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Accumulation of Fluorophores into DNA Duplexes To Mimic the Properties of Quantum Dots

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Abstract: By using perylene and pyrene as fluorophores, we have designed various fluorophore assemblies that mimic inorganic quantum dots in showing a high emission intensity, a large Stokes' shift, and a modulated emission maximum. For this purpose, we utilized two kinds of duplex motifs with D-threoninols as scaffolds: cluster and interstrand-wedged motifs. In the cluster motif, fluorophores are introduced into both strands to produce tentative pseudo-"base-pairs", in which the dyes strongly interact with each other and form dimers, trimers, or hexamers. In the interstrand-wedged motif, a base-pair is inserted between the fluorophores to suppress their direct interaction. These two motifs were applied to accumulate dyes within a DNA duplex, depending on their emission properties. Since pyrene exhibits strong excimer emission, the emission at 500 nm of a pyrene cluster motif strongly increased as the number of accumulated dyes increased, whereas the interstrand-wedged motif quenched pyrene monomer emission. In contrast, assembled perylenes, which are mostly quenched by dimerization, showed intense monomer emission in the interstrand-wedged motif, whereas perylene cluster motifs strongly suppressed perylene emission. These two motifs were then applied to the heteroassembly of pyrenes and perylenes. Both a large Stokes' shift and a modulation of the emission maximum, which are also characteristics of inorganic quantum dots, were successfully realized using fluorescent resonance energy transfer (FRET) and exciplex formation. These fluorophore assemblies thus obtained could be enzymatically ligated to longer DNA, demonstrating that this technique has the potential to be a versatile labeling agent for biomolecules.

1. Introduction

Quantum dots (QDs), which have attracted much interest in bio- and nanotechnology, are now widely used for biomolecular and cellular imaging due to their photochemical stability and their intense fluorescent emission. In addition, their large Stokes' shifts facilitate the detection of emitted light without collecting scattered excitation light. Their emission wavelength is also tunable by controlling their particle size. These properties have made QDs one of the most promising fluorescence labeling agents.[1] However, the cell-cytotoxicity of QDs has been a problem in their utility. Although numerous coating strategies have been proposed for QDs, the complete suppression of toxicity remains a serious problem to be solved.[2,3] In addition, the labeling of a single molecule by one quantum dot nanoparticle is very difficult since there are multiple reaction sites on the surface of QDs.

Fluorescent organic dyes are also commonly used for labeling biomolecules because of their usability and wide variety. However, emission from organic dyes is usually weaker than that from QDs due to their low extinction coefficients. This relatively low emission of organic dyes has prevented their application, for example, to single molecule fluorescence analysis. One solution to this problem may be to multiply the number of dyes used to raise the extinction coefficient. However, simple accumulation of fluorophores does not necessarily enhance the emission intensity because most of the dyes are self-quenched by dimerization.[4] Thus, regulation of the manner in which the fluorophores are assembled is absolutely necessary to increase the emission intensity of the accumulated dyes.

DNA is an ideal scaffold for the control of mutual interaction between dyes because DNA has a well-organized double helical structure and can accept intercalators between base-pairs. In addition, bases are strictly aligned in a DNA duplex according to a predetermined sequence. There have been several reports of strategies to accumulate fluorescent dyes by using nucleic acids as scaffolds.[5−18] For example, Kool et al. reported a modified DNA, in which fluorescent nucleosides (fluorosides) are conjugated in a single strand.[5,6] Armitage et al. reported a fluorescent nanotag, at which fluorescent dyes are intercalated

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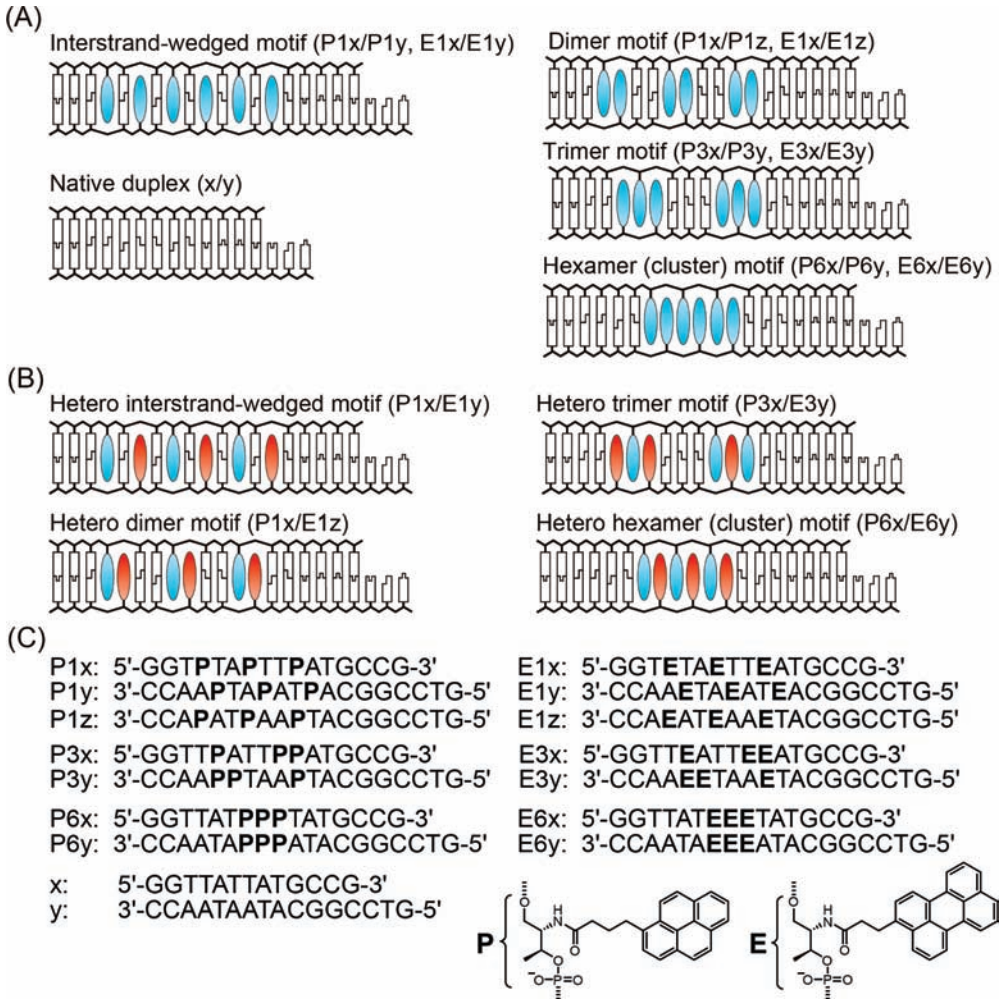
Scheme 1. Schematic Illustration of the Sequence Design of Fluorophore Assemblies; Sequence Designs of (A) Homo and (B) Hetero Combinations; (C) Sequences of the Dye Assemblies Synthesized in This Study and the Chemical Structures of Pyrene (**P**) and Perylene (**E**) Residues



