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Insulator Base Pairs for Lighting-up Perylenediimide in a DNA Duplex

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Fluorescent-labeled oligonucleotides are powerful tools for biochemical and biological research.[1] However, some fluorophores, even those with high potential, cannot be utilized for the labeling of DNA or RNA due to their strong quenching by natural nucleobases.[2] In order to protect these fluorophores from severe quenching, a new methodology that can shield these fluorophores from natural nucleobases is required. Kool et al. first reported an "insulator molecule" which enhances the emission of pyrene (Y) in a single strand.[3] They synthesized a trimer which had 5,6-dihydro-2′-deoxythymidine (DHT) inserted between pyrene and thymine (5′-T(DHT)Y-3′). This trimer showed a quantum yield about 70 times higher than that of a directly-conjugated dimer (5′-TY-3′). However, structural fluctuations in the single-stranded state still prevented complete recovery of the quantum yield of pyrene.

Here, we propose "insulator base pairs" that can shield a fluorophore from nucleobases in order to achieve a high quantum yield in a duplex. We designed two insulator molecules: *trans*-isopropylcyclohexane (**H**) and 4-cyclohexylbenzene (**I**) moieties as shown in Figure 1, each of which has one rigid cyclohexane ring with no π electrons.[4,5] The absence of π electrons should efficiently suppress electron transfer. A biphenyl moiety (**J**) containing no cyclohexane rings was used as a control. These molecules were introduced into both strands of a DNA duplex to form tentative "base pairs". Because these molecules have high hydrophobicity, they should face to the inside of the duplex and form "base pairs". These "insulator base pairs" would therefore be expected to shield a fluorophore from quenching by nucleobases.

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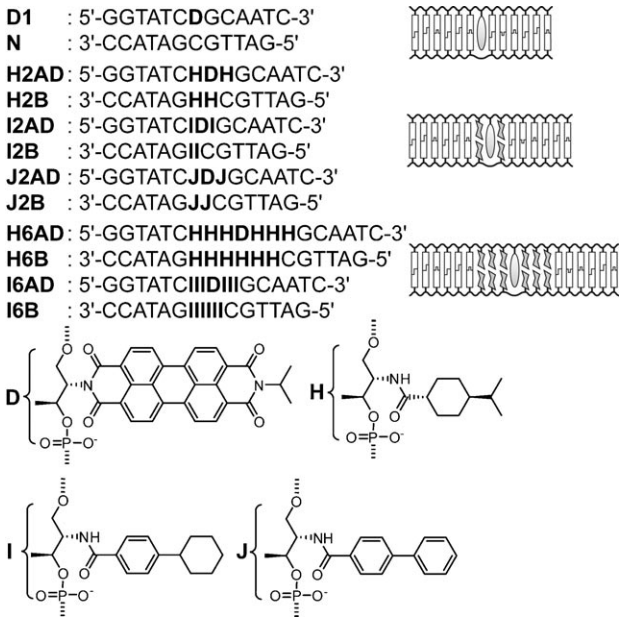

Figure 1. Sequences of the modified DNAs synthesized in this study.

We selected the fluorophore perylenediimide (PDI: **D**) to test the shielding effect of these "insulator base pairs". PDI has been widely applied to material use because of its high electron affinity, high brightness, strong π–π stacking interaction and photostability.[6–8] Furthermore, PDI has also been used for the functionalization of DNA.[9–18] However, even though the PDI monomer shows a high quantum yield and photostability, the quenching of PDI by nucleobases, due to electron transfer from nucleobases, has severely limited its application as a fluorophore.[10,17,19] Thus, we introduced "insulator base pairs" between PDI and nucleobases in order to enhance the fluorescence of PDI. Sequences of modified DNA are shown in Figure 1. Two or six "insulator base pairs" were introduced between PDI and nucleobases on D-threoninol as a scaffold.[20] DNA containing one PDI but no insulator was also synthesized as a control (**D1**).

For the hybridization of **D1** with a complementary strand (**N**), almost no fluorescence emission was detected from

PDI (see the orange line in the inset of Figure 2A). This extremely low emission is attributable to the almost complete quenching of PDI by electron transfer from nucleobases to PDI.[17] However, when two **H–H** pairs were inserted between PDI and its neighboring nucleobases (**H2AD/H2B**), distinct emission at 550 nm was observed from PDI. As summarized in Table 1, the quantum yield (Φ) of **H2AD/H2B** was 0.020 whereas that of **D1/N** was less than 0.001.

