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Biomolecular sensor based on fluorescence-labeled aptamer

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Abstract—Fluorescent DNA probes for L-argininamide were developed by a combination of DNA aptamers and fluorophore–quencher pairs. These molecules were synthesized by a combination of pre- and post-synthetic modification methods. The fluorescence-labeled aptamer could detect L-argininamide specifically. The binding affinities were defined by the binding affinity of the original aptamer to indicate that the end labeling of the aptamer did not influence the affinities.

A sensor-detected specific biomolecule has an important application in chemical biology.[1] These sensors can detect biologically active molecules and determine the concentration of these molecules. A molecular sensor consists of a recognition moiety and a signaling moiety. One of the most useful recognition molecules is an aptamer.[2] Aptamers are single-stranded nucleic acids, which bind specific ligands and are obtained by *in vitro* selection, for any compounds. Fluorescence molecules are attractive as a signaling moiety. Therefore, if the aptamers are labeled with a fluorophore, the fluorescent-labeled aptamers are used for signaling aptamers. Two kinds of signaling aptamers have been reported. One is a single fluorophore-labeled DNA.[3] This type of probe exhibits changes in fluorescence upon binding to a target molecule and this change in fluorescence is based on the change in the microenvironment around a fluorophore. The other type of probe is a doubly labeled DNA bearing a fluorophore and a fluorescence quencher. The changes in fluorescence of this probe are based on fluorescence resonance energy transfer (FRET) and reflect the global conformational change by binding to a ligand. The latter functions as a sensitive probe with a high signal-to-background ratio. Yamamoto and Kumar[4] reported the first aptamer beacon, which is an RNA aptamer bearing a fluorophore and a quencher. They developed a sensor molecule for detecting the Tat protein of HIV-1. Several other signaling aptamers have been reported for sensing the corresponding proteins,[5] but there are few reports for sensing small molecules, such as ATP[6]or cocaine.[7]

We designed a fluorescent DNA probe, which is composed of an aptamer and a pair of a fluorophore and a quencher to detect L-argininamide. The amino acid, arginine, has a charged guanidinium group and the guanidinium group interacts with several moieties of nucleic acids. The argininamide-binding DNA aptamers have been selected by Harada and Frankel[8] and the structures of the complexes of L-argininamide and its DNA aptamer have been reported by Patel et al.[9] and Wemmer et al.[10]

Design of fluorescence-labeled aptamer. The sequences of the DNA aptamer bound to L-argininamide referred to Harada's report.[8] We used the aptamer's sequence of which the structures were analyzed by NMR. The aptamer has a large hairpin loop. The binding of L-argininamide to this DNA aptamer leads to zipping up the loop through Watson–Crick base pairs, mismatch pairs, and base triplex formation. The structure of the complex of the aptamer changes from that of the free aptamer, that is to say that L-argininamide stabilizes the stem-loop structure. The stem-loop takes a random structure without the ligand if the stem is short. We attached a fluorophore and a quencher to oligodeoxyribonucleotides at the 5′-end and 3′-end, respectively. Scheme 1 shows the concept of the fluorescence-labeled aptamer. In the absence of the ligand, the aptamer cannot form the stem-loop structure depending on its length and temperature, that causes inhibition of the fluorescence of the

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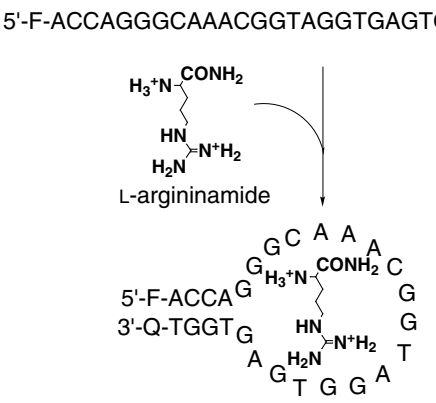
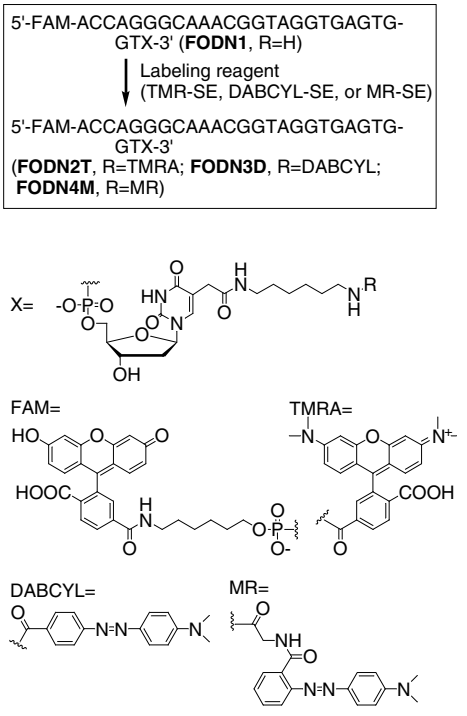