into a natural DNA duplex to prevent self-quenching of dyes.[7] Previously, we designed several kinds of motifs, in which nonfluorescent dyes on acyclic scaffolds were aligned in the DNA duplex:[19−24] these dyes are tethered on D-threoninol as a scaffold, which are then introduced into the DNA strand. Thus, alignment of the chromophores is easily and rigidly regulated by design of their sequence in the duplex. Here, we aimed to mimic the fluorescent properties of QDs by applying these motif designs to the accumulation of fluorophores in a DNA duplex. Since the interaction between fluorophores is strictly controlled, dyes are accumulated inside the DNA duplex without losing their intrinsic emission properties.

2. Results and Discussion

2.1. Strategy for Arrangement of the Fluorophores and for Sequence Design. We first designed several duplex motifs to control the interaction between fluorescent dyes as shown in Scheme 1A, using D-threoninols as scaffolds for the fluorophores. In an interstrand-wedged motif, natural base-pairs and dyes are alternately aligned so that all the dyes in the duplex are isolated. We have reported that this motif strongly stabilizes the DNA duplex due to stacking interaction between the dyes and the base-pairs.[21,22] In addition, all the dyes in this motif are intercalated between natural base pairs as evidenced by NMR

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analysis. Consequently, direct interaction among the dyes can be avoided in the interstrand-wedged motif. On the other hand, when two dyes are introduced at a base-pairing position in both strands, the fluorophores form a stacked dimer (see the dimer motifs in Scheme 1A or B). Similarly, three or six dyes can also be clustered by introducing them at the corresponding base-pairing position (trimer or hexamer motif in Scheme 1A or B, respectively). These cluster motifs were previously demonstrated using azo-dyes and were investigated in detail using melting and spectroscopic analyses.[23,24] We found that the dyes in these cluster motifs physically and optically interacted with each other and strongly stabilized a DNA duplex.

Generally, fluorescence dyes are categorized into two types. One type includes self-quenching dyes that significantly reduce their emission upon dimerization.[4] Most fluorescence dyes belong to this category.[25] The interstrand-wedged motif is suitable for the accumulation of these fluorophores, because in this motif, dyes are isolated in the duplex, and thus self-quenching is minimized. The other type includes excimer-forming dyes such as pyrene that exhibit intense excimer emission in the dimeric state.[26] The cluster motif is therefore suitable for maximization of the excimer emission of these fluorophores.

Here, due to their photochemical stability, perylenes (**E**) and pyrenes (**P**) were selected as representatives of the above two categories of dyes, respectively, and were introduced into DNA duplexes (See Scheme 1C).[27] In addition, these dyes show excimer or exciplex emission when the two dyes interact. Thus, their emission wavelength is tunable by designing sequences. D-Threoninol was used as a linker to conjugate these dyes with the DNA backbone because this linker facilitates the intercalation of dyes.[28] Accordingly, interaction between chromophores can be strictly controlled by the sequence design as shown in Scheme 1C. For example, when **P1x** and **P1y** are hybridized, each pyrene is intercalated between A-T pairs.[29] As a result, interaction between pyrenes is suppressed, and only monomer emission is expected. On the other hand, in the case of **P1x**/**P1z**, **P3x**/**P3y**, or **P6x**/**P6y**, the pyrenes interact with each other to form dimers, trimers, or a hexamer, respectively (Scheme 1A). Thus, these duplexes are expected to show excimer emission. Since all of these duplexes have the same six chromophores, we can directly evaluate the effect of the number of clustered dyes on emission properties without changing the base composition. In addition, we added a sticky end to these duplexes so that the accumulated dyes can be easily labeled on a long DNA such as a gene by enzymatic ligation.

