Biosensors

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A General Approach to the Construction of Structure-Switching Reporters from RNA Aptamers**

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Aptamers are single-stranded DNA or RNA molecules that are capable of ligand binding and have been widely investigated as molecular tools for a variety of applications.[1] For example, aptamers have become popular recognition elements in various biosensor platforms in which the aptamer–target binding event is reported by an optical, acoustic, mechanical, or electric signal.[2]

Biosensing by fluorescence is one of the most popular methods for the design of aptamer-based assays, largely because of ease of detection.[3] Our research group has previously reported a "structure-switching" approach to engineering fluorescent reporters from DNA aptamers.[4] This approach takes advantage of the ability of aptamers to form both an aptamer–target complex and a DNA–DNA duplex with a complementary sequence. Thus, a DNA duplex with a fluorophore-labeled aptamer and a quencher-labeled complementary strand can function as a reporter: the addition of target would release the complementary sequence from the aptamer, accompanied by an increase of fluorescence intensity.

The structure-switching approach was initially demonstrated using an anti-ATP aptamer (ATP = adensoine triphosphate) and an anti-thrombin aptamer,[4] and was further used to design reporters for other targets including guanosine triphsophate (GTP), cocaine, arginine, and the platelet-derived growth factor (PDGF).[5] Subsequent work has also expanded the utility of structure-switching aptamers in secondary applications to monitor enzymatic reactions, facilitate high-throughput screening, and develop nanodevices and solid-phase assays.[6] The structure-switching approach has also been exploited to design aptamer sensors that function by colorimetric, electrochemical, or other reporting mechanisms.[7]

A large number of RNA aptamers have been isolated for diverse targets[8] through systematic evolution of ligands by exponential enrichment (SELEX) experiments.[9] Moreover, many metabolite-binding RNA aptamers exist in cells as part of riboswitches to regulate gene expression.[10] The availability of versatile RNA aptamers represents a superior opportunity to develop RNA-aptamer-based biosensors. However, the potential of RNA aptamers for bioanalytical applications is still underexplored. For example, only a few studies have investigated the use of RNA aptamers in the development of fluorescence assays.[11] To expand the exploration of RNA aptamers in bioanalysis, in this study we sought to devise a generalizable, structure-switching-based approach for the rational design of fluorescent reporters from known RNA aptamers.

Since DNA oligonucleotides are more readily available and more stable than synthetic RNA oligonucleotides, our proposed strategy began with the polymerase chain reaction (PCR) to produce double-stranded (ds) DNA from a synthetic, aptamer-encoding DNA template (purple line, Figure 1) using two primers. Primer 1 contains a T7 promoter at the 5′-end so that the resultant dsDNA can be used to synthesize an RNA molecule using T7 RNA polymerase by in vitro transcription. The RNA was designed to have a sequence extension (red line) at the 5′-end so that this RNA can be used to assemble a three-component duplex structure with a fluorophore (F)-labeled DNA strand (denoted FDNA) and a quencher (Q)-labeled DNA oligonucleotide (QDNA) that has a complementary sequence to part of the RNA aptamer. This signaling duplex was expected to exhibit low fluorescence because of the proximity of the fluorophore and quencher. However, addition of the target should promote dissociation of QDNA from the duplex in favor of the target–aptamer complex formation, thus leading to the generation of a fluorescent signal. Two RNA aptamers were examined in this study: a synthetic aptamer that recognizes theophylline,[9a] and a naturally occurring aptamer that binds thiamine pyrophosphate (TPP).[12]

The theophylline aptamer (see Figure S1 in the Supporting Information for the secondary structure) was chosen as the first model aptamer. This aptamer is well-characterized, exhibits remarkable specificity, and has been used in several biosensor engineering studies.[9a,13] We assessed different combinations of FDNA, the extended theophylline RNA aptamer (TRA) and QDNA in preliminary experiments (one example is provided in Figure S2 in the Supporting Information). Based on these investigations, we decided to investigate a system consisting of FDNA1, TRA1, and QDNA2 (Figure 2). TRA1 was designed to have several sequence elements, each of which served a defined purpose (see also Figure S1 in the Supporting Information): G_1G_2 to facilitate RNA transcription, C_3-A_{22} for FDNA1 binding, U_{23} to create a suitable distance between F and Q for optimal quenching,[4] $\underline{C}_{24}$–$\underline{A}_{39}$ for QDNA2 binding, G_{33}–C_{65} (letters in blue) is the

