity to form the aptamer-target complex, the introduction of the target into the DNA mixture should cause the aptamer to release the QDNA in favor of the target, producing a large increase of fluorescence intensity.

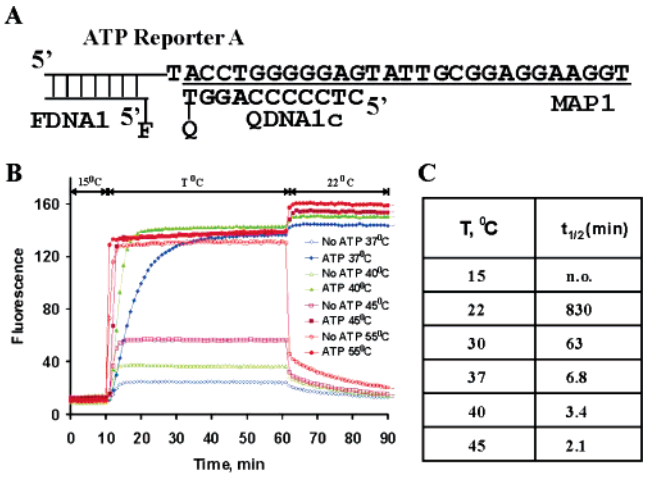
Figure 1B illustrates an ATP-binding DNA aptamer as a test case. The original DNA aptamer was created by Huizenga and Szostak through in vitro selection[18] and it was found from an NMR study that the aptamer forms a tertiary complex with two ATP molecules.[19] We added an arbitrarily chosen 15-nt GC-rich sequence onto the 5′-end of the aptamer for FDNA1 binding. FDNA1 (modified with 5′-fluorescein) and its complementary sequence were found to form a DNA duplex with a measured melting point of 68 °C (in 0.5M NaCl, data not shown). Therefore, stem-1, which is formed between FDNA1 and MAP1, is sufficiently stable in the temperature range used for aptamer binding. A single nucleotide, T16 of MAP1, was introduced to separate the FDNA1 binding domain and the aptamer domain to minimize the potential steric interference between the two domains in the folded tertiary structure.

Several 3′-DABCYL-modified oligonucleotides (QDNA1a to QDNA1d) were tested as quenchers [DABCYL: (4-(4-dimethylaminophenylazo)benzoic acid; structure shown below), and their ability to form stem-2 with MAP1 was judged by the thermal denaturation profiles shown in Figure 1C. QDNA1b (filled green triangles) and QDNA1d (open purple circles) were the two most effective quenchers in the group and had apparently equal quenching efficiency. This observation is not a surprise considering that the 12-bp stem-2 formed by both QDNAs has the same base composition. The two 11-nt QDNAs, however, exhibited different quenching efficiencies with QDNA1c being more effective than QDNA1a. This is likely due to the increased GC content of QDNA1c as it contains 8 GCs (7 GCs in QDNA1a). We chose to use QDNA1c to test the ATP induced structure switching because QDNA1c forms a stem-2 that is almost as stable as those formed by the two 12-nt QDNAs at low temperatures (20−30 °C). However, because the QDNA1c has a less stable stem-2 (whose melting point is ∼3 °C lower than those by the two 12-nt QDNAs), it should dissociate more easily from MAP1 in the presence of ATP and thus be a more sensitive reporter.



DABCYL

Evidence for Structure Switching. FDNA1-QDNA1c-MAP1 tripartite system is denoted as ATP Reporter A (Figure 2A). We used a series of temperature-changing fluorescence assays to obtain evidence for the proposed structure switching process (Figure 2B). In each experiment, the preannealed ATP Reporter A was incubated at 15 °C for 10 min, followed by a rapid temperature increase (within 1 min) from 15 °C to a designated temperature (37, 40, 45, or 55 °C), followed by a 50-minute incubation at each elevated temperature. Finally, the solution was rapidly cooled (within 1 min) to 22 °C and incubated at this temperature for 30 min.