2.2. Spectroscopic Behaviors of Homo Combination. 2.2.1. Assembly of Pyrene into the Duplex. We first assembled pyrene into the duplex to investigate the effect of motif design on emission properties. Since pyrene is categorized as a dye that forms an excimer upon dimerization and since its emission properties depend on its association state, pyrene is a good example to demonstrate the motif design. Figure 1 shows the fluorescence emission spectra of duplexes containing six pyrenes. In the **P1x/P1y** duplex with an interstrand-wedged motif, two peaks assignable to monomer emission appeared at 380 and 400 nm (see the red line in Figure 1). On the other hand, only weak excimer emission was observed at around 480 nm, demonstrating that the interaction between pyrenes was suppressed by intervening A-T pairs and thus pyrenes were essentially isolated. This result agreed with our previous report showing that an interstrand-wedged motif efficiently shielded the mutual interaction of the incorporated dyes.[21,22] In the case of **P1x/P1z** in which three sets of pyrene dimers were incorporated into the duplex, excimer emission appeared at around 480 nm as we designed. However significantly, as the number of clustered dyes increased, excimer emission was concurrently enhanced: **P3x/P3y** with two sets of trimers, exhibited a 3-fold stronger excimer emission than **P1x/P1z**. In particular, a pyrene hexamer designed by the cluster motif (**P6x**/**P6y**) drastically increased the excimer emission of pyrene (see the black line in Figure 1). Note that monomer emission was almost completely suppressed in **P6x**/**P6y** as shown in the inset of Figure 1. These results demonstrate that a cluster motif assembled using D-threoninol is favorable for enhancement of excimer emission due to the firmly stacked structure of the duplex.[30] We also determined the quantum yields (Φ's) of these duplexes as listed in Table 1. The quantum yield of **P1x**/**P1y** was as low as 0.003 due to strong quenching by nucleobases. In contrast, the clustering of pyrenes significantly raised the yield of excimer emission. The quantum yield of **P6x**/**P6y** involving six pyrenes stacked with each other was 0.05, which was 17-fold greater than pyrene monomer emission from an interstrand-wedged motif. This quantum yield is almost comparable with those of pyrene cluster reported by other groups.[31,32] Thus, it can be concluded that the cluster motif is suitable for the maximization of excimer emission of pyrene.

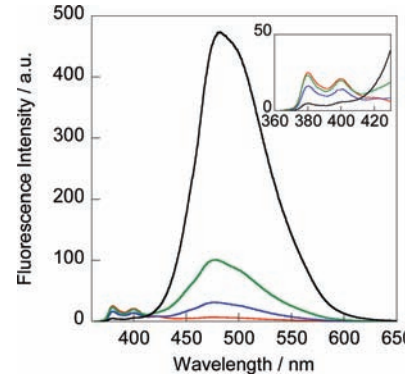

Figure 1. Fluorescence emission spectra of duplexes containing six pyrenes. The fluorescence emission spectra at 20 °C of **P1x**/**P1y** (red line), **P1x**/**P1z** (blue line), **P3x**/**P3y** (green line), and **P6x**/**P6y** (black line) are shown. Spectra at 360−430 nm are magnified in the inset. The excitation wavelength was 345 nm. Conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

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Table 1. Effect of Motif Design on the Spectroscopic Properties and Stability of DNA Duplexes

sequence	T_m/°C[a]	Φ	emission maximum/nm[e]
x/y	47.7	–	–
P1x/P1y	45.9	0.003[b]	380[f]
P1x/P1z	47.4	0.007[b]	476[f]
P3x/P3y	51.7	0.02[b]	477[f]
P6x/P6y	57.4	0.05[b]	484[f]
E1x/E1y	56.2	0.33[c]	462[g]
E1x/E1z	56.0	0.02[c]	461[g]
E3x/E3y	52.7	0.01[c]	457[g]
E6x/E6y	60.4	0.004[c]	554[g]
P1x/E1y	50.3	0.36[d]	461[f]
P1x/E1z	49.4	0.29[d]	469[f]
P3x/E3y	51.3	0.10[d]	467[f]
P6x/E6y	58.0	0.03[d]	545[f]

[a] Solution conditions: [DNA] = 1.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). [b] Quantum yield determined from the quantum yield of pyrene in N_2-bubbled cyclohexane (0.65) used as a reference. [c] Quantum yield determined from the quantum yield of perylene in N_2-bubbled cyclohexane (0.78) used as a reference. [d] Apparent quantum yield excited at 330 nm. These values were determined from the quantum yield of pyrene in N_2-bubbled cyclohexane (0.65) used as a reference. [e] The wavelength at which the most intense peak was observed. Solution conditions: [DNA] = 1.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), 20 °C. [f] Excitation wavelength: 345 nm. [g] Excitation wavelength: 425 nm.