Scheme 1. Fluorescence-labeled aptamer for L-argininamide.

fluorophore at the 5′-end. The binding of the ligand to the aptamer domain will lead to stabilizing the stem-loop structure in the aptamer–ligand complex. This will result in quenching the fluorescence. We also studied the quencher molecule and the length of the stems to explore the optimum detection system based on the aptamer.

Synthesis of doubly fluorescent labeled ODNs. 5′- and 3′-labeled ODNs were chemically synthesized by a combination of a pre-synthetic modification method and a post-synthetic modification method. In this method, 5′-fluorescence-labeled ODN bearing 5-aminoalkyl-modified 2′-deoxyuridine at the 3′-end (Scheme 2, **FODN1**) was synthesized by a pre-synthetic modification method. The 5′-fluorescence group was incorporated into ODN by use of commercially available FAM phosphoramidite. The 5-aminoalkyl-modified 2′-deoxyuridine was incorporated into ODN at the 3′-end by using 5′-DMTr-5-methoxycarbonylmethyl-2′-deoxyuridine 3′-phosphoramidite. After the assembly of the ODN on a CPG support, it was treated with 1,6-diaminohexane to attach an aminoalkyl group at the 5-position of the modified nucleoside. The nucleoside analog and the method were developed by our group.[11] The modified ODNs were labeled by the succinimide esters of quenchers at 5-aminoalkyl-modified 2′-deoxyuridine. Several quenchers were used to assess a better fluorophore–quencher pair (Scheme 2, **FODN2T**, **FODN3D**, **FODN4M**).



Scheme 2. Synthesis of doubly fluorescent labeled ODNs and structures of modified moieties in ODNs.

Detection of the conformational change by the addition of L*-argininamide.* We first investigated the nature of this fluorescence-labeled aptamer in the absence of the ligand. Figure 1 shows the temperature dependence of the fluorescence intensity for **FODN2T**. The fluorescence intensity of fluorescein (520 nm) in **FODN2T** decreased dramatically as the temperature was lowered. On the other hand, the fluorescence intensity of **FODN1** decreased somewhat at low temperature, at which both **FODN1** and **FODN2T** formed the stem-loop structure. This result indicates that the emission from fluorescein of **FODN2T** is quenched by the proximate quencher molecule, TMRA, at the 3′-end of **FODN2T**. In the presence of L-argininamide, the temperature-dependent fluorescence intensity curve shifted to higher temperature. This result indicates that L-argininamide stabilizes the stem-loop structure of this labeled aptamer. The following ligand titration experiments were performed at 40 °C, at which the difference of the fluorescence intensities of **FODN2T** with and without L-argininamide was largest.

The titration is shown in Figure 2 along with other ligand titrations. When L-argininamide was added to the solution of **FODN2T**, the fluorescence intensities of fluorescein decreased with increasing concentration of L-argininamide. The selectivity did not change from the original aptamer because this fluorescence quenching was caused for only L-argininamide and was not

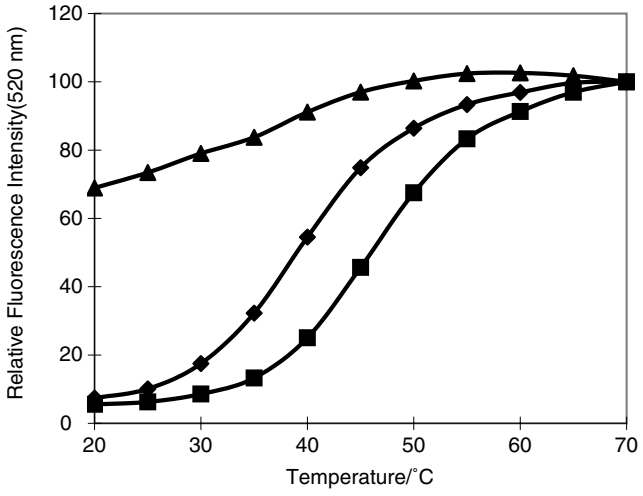


Figure 1. Temperature dependence of the fluorescence intensity of **FODN1** (triangle) in the absence of L-argininamide and **FODN2T** in the absence (diamond) and presence (square) of L-argininamide (180 mM) in 10 mM sodium phosphate buffer (pH 7.0).