T, °C	$t_{1/2}$(min)
15	n.o.
22	830
30	63
37	6.8
40	3.4
45	2.1

Figure 2. Evidence for structure switching. (A) DNA molecules used to assemble ATP Reporter A. (B) Examination of ATP Reporter A for structural transitions using temperature-changing experiments. Unfilled data points were for samples containing no ATP and filled data points were for ATP-containing solutions. Temperature settings are indicated on the top of the graph and in the embedded legends. The fluorescence intensity was measured every minute when the temperature of each solution was changed as follows: a 10-min incubation at 15 °C, followed by a temperature increase to 37, 40, 45, or 55 °C within 1 min and further incubation at the raised temperature for 50 min. Finally, each sample was cooled to 22 °C in 1 min and incubated at 22 °C for 30 more min. For the ATP-containing samples, 1 mM ATP was added to the preformed duplex DNA mixture stored at 4 °C and the resulting mixture was immediately examined. Other conditions are given in the experimental protocol section. (C) A quantitative description of temperature dependence of ATP reporter E. $t_{1/2}$ is the time required for the ATP reporter to reach the half-maximal fluorescence intensity at a given temperature.

In the absence of ATP, the reporter had a low and stable fluorescence intensity at 15 °C. When the temperature was raised from 15 °C to 37, 40, 45, or 55 °C, the intensity of the solution increased in a manner that was indicative of heat denaturation of the DNA duplex assembly. A higher incubation temperature resulted in a higher fluorescence intensity because less and less QDNA1c remained as part of a duplex assembly. At each temperature, a stable fluorescence intensity value was reestablished after a few minutes, indicating that the equilibrium between the amount of free QDNA1c and the amount of the QDNA1c bound in the DNA duplex assembly was reached. When the solution temperature was lowered to 22 °C, the fluorescence intensity dropped owing to the reassociation of some free QDNA1c molecules into the DNA duplex structure.

The introduction of 1 mM ATP into the DNA mixture (filled data points) did not cause a rapid increase in fluorescence intensity at 15 °C and 22 °C (i.e., room temperature; data not shown). However, when the temperature was raised from 15 °C to 37, 40, 45, or 55 °C, rapid intensity increases were observed. We used $t_{1/2}$ (the time required for the DNA solution to reach the half-maximal fluorescence intensity after the addition of 1 mM ATP at a designated temperature) to provide a quantitative measurement of the temperature dependence of the ATP-promoted intensity increase (Figure 2C). The $t_{1/2}$ at 22 °C was very large at 830 min; at 37 °C, $t_{1/2}$ was shortened to 6.8 min; when the temperature rose to 45 °C, the half-maximal intensity was reached in about 2 minutes. At temperature points other than 55 °C, the presence of ATP caused a marked difference in the increase of fluorescence intensity. The contrast between the intensity changes of the ATP-containing and ATP-lacking solutions was even sharper when the temperature was lowered from each of the higher temperature points to 22 °C, whereas the ATP-lacking solutions experienced a very significant decrease in fluorescence intensity, all the ATP-containing samples (including the one treated at 55 °C) registered a noticeable intensity gain.

The above observations are consistent with the structure switching mechanism shown in Figure 1A. Rapid structure switching did not occur at low temperatures (such as 15 °C) because most of the MAP1 molecules existed in the duplex form where the ATP binding site was partially occupied by QDNA1c. A rapid structural transition happened at the elevated temperatures because more QDNA1c molecules were forced to dissociate from the duplex assembly, and as a result, more free MAP molecules had their ATP-binding site freed for ATP binding. When the solution was cooled, whereas QDNA1c molecules naturally reannealed back onto the aptamer sequence in the absence of ATP, the formation of the ATP-aptamer complex in the ATP-containing solution prevented the reannealing.

The ATP-aptamer complex appeared to be very stable despite the presence of QDNA1c. This is evident from the observation that the fluorescence intensity stayed unchanged upon continuous incubation at 22 °C (from 62 to 90 min, Figure 2B). We also examined the fluorescence intensity of each solution after longer incubation times (up to 100 hours) and found virtually no reduction in fluorescence intensity (data not shown).

ATP Reporter A was then examined for sensing specificity (Supporting Figure 1). Although 1 mM ATP was able to produce ~90% of the maximum fluorescence signaling capability (as compared to the solution where the QDNA1c was omitted), CTP, UTP, or GTP at 1 mM were not able to induce significant intensity increases. The original ATP aptamer was known to bind dATP as well,[18] and indeed we found that the ATP reporter was able to bind to dATP (Supporting Figure 1). Furthermore, double mutations within the ATP binding site of MAP1 (mutant M1 and mutant M2) abolished the ATP-binding capability (Supporting Figure 2). All of these observations are consistent with the specific ligand-dependent structural transition mechanism depicted in Figure 1A.