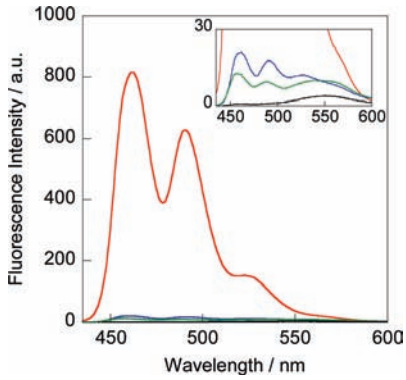

Figure 2. Fluorescence emission spectra of duplexes containing six perylenes. The fluorescence emission spectra at 20 °C of **E1x**/**E1y** (red line), **E1x**/**E1z** (blue line), **E3x**/**E3y** (green line), and **E6x**/**E6y** (black line) are shown. Spectra of **E1x**/**E1z**, **E3x**/**E3y**, and **E6x**/**E6y** are magnified in the inset. The excitation wavelength was 425 nm. Conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

2.2.2. Assembly of Perylene into DNA Duplexes. In contrast with pyrene, perylene is known to exhibit strong monomer emission with a high quantum yield whereas excimer emission is moderate,[33] indicating that perylene is categorized as a self-quenching dye. Since the behavior of perylene is completely different from that of pyrene, the effect of cluster design on its emission behavior was the exact opposite to that on pyrene. The fluorescence emission spectra of **E1x**/**E1y**, **E1x**/**E1z**, **E3x**/**E3y** and**E6x**/**E6y** are shown in Figure 2. When six perylenes were separated by A-T pairs (**E1x**/**E1y**), strong monomer emission of perylene was observed at 450−500 nm. On the other hand, when perylenes were clustered, the emission intensity drastically decreased. The emission of **E1x**/**E1z**, involving three sets of perylene dimers, was reduced to about one-fortieth that of **E1x**/**E1y** (compare the blue and red lines in Figure 2). When the number of clustered perylenes was increased from two to six (**E6x**/**E6y**), the monomer emission of perylene completely disappeared (see the black line in Figure 2) and there remained only weak fluorescence at around 550 nm that was assignable to excimer emission of perylene.

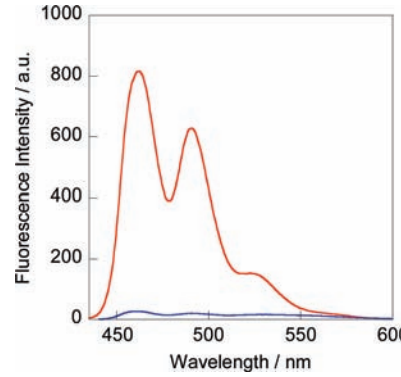

Figure 3. Duplex formation is required for enhancement of the emission intensity of perylene. Comparison of the fluorescence emission spectra at 20 °C of single-stranded **E1x** (blue line) with that of **E1x**/**E1y** (red line). The excitation wavelength was 425 nm. Conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

Quantum yields (Φ's) of these clusters are shown in Table 1. The quantum yield of **E1x**/**E1y** was as high as 0.33, clearly demonstrating that the interaction between perylenes was suppressed by intervening base pairs. On the contrary, as the number of clustered perylenes increased, the quantum yield drastically decreased. The quantum yield of **E6x**/**E6y** was only 0.004 due to the strong quenching that occurred upon cluster formation. The UV−vis spectra of **E6x**/**E6y** showed large hypochromism compared to **E1x**/**E1y** (see Supporting Information), indicating that perylenes formed a nonfluorescent complex in the ground state.

In **E1x**/**E1y**, a natural base pair between two perylenes successfully suppressed interaction between fluorophores, i.e., suppressed self-quenching. However, incorporation of a natural base between two perylenes in the single-stranded state could not suppress self-quenching as shown in Figure 3. The emission intensity of single-stranded **E1x** was as low as one-thirtieth that of **E1x**/**E1y** because of insufficient suppression of the direct interaction of fluorophores by the intervening natural nucleotide. Thus, duplex formation is absolutely required for enhancement of the emission intensity of perylene. It is known that many fluorescent dyes quench their fluorescence upon dimer formation.[4] Here, a highly fluorescent DNA was successfully developed by inserting base pairs between perylenes. By using the interstrand-wedged motif, other fluorescent dyes that are self-quenched upon dimerization could also be assembled without significantly affecting the emission intensity.

2.3. The Stacked Structure of Fluorophores. In order to investigate the effect of incorporation of dyes on the stability of these duplex motifs, the melting temperatures (T_ms) of duplexes containing pyrene or perylene were determined as listed in Table 1. For most sequences containing pyrene or perylene, the duplex was stabilized compared to native duplex without dye (**x**/**y**), which agreed with our previous results.[21,23] In particular, the duplex melting temperature significantly increased when six dyes were clustered. For example, the T_m of **E6x**/**E6y** was 60.4 °C, which was about 13 °C higher than that of **x**/**y** (47.7 °C), indicating that the duplex was stabilized

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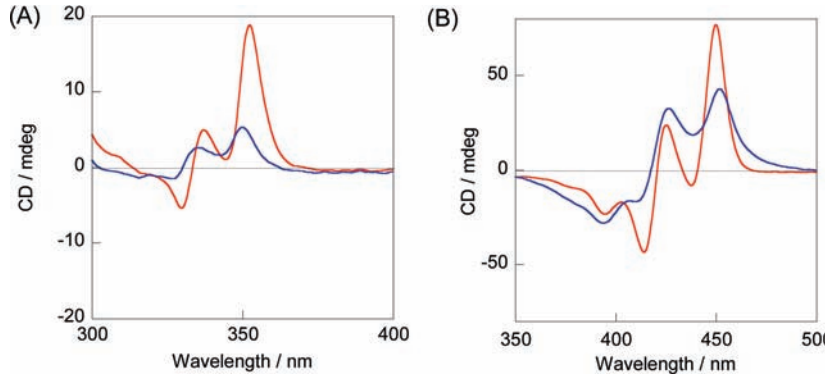

Figure 4. CD spectra of DNA duplexes containing (A) pyrene or (B) perylene. The CD spectra at 20 °C of (A) **P1x**/**P1y** (red line) and **P6x**/**P6y** (blue line), and (B) **E1x**/**E1y** (red line) and **E6x**/**E6**y (blue line) are shown. Conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 5.0 μM.