Table 1. Effect of duplex sequence on the quantum yield (Φ) and melting temperature.

Sequence		Φ[a]	T_m [°C][b]
D1/N		<0.001	41.0
H2AD/H2B		0.020	44.2
I2AD/I2B		0.025	48.2
J2AD/J2B		0.009	46.9
H6AD/H6B		0.59	62.0
I6AD/I6B		0.56	61.8
H2AD/N		0.006	39.7
D1/H2B		<0.001	39.8

[a] Quantum yield was determined using the quantum yield of rhodamine 6G in ethanol (0.94) as a reference. [b] [DNA] = 5.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

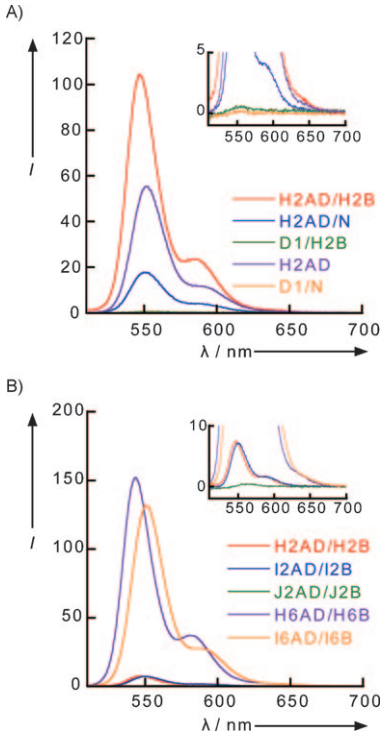

Figure 2. Fluorescence emission spectra at 20°C of A) **H2AD/H2B**, **H2AD/N**, **D1/H2B**, single-stranded **H2AD** and **D1/N** and B) **H2AD/H2B**, **I2AD/I2B**, **J2AD/J2B**, **H6AD/H6B** and **I6AD/I6B**. Solution conditions were as follows: [DNA] = 1.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). Excitation wavelength: 500 nm. Note that spectra in B) were measured at low sensitivity mode whereas those in A) were measured at medium sensitivity mode.

These results clearly demonstrated that the "insulator base pairs" successfully suppressed electron transfer from the nucleobases to PDI. On the other hand, the emission intensity of duplexes containing two **H** moieties in only one strand of the duplex (**H2AD/N** and **D1/H2B**) was far lower than that of **H2AD/H2B** (Figure 2A). Furthermore, the emission intensity of single-stranded **H2AD** was about half that of **H2AD/H2B**. Although incorporation of insulators into single-strand raised quantum yield of PDI, these data indicated that "base pairing" of insulators is desirable for the enhancement of PDI fluorescence. Incorporation of PDI into the "base-paired" insulator molecules might also lead to shielding from water as well as adjacent nucleobases.

We next examined the importance of the cyclohexane ring for the enhancement of PDI emission. **I2AD/I2B**, which has an **I–I** pair between PDI and nucleobases, showed almost the same emission intensity as **H2AD/H2B** (compare the blue line with the red one in Figure 2B). Hence, Φ of **I2AD/I2B** was almost identical to that of **H2AD/H2B**. Thus, **I–I** pairs show almost the same "insulating ability" as **H–H** pairs. In contrast, introduction of **J–J** pairs did not much enhance the fluorescence emission (green line in Figure 2B), indicating that at least one cyclohexane ring is necessary for the insulating effect.

Since the value of Φ remained still lower than 0.1 following the introduction of two insulator pairs, we determined if insertion of additional insulator pairs might enhance PDI emission. Indeed, the emission intensity of **H6AD/H6B** was dramatically higher than that of **H2AD/H2B** (Figure 2B). The Φ value of **H6AD/H6B** was as high as 0.59, which is about 30 times higher than that of **H2AD/H2B** (See Table 1). Consequently, quantum yield of PDI increased approximately several thousand-fold compared to that of **D1/N** without insulators. The Φ value of **H6AD/H6B** is comparable to those of common fluorophores,[21] indicating that this duplex can be utilized for labeling. This dramatic increase in quantum yield can also be detected even by the naked eye (see Figure 3). The solution exhibits bright orange color by incorporating six "insulator base pairs". Similarly, incorporation of six **I–I** pairs also increased Φ to 0.56

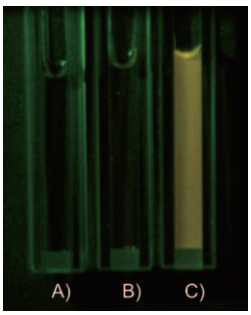

Figure 3. A photograph of A) **D1/N**, B) **H2AD/H2B** and C) **H6AD/H6B** at RT. Solution conditions were as follows: [DNA] = 1.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). Excitation wavelength: 520 nm.