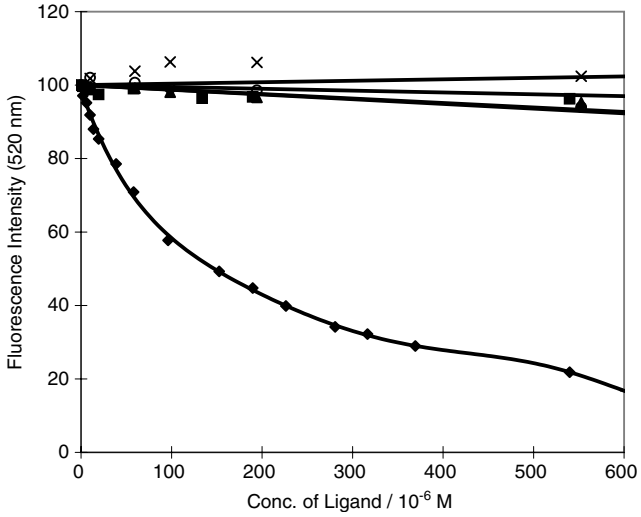


Figure 2. Fluorescence intensities of 0.2 μM **FODN2T** versus concentration of amino acids and other molecules (L-argininamide, diamond; L-arginine, square; glycine, triangle; L-lysine, × mark; guanidine, circle). Fluorescence spectra were recorded with excitation at 495 nm in 10 mM sodium phosphate buffer (pH 7.0) at 40 °C.

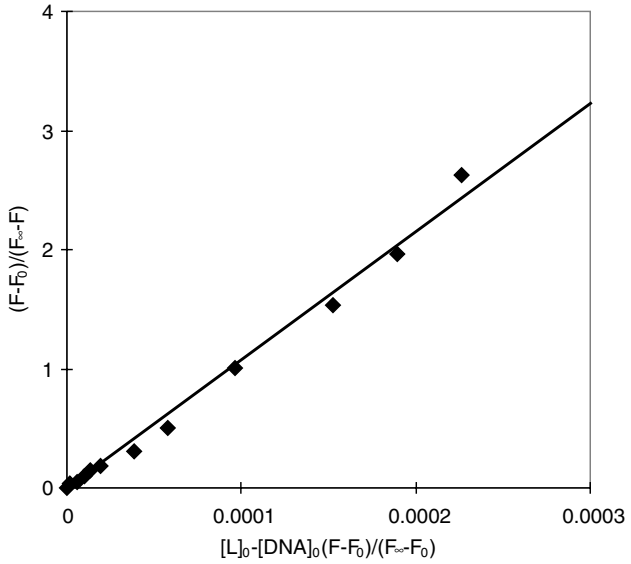


Figure 3. Determination of dissociation constant (K_d) of **FODN2T** against L-argininamide.

caused for other molecules, such as L-Arg, L-Lys, Gly, and guanidine. As this aptamer contains many guanine residues, the G-quartet structure is one of the possible structures. Takenaka et al.[12] reported a potassium-sensing ODN containing four GGG sequences. To evaluate the formation of the G-quartet structure, the effect of the potassium ion on the fluorescence intensity was investigated. The fluorescence intensities were not changed by the addition of the potassium ion (data not shown). This result suggests that the aptamer did not take the G-quartet structure under this experimental condition. This is consistent with the structures of the complexes of L-argininamide and its DNA aptamer reported previously.[9b]

The binding affinity was estimated from the change in fluorescence intensity based on Eq. 1.

$$(F - F_0)/(F_\infty - F) = K_b\{[\mathrm{L}]_0 - [\mathrm{DNA}]_0(F - F_0)/(F_\infty - F_0)\} \quad (1)$$

where F is the fluorescence signal in the presence of a ligand, F_0 is the fluorescence in the absence of a ligand, F_∞ is the fluorescence in the presence of an excess ligand, $[\mathrm{L}]_0$ is the total concentration of a ligand, $[\mathrm{DNA}]_0$ is the total concentration of DNA, K_b is the association constant, and K_d ($K_d = 1/K_b$) is the dissociation constant. Figure 3 shows the plot of $(F-F_0)/(F_\infty - F)$ versus $[\mathrm{L}]_0-[\mathrm{DNA}]_0(F - F_0)/(F_\infty - F_0)$ based on Eq. 1. From this figure, the binding affinity (K_d) of **FODN2T** against L-argininamide was 93 μM, while the binding affinity of the original aptamer was measured to be ~100 μM.[8] These results suggest that the terminal fluorescent and quenching groups did not influence the binding affinity of this aptamer.