by an intermolecular stacking interaction.[34] This duplex stabilization by incorporation of dyes into DNA substantiated that the dyes were stacked inside the DNA duplex because flipped-out chromophores often destabilize DNA duplexes.[10,13]

The CD spectra of **P1x**/**P1y**, **P6x**/**P6y**, **E1x**/**E1y**, and **E6x**/**E6y** were next measured to elucidate the structure of dye-containing duplexes, as shown in Figure 4. For **P1x**/**P1y**, typical positive couplets were observed at around 345 nm, clearly showing that pyrenes took a right-handed conformation (See the red line in Figure 4A). On the other hand, the induced CD was weaker for **P6x**/**P6y** although a positive couplet was also observed. Similarly, the induced CD of the **E6x**/**E6y** duplex containing perylene, was weaker than that of **E1x**/**E1y** (Figure 4B). We previously reported that dyes in the cluster motif are stacked in an unwound ladder-like structure resulting in a very weak induced CD.[24] Accordingly, the induced CD of dyes in cluster motifs of the present study was weaker than that in the interstrand-wedged motif although exciton coupling among the cluster motif, which can enhance the CD signal, was stronger due to the close proximity of the fluorophores.

2.4. Sequence Design of Hetero Combination. We next applied the cluster and interstrand-wedged motifs to the preparation of hetero assemblies by hybridizing a DNA tethering pyrenes (such as **P1x**) with that containing perylenes (such as **E1y**) in order to control emission wavelength and to enlarge the Stokes' shift. As demonstrated in the homo assemblies, interactions between pyrenes and perylenes can also be regulated. When pyrenes and perylenes are introduced into the interstrand-wedged motif (e.g., **P1x**/**E1y**), direct interactions between these dyes are suppressed by natural base-pairs. Instead, efficient fluorescence resonance energy transfer (FRET) should occur (see Hetero interstrand-wedged motif in Scheme 1B). On the other hand, in a hetero cluster motif (**P6x**/**E6y** in Scheme 1B), pyrenes and perylenes form an exciplex. Thus, emission should be observed at wavelengths longer than that of monomer emission.[35−37] Therefore, the emission wavelength is tunable by controlling the manner of dye clustering in the hetero dye combination.

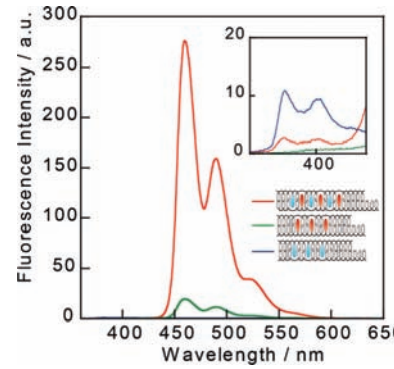

Figure 5. Fluorescence emission spectra of hetero duplexes with the interstrand-wedged motif. The fluorescence emission spectra at 20 °C of **P1x**/**E1y** (red line), **P1x**/**y** (blue line), and **x**/**E1y** (green line) are shown. Spectra are magnified in the inset. The spectra were measured at low sensitivity whereas those in the inset were measured at medium sensitivity. The excitation wavelength was 345 nm. Conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

2.4.1. Analysis of the Interstrand-Wedged Motif for Efficient FRET To Enlarge the Apparent Stokes' Shift. The fluorescent emission spectrum of **P1x**/**E1y**, in which pyrenes and perylenes were aligned in the interstrand-wedged motif, excited at 345 nm, is shown by the red line in Figure 5. Strong peaks of perylene emission were observed at 460 and 495 nm whereas peaks of pyrene emission at 380 and 400 nm were extremely small. The emission spectra of control duplexes containing three pyrenes (**P1x**/**y**) or three perylenes (**x**/**E1y**), obtained by combining natural (**x** or **y**) and modified (**P1x** or **E1y**) strands, are also shown in Figure 5. The intensity of perylene emission at around 460 nm in **P1x**/**E1y** was stronger than that in **x**/**E1y**. In contrast, the intensity of pyrene emission at around 380 nm in **P1x**/**E1y** was lower than that in **P1x**/**y** (See the inset of Figure 5). These results clearly demonstrate that strong emission of perylene in **P1x**/**E1y** can be attributed to efficient FRET from pyrene to perylene. The FRET efficiency of **P1x**/**E1y,** calculated from the emission intensity of pyrene, was as high as 0.75. Thus, efficient FRET was attained by introducing pyrene and perylene in close proximity but without direct contact.

It is worth noting that strong emission bands were observed from **P1x**/**E1y** at 460 and 490 nm despite the short excitation wavelength (345 nm). Additionally, the apparent Stokes' shift became as large as 115 nm. In addition, the excitation spectra

(34) T_m of **E3x**/**E3y** was lower than that of **E1x**/**E1z** although the number of clustered dyes increased. Generally, dyes in a cluster motif favor locations adjacent to the 5′-side of a natural nucleobase as elucidated by a solution structure from NMR (see ref 24). However, dyes of the asymmetric trimer in **E3x**/**E3y** are forced to be located adjacent to the 3′-side, which resulted in destabilization of the duplex.

