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Yuichi Ohya[a]; Kentaro Yabuki[a]; Motonari Tokuyama[a]; Tatsuro Ouchi[a]
[a] Department of Applied Chemistry, Faculty of Engineering and High Technology Research Center, Kansai University, Suita, Osaka, Japan

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Construction and Energy Transfer Behavior of Sequential Chromophore Arrays on an Oligo-DNA Assembly

YUICHI OHYA*, KENTARO YABUKI, MOTONARI TOKUYAMA and TATSURO OUCHI

Department of Applied Chemistry, Faculty of Engineering and High Technology Research Center, Kansai University, Suita, Osaka 564-8680, Japan

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Sequential arrays of chromophores at regulated distances were constructed on a non-covalent DNA molecular assembly system in aqueous media. Photo-induced energy transfer behaviors were then observed. We designed a number of chromophore/oligo-DNA conjugates with varying residue sequences. The chromophores eosin (Eo), Texas Red (TR) and tetramethylrhodamine (TMR) were employed as energy donor, acceptor and mediator, respectively, based on overlapping excitation and emission spectra. The chromophores were attached to the 5′-terminals of 10 residue oligo-DNAs using aminolinkers. Arrangements of Eo–TMR or TMR–TR were constructed by duplex formation of conjugates with a 20 mer matrix oligo-DNA comprising complementary sequences to the conjugates. Single-step photo-induced energy transfer from Eo to TMR and from TMR to TR was confirmed for the duplex. The three kinds of chromophore conjugates were then mixed with longer matrix oligo-DNAs (30 or 40 mer) consisting of complementary sequences to the conjugates, producing Eo–$(TMR)_n$–TR ($n = 1$ or 2) arrays. Multi-step photo-induced energy transfer from Eo to TR through the TMR mediator(s) was observed on the molecular assemblies. This photo-energy transmission system offers a good model for artificial photosynthetic systems.

Keywords: Fluorescence resonance energy transfer; DNA; Complementary hydrogen bond; Molecular assembly

INTRODUCTION

Supramolecular assembly via non-covalent interactions has become an area of intense interest, not only in organic, inorganic and biological chemistry, but also in materials science. Well-organized structures on the nanometer scale constructed by self-assembly are extremely useful in the development of new systems and materials for nanotechnology. This area of science has progressed by taking natural systems as ultimate models. In natural photosynthetic systems, arrangements of porphyrin derivatives with regulated distances and geometry provide highly efficient photo-induced energy transfer in non-covalent assembly systems [1,2]. Sequential arrangement of chromophores using non-covalent interactions in aqueous media would therefore provide a good model for artificial photosynthetic systems. The biological tasks of DNA are genetic information storage and propagation. The DNA duplex contains a rich π-electron system comprising four bases stacked upon each other. A number of researchers have proposed the kinetics of electron transfer through DNA duplex or conductivity of DNA duplex [3–7]. In recent years, labeling of DNA using fluorescent probes and measurement of energy transfer behaviors between probes have been investigated for detection of duplex or triplex formation of DNA [8,9], structural analyses of DNA [10,11], diagnostic detection of specific genes [12,13] and other purposes [14,15]. On the other hand, a wide variety of non-covalent binding pairs, binding donors and binding acceptors with high specificity and stability can easily be provided by varying sequences of oligo-DNAs. Oligo-DNAs are therefore useful molecules for constructing molecular assembly systems. In fact, high-molecular-weight supramolecular polyassembly systems using complementary oligo-DNAs as binding groups have been reported by our group [16] and Takenaka *et al.* [17]. Using such characteristics of oligo-DNAs, sequential arrangement of functional molecules (groups) can be easily provided. For example, specific chromophores with overlapping excitation and emission spectra

*Corresponding author. Tel.: +81-6-6368-0818. Fax: +81-6-6339-4026. E-mail: yohya@ipcku.kansai-u.ac.jp