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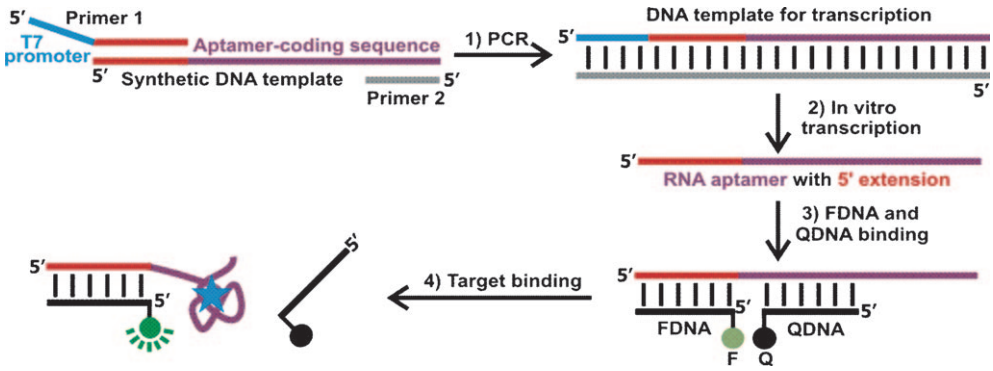

Figure 1. The proposed strategy for designing fluorescent RNA aptamer reporters that switch structures from a weakly fluorescent duplex to strongly fluorescent complex upon target binding. The aptamer sequence, shown in purple and produced by PCR and in vitro transcription, is linked to a sequence element that binds FDNA. The blue star represents the target for the aptamer.

a)

FDNA1: 5′ F-TAGCGTCGGAGCGTGGCAGG
QDNA1: 5′ ATCGCCCATAGAGTG-Q
QDNA2: 5′ TATCGCCCATAGAGTG-Q
QDNA3: 5′ GTATCGCCCATAGAGTG-Q
QDNA4: 5′ GGTATCGCCCATAGAGTG-Q
TRA1: 5′ GG*CCUGCCACGCUCCGACGCUA*UCACUCUAUGGGCGAU
ACCAGCCGAAAGGCCCUUGGCAGCGUCcaacacaucg

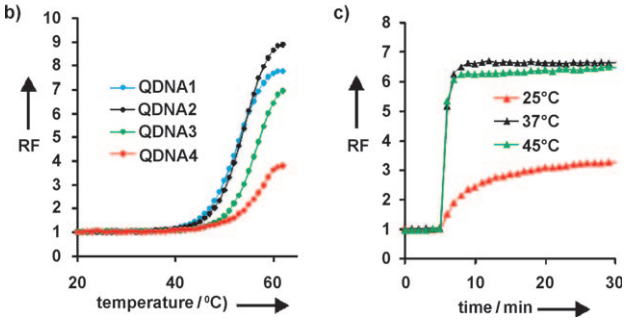

Figure 2. Structure-switching reporter for theophylline. a) Sequences of FDNA1, TRA1, and QDNA1–4. b) Thermal denaturation profiles of the duplexes made from QDNA1–4 with TRA1 and FDNA1. c) Target-induced fluorescence response at 25 °C, 37 °C, and 45 °C. RF = relative fluorescence. The duplex was incubated for 5 min followed by addition of 1 mM theophylline. The average values of two independent experiments (with less than 20% variations) are shown (the same applies to other figures below if not specified otherwise). Experimental details are provided in the Supporting Information.