Diverse Duplex Design. Our engineering strategy can be easily expanded to include a variety of modification choices and Figure 3A lists three more duplex configurations. ATP Reporters B and C are both bipartite systems involving the use of a fluorescein-dT (T1 and T15, respectively) as the fluorophore and a separate QDNA as the quencher. ATP Reporter D is another tripartite system where FDNA and QDNA were designed to bind two adjacent stretches of the unmodified DNA aptamer. The relevant QDNA and FDNA molecules were chosen for each configuration following the examination of thermal denaturation profiles of several constructs for each system (data not shown). All these four ATP reporters were tested for signaling capability and specificity (Figure 3B). Without exception, each of them was able to report specifically on the presence of ATP without false signaling for GTP (as well as CTP and UTP, data not shown). The ability to design alternative structure-switching configurations should help expand the usefulness of our strategy to allow the preparation of optimized signaling species for different aptamers.

Reporters for Real-time Sensing at Low Temperature. It is apparent that ATP Reporter A can only be used to perform real-time detection at elevated temperatures such as 40 °C or

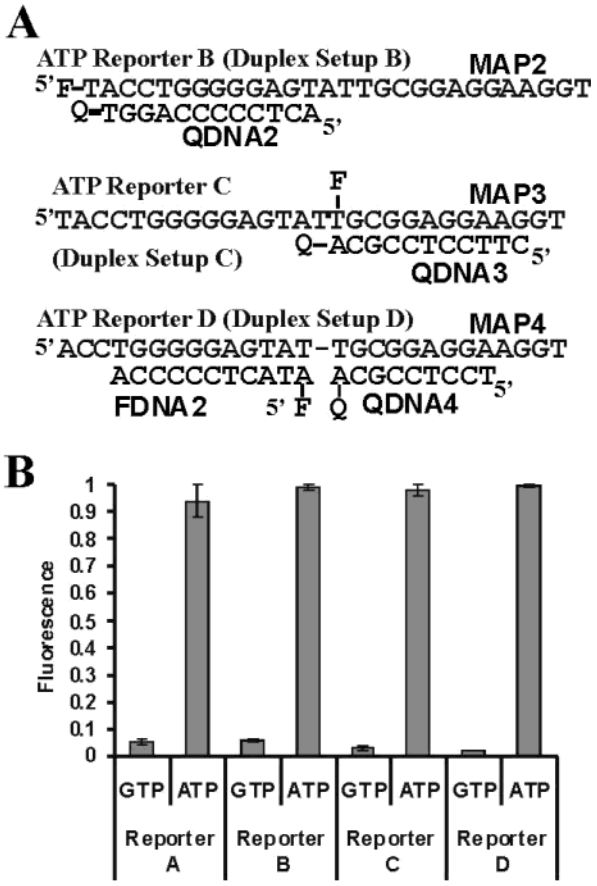


Figure 3. Different duplex configurations. (A) ATP Reporter B is a bipartite system consisting of the 5′-fluorescein labeled aptamer MAP2 and the 12-nt QDNA2. ATP Reporter C is another bipartite system made of aptamer MAP3 (internally labeled with fluorescein on T16) and the 11-nt QDNA3. ATP Reporter C is a tripartite system with the unmodified aptamer (MAP4), the 11-nt FDNA2 and 9-nt QDNA3. (B) Each reporter was examined without a target as well as in the presence of either ATP or GTP (in triplicate). The experiments were carried out as follows: A stock solution of a relevant MAP, QDNA and FDNA was combined with water, ATP or GTP and the resulting mixture was first incubated at 40 °C for 10 min, followed by incubation at 22 °C for 30 min before its fluorescence was measured. The final DNA concentrations were as follows: for ATP Reporter A, FDNA1 at 40 nM, MAP1 at 80 nM, QDNA1c at 120 nM; for ATP Reporter B, MAP2 at 40 nM, QDNA2 at 80 nM; for ATP Reporter C, MAP3 at 40 nM, QDNA3 at 80 nM; for ATP Reporter D, FDNA2 at 40 nM, MAP4 at 80 nM, QDNA4 at 120 nM. The final ATP or GTP concentration was 1 mM. The fluorescence intensities were normalized using the following equation: $(F - F_0)/(F_{max} - F_0)$, where F is the fluorescence intensity of each sample, F_0 and F_{max} are for the samples with the lowest and highest fluorescence intensities, respectively.

above but not at lower temperatures (Figure 2B). A system that is only able to perform real-time detection at elevated temperatures has two key drawbacks for practical sensing applications. First, our tripartite or bipartite structure-switching reporters have increased background intensity at higher temperatures, producing a smaller signaling magnitude upon the binding of target. Second, because most aptamers are created at room temperature, their binding affinities are usually at their best near this temperature, and therefore, the detection at higher temperatures may significantly reduce the sensitivity of the aptamer reporters. Thus, it is very desirable to engineer aptamer reporters that are capable of real-time sensing at room temperature.