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(36) Lindegaard, D.; Madsen, A. S.; Astakhova, I. V.; Malakhov, A. D.; Babu, B. R.; Korshun, V. A.; Wengel, J. *Bioorg. Med. Chem.* **2008**, *16*, 94–99.
(37) Kashida, H.; Takatsu, T.; Sekiguchi, K.; Asanuma, H. *Chem.—Eur. J.* **2010**, *16*, 2479–2486.

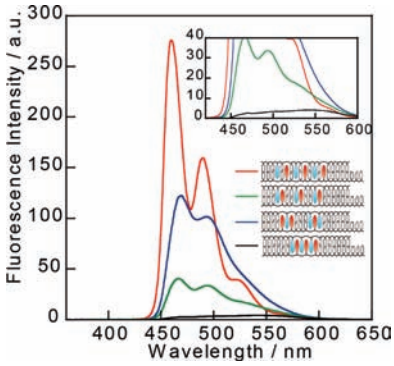

Figure 6. Fluorescence emission spectra of hetero duplexes with the cluster motif. The fluorescence emission spectra at 20 °C of **P1x**/**E1y** (red line), **P1x**/**E1z** (blue line), **P3x**/**E3y** (green line), and **P6x**/**E6y** (black line) are shown. The spectrum of **P6x**/**E6y** is magnified in the inset. The excitation wavelength was 345 nm. Conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

of **P1x**/**E1y** showed absorption bands at 300−470 nm (see Supporting Information), demonstrating that hetero assembly could be excited at a wide range of wavelengths. QDs have broad absorption bands that range from UV to visible regions which means that a wide range of excitation wavelengths are available. In the hetero fluorophore assemblies proposed here, we mimic these properties by using FRET.

2.4.2. Analysis of Exciplex Emission of the Cluster Motif. Although homoclustering of perylenes did not exhibit excimer emission behavior but instead induced drastic quenching, heteroclustering of perylenes with pyrene is expected to show exciplex behavior.[16,38] Simple combination of **P*n*x** with **E1z** or **E*n*y** results in various heteroclusters of predetermined size, whose emission spectra are shown in Figure 6. A **P1x**/**E1z** duplex consisting of heterodimers of pyrene and perylene shifted its emission band to a longer wavelength than that of the **P1x**/**E1y** (FRET pair) duplex due to exciplex formation (compare the blue and red lines). **P3x**/**E3y,** involving two sets of trimer heteroclusters, also showed emission bands at almost the same wavelength as **P1x**/**E1z**. When the number of clustered dyes was increased to six, i.e., **P6x**/**E6y**, monomer emission of perylene (460 and 495 nm) completely disappeared leaving an exciplex emission at around 550 nm. Note that Stokes' shift was further enlarged to about 200 nm under these conditions: exciplex emission was observed at around 550 nm whereas the excitation wavelength was 345 nm.

2.4.3. Evaluation of the Quantum Yields of Hetero Assemblies. For quantitative evaluation of emission intensity the quantum yields of these hetero assemblies were calculated (Table 1). The apparent quantum yield of the interstrand-wedged **P1x**/**E1y**, excited at 345 nm, was as high as 0.36, which was almost comparable to that of homoassembly of the interstrand-wedged homo dimer **E1x**/**E1y** involving six isolated perylenes. Although the quantum yield of the interstrand-wedged **P1x**/**P1y** involving isolated six *pyrenes* was as low as 0.003, intense emission of **P1x**/**E1y** was realized by locating acceptor perylenes in close proximity to donor pyrenes resulting in efficient energy transfer from excited pyrenes to perylenes (note that the energy transfer efficiency was 0.75). On the other hand, when pyrene and perylene formed a hetero dimer (**P1x**/**E1z**), the quantum yield slightly decreased (0.29). Although the quantum yield further decreased as the number of clustered dyes increased, the severe quenching that was observed with **E6x**/**E6y** did not occur: the quantum yield of **P6x**/**E6y** was still 0.03, which was comparable to that of the **P6x**/**P6y** pyrene excimer.[39]

These changes in fluorescence spectra were easily detectable even by the naked eye (Figure 7). The **P1x**/**E1y**, in which pyrenes and perylenes were isolated by intervening A-T pairs, appeared as a bright blue color under the transilluminator as shown at the left of Figure 7. As the number of clustered dyes increased, the color changed to green (**P1x**/**E1z** and **P3x**/**E3y**). In the case of **P6x**/**E6y** in which three pyrenes and perylenes were clustered, the color of the solution became orange, as shown at the right of Figure 7. These results show that the emission color is tunable by controlling the arrangement of the dyes assembled in the duplex. The emission colors of inorganic quantum dots are regulated by control of the size of the particles used. Here, we could also mimic this property by controlling the clustering manner.