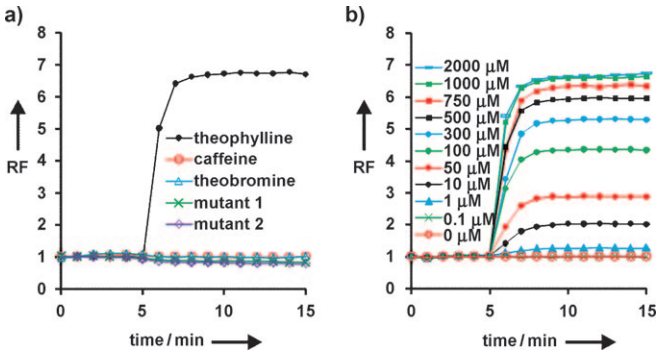

Figure 3. Specificity and sensitivity of the theophylline reporter. a) Specificity test. The signaling duplex was incubated at 37 °C for 5 min before addition of 1 mM theophylline (black), caffeine (red), or theobromine (light blue). Two mutant aptamers (green: mutant 1; purple: mutant 2) were also tested with addition of 1 mM theophylline. b) Signal response of the theophylline reporter to theophylline concentrations.

actual recognition element, and c_{66}–g_{75} (lowercase letters) as part of 3′-primer binding site in the DNA template for PCR. C_{24}–G_{32} were added as a "extension domain" to allow the formation of a stable QDNA–TRA duplex without sequestering many nucleotides in the actual aptamer sequence, a feature modeled after the design of structure-switching DNA aptamers.[4]

To derive an optimal QDNA, four different QDNA sequences (Figure 2a) were examined for thermal denaturation profiles in the duplex with FDNA1 and TRA1 (Figure 2b). The FDNA1–TRA1–QDNA2 duplex produced the best fluorescence enhancement and a relatively low melting point; thus, QDNA2 was selected for further experiments. We also found that for this signaling duplex, the theophylline-induced fluorescence increase progressed rapidly at both 37 °C and 45 °C, and reached the maximal enhancement after 3 minutes (Figure 2c). In contrast, at 25 °C, both the rate of signal increase and the maximal signal were considerably poorer. Since the signaling performance was at its best at 37 °C, this temperature was chosen for the remaining experiments.

The FDNA1–QDNA2–TRA1 reporter was found to be highly specific for theophylline; the addition of theobromine and caffeine (two structural derivatives of theophylline) did not generate significant signal output (Figure 3a). This observation is consistent with the previous report that the theophylline-binding aptamer exhibits much higher affinity for theophylline than for theobromine and caffeine.[9a]

Two mutant aptamers with altered nucleotides critical to target recognition[9a] were also examined. In mutant 1, G_{41} and A_{60} were mutated to A_{41} and U_{60}; in mutant 2, $A_{40}G_{41}$ were replaced with $G_{40}A_{41}$. The signaling duplexes from both mutants did not produce signal increase upon theophylline addition (Figure 3a). This observation verifies the importance of the specified nucleotides for theophylline binding and further validates the specificity data obtained for this reporter. To assess the sensitivity of the theophylline reporter, the relative fluorescence was determined at various concentrations of theophylline (Figure 3b). The detection limit was found to be 1 μM and the dynamic detection window was between 1–1000 μM (Figure 3b; the relative fluorescence observed at 20 min versus the theophyline concentrations can be found in Figure S3 in Supporting Information).

We then turned our attention to the TPP-binding RNA aptamer, which is part of natural riboswitches that regulate gene expression in response to intracellular TPP concentrations.[12] This aptamer has been the subject of extensive structural and functional analyses.[14] We chose to examine this aptamer for the structure-switching design mainly because it is much larger than the theophylline aptamer (87 vs. 33 nucleotides (nt)) and structurally more complex (Figure S1 in the Supporting Information).

The design of the TPP reporter was based on the information acquired with the theophylline reporter. Briefly, the 20-nt FDNA1 was retained as the FDNA and 16-nt QDNA5 was used as the new QDNA (Figure 4a and Figure S1 in the Supporting Information). The following features were designed into the extended TPP RNA aptamer (TPPRA): G_1G_2 for high transcription efficiency, C_3–A_{22} for FDNA1 binding, U_{23} for optimal F–Q distance, C_{24}–G_{39} for QDNA binding, and C_{33}–G_{119} for TPP recognition. Similar to the theophylline reporter, it was determined that the optimal QDNA length was 16 nt and sensing was most optimal at 37 °C.

