Fluorescent triplex-forming DNA oligonucleotides labeled with a thiazole orange dimer unit

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Fluorescent probes for the detection of a double-stranded DNA were prepared by labeling a triplex-forming DNA oligonucleotide with a thiazole orange (TO) dimer unit. They belong to ECHO (exciton-controlled hybridization-sensitive fluorescent oligonucleotide) probes which we have previously reported. The excitonic interaction between the two TO molecules was expected to effectively suppress the background fluorescence of the probes. The applicability of the ECHO probes for the detection of double-stranded DNA was confirmed by examining the thermal stability and photophysical and kinetic properties of the DNA triplexes formed by the ECHO probes.

Introduction

Triplex-forming DNA oligonucleotides (TFOs) bind specifically to polypurine tracts of double-stranded DNA.^{1,2} Triplex formation for the DNA recognition has the remarkable advantage of not requiring strand opening of the target DNA duplex. TFOs have been used to recognize DNA in a living cell for the purposes such as regulation of transcription,³ promotion of homologous recombination⁴ and mutation of genomic DNA.⁵ Fluorescent probes might become a strong tool to obtain spatiotemporal information on the behavior of such TFOs in living cells. Triplex-based fluorescent probes which exhibit changes in fluorescence intensities or wavelengths upon hybridization with a double-stranded DNA have been reported to detect a DNA triplex formation or facilitate the analysis of the thermal stability of DNA triplexes.^{6–14} Although the probes utilizing Förster resonance energy transfer (FRET) such as molecular beacons could control the emission of the fluorescence, they normally require two kinds of dyes, a fluorophore and quencher, and an extra structure in addition to the sequence-recognition moiety.^{6–9} In contrast, the probes labeling with a single kind of dye have advantages in the facile synthesis and design.^{10–15} Pyrimidine TFOs labeled with thiazole orange (TO) exhibited comparably larger changes in fluorescence intensities before and after triplex formation^{14,15} because the fluorescence intensity of TO molecules is low in the presence of poly-pyrimidine single-stranded DNA¹⁶ and high in the presence of DNA triplexes.^{15,17}

To suppress the fluorescence of the TO molecules covalently attached to DNA oligonucleotides, the utilization of excitonic interaction between two molecules of TO is effective.^{18,19} Exciton theory explains that the fluorescence of two dye molecules forming an H-dimer is strongly suppressed.²⁰ This effect is caused by

a splitting of the excited state of dye molecules into two energy levels, and excitation to the upper energy level is followed by internal conversion to the lower energy level. Radiative transitions from the lower energy level to the ground state are forbidden. We have reported fluorescent oligonucleotide probes labeled with a TO dimer unit, which we call ECHO (exciton-controlled hybridization-sensitive fluorescent oligonucleotide) probes, for the detection of single-stranded DNA and RNA.^{21–32} ECHO probes emitting low background fluorescence were expected to be also applicable to detect double-stranded DNA as TO molecules emit intense fluorescence in the presence of DNA triplexes.^{15,17} However, DNA triplexes formed by ECHO probes have not been reported and the photophysical properties are unknown. Besides the photophysical properties, the effects on the thermal stability and kinetic parameters of the DNA triplexes formed by the ECHO probes, which are caused by the interaction of the two TO molecules with the DNA triplexes, are also important, because DNA triplexes intrinsically have a low thermal stability and slow formation rate.^{33–42} Higher thermal stability and larger formation rate than those of the natural DNA triplexes are favorable for the detection of DNA duplexes.

In this study, we investigated the photophysical properties of the DNA triplexes formed by ECHO probes by measuring the absorption and fluorescence spectra of the probes in a single-stranded and DNA triplex form at pH 6.0, 7.0 and 8.0. The dissociation temperatures of the ECHO probes were determined from the melting curves obtained by monitoring the absorptions at 260 and 510 nm. The association rate constants of the DNA triplexes formed by the ECHO probes were calculated by measuring the fluorescence intensities at the elapsed times from the mixing of the probes and the target DNA duplex.