We reasoned that the inability of ATP Reporter A to perform low-temperature real-time sensing was caused by the occupancy of a long stretch of aptamer sequence by QDNA, because the

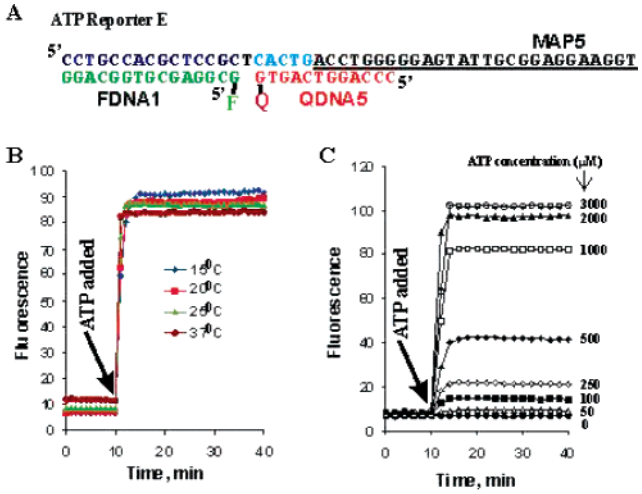


Figure 4. ATP reporter with real-time sensing capability at low temperature. (A) The DNA sequences used for the construction of ATP Reporter E. The modified aptamer contained the FDNA-binding domain (in dark blue), the original aptamer sequence (in black), and an inserted 5-nt domain (in medium blue) as part of the QDNA-binding domain. (B) Real-time sensing results. ATP reporter E was incubated in the absence of ATP for 10 min at an indicated temperature (15, 20, 25, or 37 °C), followed by the addition of ATP to 1 mM and a further incubation at the same temperature for 30 more min. (C) The real-time sensing capability of ATP Reporter E was examined as a function of ATP concentration at 20 °C. ATP was used at 0, 50, 100, 250, 500, 1000, 2000, and 3000 μM. The experiments were carried out the same way as in (B).

reporter was constructed with an 11-nt QDNA1c that was designed to block the first 11 nucleotides of the original 27-nt aptamer sequence. We hypothesized that if we could reduce the number of the blocked nucleotides in the aptamer sequence from 11 to a smaller number (such as 6 or 7), we might then be able to produce a low-temperature reporter. Because the reduction in the number of blocked nucleotides in the aptamer cannot be achieved simply by using a smaller QDNA (because this would produce a less stable duplex structure and a larger background signal), we decided to introduce additional nucleotides between the aptamer sequence and the FDNA-binding motif. Thus, we redesigned ATP Reporter A into the new tripartite system shown in Figure 4A (denoted ATP Reporter E) by inserting an arbitrary 5-nt sequence, CACGT, between the FDNA-binding domain and the aptamer motif. A 12-nt QDNA5 was used as the new quencher (it forms base pairs with both the five inserted nucleotides and the first seven nucleotides in the aptamer sequence).

ATP Reporter E was tested for real-time signaling at 15, 20, 25, and 37 °C and the data are shown in Figure 4B (the signaling DNA mixture was incubated at a designated temperature in the absence of ATP for 10 min, followed by the addition of 1 mM ATP and further incubation for 30 more min). ATP Reporter E was found to switch very quickly at all tested temperatures including 15 °C (the $t_{1/2}$ for ATP Reporter E at all these temperatures was all less than 1 min). In contrast, the ATP-promoted intensity increase of ATP Reporter A at 15 °C was too slow to determine a $t_{1/2}$ (Figure 2C). These data indicated that ATP Reporter E indeed had a much improved low-temperature real-time sensing capability.

