Fluorescent Probes

In Vitro Selection of Structure-Switching Signaling Aptamers**

*Razvan Nutiu and Yingfu Li**

Signaling aptamers are aptamer probes that couple target binding to fluorescent-signal generation. These molecular sensors have potential applications in biosensing, proteomics, and drug discovery.[1] Here we describe a new in vitro selection approach for generating unmodified DNA aptamers that can be immediately transformed into effective signaling probes without the need for further optimization.

Standard aptamers can be easily generated by the technique of in vitro selection.[2] However, for biosensing applications, postselection modifications must be carried out in order to convert the aptamer concerned into a signaling probe. Although several conversion strategies have been reported,[3] the modification processes often require lengthy optimization steps to ensure that the affinity and specificity of the original aptamer is not lost upon labeling and that the modified signaling aptamer is able to significantly modulate the fluorescent signal upon target binding.

An alternative method is to create signaling aptamers directly by in vitro selection. Jhaveri et al. have reported the only signaling-aptamer selection method by which signaling aptamers labeled with a single fluorophore can be created.[4] However, their results showed that even if the selected aptamers were capable of target binding, a significant number of them failed to induce a concomitant fluorescence change upon target binding, presumably due to the insubstantial alteration of fluorescence properties of the attached fluorophore. More recently, the same group published a selection method for the optimization of signaling properties of molecular beacons containing a fluorophore–quencher pair.[5] It is conceivable, as the authors have already suggested in their report, that this method could be adapted to the selection of signaling-aptamer probes that detect nonnucleic acid targets. Encouraged by their work, we conducted an in vitro selection study for deriving signaling aptamers that function by the structure-switching mechanism previously described by us for the rational design of signaling aptamers.[6]

The structure-switching idea takes advantage of the universal ability of any aptamer to adopt two different structures: a duplex structure with an antisense DNA and a complex structure with the cognate target. We have shown that an aptamer can switch structures from duplex to complex upon target addition and, if the aptamer is labeled with a fluorophore and the antisense DNA is labeled with a quencher (denoted QDNA), the structure-switching process can be synchronized to fluorescence signaling.[6] However, fine-tuning of the QDNA represents a major challenge in the design of structure-switching signaling aptamers, as many QDNA sequences must be tested before a suitable signaling-aptamer system can be established. Therefore, we sought to overcome this problem by utilizing the power of in vitro selection. The strategy described in this report permits the creation of standard aptamers that are encoded with a duplex-to-complex switching capability and can be converted into signaling probes immediately upon their isolation.

The selection strategy is shown in Figure 1. A special DNA library (Figure 1a) was used that contained a central 15-nucleotide fixed-sequence domain (red) flanked by two random-sequence domains (green) of 10 and 20 nucleotides, each further flanked by primer-binding sequences (black). The central fixed-sequence domain was designed to be complementary to an antisense oligonucleotide biotinylated at its 5′-end (denoted BDNA). This arrangement permitted immobilization of the DNA library onto avidin-coated beads through DNA hybridization (step 1 of Figure 1b; see the Supporting Information for more details regarding the library design). We also added two short oligonucleotides, P1 and P2, to prevent the involvement of the primer-binding sequences in the tertiary folding of eventual aptamers. To demonstrate our concept, we used four standard NTPs (nucleoside triphosphates; blue star) as the potential aptamer targets. The immobilized DNA assembly was then exposed to a solution of a mixture of NTPs, each at 0.1 mM (step 2). Aptamers able to form the DNA/target complex should switch from the bead-bound state to the solution. These molecules were then collected and amplified by PCR (step 3). The reverse primer contained a ribonucleotide (R) at its 3′-end to create a chimeric antisense strand prone to NaOH-mediated cleavage, which permitted the isolation of the sense strand by gel electrophoresis (step 4). The recovered sense DNA was reannealed to BDNA, P1, and P2 and used for the

[*] R. Nutiu, Prof. Dr. Y. Li
Department of Biochemistry and Department of Chemistry
McMaster University
Hamilton, ON, L8N 3Z5 (Canada)
Fax: (+1) 905-522-9033
E-mail: liying@mcmaster.ca

[**] We thank the entire Li group as well as Dr. Eric Brown and Dr. Gerard Wright for insightful discussions. This study was funded by the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada. Y.L. holds a Canada Research Chair. R.N. received a CIHR Doctoral Research Award.