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Figure 1. (A) DNA sequences used in this study. "M" denotes 5-methylcytosine. (B) Chemical structure of the TO-labeled thymidine derivative described as D₅₁₄.

Figure 2. Absorption spectra of the ECHO probes in the absence (gray line) and presence (black line) of the D₁/D₂ duplex at pH 7.0. The insets show the absorption spectra from 400 to 600 nm. (A) Absorption spectra of T₂. (B) Absorption spectra of T₃. (C) Absorption spectra of T₄.

Results and Discussion

The sequences of the DNA oligonucleotides used in this study are shown in **Figure 1A**. T₁ was a triplex-forming DNA 15-mer containing 5-methylcytosine (described as M). 5-methylcytosine has a slightly higher pK_a value than that of cytosine, and DNA triplexes containing 5-methylcytosine are more stable at higher pH than are DNA triplexes containing cytosine.⁴³ T₂, T₃, T₄, and T₅ were TO-labeled TFOs. The TO-labeled thymidine residue was termed D₅₁₄ (**Fig. 1B**). The preparation of T₂, T₃, T₄ and T₅ was performed as described in our previous reports.^{28,31,32} D₁ and D₂ were DNA 22-mers that form a double-stranded DNA. The D₁/D₂ duplex is a target of T₁, T₂, T₃, T₄ and T₅ for the formation of DNA triplexes. The DNA 15-mer, D₃, was complementary to T₁, T₂, T₃, T₄ and T₅ and formed double-stranded DNA duplexes with these molecules. The DNA oligonucleotides shown in **Figure 1A** were identified using MALDI-TOF mass spectrometry.

The connection of a TO dimer unit covalently to a thymidine residue in DNA oligonucleotides led to the occurrence

of a structural change in the TO groups from an H-dimer to monomeric units upon the hybridization with a DNA or RNA complementary strand, which was deduced from the red shift of the visible light absorption derived from the TO moiety.³⁰⁻³² Similarly, the wavelengths of the absorption maximum of the two TO molecules on the TFOs might be changed by the DNA triplex formation. To confirm this, the absorption spectra of T₂, T₃ and T₄ in the absence and presence of a D₁/D₂ duplex were measured in buffers at pH 6, 7 and 8. The absorption spectra of T₂, T₃ and T₄ at pH 7 are shown in **Figure 2**. In the absence of the D₁/D₂ duplex, the wavelength of the absorption maximum derived from the TO moiety was 477 or 478 nm. In the presence of the D₁/D₂ duplex, the wavelength of the absorption maximum was red shifted to 510 or 511 nm for T₂ and T₃. The shift of the absorption maximum suggested that the dissociation of the H-dimer occurred upon the DNA triplex formation. However, the spectra of T₄ in the presence and absence of the D₁/D₂ duplex exhibited almost the same shape. In a buffer at pH 6, all spectra of T₂, T₃ and T₄ showed a red shift in the presence of the D₁/

Figure 3. Fluorescence spectra of the ECHO probes in the absence (gray line) and presence (black line) of the D_1/D_2 duplex at pH 7.0. (A) Fluorescence spectra of T_2 . (B) Fluorescence spectra of T_3 . (C) Fluorescence spectra of T_4 .

D_2 duplex similar to what was observed for T_2 and T_3 at pH 7 (Fig. S1A). In a buffer at pH 8, none of the spectra exhibited the red shift of the absorption maximum (Fig. S1B). These pH-dependent findings were consistent with the general property of DNA triplexes. As an acidic condition favors for the protonation of 5-methylcytosine, T_2 , T_3 and T_4 were thought to form thermally more stable DNA triplexes at pH 6 than at a higher pH. At pH 8, the difficulty in the protonation of 5-methylcytosine would weaken the thermal stabilities of the DNA triplexes. At pH 7, T_4 might form a thermally less stable DNA triplex compared with the DNA triplexes formed by T_2 or T_3 .

