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Ratiometric Fluorescent Signaling of Small Molecule Environmentally Sensitive Dye Conjugate for Detecting Single-Base Mutations in DNA

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Since the report on Ca^{2+} indicators by Tsien and co-workers,[1] ratiometric fluorescent probes have come to be recognized as a promising tool for biosensing and bioimaging studies.[2] Significant efforts have been devoted to develop this class of probes for a wide range of analytes including metal ions,[3] inorganic and organic anions,[4] enzyme activities,[5] and nucleic acids.[6–8] A typical way to produce the ratiometric signal is by using double fluorescent dyes, in which techniques based on fluorescence resonance energy transfer (FRET) are widely used.[2] For example, for the analysis of single-nucleotide polymorphism (SNP) in genomic DNAs, two-fluorophore-labeled hybridization probes have been developed based on the FRET strategy,[6,7] in which pairs of Alexa 488-Cy5,[6] 6-carboxyfluorescein-coumarin,[7a] carboxy-x-rhodamine-Cy3,[7b] and Cy3-Cy5[7c] molecules were utilized. Another major approach to produce the ratiometric signal is based on the formation of excimers,[6,8] and ratiometric fluorescent detection of SNP has been demonstrated by using pyrene-[6,8a,b] or perylene-bisimide[8c]-modified hybridization probes, and bispyrene diamines.[8d]

Herein, we report on a different way to design ratiometric fluorescent probes for SNP genotyping. In contrast to most approaches based on the interaction between two fluorescent dyes in hybridization probes,[6–8] we focused on the unique structure of the helical duplex to produce the ratiometric signaling. For this purpose, an environmentally sensitive fluorescent dye to probe hydrophobic grooves of the DNA helix was used,[9,10] in which the dye is conjugated with a fluorescent ligand developed by our group to recognize an orphan nucleobase opposite an abasic (AP) site in the DNA duplex.[11] Herein, a benzofurazan derivative, 4-(*N*,*N*-dimethylaminosulphonyl)-1,2,3-benzoxadiazole (DBD),[12] was utilized as the environmentally sensitive fluorescent dye, which is linked through an alkyl spacer to 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND).[11f] The resulting conjugate with an ethylene linker, ATMND–DBD (Figure 1), was used simultaneously with an AP-containing DNA probe as following two steps: 1) a target single-stranded DNA is hybridized with the AP-containing DNA probe

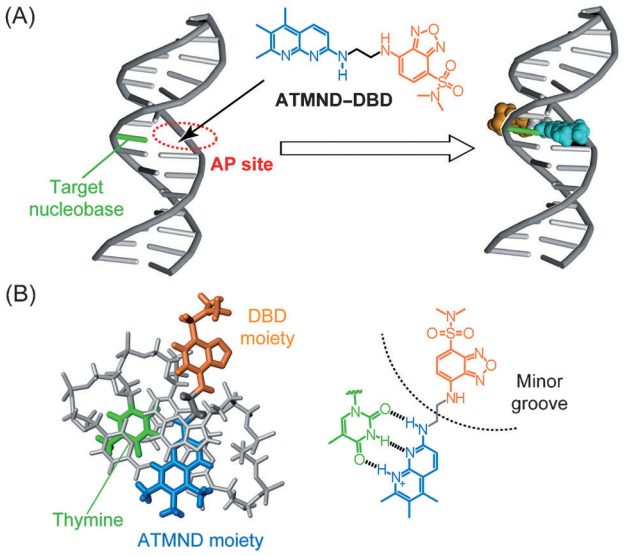

Figure 1. A) Schematic illustration of the binding of ATMND–DBD to the target nucleobase opposite an AP site in a DNA duplex. B) One of the possible binding modes of ATMND–DBD with thymine in the DNA duplex obtained with software MacroModel (Ver. 9.0, AMBER* force field, GB/SA solvent model). Thymine is shown in green, ATMND moiety in blue, and DBD moiety in orange. Pseudo-base pairing with thymine is also shown. In this binding mode, DBD moiety protrudes into the minor groove of the DNA duplex.

to place the AP site opposite a nucleobase to be detected; 2) the ligand (ATMND moiety) selectively binds to the orphan (target) nucleobase in the duplex through complementary hydrogen bonding, and the DBD moiety becomes located at the surface of the minor or major groove of the duplex (cf. also Figure S1 in the Supporting Information). Indeed, the binding of ATMND–DBD is highly selective to pyrimidine (C, T) over purine (G, A) bases, and a clear ratiometric response was obtained based on quenching of the ATMND emission (403 nm) and enhancing of the DBD emission (615 nm). Although the sensing ability depends on the DNA sequence to some extent, the limit of detection (LOD) reaches to nanomolar level, and the ratiometric response to 500 nM samples can be judged even with the naked eye. Therefore, our approach offers a convenient and