Supporting information for this article (library design and experimental details) is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200461848

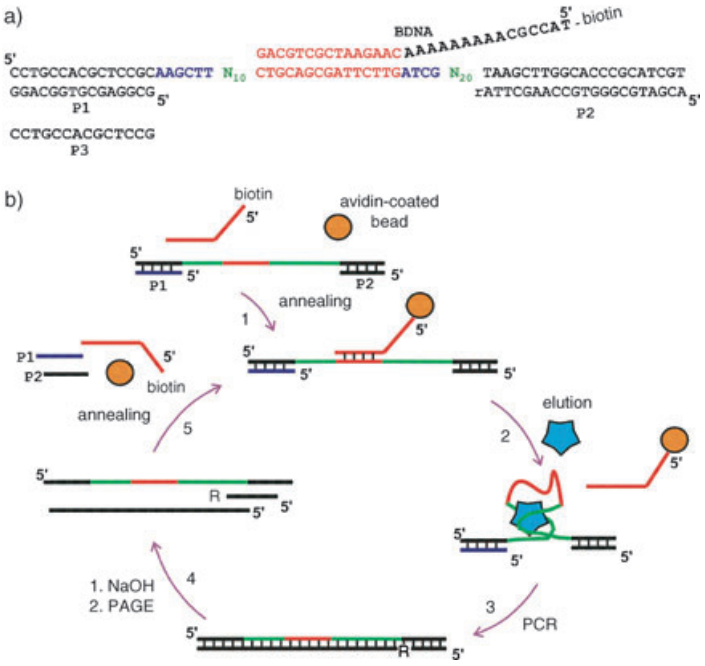

Figure 1. In vitro selection of structure-switching aptamers. a) DNA library design. b) In vitro selection scheme.

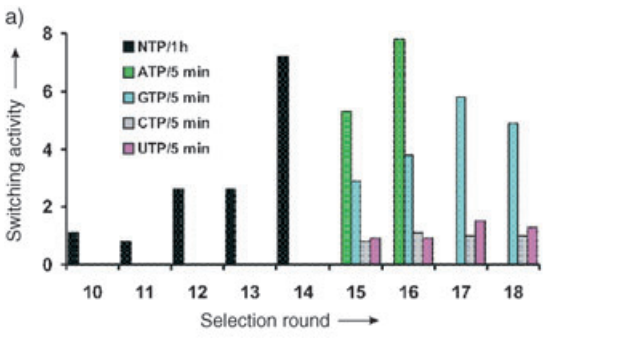

Figure 2. In vitro selection results. a) Progression of in vitro selection. b) The isolated aptamers. The primer binding sites are not shown; "x" represents deletions. Black letters in the central constant region represent mutations relative to the original sequence. The green letters represent the random domain, the red letters represent the central antisense-binding domain, and the blue letters represent initially present fixed nucleotides. ATP = adenosine 5′-triphosphate, GTP = guanosine 5′-triphosphate, CTP = cytidine 5′-triphosphate, UTP = uridine 5′-triphosphate.

next round of selection (step 5). It is noteworthy that BDNA and P1 were intended as the eventual sequences for carrying a quencher and a fluorophore, respectively, so that upon completion of in vitro selection, the selected aptamers could be immediately converted into signaling molecules.

The switching activity, defined as the ratio of the fraction of DNA eluted by the targets over that by selection buffer only, was measured for each selection round (Figure 2a). By the 14th round, the DNA population exhibited significant switching activity. Upon testing, generation 14 (G14) was found to be responsive only to ATP. Next, G14 was subjected to four parallel selections, one for each NTP (at 0.1 mM). After two more rounds, the ATP-binding population (ATP-G16) showed an eightfold ATP-dependent switching activity. Similarly, a sixfold GTP-dependent switching activity was observed for GTP-G17. In contrast, both the CTP and UTP populations failed to register any significant activity after several more rounds of selection. Subsequently, ATP-G16 and GTP-G18 were cloned and sequenced.