To examine the enhancement of the fluorescence intensity of the ECHO probes by the formation of the DNA triplexes, the fluorescence spectra of T_2 , T_3 and T_4 in the absence and presence of the D_1/D_2 duplex were measured in buffers at pH 6, 7 and 8. The fluorescence spectra of T_2 , T_3 and T_4 at pH 7 obtained by excitation with 488 nm light are shown in Figure 3. All the ECHO probes emitted negligible fluorescence in the absence of the D_1/D_2 duplex, in contrast, the fluorescence of T_2 and T_3 was highly enhanced in the presence of the D_1/D_2 duplex. T_4 exhibited a moderate enhancement of the fluorescence, which might be caused by the H-dimer remained in the presence of the D_1/D_2 duplex. The fluorescence enhancement of T_2 , T_3 and T_4 was higher at lower pH buffer solutions (Fig. S2). Lower pH conditions facilitate the protonation of 5-methylcytosine, which causes the thermal stabilization of the DNA triplexes. The pH dependence of the fluorescence intensities indicated that the thermal stability of the DNA triplex influences the enhancement of the fluorescence intensities emitted from the ECHO probes upon the DNA triplex formation. The results of the absorption and emission spectrometry are summarized in Table 1. The ratio of the fluorescence intensities after and before the DNA triplex formation at pH 6.0, at which stable DNA triplexes were thought to be formed, was 46–97. These values suggested that the intramolecular excitonic interactions between the two molecules of TO worked effectively to suppress the fluorescence intensity of the ECHO probes in the absence of the D_1/D_2 duplex. The quantum yields of the ECHO probes forming the DNA triplexes at pH 6.0 were 0.44–0.51, which indicated that the ECHO probes exhibited almost the same or

higher fluorescence emission efficiency after the formation of the thermally stable DNA triplexes compared with the formation of DNA or RNA duplexes.^{30–32}

To investigate the thermal stability of the DNA triplexes formed by the ECHO probes, thermal melting experiments of the DNA triplexes were performed and the absorbance at 260 nm of the DNA triplexes was monitored with increasing temperature. The melting curves of T_1 and T_2 in the presence of the D_1/D_2 duplex at pH 6, 7 or 8 are shown in Figure 4A and B. The melting curve of T_1 showed two transitions and the dissociation temperature of T_1 and the melting temperature of the D_1/D_2 duplex were determined separately from the melting curves at pH 6, 7 or 8. In contrast, the melting curves of T_2 at pH 6 or at pH 8 seemed to have only one transition and the determination of the dissociation temperature of T_2 was difficult. Therefore, the absorbance at 510 nm derived from the TO molecules was measured to monitor the dissociation of T_2 . As the UV experiments indicated that the dissociation of the H-dimer occurred upon the DNA triplex formation, it should be possible to monitor the dissociation of the ECHO probes from the D_1/D_2 duplex by measuring the visible light absorption derived from the TO molecules. The melting curves plotted by using the absorbance at 510 nm are shown in Figure 4C. Although the absorption at 260 nm reflects the local structures of all the DNA bases, the absorption at 510 nm derived from the TO molecules reflects the structural changes around the TO-labeled nucleotide. Consequently, if the length of the ECHO probes is longer or the two molecules of TO are attached at the end of the TFOs, the melting curves obtained by monitoring the absorptions at 260 and 510 nm might exhibit a large difference. Nevertheless, in the cases for which the melting temperature of the DNA triplex could be determined from the absorption at 260 nm, the differences of the melting temperatures derived from the absorptions at 260 and 510 nm were, at most, 2°C for the ECHO probes used here. The melting temperatures are summarized in Table 2.

Here, for the analysis of the dissociation of the ECHO probes from the D_1/D_2 duplex, we adopted the melting temperatures derived from the absorption at 510 nm. The melting temperatures of the D_1/D_2 duplex were ~66°C at pH 6, 7 and 8. The dissociation temperatures of T_2 , T_3 and T_4 from the D_1/D_2 duplex

