Pyrene-modified DNA Aptamer as a Fluorescent Biosensor with High Affinity and Specificity for ATP Sensing

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The fluorescence properties of anti-ATP apatmers labeled with a pyrenylmethyl group at the 2′-positin were investigated. The labeling of the nucleoside residue at the stem region adjacent to the ligand-binding pocket gave a fluorescent biosensor having high affinity yet retaining its specificity for ATP.

Aptamer-based biosensors have attracted current research interest, because of their wide applicability in diagnostics and protein filings.[1] Methods for generation of aptamer-biosensors can be classified into several approaches. One approach is the production of randomized nucleic acid sequence libraries containing fluorescent nucleotides and the selection of the optimized sequences that transduce the ligand-binding to a measurable emission for sensing.[2] The other approach is based on the FRET method for producing signaling aptamers.[3] In this approach, the aptamers are designed to induce large conformational changes on binding to cognate ligands, that differentiates the distance between FRET fluorophores to give a sensing emission.

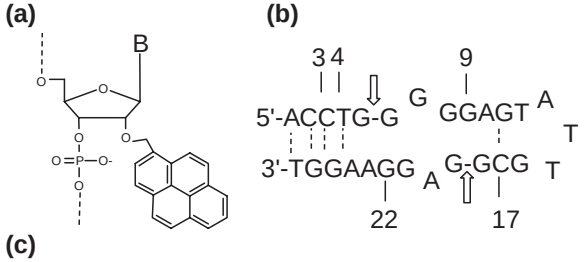
Another possible approach is site-directed fluorescence labeling of aptamers.[4] Binding of the labeled aptamers to ligands induces global and/or local conformational changes that yield the signal emission for sensing. The appropriate site for fluorescent labeling to generate signaling aptamers could usually be rationalized on the basis of aptamer sequence, structure, and ligand binding.[1,4]

In our initial attempt to design a signaling aptamer, we used a bis-pyrene fluorophore as a fluorescent label, since the excimer and monomer fluorescence of the bis-pyrene are sensitive to local structural changes near the bis-pyrene label attached to oligonucleotides.[5] The site-directed incorporation of the bis-pyrene fluorophore into anti-ATP DNA aptamer provided a fluorescent sensor showing high signal intensity and specificity for the target ligand.[6] Although the generation of a signaling aptamer using a bis-pyrene label was realized, there still remain a problem that the generated aptamer has significantly reduced affinity for the cognate ligand.

In this communication, we demonstrate that the pyrene-modification at the 2′-position[7] of DNA aptamer can solve the problem. We found that the signaling aptamers possessing high affinity yet retaining its specificity for a given ligand can be generated with the single pyrene label at the 2′-nucleoside residue adjacent to the ligand-binding pocket. The 2′-pyrene labeling method would be widely applicable in the synthesis of a fluorescent aptamer biosensor.

The anti-ATP DNA aptamer was employed as a model system that was already tested in the bis-pyrene label.[6] Moreover, this aptamer has been widely used for a benchmark for generating a signaling aptamer[3a,3b,4] and three-dimensional structure of the aptamer-ligand complex was established.[8] In the aptamer-ligand complex, two adenine moieties are intercalated at the adjacent sites between G5 and G6 and between G18 and G19 through direct contact with G9 and G22. Figure 1 indicates the 2′-pyrene modified nucleosides and their incorporation into several positions of the aptamer. The modified cytidine was incorporated into the position of 3 and 17, yielding the fluorescence-modified aptamers (**I** and **III**). Similarly, the modified uridine and adenosine were placed at the designated positions (4, 20, 23, and 24), giving the modified aptamers (**II** and **VI**–**IX**). The synthesis of the modified aptamers was accomplished by an established procedure as previously described.[7]

The fluorescence responses of all the 2′-pyrene-modified aptamers in the presence of ATP were investigated at room temperature in the buffer containing 300 mM NaCl and 5 mM $MgCl_2$. Among these aptamers, only the modified aptamers possessing a pyrene at the stem region adjacent to the ligand-binding site (**II** and **III**) exhibit the fluorescence responses for the cognate ligand. Figure 2 shows the fluorescence changes of aptamer **III** upon addition of ATP. The fluorescence intensity at 380 nm was considerably increased in responding to ATP. Similar fluorescence changes were also observed for the aptamer **II**. As shown in the inset of Figure 2, the fluorescence data were best fitted with the theoretical equation based on the cooperative binding of two molars of ATP to the aptamer.[9] The dissociation constants (K_d values) for aptamer **II** and **III** thus obtained and those for the bis-pyrene-modified aptamer[6] are summarized in Table 1. Based on the K_d values, the present fluorescent aptamers bind to ATP at least 10-times stronger than the bis-pyrene aptamer does, which is almost compatible to the original



(c)

I: 5′–ACC(pyr)TGGGGGAGTATTGCGGAGGAAGGT
II: 5′–ACCU(pyr)GGGGGAGTATTGCGGAGGAAGGT
III: 5′–ACCTGGGGGAGTATTGC(pyr)GGAGGAAGGT
IV: 5′–ACCTGGGGGA(pyr)GTATTGCGGAGGAAGGT
V: 5′–ACCTGGGGCAGTATTGCGGA(pyr)GGAAGGT
VI: 5′–ACCTGGGGGAGTATTGCGGAGCA(pyr)AGGT
VII: 5′–ACCTGGGGCAGTATTGCGGAGCAA(pyr)GGT

Figure 1. (a) The structure of 2′-pyrene-modified nucleoside. (b) The sequence of anti-ATP DNA aptamer. The arrows indicate the site of the ATP-binding. (c) The sequences of pyrene-modified DNA aptamers. The 2′-pyrene-modified cytosine, uracil, and adenine nucleosides are indicated by C(pyr), U(pyr) and A(pyr), respectively.