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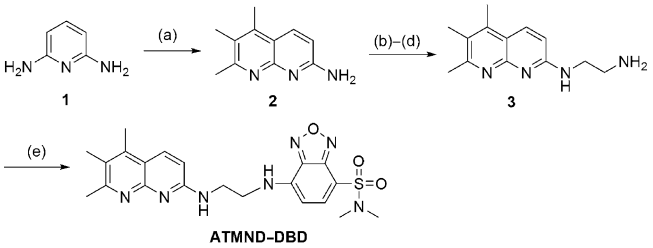
label-free method for detecting SNP as has been reported previously,[11] and this study further offers a basis for the advanced design of ratiometric fluorescent probes for detecting DNAs, especially for SNP genotyping.

ATMND–DBD was synthesized in five steps from 2,6-diaminopyridine (Scheme 1 and the Supporting Information).[13] The introduction of the ethylene linker (1,2-diaminoethane) to the naphthyridine ring was done according to



Scheme 1. Synthesis of ATMND–DBD. Reagents and conditions: a) 3-methylpentane-2,4-dione, H_3PO_4, 48%; b) $NaNO_2$, H_2SO_4; c) $POCl_3$; d) 1,2-diaminoethane, 31% (three steps); e) DBD-F, Et_3N, DMF, 36%.

the literature,[13b] and then the DBD moiety was attached. For comparison, two conjugates with different lengths of the linker (see the Supporting Information: C3, trimethylene; C4, tetramethylene) were also prepared similar to ATMND–DBD.

Figure 2A shows the fluorescence responses of ATMND–DBD (2.0 μM) upon addition of 21-meric AP-site-containing DNA duplexes (2.0 μM; 5′-GCA GCT CCC GXG GTC TCC TCG-3′/3′-CGT CGA GGG CNC CAG AGG AGC-5′, X = AP site; SpacerC3 (a trimethylene residue), N = G, C, A, or T) at 20 °C in solutions buffered to pH 7.0. ATMND moiety exhibited the emission band with a maximum at 420 nm when excited at 376 nm (the absorption maximum wavelength of ATMND moiety), and the emission is quenched in the presence of DNA duplexes. As has been observed for parent ATMND,[11f] the responses are significant for pyrimidine bases (N = T and C), whereas moderate responses were observed for purine bases (N = G and A). It is highly likely that ATMND moiety binds to the AP site of the DNA duplex, and the binding is promoted selectively to pyrimidine bases by three-point hydrogen bonding (Figure 1B).[11f]

The fluorescence response of DBD moiety was clearly observed and is also highly selective to pyrimidine bases over purine bases (Figure 2A, right). In the absence of DNA duplexes, the weak emission with a maximum at 615 nm was observed when excited at 451 nm (the maximum absorption wavelength of DBD moiety), and the binding to thymine (N = T) or cytosine (N = C) resulted in the enhancement of fluorescence intensities. The binding to these two nucleobases was also accompanied by a 30 nm blueshift of the emission-maximum wavelength, indicating a change in the microenvironment of the DBD moiety. A hypothetical binding of DBD moiety to the AP site is exclusive, because a control ligand, a DBD derivative that lacks ATMND moiety,