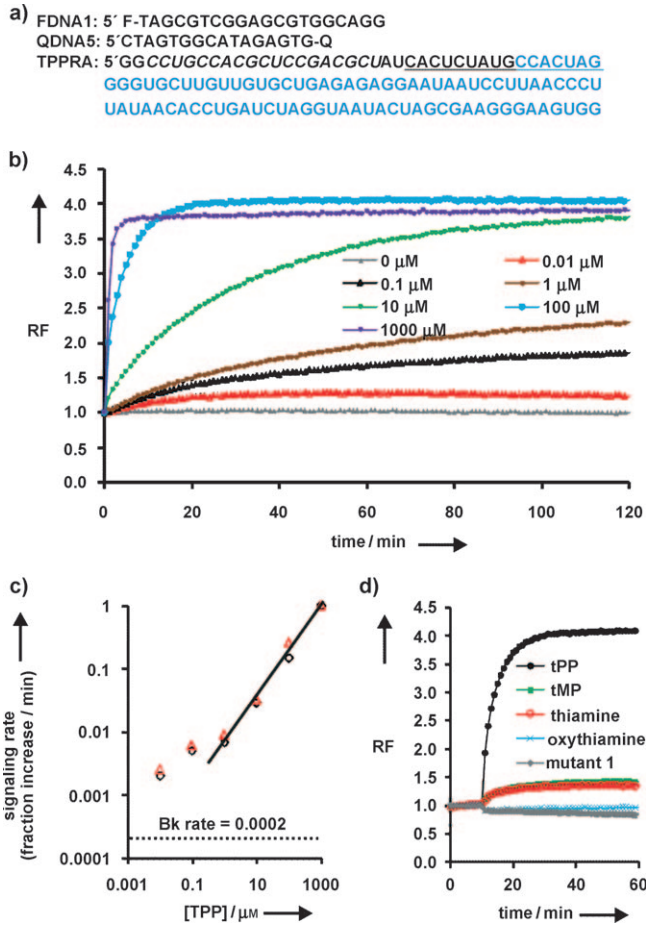

Figure 4. Structure-switching reporter for TPP. a) Sequences of FDNA and QDNA, and the extended RNA aptamer for TPP (TPPRA). b) Target-induced fluorescence increase of the reporter where the TPP concentration was varied between 0–1000 μM. c) Signaling rate versus TPP concentration (the method for deriving signaling rate (fraction increase/min) is provided in the Supporting Information) Bk rate = background rate, produced by the signaling duplex in the absence of TPP. d) Specificity test. The TPP signaling duplex was incubated for 10 min before 100 μM TPP (black), TMP (green), thiamine (red), or oxythiamine (light blue) was introduced (all 100 μM). One mutant aptamer (gray) was also tested with the addition of 100 μM TPP.

The TPP reporter produced a signal enhancement of around fourfold upon addition of 1 mM TPP. At this concentration, the reporter reached maximal enhancement after approximately 5 min. At lower concentrations (10 nM–100 μM), however, the rate of signal increase was reduced (Figure 4b). This reporter exhibited a linear dynamic range between 1–1000 μM when the signaling rate was plotted against TPP concentration, although the detection limit can reach 10 nM (Figure 4c). The slightly reduced maximal signal enhancement at 1000 μM in comparison to 100 μM may be caused by the fluorescence quenching of TPP at this concentration.

The TPP reporter also showed high specificity for TPP: only a subtle signal was seen with thiamine monophosphate (TMP) and thiamine, and no signal increase was observed with oxythiamine (Figure 4d). These observations are consistent with previous results.[14b,e] One mutant aptamer (sequence shown in Figure S4 in the Supporting Information) was also examined, and did not produce signal enhancement upon TPP addition (Figure 4d).

A key difference between theophylline and TPP reporters is the signal increase speed in relation to the target concentration. For the theophylline reporter, the signal increase was rapid (reaching the maximal signal after approximately 5 min) at all responsive target concentrations (Figure 3b). In contrast, the signaling rate of the TPP reporter was significantly dependent on the target concentration (Figure 4b): approximate times required to reach the maximal signal were 5 min for 1 mM TPP, 15 min for 0.1 mM TPP, 120 min for 10 μM TPP, and much longer times for lower concentrations. We speculate that this natural aptamer may have been evolved to have the necessary ability to respond accurately to the level of TPP in cells. Since the TPP aptamer is proposed to function in cells as TPP-responsive genetic OFF switch (to terminate the transcription of downstream genes that code for proteins involved in TPP biosynthesis),[12c] it would be most desirable for cells to have an aptamer that shows exquisite response to the cellular TPP level: very quick response when the TPP concentration is very high (thus no need for more TPP biosynthetic proteins) and progressively reduced response at lower levels of TPP (to reach an optimal biosynthetic proteins/TPP ratio).