We also investigated the effect of the stem length. **FODN2T** has four base pairs in the stem region. The fluorescence intensities of an ODN, which has six base pairs in the stem, were lower than that of **FODN2T** in the absence of the ligand (data not shown). As the fluorescence intensities of both ODNs were almost the same in the presence of ca. 700 μM L-argininamide, **FODN2T** shows a larger fluorescence change in addition of L-argininamide than the ODN which has six base pairs. This result suggests that the stem region must have moderate stability for switching the conformation.

Fluorescence quenchers. Three kinds of quenchers were attached to the aptamer at the 3′-position. The structures of quenchers (TMRA, DABCYL, and MR) are represented in Scheme 2. Fluorescence intensities of these aptamers versus concentrations of L-argininamide are plotted in Figure 4. **FODN3D** bearing DABCYL

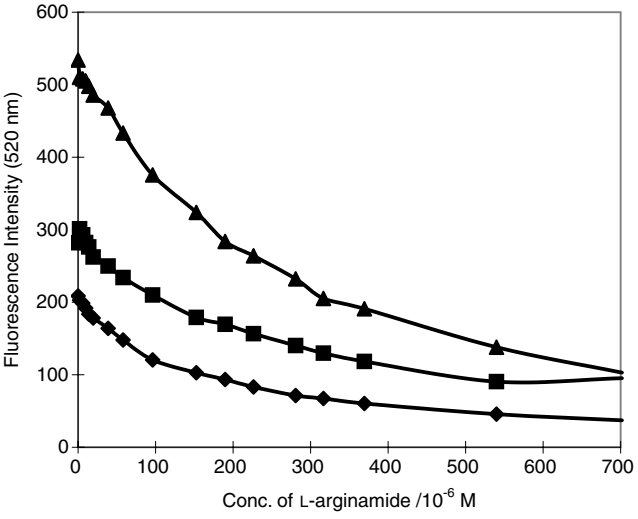


Figure 4. Effect of quenchers on fluorescence intensities versus concentration of L-argininamide. Fluorescence spectra of 0.2 μM **FODN2T** (diamond), **FODN3D** (square), and **FODN4M** (triangle) were recorded with excitation at 495 nm in 10 mM sodium phosphate buffer (pH 7.0) at 40 °C.

was similar to **FODN2T** except that the fluorescence intensities of **FODN3D** were higher throughout the entire region than that of **FODN2T**. On the other hand, **FODN4M** bearing methyl red represented a large fluorescence change according to the concentration of the ligand. From this figure, the fluorescence intensity of **FODN4M** was larger than that of **FODN2T** and **FODN3D** in the absence of L-argininamide. This result suggests that TMR and DABCYL quenched the fluorescence of fluorescein larger than MR at the open structure. The spectral overlap of the absorption spectra of TMR (λ_{max} = 540 nm) or DABCYL (λ_{max} = 453 nm) and the emission spectrum of FAM is larger than that of the absorption spectrum of MR (λ_{max} = 419 nm) and the emission spectrum of FAM. Therefore, Föster-type of energy transfer by an induced dipole mechanism occurs for **FODN2T** and **FODN3D** even at the open structure. When FAM is brought closer to the quenchers in the presence of L-argininamide, the fluorescence is quenched by an induced dipole mechanism, a collisional mechanism, and static quenching, which arise from a ground state intramolecular heterodimer.[13] MR may work mainly as the quencher by a collisional mechanism and static quenching rather than by an induced dipole mechanism. Therefore, the fluorescence intensity of **FODN4M** in the absence of the ligand was larger than that of **FODN2T** and **FODN3D**. Then the fluorescence-labeled aptamer bearing FAM as a fluorophore and MR as a quencher is the most suitable among the systems.

The fluorescence-labeled aptamers for L-argininamide were synthesized by a combination of pre- and post-synthetic modification methods which was useful to change the reporter groups. The designed and synthesized aptamers responded to the concentration of L-argininamide through changes in fluorescence. These end modifications did not affect the affinity of the aptamer. The stem length of the aptamer affected sensitivity but did not affect the binding affinity of this aptamer. The selection of quenchers for a fluorophore–quencher pair was important in terms of sensitivity for the detection of L-argininamide. This fluorescence-labeled aptamer will be easily adapted to a microtiter plate assay for high-throughput analysis.

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