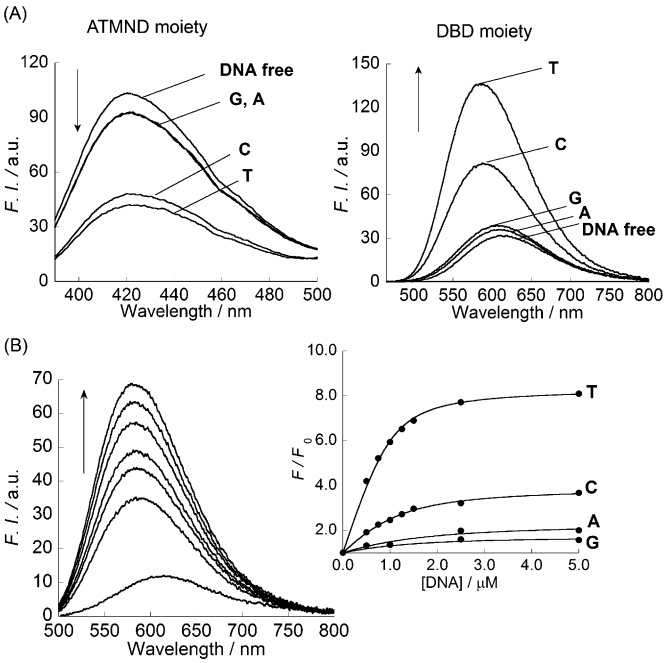


Figure 2. A) Fluorescence responses of ATMND–DBD (2.0 μM) to target nucleobases (N = G, C, A, or T) in 21-meric AP-site-containing DNA duplexes (2.0 μM) in solutions buffered to pH 7.0 (10 mM sodium cacodylate) and containing NaCl (100 mM) and EDTA (1.0 mM, water/ethanol 97.2:2.8). Excitation: ATMND moiety, at 376 nm; DBD moiety, at 451 nm; $T = 20$ °C. Duplex: 5′-GCAGCTCCC GXG GTCTCCTCG-3′/3′-CGTCGAGGG CNC CAGAGGAGC-5′, X = AP site; SpacerC3 (a trimethylene residue), N = G, C, A, or T. B) Fluorescence titration for the binding of ATMND–DBD (1.0 μM) to thymine (N = T) in the 21-meric AP-site-containing DNA duplex (0–5.0 μM). Other conditions are the same as those for Figure 2A. Inset: nonlinear regression analysis of the fluorescent intensity ratio at 585 nm based on a 1:1 binding isotherm model. F and F_0 denote the intensities of ATMND–DBD in the presence and absence of duplexes, respectively.

showed no fluorescence responses to 21-meric AP-site-containing DNA duplexes (Figure S2 in the Supporting Information). Therefore, it is feasible that the observed response of DBD moiety is due to its being in the hydrophobic microenvironment of a surrounding DNA molecule, namely, the major or minor groove of the duplex DNA, when ATMND–DBD binds to the AP site (Figure 1B and Figure S1 in the Supporting Information).

The binding affinity of ATMND–DBD was then quantitatively determined by fluorescence-titration experiments, for which the responses of DBD moiety were utilized. As can be seen from the typical response to thymine (Figure 2B), the response is concentration dependent, which is well fitted by a nonlinear least-square regression analysis based on a 1:1 binding isotherm (Figure 2B, inset). ATMND–DBD showed the strongest binding affinity for thymine with a 1:1 binding constant K_{11} of $6.7(\pm0.6)\times10^6\,\text{M}^{-1}$ ($n = 3$). The affinity for cytosine ($K_{11} = 2.2(\pm0.6)\times10^6\,\text{M}^{-1}$, $n = 3$) is comparable to the affinity for thymine, although it is two orders of magnitude lower for guanine and adenine ($K_{11}/10^6\,\text{M}^{-1}$: G, 0.05; A, 0.06). We note that the binding selectivity and affinity of ATMND–DBD are comparable to those of the parent

ligand, ATMND,[11f] indicating that conjugation with the DBD moiety has a small influence on the original binding properties of the pyrimidine-selective ATMND.

By keeping such binding properties, ATMND-DBD can provide ratiometric fluorescence signaling to detect pyrimidine nucleobases (Figures 3A and S3 in the Supporting Information). We assessed the binding-induced change in the ratio of the two emission intensities (F_{585}/F_{420}), that is, the emission of DBD moiety at 585 nm (F_{585}) and the emission of ATMND moiety at 420 nm (F_{420}). In the case of detection of thymine in the 21-meric DNA duplex, a linear response of the emission ratio (F_{585}/F_{420}) was obtained in the concentration range from 0 to 500 nM. LOD was estimated to be 16 nM. A linear response was also obtained for cytosine with LOD of 32 nM. In contrast, the responses are almost negligible for guanine and adenine in this concentration range. Therefore, ATMND–DBD will provide analysis of polymerase-chain reaction (PCR)-amplification products with a sufficient sensitivity and selectivity for pyrimidine bases (see below).

The ratiometric response of ATMND–DBD can be effectively obtained irrespective of the nucleobases flanking the AP site (N′XN′, N′ = G, C, A, or T), and the response is highly specific to pyrimidine over purine bases (Figure S4 in the Supporting Information). However, the response selectivity between cytosine and thymine depends on the flanking nucleobases. Although the response is selective to thymine for GXG, the cytosine-selective response is obtained for TXT and AXA, and the response is comparable for CXC. More importantly, the response of DBD moiety becomes larger than that for GXG, probably due to the difference in the microenvironment surrounding the DBD moiety. As for the response to cytosine (2.0 μM), the largest increase in the intensity by 61-fold is observed for AXA with a maximum at 565 nm, and the response follows the order of AXA > TXT (35-fold, 566 nm) > CXC (12-fold, 579 nm) > GXG (3.6-fold, 585 nm). The response to thymine is the largest also for AXA (50-fold, 563 nm), and follows the order of AXA > TXT (17-fold, 567 nm) > CXC (15-fold, 579 nm) > GXG (7.5-fold, 581 nm). Therefore, as compared with pyrimidine detection demonstrated for GXG (Figure 3A), sensitivity would be better for the other three sequences. Indeed, for the analysis of AXA/TNT (Figures 3B and S3 in the Supporting Information), the LOD for cytosine and thymine was estimated to be 1.4 and 2.1 nM, respectively, and the ratiometric responses to 500 nM samples can be judged even with the naked eye—the change in the emission color is clearly seen for pyrimidine bases under UV irradiation (Figure 3C).