Table 1. Photophysical properties of the ECHO probes

			$\lambda_{\max}$ (Abs) ^a	$\lambda_{\max}$ (Ex) ^b	$\lambda_{\max}$ (Em) ^c	Φ ^d	F/F _{ss} ^e
T ₂	pH 6	Single-strand	477 nm (64,000), 508 nm (44,000)	515 nm	554 nm	0.011	-
		+D ₁ and D ₂	485 nm (48,000), 511 nm (60,000)	517 nm	535 nm	0.51	97
	pH 7	Single-strand	477 nm (59,000), 505 nm (39,000)	512 nm	558 nm	0.0060	-
		+D ₁ and D ₂	482 nm (51,000), 510 nm (54,000)	517 nm	535 nm	0.36	67
	pH 8	Single-strand	478 nm (57,000), 506 nm (41,000)	517 nm	569 nm	0.008	-
		+D ₁ and D ₂	478 nm (61,000), 507 nm (46,000)	514 nm	535 nm	0.023	4.8
T ₃	pH 6	Single-strand	478 nm (59,000), 505 nm (42,000)	510 nm	532 nm	0.031	-
		+D ₁ and D ₂	486 nm (47,000), 511 nm (62,000)	517 nm	534 nm	0.49	46
	pH 7	Single-strand	478 nm (58,000), 505 nm (38,000)	513 nm	531 nm	0.019	-
		+D ₁ and D ₂	486 nm (44,000), 511 nm (56,000)	517 nm	534 nm	0.46	80
	pH 8	Single-strand	478 nm (57,000), 506 nm (36,000)	516 nm	568 nm	0.012	-
		+D ₁ and D ₂	478 nm (52,000), 507 nm (45,000)	516 nm	532 nm	0.12	23
T ₄	pH 6	Single-strand	478 nm (80,000), 505 nm (56,000)	515 nm	558 nm	0.016	-
		+D ₁ and D ₂	485 nm (63,000), 510 nm (70,000)	517 nm	536 nm	0.44	79
	pH 7	Single-strand	478 nm (68,000), 507 nm (50,000)	513 nm	549 nm	0.013	-
		+D ₁ and D ₂	479 nm (59,000), 509 nm (54,000)	516 nm	534 nm	0.15	23
	pH 8	Single-strand	478 nm (62,000), 508 nm (53,000)	513 nm	548 nm	0.011	-
		+D ₁ and D ₂	478 nm (63,000), 507 nm (59,000)	513 nm	531 nm	0.029	4.8

^aAbsorption $\lambda_{\max}$ of the ECHO probes. The values in the parenthesis are the molar extinction coefficients. ^bExcitation $\lambda_{\max}$ of the ECHO probes. ^cEmission $\lambda_{\max}$ of the ECHO probes. ^dQuantum yield of the ECHO probes. ^eRatio of the fluorescence intensities of the ECHO probes in the presence and absence of the D₁/D₂ duplex. Those values were calculated by using the fluorescence intensities at the emission $\lambda_{\max}$.

were higher than those of T₁ at pH 6 and 7. Therefore, the thermal stability of the DNA triplex at pH 6 and 7 was enhanced by the incorporation of two molecules of TO on the TFO. The dissociation temperatures of the TFOs, T₁, T₂, T₃ and T₄, from the D₁/D₂ duplex were higher at pH 6 than at pH 7, as the protonation of 5-methylcytosine is favored in acidic conditions. At pH 8, as for T₂, T₃, and T₄, the melting curves derived from the absorption at 260 nm showed a single transition and the melting curves derived from the absorption at 510 nm showed no transition. The results indicated that the ECHO probes do not form a triplex at pH 8. The TFOs exhibited an increase in the dissociation temperatures in the following order, T₃ > T₂ > T₄ > T₁, at pH 7. Although the thermal stabilization effect of the TO-labeling on the TFOs was different among T₂, T₃ and T₄, this difference regarded only the position of the TO-labeling. The difference of the dissociation temperatures of the TFOs might be caused by the differences in the sequences surrounding the TO-labeling sites. T₄ has 5-methylcytosine residues on both sides of D₅₁₄, a thymidine residue labeled with TO. When protonated 5-methylcytosine with a positive charge is close to the intercalation sites of TO, the electric repulsion between the protonated 5-methylcytosine and a TO moiety containing a positive charge might inhibit the interaction between the TO and the DNA triplex, resulting in the reduction of the thermal stabilization effect of the TO on the TFOs. Therefore, it is suggested that the TO-labeling site on TFOs should be located away from cytosine or 5-methylcytosine, for the effective stabilization via the introduction of TO molecules on TFOs.

