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Bis-Pyrene Labeled DNA Aptamer as an Intelligent Fluorescent Biosensor

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Abstract—The site-directed incorporation of bis-pyrenyl fluorophore into anti-ATP DNA aptamer results in a creation of an intelligent fluorescent sensor with high signal intensity and specificity for detecting the target ligand in a homogeneous system.

The generation of specific nucleic acid binding species such as aptamers that exhibit a specific signal for the detection of target ligands has been a subject of intense research, since aptamers that function as biosensors would have a large potential in numerous biochemical and clinical applications such as protein profiling and diagnostics.[1–4] Ellington et al. have succeeded to create such signaling aptamers by in vitro selection using a fluorescent nucleoside.[2] While this method is promising to provide signaling aptamers of high sensitivity and specificity, the procedures require several complicated steps which are unfamiliar to most chemists. Based on the ternary structures of aptamer–ligand complexes, it is feasible to modulate their function chemically. A molecular beacon technique has already proved to be useful in generation of aptamer-based biosensors.[3] Although this approach is quite attractive for designing signaling aptamers, it may be limited to the aptamers that induce drastic changes in their structures upon binding to cognate ligands. In contrast, it has been reported that a direct chemical labeling of aptamers can also lead to the signaling aptamers.[4] However, fluorescein- and acridine dye-conjugated aptamers showed only small fluorescence responses in the detection of a target ligand.[4]

We have focused on a bis-pyrene labeled fluorophore[5] (Fig. 1a) in our attempt to design a signaling aptamer. This novel bis-pyrene fluorophore is easily incorporated as a fluorescent non-nucleosidic linker into internal or terminal positions of aptamers, enabling us to seek an appropriate position of the fluorescence label in the aptamer. Since it has been established that the excimer (480 nm) and monomer (380 nm) fluorescence emission of bis-pyrene fluorophore are highly sensitive to the local structural change caused by base-pairing and/or nucleotide sequence variations near the bis-pyrene label attached to oligonucleotide duplexes,[5] it is anticipated

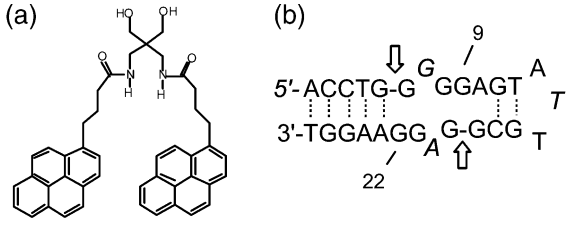

Figure 1. (a) The structure of bis-pyrene label (BP). (b) The sites (shown by italic) of bis-pyrene-label incorporated into anti-ATP DNA aptamer. The arrows indicate the ATP binding sites. (c) The sequences of bis-pyrene labeled aptamers. BP donates the bis-pyrene label.

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that bis-pyrene labeled aptamers would exhibit a drastic fluorescence change upon binding to a cognate ligand. In this communication, we demonstrate that the site-directed incorporation of bis-pyrenyl fluorophore into a DNA aptamer results in a creation of an intelligent fluorescent sensor with high signal intensity and specificity for detecting target ligands in a homogeneous system.

We have used the structures of anti-ATP aptamer selected from a DNA pool[6] as a model system for the design of pyrene excimer forming aptamers, since three dimensional structure of the aptamer complex has been already established.[7] In the DNA aptamer 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.[7] The excimer label (BP) was therefore incorporated into several different nucleoside positions in the sequence of anti-ATP aptamer as shown in Figure 1b. The sequences of the resulting fluorescent aptamers are indicated in Figure 1c. The aptamers (**II** and **IV**) contain the excimer label that is replaced with the nucleoside of position 7 for **II** and 20 for **IV** between the crucial residues (G5, G6 bases and G18, G19 bases) for the ATP-binding. In contrast, aptamers (**I** and **III**) possess the pyrene label at the position far from the contacting nucleoside residues. Aptamers **V**–**VII** are mutants of **IV** where G was replaced by C at the position of 9 and/or 22 that is the putative contacting site of ATP. The fluorescent aptamers were synthesized by using a protected phosphoramidite derivative of BP according to the established procedure[5] and the structures of the aptamers were verified by electron-spray mass spectral analysis.