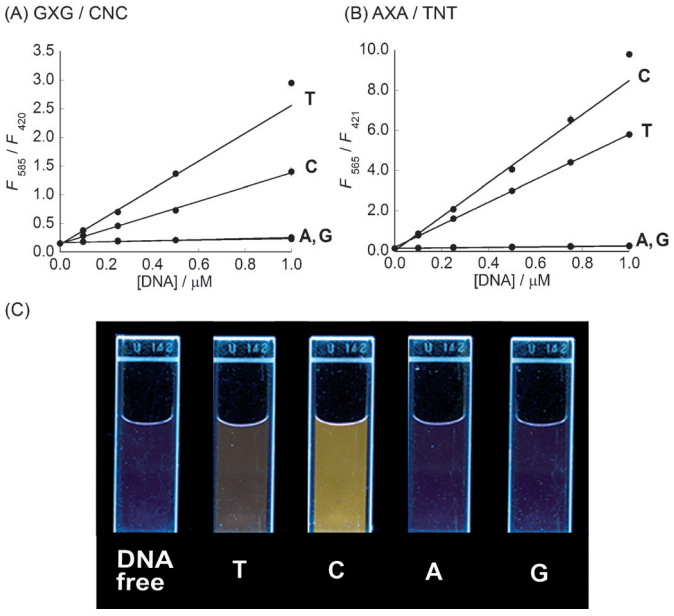


Figure 3. Ratiometric responses of ATMND–DBD (2.0 μM) to detect nucleobases (N = G, C, A, or T) in 21-meric AP-site-containing DNA duplexes (5′-GCA GCT CCC N′XN′ GTC TCC TCG-3′/3′-CGT CGA GGG N′NN′ CAG AGG AGC-5′, X = AP site; SpacerC3, N′ = G or A): A) GXG/CNC; B) AXA/TNT. Sample solutions containing NaCl (100 mM) and EDTA (1.0 mM, water/ethanol 97.2:2.8) were buffered to pH 7.0 (10 mM sodium cacodylate). Excitation: ATMND moiety, at 376 nm; DBD moiety, at 451 nm. Analysis: 420 (F_{420}) or 421 (F_{421}), 585 (F_{585}), or 565 nm (F_{565}). T = 20 °C. LOD was estimated from LOD = 3 (S_{blank}/slope). C) Visual detection of nucleobases (500 nM, AXA/TNT, N = G, C, A, or T) by ATMND–DBD (500 nM). Samples were excited with a UV lamp at 365 nm and photographed with a digital camera. T = 23 °C (RT). Other conditions are the same as those for Figure 3A and B.

These useful ratiometric responses cannot be obtained by conjugates with a longer linker between ATMND and the DBD moiety (Figure S5 in the Supporting Information). In the case of the trimethylene-linker-containing conjugate (C3), the response of the DBD moiety becomes smaller compared with that of ATMND–DBD with an ethylene linker, and the resulting ratiometric response is only moderate even in the presence of 2.0 μM DNA samples (F_{585}/F_{420}: T, 1.3 ± 0.17; C, 0.8 ± 0.01; A, 0.2 ± 0.01; G, 0.2 ± 0.01; DNA free, 0.17 ± 0.01). In the case of the conjugate with the longer tetramethylene linker (**C4**), the ligand has almost no responses attributable to the DBD moiety. Thus, the longer linker in the conjugates resulted in the decreased ratiometric fluorescence response. In addition, the binding affinities of these conjugates for thymine are one order of magnitude lower than that of ATMND–DBD (Figure S6 in the Supporting Information; $K_{11}/10^6\,M^{-1}$: C3, 0.53 ± 0.03; C4, 0.11 ± 0.02). Apparently, the binding and sensing properties strongly depend on the spacer, and the choice of the linker length is crucial for the design of this type of conjugates.