To estimate the association rate constant of the DNA triplexes, the fluorescence intensities of the ECHO probes were monitored. Although they emitted negligible fluorescence in the absence of D₃ or the D₁/D₂ duplex, they exhibited intense fluorescence in the duplex or triplex form. The fluorescence intensity of the ECHO probes was proportional to the concentration of the DNA triplex (Fig. S3), assuming that all the TFOs bound to the D₁/D₂ duplex after 16 h equilibration. Hence, it is possible to monitor the increase in the DNA triplex concentration by measuring the fluorescence intensity of the samples containing the ECHO probes and the D₁/D₂ duplex. The fluorescence intensities of T₂, T₃, T₄, and T₅ at 25°C and pH 6 in the presence of D₃ or the D₁/D₂ duplex were measured over time (Fig. 5). The time course of the fluorescence intensities of the DNA duplex formation indicated that the hybridization of T₂, T₃, T₄ or T₅ with D₃ was mostly completed within 60 min. In contrast, the increase rates of the fluorescence intensity after mixing T₂, T₃, T₄ and T₅ with the D₁/D₂ duplex for the formation of the DNA triplexes were relatively slower than those observed in the cases of DNA duplex formation. A simple model in which the dissociation rate constant was assumed as zero was used to estimate the association rate constant similarly to a previously reported method,³⁸ as the most of the reported dissociation rate constants of the TFOs were 7–9 orders of magnitude smaller than the association rate constants.^{38–42} The association rate constant k_1 values for the DNA triplexes calculated by curve-fitting are shown in Figure 5B. The k_1 values of the TFOs labeled with two molecules of TO were 1,430–2,190

Figure 4. Melting curves of the DNA triplexes at pH 6, 7 and 8. (A) Melting curves of the DNA triplex formed by T_1 . The absorbance at 260 nm was monitored. (B) Melting curves of the DNA triplex formed by T_2 . The absorbance at 260 nm was monitored. (C) Melting curves of the DNA triplex formed by T_2 . The absorbance at 510 nm was monitored.

$M^{-1}s^{-1}$. These values were larger than k_1 value of T_1 which was calculated as $690 M^{-1}s^{-1}$ by monitoring UV absorption at 260 nm (Fig. S4).³⁸

In contrast, T_5 labeled with four molecules of TO at two thymidine residues exhibited a much larger k_1 value ($9,490 M^{-1}s^{-1}$). The rate constant of the DNA triplex formation was largely increased when the number of TO molecules attached to TFOs increased. The reason might be the sequence-independent interaction of the TO moiety with double-stranded DNA. The binding constant of TO with double-stranded DNA was reported to be $\sim 10^5 M^{-1}$.¹⁶ While the association rate constant was improved by increasing the number of the TO molecules for the labeling, the fluorescence of T_5 labeled with four molecules of TO was not enhanced compared with that of T_3 labeled with two molecules

of TO (Fig. S5A and B). The reason is not clear. A similar phenomenon was observed for the fluorescent probes labeled with one or two molecules of TO.³² Moreover, the labeling with the TO dimers at the two thymidine residues caused a problem in the determination of the dissociation temperature of T_5 . The melting curves derived from the absorption at 510 nm were not sigmoidal, which might be caused by the overlap of the subtly different two sigmoidal curves. The midpoint of the transition indicated that the thermal stability of the DNA triplex formed by T_5 is not higher than that of the DNA triplex formed by T_3 (Fig. S5C).