2.5. Labeling of the Insulin Chain-A Gene. One of the advantages of fluorophore assemblies presented here over quantum dots is that DNA scaffolds facilitate the labeling of biomolecules. Labeling of one DNA molecule by a quantum dot is difficult because there are multiple reaction points on the particle as mentioned above. However, since the sequence of the sticky end of the dye assembly can be designed to be complementary to the fragment of restriction enzyme, these dye assemblies can be specifically conjugated with native DNA by enzymatic ligation. Here, we demonstrated this advantage by labeling the 72 bp native DNA encoding the insulin chain-A gene[40] with **P1x**/**E1y,** whose sequence showed a high quantum yield (0.36) with a large Stokes' shift. For this model, the insulin chain-A gene was chemically synthesized with a 3-base sticky end (5′-GAC-3′: corresponding to the cleavage fragment of Cpo I). **P1x**/**E1y** was then ligated to the gene using T4 DNA ligase and the ligation efficiency was analyzed by agarose gel electrophoresis as shown in Figure 8. When this dye assembly was ligated with the model insulin gene, a new DNA band with a length longer than 75 bp appeared (lane 4 in Figure 8A). This novel band showed a blue fluorescence emission when excited at 365 nm (lane 4 in Figure 8B), which confirmed that this band represented the ligation product of the dye assembly and the insulin A-chain gene. It is noteworthy that no other byproducts were observed demonstrating that the present dye assembly can be selectively ligated with native DNA. Insertion of five natural base pairs between the sticky end and the artificial moiety (motif) was sufficient for efficient ligation of a modified duplex to a native duplex using T4 ligase. Thus, for the development of DNA-based labeling, a wide variety of molecular designs is available.

3. Conclusions

In conclusion, we have successfully developed new fluorophore assemblies in DNA duplexes. The use of various motifs with D-threoninols as scaffolds could mimic most of the properties of inorganic QDs as follows.

(1) The extinction coefficient of duplexes was enhanced by introducing multiple fluorophores into DNA. Here, by using interstrand-wedged or cluster motifs depending on the properties

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(39) In order to use the exciplex emission for labeling, further optimization of dye structure would be necessary to overcome low quantum yield.

(40) Crea, R.; Kraszewski, A.; Hirose, T.; Itakura, K. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5765–5769.

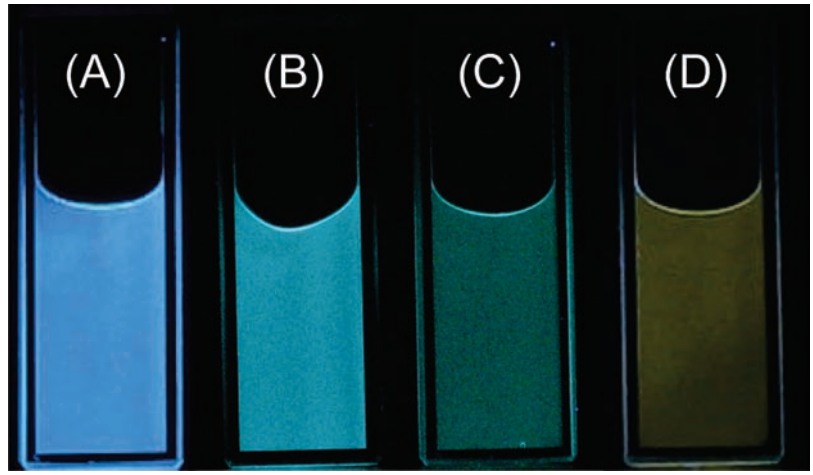

Figure 7. Visible changes in the fluorescence spectra of duplexes. Photograph of (A) **P1x**/**E1y**, (B) **P1x**/**E1z**, (C) **P3x**/**E3y**, and (D) **P6x**/**E6y** is shown. The excitation wavelength was 340 nm. The solution conditions were as follows: [DNA] = 1.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), rt.

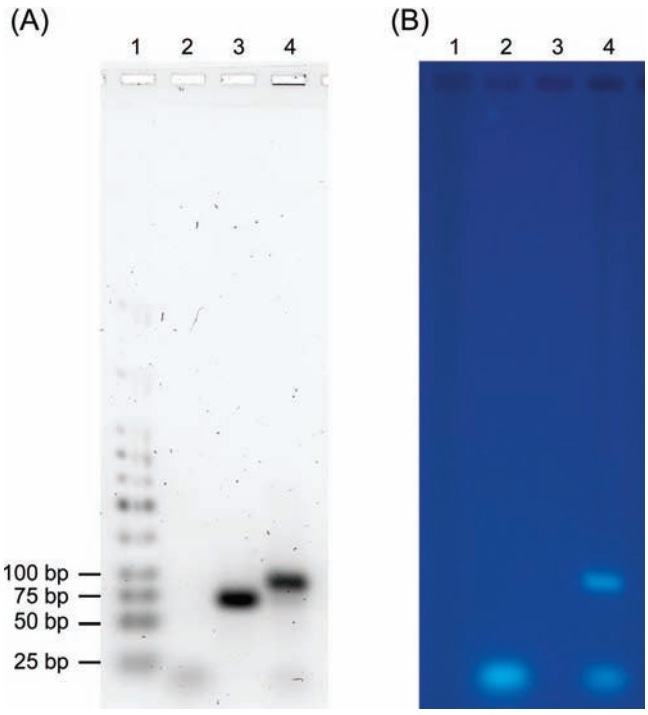

Figure 8. Ligation of a modified duplex to the insulin A-chain gene. (A) Agarose gel electrophoresis patterns for the ligation product stained by SYBR Green I. (B) A photograph of the same gel in (A) before staining under a transilluminator (excitation wavelength: 365 nm). Lane 1: DNA marker. Lane 2: dye assembly (**P1x**/**E1y**). Lane 3: insulin A-chain gene (72 bp). Lane 4: ligation product.

of the fluorophore, emission was maximized without self-quenching of the dyes.

(2) Fused aromatic compounds such as pyrene and perylene were used due to their photochemical stabilities.

(3) A large Stokes' shift was attained by using FRET from pyrene to perylene or by exciplex emission of hetero assemblies in the duplex. The large band of UV−vis absorption of these hetero assemblies allowed excitation over a wide range of wavelengths (300−470 nm).