The ratio of excimer fluorescence intensity at 480 nm (**Ie**) versus monomer intensity at 380 nm (**Im**) was used to test the aptamers with the excimer label as a sensor for ATP ligand.[8] Figure 2a shows the change in the ratio of **Ie**/**Im** for aptamers **I**–**IV** upon addition of four nucleoside triphosphates (ATP, GTP, UTP, and CTP). The aptamers (**I** and **III**) having the bis-pyrene label at the position far from the ATP binding sites showed little or no significant change in the fluorescence in the presence of ATP and other nucleoside triphosphates. Of two aptamers (**II** and **IV**) containing the bis-pyrene label at the adjacent site of the ATP binding, only aptamer **IV** exhibited 3.3-fold increase in the fluorescence signal in the presence of 3 mM ATP. The fluorescence changes observed in the aptamer **IV** should be derived from the local structural alteration that affects largely the pyrene interactions with nearby bases upon binding to the cognate ligand. That similar fluorescence changes were not observed in the aptamer **II** may be likely due to the small effect of the structural alteration on the pyrene interactions or the significant loss of affinity for the cognate ligand.

To confirm the specificity of the signaling aptamer **IV**, changes in the excimer versus monomer fluorescence ratio (**Ie**/**Im**) were also measured in the presence of GTP, CTP, and UTP. No significant increase in the fluorescence intensity was observed in the presence of these non-specific ligands. To further demonstrate the ligand specificity for the signaling aptamer, mutation was introduced into the position of 9 or/and 22 of **IV**. As shown in Figure 2b, the ATP-dependent change in the fluorescence signal for the mutant aptamer **V** was smaller than that for the original aptamer **IV**, while the other mutants (**VI** and **VII**) showed little or no fluorescence change upon addition of ATP.

To determine whether the fluorescence of bis-pyrene labeled aptamer **IV** is responsible for the binding of two ATP molecules, the ratio of the change in the fluorescence to the concentration of ATP, $\{(F-F_0)/F_0\}/[ATP]$, was plotted against the fluorescence change, F/F_0. If the Scatchard plot[9] exhibited linear, the labeled aptamer would have a single binding site. However, as shown in Figure 3a, the resulting plot exhibited non-linear or biphasic, which strongly suggests the multiple binding sites were retained even in the aptamer containing the bis-pyrene label near the ATP-binding sites. We therefore attempted to determine the dissociation constants for the aptamer **IV** according to a cooperative binding model.[9] Figure 3b shows the fluorescence responses in the binding of the aptamer **IV** to ATP and its fitting curves to the model for the aptamer having two binding sites. The analysis of the fitting curves gave two dissociation constants, K_{d1} ($1/K_1$) of 1.17 ± 0.07 mM and K_{d2} ($1/K_2$) of 1.64 ± 0.21 mM.[10] The contribution of the first ATP binding to the relative fluorescence change is calculated to be 0.23 that is smaller than the relative fluorescence change due to the formation of the ternary complex (0.73). These results strongly suggest that bis-pyrene labeled aptamer **IV** enables the detection of