(**I6AD/I6B**). These results clearly show that incorporation of multiple "insulator base pairs" between PDI and nucleobases is effective for enhancement of the quantum yield. In addition, emission intensity of **H6AD/H6B** was still higher than that of single-stranded **H6AD** (see Figure S1 in Supporting Information), demonstrating that "base pairing" of insulators is also effective for the enhancement in quantum yield.

The melting temperatures (T_m values) of the duplexes are also summarized in Table 1. Incorporation of insulator pairs into DNA unexpectedly stabilized the duplex even though insulators have non-planar structures. For example, the T_m of **H2AD/H2B** was 44.2 °C, which was 3.2 °C higher than that of **D1/N**. Furthermore, the introduction of six **H–H** pairs greatly enhanced the thermal stability of the duplex: the T_m of **H6AD/H6B** was as high as 62.0 °C. It can be concluded that **H–H** pairs strongly stabilize the duplex probably due to hydrophobic interactions. This high stability of **H–H** pairs supports the idea that insulator moieties form "base pairs" in a DNA duplex and disturb π–π stacking between PDI and the nucleobases. Similarly, incorporation of **I–I** and **J–J** pairs showed similar stabilization compared to **D1/N**. Shielding of PDI from natural nucleobases was also substantiated by the UV/Vis spectra; absorption maximum of **H2AD/H2B** was 535 nm whereas that of **D1/N** was 546 nm (See Figure S2 in Supporting Information). Concurrently, absorbance of **H2AD/H2B** was larger than that of **D1/N**. These blue-shift and hyperchromic effect show that interactions between PDI and nucleobases were disturbed by insulator base pairs.

In conclusion, the quantum yield of PDI dramatically increased following the introduction of "insulator base pairs" with cyclohexane moieties. The quantum yield of PDI was increased from <0.001 to as high as 0.59 when six **H–H** pairs were introduced between PDI and nucleobases. Thus, this duplex has the potential to be used for labeling of DNA or RNA. Because even PDI, whose fluorescence is completely quenched by natural nucleobases, showed high quantum yield, the "insulator base pairs" could be utilized for the enhancement of quantum yield of other fluorophores. We recently reported the assembly of fluorophores in DNA duplexes, in which the fluorophores and the natural base pairs were alternately introduced.[22] The "insulator base pairs" can also be utilized to assemble fluorophores without decreasing the quantum yield.

Experimental Section

Synthesis of the DNA modified with perylenediimide and insulators: The synthesis of phosphoramidite monomer bearing non-natural residues is described in Supporting Information. All modified DNAs were synthesized on an automated DNA synthesizer (ABI-3400 DNA synthesizer, Applied Biosystems). 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 work-up, they were purified by reversed phase (RP)-HPLC and were characterized by MALDI-TOFMS (Autoflex, Bruker Daltonics).

The MALDI-TOFMS data for the DNA were as follows: *m*/*z*: **H2AD**: calcd for [**H2AD**+H$^+$]: 4865; found: 4864; **H2B**: calcd for [**H2B**+H$^+$]: 4283; found: 4281; **H6AD**: calcd for [**H6AD**+H$^+$]: 6142; found: 6141; **H6B**: calcd for [**H6B**+H$^+$]: 5560; found: 5560; **I2AD**: calcd for [**I2AD**+H$^+$]: 4933; found: 4935; **I2B**: calcd for [**I2B**+H$^+$]: 4351; found: 4351; **I6AD**: calcd for [**I6AD**+H$^+$]: 6346; found: 6346; **I6B**: calcd for [**I6B**+H$^+$]: 5764; found: 5764; **J2AD**: calcd for [**J2AD**+H$^+$]: 4921; found: 4921; **J2B**: calcd for [**J2B**+H$^+$]: 4339; found: 4341; **D1**: calcd for [**D1**+H$^+$]: 4227; found: 4227.

Spectroscopic measurements: Fluorescence spectra were measured on a JASCO model FP-6500 with a microcell. The excitation wavelength was 500 nm. The sample solutions were as follows: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 1.0 μM. Quantum yield were determined from the quantum yield of Rhodamine 6G in ethanol (0.94) as a reference.

Measurement of the melting temperature: The melting curve of duplex DNA was obtained with a Shimadzu UV-1800 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] = 5.0 μM.

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