(4) The emission wavelength was tunable by controlling the manner of dye clustering.

These DNAs could be efficiently ligated with native DNA using natural T4 ligase. In addition, cytotoxicity should be considerably improved compared to inorganic QDs because the present DNAs are composed mainly of natural nucleotides and involve less toxic elements. Accordingly, fluorophore assemblies presented here have the potential to be a versatile labeling agent of other biomolecules such as proteins.

4. Experimental Section

4.1. Materials. All the conventional phosphoramidite monomers, CPG columns, reagents for DNA synthesis and Poly-Pak II cartridges were purchased from Glen Research. Other reagents for the synthesis of phosphoramidite monomers were purchased from Tokyo Kasei Co., Ltd., and Aldrich.

4.2. Synthesis of the DNA Modified with Pyrene and Perylene. All of the modified DNAs were synthesized on an automated DNA synthesizer (ABI-3400 DNA synthesizer, Applied Biosystems) by using phosphoramidite monomers bearing dye molecules. Procedures for the synthesis of pyrene and perylene monomers were reported previously.[37,41] The coupling efficiency of the monomers corresponding to the modified residues was as high as that of the conventional monomers, as judged from the coloration of the released trityl cation. After the recommended workup, they were purified by reversed phase (RP)-HPLC and were characterized by MALDI-TOFMS (Autoflex, Bruker Daltonics).

The MALDI-TOFMS data for the DNAs were as follows:

P1x: Obsd. 5292 (Calcd. for [**P1x**+H^+]: 5291). **P1y**: Obsd. 6160 (Calcd. for [**P1y** +H^+]: 6160). **P1z**: Obsd. 6161 (Calcd. for [**P1z** +H^+]: 6160). **P3x**: Obsd. 5292 (Calcd. for [**P3x** +H^+]: 5291). **P3y**: Obsd. 6160 (Calcd. for [**P3y**+H^+]: 6160). **P6x**: Obsd. 5291 (Calcd. for [**P6x**+H^+]: 5291). **P6y**: Obsd. 6160 (Calcd. for [**P6y**+H^+]: 6160). **E1x**: Obsd. 5399 (Calcd. for [**E1x**+H^+]: 5399). **E1y**: Obsd. 6269 (Calcd. for [**E1y**+H^+]: 6268). **E1z**: Obsd. 6268 (Calcd. for [**E1z**+H^+]: 6268). **E3x**: Obsd. 5401 (Calcd. for [**E3x**+H^+]: 5399). **E3y**: Obsd. 6268 (Calcd. for [**E3y**+H^+]: 6268). **E6x**: Obsd. 5400 (Calcd. for [**E6x**+H^+]: 5399). **E6y**: Obsd. 6268 (Calcd. for [**E6y**+H^+]: 6268).

4.3. Spectroscopic Measurements. CD spectra were measured on a JASCO model J-820 with a 10-mm quartz cell that was equipped with programmed temperature controllers. The sample

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(42) Sapsford, K. E.; Berti, L.; Medintz, I. L. *Angew. Chem., Int. Ed.* **2006**, *45*, 4562–4589.

solutions were as follows: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 5.0 μM.

Fluorescence spectra were measured on a JASCO model FP-6500 with a microcell. The excitation wavelength was 345 or 425 nm. The sample solutions were as follows: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

4.4. Measurement of the Melting Temperature. The melting curve of duplex DNA was obtained with a JASCO V-530 by measurement of the change in absorbance at 260 nm versus temperature. The melting temperature (T_m) was determined from the maximum in the first derivative of the melting curve. Both the heating and the cooling curves were measured, and the calculated T_ms agreed to within 2.0 °C. The temperature ramp was 0.5 °C min^{-1}. The sample solutions were as follows: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM.

4.5. Calculation of FRET Efficiencies. FRET efficiencies (Φ_T) were calculated according to the following equation:[42]

$$\Phi_T = 1 - \frac{I_{AD}}{I_D}$$

where I_{AD} and I_D are the emission intensities at 380 nm of sequences with (**P1x/E1y)** and without acceptors (**P1x/y)**, respectively. The emission intensities were measured at 20 °C. The excitation wavelength was 345 nm.

4.6. Ligation with the Insulin A-Chain Gene. The insulin A-chain gene, which is composed of 75 mer (A1) and 72 mer DNA (A2), was chemically synthesized. To phosphorylate the 5′-ends of **A1** and the modified DNA (**E1y**), T4 polynucleotide kinase (10 U μL^{-1}, Takara) was added to the mixture of **A1** and **E1y**. The mixture was incubated for 30 min at 37 °C. The kinase was then inactivated for 30 min at 70 °C. After addition of **A2** and **P1x**, the solution was cooled to 16 °C for 15 min. T4 DNA ligase and the reaction buffer (both from the DNA Ligation Kit, Invitrogen) were added to the mixture, which was then incubated for 2 h at 16 °C. The ligation conditions were: [**A1**] = [**A2**] = 1.3 μM, [**P1x**] = [**E1y**] = 2.7 μM, [T4 DNA ligase] = 0.5 U/μL. The product was analyzed by nondenaturing 2.5% agarose gel electrophoresis. The photograph shown in Figure 8B was taken over a transilluminator (excitation wavelength: 365 nm). The bands were further detected by staining with SYBR Green I (Lonza).

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Supporting Information Available: UV−vis, CD, and fluorescence excitation spectra of duplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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