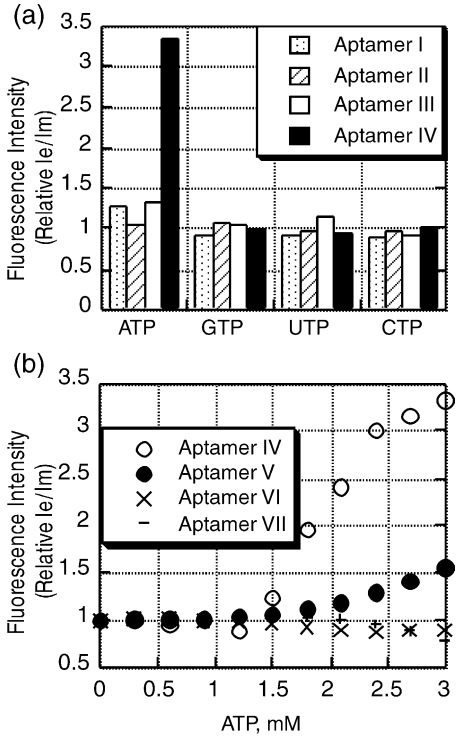

Figure 2. (a) The relative ratio of excimer (**Ie** at 480 nm) versus monomer (**Im** at 380 nm) fluorescence intensity for bis-pyrene labeled aptamers (**I**–**IV**) in the prescence (3 mM) of nucleoside triphosphates (ATP, GTP, UTP, and CTP); (b) the relative ratio of excimer (**Ie** at 480 nm) and monomer (**Im** at 380 nm) fluorescence intensity for bis-pyrene labeled aptamers (**IV**–**VII**) at various concentrations of ATP.

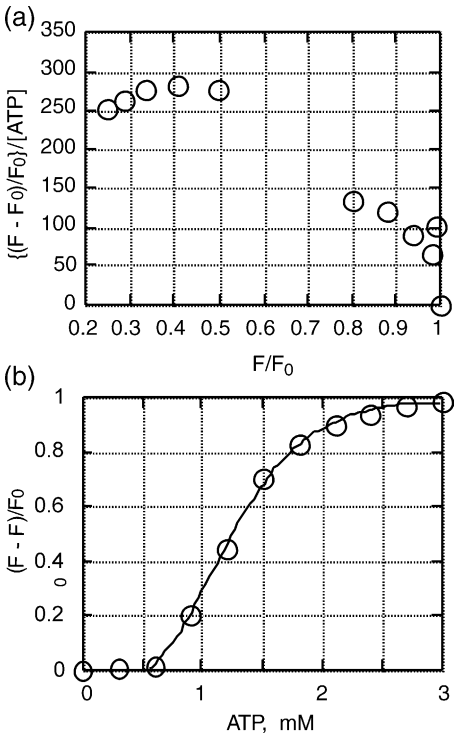

Figure 3. (a) The Scatchard plot for aptamer **IV** derived from the fluorescence changes in the presence of ATP; (b) the fractional change in the fluorescence $(F-F_0)/F_0$ for aptamer **IV** at various concentrations of ATP.

the successive binding of two ATP molecules by monitoring the response of pyrene excimer and monomer fluorescences.

Owing to the pyrene excimer and monomer fluorescenes that are highly sensitive to the local structures of DNA, the present signaling aptamer exhibits a large fluorescence response yet retains its binding specificity to the cognate ligand. Therefore, the bis-pyrene label has proved to be useful for generating an aptamer-based biosensor possessing a high specificity.

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8. All fluorescence measurements were carried out at room temperature with excitation at 350 nm in a buffer containing 300 mM NaCl, 20 mM Tris–HCl and 5 mM $MgCl_2$ (pH = 7.6) in which the aptamer concentration was 30 μM.
9. In order to determine the dissociation constants for the aptamer, the fluorescence data were analyzed by following equation:

$$(F - F_0)/F_0 = \{f_1\ K_1[L] + f_2\ K_1K_2[L]^2\}/(1 + K_1[L] + K_1K_2[L]^2)$$

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. The fluorescence data were also analyzed on the basis of the assumption that the aptamer contained only a single-binding site, which is according to the following equation to give Scatchard plot:

$$\{(F - F_0)/F_0\}/[L] = fK - K\ (F/F_0)$$

10. Although the dissociation constant of 6 ± 3 μM for the original aptamer was reported,[6] the fluorescein labeled aptamer[4] showed the two dissociation constants of K_{d1} $(1/K_1)$ of 0.03 ± 0.02 mM and K_{d2} $(1/K_2)$ of 0.05 ± 0.03 mM that were obtained by the similar fluorescent titration experiments.