ATP Reporter E also displayed a very large signaling magnitude (i.e., S/B, defined as the fluorescence intensity in the presence of ATP over that in the absence of any target) and the S/B values were found to be 14.1, 13.0, 10.4, and 7.1 at 15,

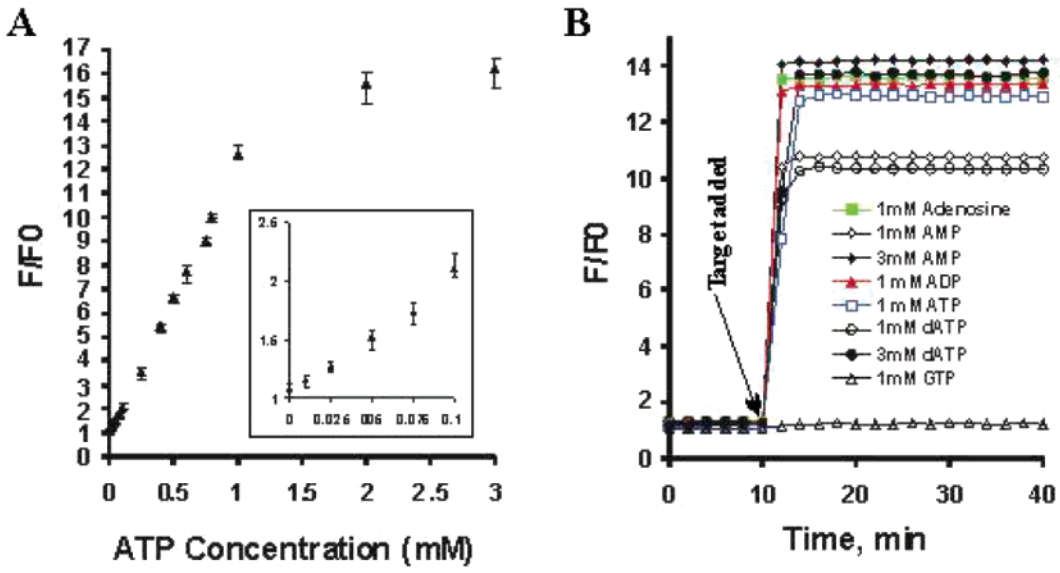


Figure 5. Target detection range and sensing specificity of ATP Reporter E. (A) The fluorescence intensity of ATP Reporter E was recorded in real-time at 20 °C with different concentrations of ATP (in triplicate) using the same experimental setting as in Figure 4B. The data obtained at the 20th minute were normalized using the following equation: F/F_0, where F is the fluorescence intensity of each sample, F_0 is for the sample with the lowest fluorescence intensity. ATP concentrations were 0, 10, 25, 50, 75, 100, 250, 400, 500, 600, 750, 800, 1000, 2000, and 3000 μM. Each data point represents the average value of three independent experiments with error bars indicated. (B) ATP Reporter E was examined for real-time sensing behavior in the presence of GTP (1 mM), ATP (1 mM), ADP (1 mM), AMP (1 mM and 3 mM), adenosine (1 mM) or dATP (1 mM and 3 mM). The data were normalized using the same equation as in (A), F/F_0, where F is the fluorescence intensity of each sample and F_0 is the initial reading for the reporter in the absence of any target.

20, 25, and 37 °C, respectively, upon the addition of 1 mM ATP. ATP Reporter E was also examined for intensity response to the increase of ATP concentration at 20 °C in real time (Figure 4C). Its signaling intensity increased linearly as the ATP concentration was raised between 0.01 and 1 mM (Figure 5A). This ATP reporter was also tested for target specificity (Figure 5B). Although the reporter registered a large signaling magnitude in the presence of 1 mM ATP, ADP, and adenosine, the addition of 1 mM CTP (data not shown), 1 mM UTP (data not shown) or GTP was not able to induce a change in the fluorescence signal. We found that 1 mM dATP and 1 mM AMP induced a smaller but still substantial fluorescence intensity increase (10-fold vs 13-fold for 1 mM ATP). We confirmed that the intensity reduction was not caused by the inaccuracy of target concentrations (the concentration of each target was carefully determined by the standard spectroscopic methods). We further found that the reduced signaling magnitude seen with 1 mM dATP and AMP was due to a shift in the saturating target concentration because the maximum fluorescence enhancement was achieved when 3 mM dATP or 3 mM AMP was used (Figure 4C). This later experiment suggests that the affinity of ATP Reporter E for AMP (and dATP) is noticeably lower than that for ATP, ADP, and adenosine. The above target specificity pattern is in good agreement with that observed for the original aptamer.[18] The observation of the reduced affinity for AMP by ATP Reporter E (in comparison to its affinity for ATP, ADP, and adenosine) is quite intriguing considering that these compounds have identical chemical structures except the 5′-phosphate groups.[21]