It was noted herein that two fluorescent moieties (ATMND and DBD) can be excited simultaneously to give the ratiometric response. This can be seen from the change in the emission color (Figure 3C, excitation at 365 nm), and also from the whole emission spectra upon excitation at 375 nm, Figure S7 in the Supporting Information). However, as shown in Figure 2, the excitation at longer wavelength (451 nm, absorption maximum of DBD moiety) makes the response of DBD moiety more significant compared with that obtained by the excitation at shorter wavelength (375 nm). Thus, in the case of ATMND–DBD, doubled irradiation is better way to obtain more effective ratiometric re-

sponses and would be not always drawback to the analysis of in vitro samples.

Finally, ATMND–DBD was applied to the analysis of the single-base mutation present in 107-meric DNAs (*K-ras* gene, sense trand, codon 12: GNT, N = G, C, A or T).[14] After PCR amplification (see the Supporting Information), an aliquot from the PCR product was analyzed in buffer solution (pH 7.0) containing ATMND–DBD and 20-meric AP-site-containing probe DNA (Figure S8 in the Supporting Information). ATMND–DBD showed the all-or-none responses to pyrimidine over purine bases (F_{585}/F_{420}: T 1.0; C 1.5; A 0.6; G, 0.6; control 0.5) so as to distinguish between the two types of nucleobases. ATMND–DBD would be therefore applicable to the analysis of transversion, that is, replacements of purine for pyrimidine bases (or vice versa), such as A/C, C/G, A/T, and G/T SNPs. Herein, the analysis requires no time-consuming steps, such as purification of PCR products and careful control of temperature, and the result is readily obtained after PCR.

In summary, a ratiometric fluorescent signaling probe, ATMND–DBD, for SNP genotyping was successfully developed. In contrast to conventional approaches based on FRET or excimer formations in hybridization probes, the design of small ligand-based probes with the environmentally sensitive fluorescent dye was demonstrated. The ratiometric signal was indeed effective, and the strategy would be sufficiently flexible for further design of this class of probes to detect all possible mutations. Also, because the naphthyridine (ATMND) moiety can bind to the other non-Watson–Crick base-pairing sites in DNA duplex, as was demonstrated in our previous work,[15–17] ATMND–DBD would have a potential for further applications, such as gap–site binding,[15] detecting mismatches,[16] and affinity labeling in aptamer assays or molecular beacons.[17] We are now undertaking further studies in this direction.

Experimental Section

Synthesis of ATMND–DBD: DBD-F (4-(*N*,*N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole, 50.3 mg, 0.205 mmol) and triethylamine (2 mL) were added to a solution of **3** (50.1 mg, 0.205 mmol) in DMF (8 mL). The reaction mixture was stirred under nitrogen gas at RT for 24 h and then concentrated in vacuo. The crude product was purified by FPLC ($CHCl_3$/MeOH) on a NH_2-modified silica gel with the gradient elution system to give ATMND–DBD (33.6 mg, 0.0737 mmol, 36%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.36 (s, 3H), 2.52 (s, 3H), 2.71 (s, 3H), 2.84 (s, 6H), 3.77 (t, 2H, J = 5.5 Hz), 4.06 (t, 2H, J = 5.5 Hz), 6.26 (d, 1H, J = 8.0 Hz), 6.62 (d, 1H, J = 9.0 Hz), 7.86 (d, 1H, J = 8.0 Hz), 8.03 ppm (d, 1H, J = 9.0 Hz); HRMS (ESI) for calcd for $C_{21}H_{26}N_7O_3S$: 456.1818 $[M+H]^+$; found: 456.1812.

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Keywords: DNA recognition · fluorescent probes · ligand design · noncovalent interactions · nucleobases

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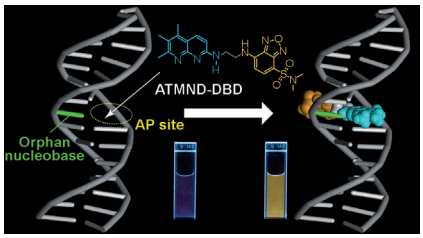
Bind it! A ratiometric fluorescent probe based on conjugation of a DNA-binding ligand with an environmentally sensitive dye to probe hydrophobic grooves of the DNA helix was developed. The resulting conjugate ATMND–DBD can bind to an orphan nucleobase opposite an abasic (AP) site with high selectivity and affinity for pyrimidine bases (see figure).



Fluorescent Probes

C.-x. Wang, Y. Sato, M. Kudo, S. Nishizawa, N. Teramae .* ■■■■–■■■■

Ratiometric Fluorescent Signaling of Small Molecule Environmentally Sensitive Dye Conjugate for Detecting Single-Base Mutations in DNA