To examine the sequence specificity of the ECHO probes, fluorescence spectra of T_2 and T_3 in the presence of non-specific DNA were measured (Fig. 6). When the amount of the

Table 2. Melting temperatures of the DNA triplexes

	pH 6.0		pH 7.0	
	260 nm ^a	510 nm ^a	260 nm ^a	510 nm ^a
T ₁	40	n/a	27	n/a
T ₂	n/a ^b	57 (+ 17) ^c	44 (+ 17) ^c	42 (+ 15) ^c
T ₃	n/a ^b	62 (+ 22) ^c	n/a ^b	48 (+ 21) ^c
T ₄	46 (+ 6) ^c	46 (+ 6) ^c	35 (+ 8) ^c	34 (+ 7) ^c

^aWavelength used to monitor the absorbance for the determination of the melting temperatures. ^bThe melting curve analysis was difficult. The value in the parenthesis is the difference of the dissociation temperature from that of T₁ at the same pH.

non-specific DNA was increased, the fluorescence intensities of T₂ and T₃ decreased in the presence of the D₁/D₂ duplex and increased in the absence of the D₁/D₂ duplex. The ratio of the ECHO probes which bound to the non-specific DNA might be raised by the increase of the amount of the non-specific DNA because more diverse sequences would be included in the non-specific DNA. It was indicated that the interactions with the non-specific DNA enhanced the fluorescence of the ECHO probes to a lesser degree than that with the D₁/D₂ duplex. However, there were the clear differences between the fluorescence intensities between in the presence and absence of the D₁/D₂ duplex even though 100 times base concentration of the non-specific DNA was used. The results indicated that a certain ratio of the ECHO probes could bind to the D₁/D₂ duplex even in the presence of the excess amount of the non-specific DNA. To achieve a high sequence-specificity, it would be required to reduce the affinity to the non-specific DNA and suppress the enhancement of the fluorescence upon binding to the non-specific DNA.

In conclusion, ECHO probes were applicable to the fluorescent detection of double-stranded DNA, although the recognition sequences were restricted to polypurine/polypyrimidine tracts because of the sequence requirement for triplex formation. Thermal stabilization of the DNA triplexes formed by the ECHO probes was achieved by the interaction of TO molecules with the DNA triplexes. Binding of the TO molecules to the DNA triplexes also led to the fluorescence enhancement. The fluorescence signals exhibited high S/N ratios owing to the low background fluorescence of the ECHO probes caused by the excitonic interaction between the two TO molecules. Increase of the number of the TO molecules for the labeling from two to four resulted in the large increase in the association rate constant of the ECHO probe for the triplex formation. Therefore, ECHO probes are now added to the repertoire of the fluorescent probes for the detection of double-stranded DNA by triplex formation.

Material and Methods

General. MALDI-TOF mass spectra were acquired using a Bruker Daltonics Reflex instrument. UV and fluorescence spectra were recorded on a Shimadzu UV-2550 spectrophotometer and RF-5300PC spectrofluorophotometer, respectively.

Internal labeling of TFOs with TO. The synthetic procedures used to label TFOs with TO were the same as those

Figure 5. Time course of the fluorescence intensities of the TO-labeled TFOs, T₂, T₃, T₄ and T₅, in the presence of D₃ (A) or the D₁/D₂ duplex (B) at 25°C and pH 6.0. Association rate constants k_1 (M⁻¹s⁻¹) for the DNA triplex formation at 25°C and pH 6.0 were calculated from the time course data of the fluorescence intensities.

reported previously.^{28,31,32} Purified DNA oligonucleotides labeled with TO were identified using MALDI-TOF mass spectrometry. “M” indicates 5-methylcytosine. DNA labeled with two molecules of TO was positively charged with two-electron deficiency, as TO has a positive charge on its heterocyclic moiety. Therefore, the electric charge of [M-H] of the DNA labeled with two molecules of TO is +1. Similarly, the electric charge of [M-3H] of the DNA labeled with four molecules of TO is +1. 5'-TTTTTMTD₅₁₄TMTMTMT-3' (T₂), calculated for C₂₀₄H₂₅₈N₄₂O₁₀₂S₂P₁₄: 5427.2 [M-H]⁺; found: 5426.9; 5'-TTTDD₅₁₄TMTTTMTMTMT-3' (T₃), calculated for C₂₀₄H₂₅₈N₄₂O₁₀₂S₂P₁₄: 5427.2 [M-H]⁺; found:

Figure 6. Fluorescence spectra of the ECHO probes with non-specific DNA in the absence (gray line) and presence (black line) of the D_1/D_2 duplex at pH 6.0. The samples contained DNA from salmon testes at base concentration 16.8 (left), 168 (middle), or 1680 μM (right). **(A)** Fluorescence spectra of T_2 . **(B)** Fluorescence spectra of T_3 .