Based on sequence alignment, three sequence classes were found in the ATP population and one class in the GTP population (Figure 2b). Four individual ATP-binding (ATP1.1, ATP2.1, ATP2.5, and ATP3.1) and three GTP-binding (GTP1.1, GTP1.2, and GTP1.5) molecules were chemically synthesized and assessed for signaling abilities. At this point, the BDNA was modified with a 4,4-dimethylaminoazobenzene-4-′′-carboxylic acid (DABCYL) moiety (Q) at the 3′-end and renamed QDNA, and P1 was labeled with fluorescein (F) at the 5′-end and renamed FDNA (Figure 3a). Each DNA assembly (made of the synthetic aptamer, FDNA, QDNA, and P2) successfully signaled the presence of the cognate target, with an increase in fluorescence intensity upon target addition (Figure 3b). For each aptamer, the fluorescence intensity increased markedly when the cognate NTP target was introduced while it remained unchanged upon the addition of the noncognate target. (For clarity, the noncognate target data are only shown for ATP1.1 and GTP1.2.) We found that ATP1.1 and GTP1.2 have the best signaling performance within each relevant group.

To further confirm the structure-switching nature of each aptamer, we conducted an equilibrium-shifting experiment, as shown in Figure 4 for ATP1.1 as an example. Two aptamer-containing solutions A and B (data series in gray and black, respectively) were used. For both solution A and solution B, we first incubated FDNA, P2, and ATP1.1 for 10 min in the absence of QDNA and ATP. Because of the lack of QDNA, the fluorescence intensity of each solution was at its maximum. Then, 1 mM ATP was added to solution B while no ATP was added to solution A. The fact that both solutions maintained the same level of fluorescence intensity indicates that the binding of ATP to the aptamer did not alter its fluorescence intensity. Upon the addition of QDNA after 20 minutes, the fluorescence intensity of both solutions decreased. However, the target-containing solution (solution B) experienced a much smaller reduction in fluorescence

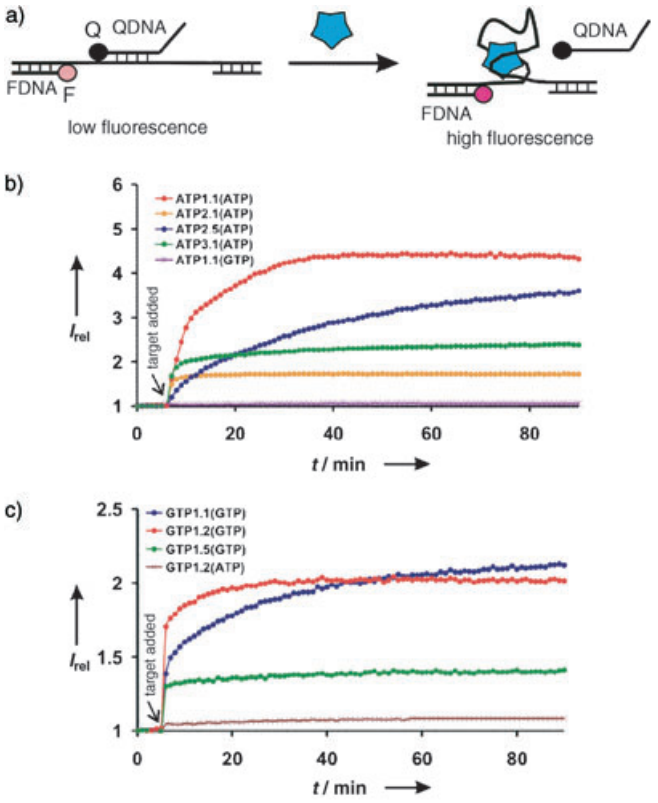

Figure 3. Converting selected aptamers into fluorescent reporters. a) DNA modification and signaling scheme. b) and c) Signaling profiles of four ATP-binding and three GTP-binding aptamers, respectively. I_{rel} relative fluorescence intensity.