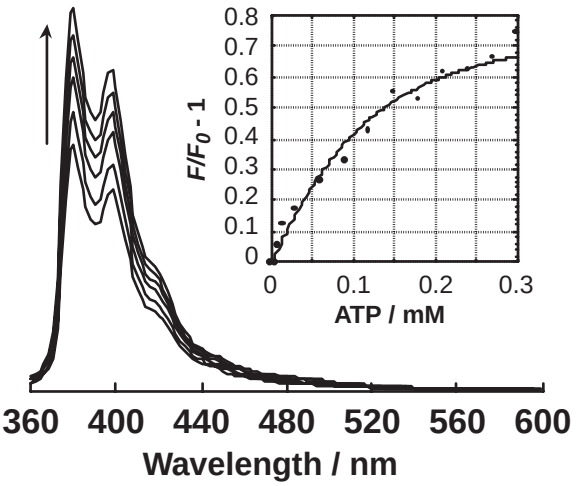


Figure 2. Fluorescence changes of pyrene-modified DNA aptamer **III** in the presence of various amounts of ATP (0, 60, 120, 180, 240, and 300 μM). Inset shows the fluorescence data and fitting curve based on the binding of one molar aptamer to two molars of ATP. Measurements were carried out at room temperature for the aptamer solution (30 μM) in 20 mM Tris buffer containing 300 mM NaCl and 5 mM $MgCl_2$ adjusted to pH 7.6. Excitaion wavelength was 350 nm.

Table 1. K_d values of pyrene-modified DNA aptamers **II**, **III**, and bis-pyrene-modified aptamer in binding to ATP

	K_{d1}/mM	K_{d2}/mM
Aptamer **II**	0.12	0.16
Aptamer **III**	0.12	0.14
Bis-pyrene apatmer[a]	1.7	1.68

[a]The sequence of the bis-pyrene apatmaer: 5′-ACC TGG GGG AGT ATT GCG G(BP)G GAA GGT, where the BP donates the bis-pyrene unit.[6]

DNA aptamer.[4]

For the aptamers **II** and **III**, the fluorescence changes were observed upon binding to ATP but not observed on binding to other nucleoside tri-phosphates (Figure 3a). Figure 3b shows the fluorescence responses of the apatamer **III** in the presence of ATP, ADP, and AMP. This aptamer gave the specific fluorescence responses for all the adenine-containing nucleotides. K_d values obtained for the aptamer-ADP (K_{d1}: 0.1 mM; K_{d2}: 0.1 mM) and the aptamer-AMP (K_{d1}: 0.08 mM; K_{d2}: 0.07 mM) were slightly lower than those for the aptamer-ATP complex. These results provide the clear evidences that the pyrene-modified signaling aptamers retain their specificity similar to the original aptamer in recognition of the cognate ligand.

A fluorescent biosensor having high affinity and specificity for a given ligand can be generated by single pyrene labeling at the 2′-sugar of DNA aptamer. Important findings are that the pyrene label should be placed at the nucleoside residue in the stem region adjacent to the ligand-binding pocket to yield the signaling aptamers. Since many aptamers have hairpin-like structures containing stem region near the ligand-binding site, the present labeling method might open a general way to design a fluorescent-signaling aptamer.

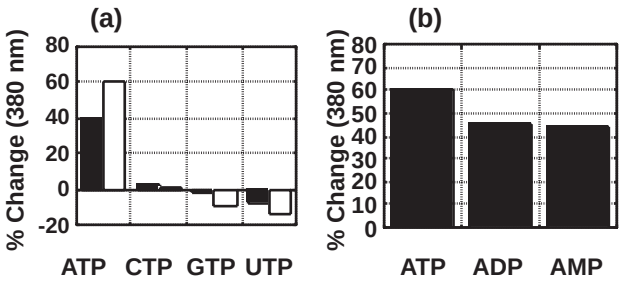


Figure 3. (a) % Change in fluorescence intensity at 380 nm of aptamer **II** (black bar), and **III** (white bar) in the presence of nucleoside 5′-tri-phosphates. (b) % Change in fluorescence intensity at 380 nm of aptamer **III** in the presence of ATP, ADP, and AMP. The aptamer and nucleotide concentrations were 30 and 300 μM, respectively. The other conditions for the fluorescence measurements were the same as described in Figure 2.

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References and Notes

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9 The dissociation constants for the aptamer were obtained from the analysis of the fluorescence data using a following equation:

$$F/F_0 - 1 = \{f_1K_1[\mathrm{L}] + f_2K_1K_2[\mathrm{L}]^2\}/(1 + K_1[\mathrm{L}] + K_1K_2[\mathrm{L}]^2)$$

where F is the fluorescence signal in the presence of a ligand, F_0 is the fluorescence in the absence of a ligand, f_1 is the relative fluorescence for the singly bound substrate, f_2 is the relative fluorescence for the doubly bound substrate, K_1 is the association constant for the first-order complex, and K_2 is the association constant for the second-order complex. It is noted that the present fluorescence data were not fitted well with the assumption that the aptamer contained only a single-binding site.

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