5428.2; 5'-TTTTTMTTTTMD₅₁₄MTMT-3' (T_4), calculated for $C_{204}H_{258}N_{42}O_{102}S_2P_{14}$; 5427.2 [M-H]⁺; found: 5428.7; 5'-TTTDD₅₁₄TMTTTTMD₅₁₄MTMT-3' (T_5), calculated for $C_{258}H_{316}N_{50}O_{105}S_4P_{14}$; 6356.4 [M-3H]⁺; found: 6356.5.

Absorption and fluorescence measurements. Absorption and fluorescence spectra of the ECHO probes (0.4 μM strand concentration) in the absence and presence of their target DNA duplex, D_1 and D_2 (0.4 μM strand concentration), were measured in 100 mM sodium phosphate buffer (pH 6, 7 or 8) containing 100 mM sodium chloride using a cell with a path length of 1 cm. All the samples were left at 25°C for 16 h before the recording of their spectra, for equilibration. The excitation and emission bandwidths were 1.5 nm. The fluorescence quantum yield (Φ) was determined using 9,10-diphenylanthracene as a reference, with a known value of $\Phi = 0.95$ in ethanol.

Measurements of melting curves. Absorbance of the samples at 260 and 510 nm was monitored from 10 to 90°C with a heating rate of 1°C min⁻¹. The samples contained TFOs (1.0 μM strand concentration), their target DNA duplex, D_1 and D_2 (1.0 μM strand concentration), 100 mM sodium phosphate buffer (pH 6.0, 7.0 or 8.0), and 100 mM sodium chloride. All the samples were left at 25°C for 16 h before the measurement of the melting curve for equilibration. Melting temperatures were determined by using the first derivatives of the melting curves.

Time course of fluorescence intensities. Time course of the fluorescence intensity of the ECHO probes were measured at 25°C. The fluorescence intensities at 532 nm by excitation at 514

nm were recorded at the elapsed times from the sample preparation. Samples contained the ECHO probes (0.2 μM strand concentration) with D_3 or the D_1/D_2 duplex (0.2 μM strand concentration), 100 mM sodium phosphate buffer (pH 6.0), and 100 mM NaCl. Three samples were prepared for the measurements of each TFO.

Kinetic analysis. Rate constants for the triplex formation and dissociation were described as k_1 and k_2 , respectively. When the k_2 value was assumed as zero, triplex concentration [Triplex] is described in Equation 1 using the initial concentrations (C_0) of the D_1/D_2 duplex and the TFOs, the rate constant (k_1), and time (t). Here, the dissociation of the DNA duplex was ignored, for the simplification of the calculation.

$$[\text{Triplex}] = C_0 [1 + (C_0 k_1 t)^{-1}]^{-1} \quad (1)$$

DNA triplex concentration at the elapsed times was calculated from the values of C_0 , k_1 and t (s) using Equation 1. Conversion of triplex concentrations to fluorescence intensities was performed using the supposition that the experimental value of the fluorescence intensity at 720 min (43,200 sec) was the same as the calculated value. Curve-fitting to estimate the k_1 values was performed by using gnuplot.

Sequence-specificity of the ECHO probes. DNA from salmon testes (Sigma-Aldrich) was added to the solution of the D_1/D_2 duplex (0.4 μM duplex concentration) containing 100 mM sodium phosphate buffer (pH 6) containing 100 mM sodium chloride to be 1, 10 or 100 times base concentration of that of the D_1/D_2 duplex (16.8 μM base concentration). T_2 or T_3

was lastly added to the solutions to be 0.4 μM strand concentration. All the samples were left at 25°C for 24 h before the recording of their fluorescence spectra, for equilibration. The excitation bandwidth was 3.0 nm.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/artificialdna/article/24102

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