In summary, we have demonstrated an approach that can be generally applied for the design of fluorescent reporters from RNA aptamers. Our method is based on a three-component system made of two chromophore-labeled DNA oligonucleotides obtained from chemical synthesis and an aptamer-containing RNA strand produced by in vitro transcription. Since the chemical synthesis of RNA is less efficient than that of DNA, and RNA is more prone to degradation, the production of RNA aptamers by in vitro transcription is an attractive option. The strategy to tag the aptamer with a fluorophore-labeled DNA oligonucleotide by duplex formation also circumvents the need of chemical synthesis of modified RNA and alleviates the probe degradation during

storage, which would produce higher background fluorescence.

Three adjustments were made in the design of structure-switching reporters for RNA aptamers when compared to the design for DNA aptamers. Firstly, the number of base pairs in the duplex between FDNA and the extended aptamer sequence was increased from 15 to 20, as we observed that 15 base pair DNA/RNA duplex had a higher background signal and smaller signal enhancement upon target addition (Figure S2 in the Supporting Information). Secondly, the optimal length of QDNA (which produce the most desirable balance of background signal, fluorescence enhancement, and signaling rate) was found to be 16 nt for the RNA reporters and 12 nt for the DNA reporters.[4] This modification was made based on the observation that the reporters constructed with QDNA of reduced length had higher background signal and reduced signal enhancement. Thirdly, with the increased size of QDNA, we increased the size of the extension domain from 5 nt in the DNA reporters to 9 nt in the RNA reporters (the extension domain consists of nucleotides that participate in the binding of QDNA but not in the binding of the target).

We examined two model aptamers with different origins, sizes, and affinities. The theophylline-binding RNA aptamer was obtained in a SELEX experiment, is 33 nt long and has a dissociation constant K_d of 0.1 μM.[9a] The TPP aptamer is found in cells, is considerably larger (87 nt) and has a K_d value of 0.85 nM.[12c] The successful conversion of the two very different RNA aptamers into structure-switching reporters using the same design demonstrates the general utility of the method. Furthermore, to the best of our knowledge, our work is the first reported attempt of exploring a naturally occurring aptamer for the design of a fluorescent biosensor for bioanalytical applications.

The designed structure-switching reporters retained the binding specificity of the original aptamer. For example, the original theophylline aptamer can distinguish theophylline (cognate target) from caffeine and theobromine that have very similar chemical structures.[9a] As shown in Figure 3a, the structure-switching reporter displayed the same specificity. Similar observations were made with the TPP reporter (Figure 4d).

The detection limit of the theophylline reporter was found to be 1 μM, which is 10 times higher than the K_d value of the original aptamer. Similarly, the detection limit of the TPP reporter was 10 nM, which is also 10 times higher than the K_d value of the original aptamer. The fact that structure-switching reporters did not produce better detection sensitivity may have two possible explanations. Firstly, the use of a complementary sequence (i.e., QDNA) to partially block the binding site of an aptamer has reduced its affinity for its target because in this modified system, the target needs to compete with QDNA for binding to the aptamer. Secondly, since the starting signaling duplex itself also exhibits some level of fluorescence, the small signal increase induced by the target at very low concentrations can easily be obscured within the background signal associated with large excess of unswitched duplex. These two inherent problems imply that it may be difficult to achieve extremely high sensitivity with structure-switching fluorescent aptamer reporters, particularly in the real-time sensing mode (i.e., detection without a separation step). Similar observations have also been reported for structure-switching fluorescent reporters based on DNA aptamers.[4] To achieve better sensitivity, alternative methods that can offer reduced background signal should be considered. One such method is electrochemical sensing,[15] which has already been applied to achieve improved detection sensitivity with structure-switching DNA aptamers.[7d,e]

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