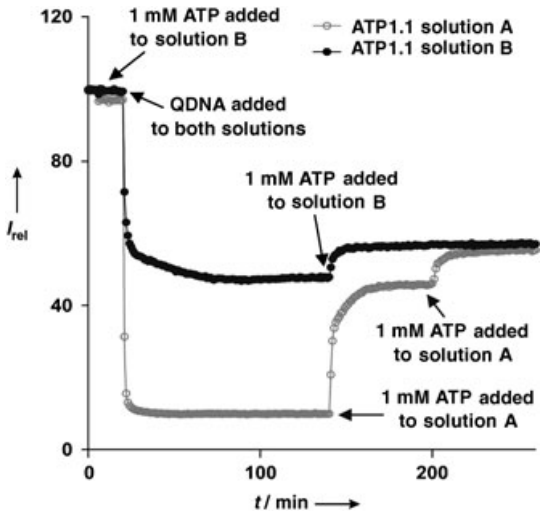

Figure 4. Proof of structure switching by an equilibrium-shifting experiment.

intensity than the target-lacking solution (solution A). The data are consistent with the formation of the aptamer–ATP complex in solution B and the significant reduction in the number of QDNA–aptamer duplexes in solution B as compared to solution A. After 140 minutes, 1 mM ATP was added to each solution. For solution B, only a small increase of fluorescence intensity was observed, a fact suggesting that the aptamer's target-binding site was largely saturated prior to the addition of the extra ATP. In stark contrast, a significant increase in fluorescence intensity was observed in solution A, which is indicative of a structure transition from the aptamer–QDNA duplex to the aptamer–ATP complex. Finally, when further 1 mM ATP was added to solution A (therefore both solutions contained the same concentrations of ATP and DNA species), solution A reached the same level of fluorescence seen with solution B. The same level of fluorescence reached by both solutions in the end with different orders of ATP and QDNA additions indicates that the switch between the duplex structure and the complex structure is an equilibrium process.

To explore the possibility of multicolor detection of different analytes in the same solution, we kept ATP1.1 unchanged and altered the sequence of the 5′-primer-binding domain of GTP1.2 (the new construct is now named GTP1.2Cy3), to bind a different FDNA labeled with indodicarbocyanine 3 (Cy3; FDNA was renamed Cy3DNA; see the sequences in the Supporting Information). Next, we exposed the mixture of FDNA, Cy3DNA, ATP1.1, GTP1.2Cy3, QDNA, and P2 to 1 mM ATP and 1 mM GTP in different orders (Figure 5a–c). First, we incubated the mixture without any target to get a background reading. Then we added the ATP (Figure 5a) or the GTP (Figure 5c) or both (Figure 5b) and recorded the fluorescence for 30 minutes. Finally, we added the second target (GTP in Figure 5a and ATP in Figure 5c) and recorded the signal for a further 30 minutes. In all three cases, both aptamers correctly indicated the cognate target independently of the presence of the second, uncognate target.

To conclude, we have demonstrated that in vitro selection can be carried out to generate structure-switching aptamers that can be immediately transformed into probes able to report target binding by fluorescence signaling. Moreover, we have demonstrated that two different signaling aptamers can work simultaneously in the same solution, thereby offering the possibility of multiplex detection. It is also worth commenting that although our aim in this study was to create signaling aptamers, the same selection method is equally attractive for standard aptamer selections, especially in cases where small molecules serve as aptamer targets (for which filter-based selection cannot be used) because our new method eliminates the need for target immobilization for creation of an affinity column.

All our selected aptamers share a common structural feature due to the design of the initial library: the nucleotides critical for target binding are arranged into two sequence motifs spanning the central fixed-sequence domain (Figure 2b). This arrangement closely resembles the "self-assembly" aptamers previously reported.[3b,c]

Interestingly, all our ATP-binding aptamers contain two stretches of nucleotides (underlined green nucleotides in Figure 2b) that constitute the ATP-binding site of a previously isolated ATP-binding DNA aptamer.[7,8] This observation indicates that we have rediscovered the same ATP-binding aptamer. This is not completely surprising as other studies have revealed that in vitro selection often offers a repeated solution to a given problem.[9] The isolation of the