Signaling Complex for Protein Detection. To demonstrate the general applicability of the above design strategy, we engineered a new reporter by using a DNA aptamer previously isolated for thrombin binding.[20] We used a modified aptamer sequence, MAP6 (Figure 6A) that contained the same FDNA1-binding domain so that FDNA1 could again be used as the source of fluorophore. Seven nucleotides were inserted between the FDNA-binding domain and the aptamer sequence and the 12-nt QDNA6 was used as the quencher.

Evidence supportive of structure switching was also obtained using temperature-variation experiments similar to the ones shown in Figure 2B (data not shown). The real-time signaling ability of the thrombin reporter was assessed and the data are shown in Figure 6B. Rapid signal generation was observed upon the addition of thrombin at 30 °C ($t_{1/2}$ = 1.4 min) and 37 °C ($t_{1/2}$ = 1.2 min). The reporter also exhibited a fairly rapid change in signal at 25 °C ($t_{1/2}$ = 4.6 min). However, $t_{1/2}$ increasingly lengthened when the detection temperature was decreased further. For example, at room temperature (22 °C), the $t_{1/2}$ increased to 9.5 min. The need for a higher temperature for real-time sensing of thrombin (as compared to the ATP sensing with ATP Reporter E, which was capable of rapid real-time sensing even at 15 °C; see Figure 4C) might be caused by one of the following two factors (or combination of both): (1) the increased percentage of the blocked nucleotides for the thrombin reporter as 6 of the 15 nucleotides (40%) in the thrombin-binding aptamer domain were blocked by QDNA6 while only 7 of the 27 nucleotides (~26%) in the ATP-binding aptamer domain were occupied by QDNA5, and (2) the nonoptimal metal ion concentrations. The later factor might be important for the thrombin aptamer because it has a guanine-quartet based tertiary structure that is known to be sensitive to both metal ion identities and metal ion concentrations. A previous study has shown that although K^+ promotes the formation of stable aptamer-thrombin complex, other metal ions such as Mg^{2+} and Ca^{2+} do not support

(21) One possible explanation that we would like to offer is that the electronic environment of the 5′-end of adenosine and its phosphate analogues affects the binding affinity. More specifically, whereas the anti-ATP aptamer can tolerate one negative charge on the alpha-phosphate group of ATP and ADP, the two negative charges on the monophosphate group of AMP interferes the aptamer-target binding. Although interesting, this point is not pursued further as the focus of the current investigation is directed at the designs of structure-switching signaling aptamer.

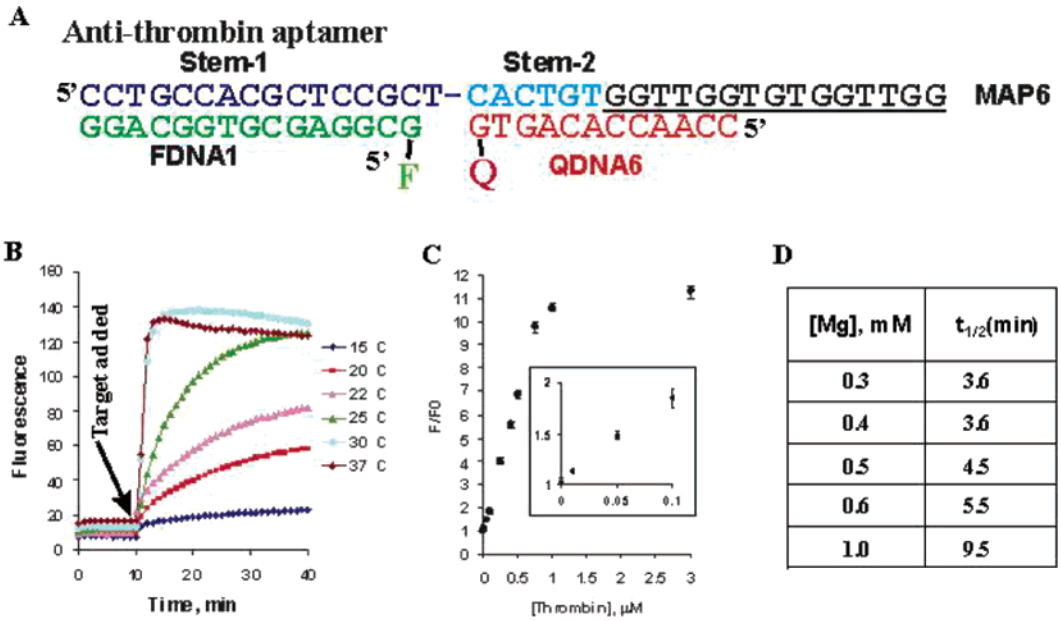


Figure 6. Structure-switching reporter for thrombin. (A) A previously isolated DNA aptamer for thrombin binding was configured into the tripartite signaling system using the similar design strategy employed for ATP Reporter E (see Figure 4A; the original thrombin-binding aptamer sequence is underlined). (B) Real-time sensing capability of the thrombin reporter was examined in a similar way to that shown in Figure 4A except that thrombin (1 μM) was used as the target and 5 mM KCl and 1 mM $MgCl_2$ as the metal ions. (C) The fluorescence intensity of the thrombin reporter was recorded in real time at 30 °C with different concentrations of thrombin (in triplicate) using the same experimental setting as in Figure 5A. The data point at the 20th minute for each sample was normalized using the following equation of F/F_0, where F is the fluorescence intensity of each sample, F_0 is for the sample with the lowest fluorescence intensity. The thrombin concentrations were 0, 10, 50, 100, 250, 400, 500, 750, 1000, and 3000 nM. Each data point represents the average value of three independent experiments with error bars indicated. (D) Influence on $t_{1/2}$ by magnesium concentration. $t_{1/2}$ is the time required for the thrombin reporter to reach the half-maximal fluorescence intensity at a given magnesium concentration at 22 °C.

the complex formation.[22] Our initial assaying mixture contained 1 mM $MgCl_2$ and 5 mM KCl and this condition was similar to that used in previous studies.[12,20] To determine whether the concentrations of potassium and magnesium ions might affect the real-time reporting capability of our thrombin reporter, we performed a series of real-time sensing measurements under different concentrations of KCl and $MgCl_2$. Although changing potassium concentration between 1 and 5 mM did not significantly affect the real-time sensing ability of the reporter (data not shown), lowering magnesium concentration enhanced the reporter's real-time detection capability at room temperature considerably (Figure 6D).

The signaling intensity of the thrombin reporter had a linear response to thrombin concentration over the range of 10−1000 nM (Figure 6C) and the maximum fluorescence enhancement reached nearly 12-fold. Once again, the target reporting was found to be very specific as other proteins including bovine serum albumin (BSA), and human factors Xa and IXa were not be able to generate fluorescence signals that were significantly above background (Supporting Figure 3). The successful engineering of two DNA aptamer reporters based on the same principle suggests that our engineering strategy indeed is general and can be easily adapted for the conversion of any DNA aptamer into a fluorescence reporter.

Discussion

Base-pairing is fundamental to nucleic acids and appropriate exploitation of this interaction (such as sense-antisense binding) has become a unique way to manipulate nucleic acid structure and function. Several recent reports have utilized antisense oligonucleotide binding to regulate nucleic acid functions such as inactivation of aptamer activity in vivo,[23] modulation of ribozyme and allosteric ribozyme activities in vitro.[24,25] In this study, we have shown that antisense oligonucleotide binding can also be explored as a general strategy to design fluorescent aptamers as real-time reporters to detect small molecules and proteins. We have demonstrated that such reporters can be easily engineered from existing DNA aptamers using a simple structure-switching/fluorescence-dequenching mechanism. Because our strategy exploits the common dual-structure-forming capability associated with each DNA aptamer, the methodology should be easy to generalize.

Two important factors need to be considered in the establishment of a generalizable method for converting aptamers into fluorescence reporters. First, the method should be broadly applicable for aptamers with different binding affinities. It is well documented that aptamers have typical affinities (K_d) in the nM range (or lower) for protein targets and in the μM range (or lower) for small-molecule targets. Therefore, we have chosen two representative aptamers for our study: the anti-ATP aptamer with a K_d of ~10 μM[18] and the anti-thrombin aptamer with a K_d of ~200 nM.[20] The successful generation of effective reporters using the same engineering principle but two different aptamers with substantial affinity difference indicates that our strategy is indeed generalizable for aptamers with a broad range of binding affinities. Second, the method should work well for

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aptamers of different sizes (typically ranging from a dozen nucleotides to several dozen nucleotides). Our design strategy, which is particularly flexible with variable choices of duplex configurations, is well suited to deal with both large and small aptamers. Our designing strategy does not rely on the availability of detailed tertiary or even secondary structure information. For example, the only prerequisite for constructing a reporter according to Duplex Setup A (or B) is the simple truncation of nonessential nucleotides from the 5′-end of the relevant original aptamer sequence so that the QDNA molecule can be designed to block the first few essential nucleotides. Therefore, our approach is very easy to generalize.