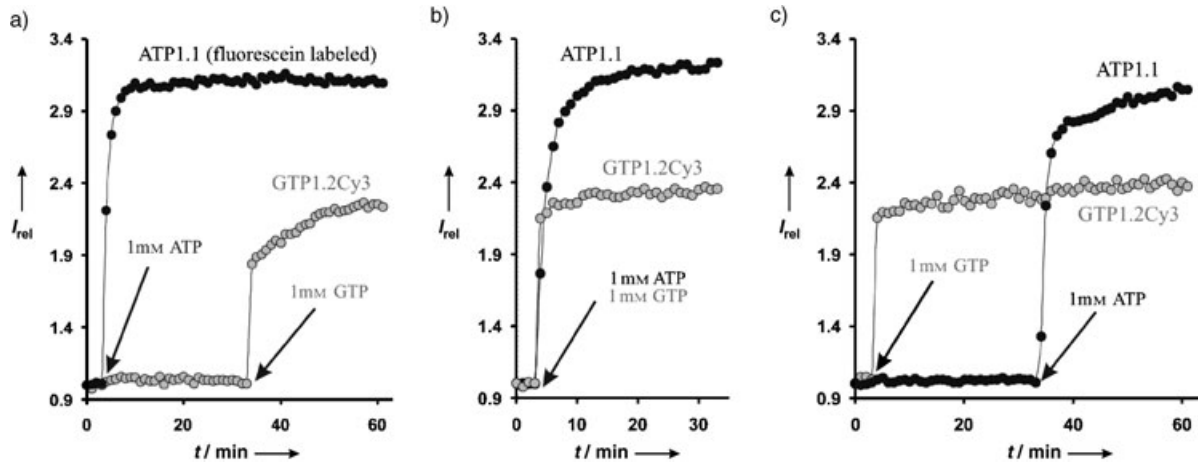

Figure 5. Two-color detection of ATP and GTP in a single solution. ATP1.1 is labeled with fluorescein, GTP1.2 is labeled with Cy3, and QDNA contains a DABCYL moiety as a quencher for both fluorophores. a) Addition of ATP followed by addition of GTP. b) Simultaneous addition of ATP and GTP. c) Addition of GTP followed by addition of ATP.

same aptamer motif in different laboratories from different library designs (a continuous stretch of random-sequence nucleotides in the previous study; two stretches of random-sequence nucleotides flanking a constant-sequence region in the current study) and two different selection strategies (the target ATP was immobilized on a column to retain the aptamer sequences from a DNA library in the previous study; the DNA library was immobilized on a column through DNA hybridization and ATP was used to elute the aptamer sequences in the current study) may suggest that these underlined nucleotides form a small but highly optimized binding site for ATP. The small size of the aptamer means its high abundance in the initial library and the optimal binding site should make it fit for survival during the selection process. The recurrence of the aptamer could also be a result of our choice to use a small-size library that could not lead to isolation of larger and more complex aptamers. We also notice that each selected structure-switching aptamer family shows a very high degree of conservation in most nucleotides located outside of the target-binding motifs, a result suggesting that these nucleotides may help the structure-switching process.

All of our aptamers were able to generate an easily detectable fluorescence signal; however, these directly converted signaling aptamers could achieve only limited fluorescence enhancement (4.5-fold with the best ATP-binding aptamer ATP1.1 and twofold with the best GTP-recognizing aptamer GTP1.2). Although our best signaling aptamers exhibited a signaling magnitude comparable to some of the known aptamer sensors obtained by rational design strategies (1.5-fold in ref. [3a], threefold in ref. [3d] and ref. [3h], fivefold in ref. [3j]), the best rationally designed signaling aptamers can produce a fluorescence enhancement as high as 14-fold.[3b],[6a]). It is conceivable that the signaling performance of our signaling aptamers can be improved by carrying out optimization experiments (such as using an extended FDNA or QDNA that can place F and Q in close proximity). It is also important to point out that all the selected aptamers involuntarily acquired one or two mutations or deletions in the BDNA-binding domain during the in vitro selection process (Figure 2b), which obviously leads to the formation of a weaker aptamer–BDNA duplex for easier structure switching. However, it also gives a higher fluorescence background for the signaling system. Based on this observation, we believe that better signaling aptamers could be derived in future in vitro selection efforts by conducting the duplex-formation step (steps 1 and 5 in Figure 1b) at an elevated temperature (22 °C was used in the current study) to eliminate the sequences with weakened duplex structures.