The use of QDNA to partially block the binding site of an aptamer was expected to reduce the affinity of an aptamer for its target. This is because the aptamer-target complex is not directly formed between the target and the free target-binding site of the aptamer but rather between the target and the aptamer that is partially occupied by QDNA. We found that ATP Reporter E had an apparent K_d (taken as the target concentration that induced half-maximal fluorescence intensity change) of ~600 μM for ATP (estimated with the data shown in Figure 5A), which is about 60-fold higher than that reported for the original ATP aptamer.[18] In contrast, the thrombin reporter had an apparent K_d of ~400 nM for the human thrombin (Figure 6B), representing only a 2-fold affinity reduction in comparison to the original thrombin aptamer.[20] These observations seem to suggest that our QDNA-binding-and-release strategy causes more reduction in the target-binding affinity to signaling reporters constructed with low-affinity aptamers than to those built with high-affinity aptamers. On the basis of this observation, we hypothesize that the magnitude of the apparent K_d increase (i.e., the reduction of affinity for a target) is dependent on the affinity of the original aptamer. For a high-affinity aptamer (e.g., the thrombin-binding aptamer), the strong binding interaction between the aptamer and the target competes favorably with the QDNA-aptamer duplex binding interaction. As a result, the use of QDNA has little effect on the affinity of the aptamer reporter. However, with a low-affinity aptamer (e.g., the ATP-binding aptamer), the relatively weak target-aptamer interaction does not compete favorably with the QDNA-aptamer interaction, and therefore higher target concentrations are required to shift the following equilibrium to the right

$$\text{FDNA} \cdot \text{MAP} \cdot \text{QDNA} + \text{Target} \rightleftharpoons \text{FDNA} \cdot \text{MAP} \cdot \text{Target} + \text{QDNA} \quad (1)$$

Consequently, the apparent K_d of the reporter is significantly increased. However, having studied only two signaling aptamers, there is not enough experimental evidence for us to make a general conclusion. More signaling aptamers will be examined in future experiments.

The structure-switching signaling aptamers also exhibit a large signaling magnitude. Both the anti-ATP and anti-thrombin reporters exhibited a maximum S/B value in excess of 10. The signaling aptamers retain the binding specificities reported for the original aptamers. For example, the original anti-ATP aptamer was reported to bind ATP, ADP, AMP, adenosine, and dATP but not CTP, GTP, or UTP;[17] the engineered ATP reporters displayed the same target-recognition properties. The structure-switching reporters can be used for real-time sensing although the temperature suitable for real-time detection is greatly influenced by the number of nucleotides in QDNA that block the nucleotides essential for the formation of the DNA-target complex. Although a structure-switching signaling aptamer constructed with a long QDNA that binds solely to the aptamer domain can only be used for real-time sensing at elevated temperatures (such as 37 °C), reporters built with the same size of QDNA that blocks fewer nucleotides in the aptamer domain can perform real-time detection at very low temperatures (as low as 15 °C). Metal ion concentrations may also influence a reporter's low-temperature sensing ability as we found that the lower Mg^{2+} concentration supported the low-temperature real-time sensing of the guanine quartet-containing anti-thrombin aptamer reporter.

DNA is easy to prepare and has exceptional chemical stability. DNA aptamers can be easily created by in vitro selection for binding to a diverse range of targets with both high binding affinity and specificity. The simplicity of our modification strategy and the effectiveness of resultant fluorescence reporters described herein should help promote the exploitation of DNA aptamers as molecular tools for the solution-based detection of biological cofactors, metabolites, proteins, and a variety of other ligands of interest.

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Supporting Information Available: Signaling specificity of ATP Reporter A (Supporting Figure 1), signaling behaviors of two reporters made of mutant anti-ATP aptamers (Supporting Figure 2), and signaling specificity of the thrombin reporter (Supporting Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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