Our selection failed to generate DNA aptamers that bind CTP or UTP. Although precise reasons for this failure could not be determined at this point, we speculate that this might have been caused by our choice of a DNA library containing only 30 random nucleotides. It is quite possible that potential CTP- and UTP-binding aptamers require considerably more nucleotides and our starting library simply did not meet this requirement. Another possibility is that these two nucleotides may inherently be poor aptamer targets. It remains to be determined whether CTP- and UTP-binding aptamers can be derived by using more complex libraries.

Received: August 31, 2004
Revised: October 18, 2004
Published online: January 11, 2005

Keywords: aptamers · fluorescence · in vitro selection · nucleic acids · sensors

[1] a) S. D. Jayasena, *Clin. Chem.* **1999**, *45*, 1628–1650; b) L. S. Green, C. Bell, N. Janjic, *Biotechniques* **2001**, *30*, 1094–1110; c) M. Famulok, M. Blind, G. Mayer, *Chem. Biol.* **2001**, *8*, 931–939; d) P. Burgstaller, A. Jenne, M. Blind, *Curr. Opin. Drug Discov. Dev.* **2002**, *5*, 690–700; e) X. Fang, A. Sen, M. Vicens, W. Tan, *ChemBioChem* **2003**, *4*, 829–834; f) R. Nutiu, J. M. Yu, Y. Li, *ChemBioChem* **2004**, *5*, 1139–1144; g) J. Srinivasan, S. T. Cload, N. Hamaguchi, J. Kurz, S. Keene, M. Kurz, R. M. Boomer, J. Blanchard, D. Epstein, C. Wilson, J. L. Diener, *Chem. Biol.* **2004**, *11*, 499–508.

[2] a) C. Tuerk, L. Gold, *Science* **1990**, *249*, 505–510; b) A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818–822.

[3] a) S. Jhaveri, R. Kirby, R. Conrad, E. Maglott, M. Bowser, R. T. Kennedy, G. Glick, A. D. Ellington, *J. Am. Chem. Soc.* **2000**, *122*, 2469 – 2473; b) R. Yamamoto, T. Baba, P. K. Kumar, *Genes Cells* **2000**, *5*, 389 – 396; c) M. N. Stojanovic, P. de Prada, D. W. Landry, *J. Am. Chem. Soc.* **2000**, *122*, 11547 – 11548; d) N. Hamaguchi, A. Ellington, M. Stanton, *Anal. Biochem.* **2001**, *294*, 126 – 131; e) M. N. Stojanovic, P. de Prada, D. W. Landry, *J. Am. Chem. Soc.* **2001**, *123*, 4928 – 4931; f) J. J. Li, X. Fang, W. Tan, *Biochem. Biophys. Res. Commun.* **2002**, *292*, 31 – 40; g) J. R. Babendure, S. R. Adams, R. Y. Tsien, *J. Am. Chem. Soc.* **2003**, *125*, 14716 – 14717; h) K. Yamana, Y. Ohtani, H. Nakano, I. Saito, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3429 – 3431; i) E. J. Merino, K. M. Weeks, *J. Am. Chem. Soc.* **2003**, *125*, 12370 – 12371; j) M. N. Stojanovic, D. M. Kolpashchikov, *J. Am. Chem. Soc.* **2004**, *126*, 9266 – 9270.
[4] S. Jhaveri, M. Rajendran, A. D. Ellington, *Nat. Biotechnol.* **2000**, *18*, 1293 – 1297.
[5] M. Rajendran, A. D. Ellington, *Nucleic Acids Res.* **2003**, *31*, 5700 – 5713.
[6] a) R. Nutiu, Y. Li, *J. Am. Chem. Soc.* **2003**, *125*, 4771 – 4778; b) R. Nutiu, Y. Li, *Chem. Eur. J.* **2004**, *10*, 1868 – 1876.
[7] D. E. Huizenga, J. W. Szostak, *Biochemistry* **1995**, *34*, 656 – 665.
[8] C. H. Lin, D. J. Patel, *Chem. Biol.* **1997**, *4*, 817 – 832.
[9] a) K. Salehi-Ashtiani, J. W. Szostak, *Nature* **2001**, *414*, 82 – 84; b) D. Saran, J. Frank, D. H. Burke, *BMC Evol. Biol.* **2003**, *3*, 26; c) R. P. Cruz, J. B. Withers, Y. Li, *Chem. Biol.* **2004**, *11*, 57 – 67.