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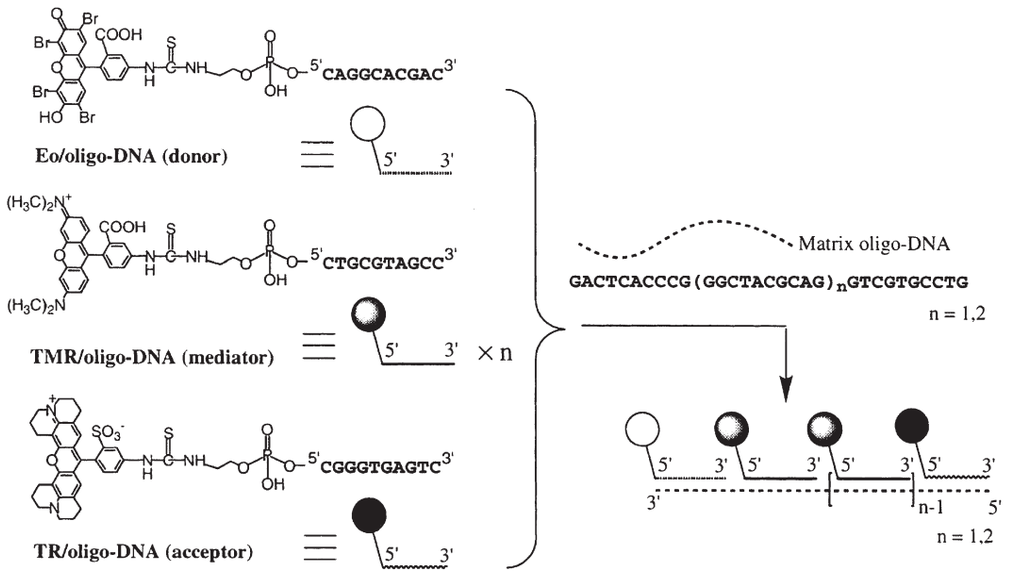
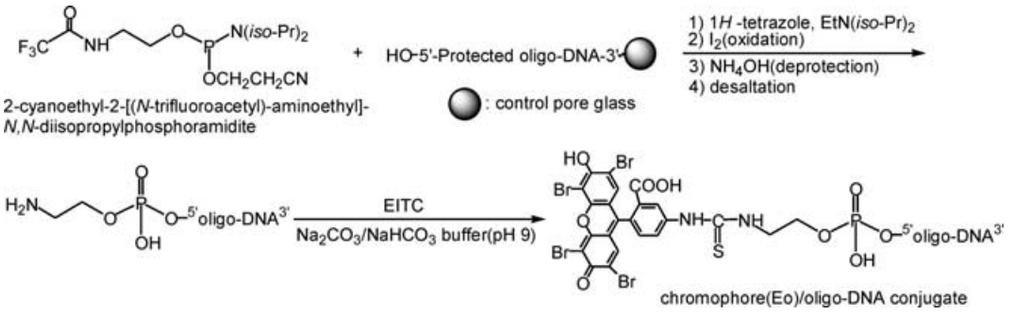


FIGURE 1 Structures of the chromophore/oligo-DNA conjugates and schematic illustration for the construction of an array of chromophores by mixing with complementary matrix oligo-DNA.

and arranged in order of excitation energy level should offer a "photo-energy transmission pathway". Such systems would provide a good model for artificial photosynthetic systems and assist our understanding of the natural photosynthetic system. Previously, we utilized DNA duplex formation to construct sequential arrangements of the chromophores fluorescamine, rhodamine B and fluorescein as photo-energy donor, acceptor and mediator, respectively. We then reported their photo-induced energy transfer behavior in aqueous media [18]. Multi-step photo-induced energy transfer from fluorescamine to rhodamine B through one or more fluorescein mediators was observed. However, the thermodynamic stability of the system was less than ideal, as only five residues were used for complementarity-determining regions and measurements therefore had to be performed at low temperature (5°C).

In the present study, we designed a new series of chromophore/oligo-DNA conjugates with 10 nucleotide residues comprising complementarity-determining regions. Using these, we constructed a stable sequential arrangement of chromophores separated by a regulated distance, to produce multi-step photo-induced energy transfer in a non-covalent molecular assembly system in aqueous media. Three different chromophores with overlapping excitation and emission spectra were utilized: eosin (Eo); Texas Red (TR); and tetramethylrhodamine (TMR), as photo-energy donor, acceptor and mediator, respectively. These were attached to the 5′-terminals of oligo-DNAs displaying differing sequences of 10 residues. The resulting conjugates were mixed with matrix oligo-DNA (30 or 40 residues) consisting of complementary sequences for the conjugates (Fig. 1), to construct a sequential Eo–$(TMR)_n$–TR array (where $n = 1$ or 2) in a DNA



SCHEME 1

duplex with 10 residues separating each chromophore component. The most common conformation of the DNA duplex is the B-form, where 10 residues form one turn. Chromophores on the DNA duplex were thus arranged on the same side of the DNA duplex separated by one helical pitch (34 Å). Energy transfer behavior between chromophores was then investigated by monitoring fluorescence spectra in buffer solution.

RESULTS AND DISCUSSION

The syntheses of chromophore/oligo-DNA conjugates were successfully performed using 5′-aminoethyl-oligoDNAs and amine-reactive chromophores, eosin isothiocyanate (EITC), tetramethylrhodamine isothiocyanate (TRITC) and Texas Red sulfonyl chloride (TRS-Cl), according to Scheme 1. The purity and structure of the conjugates obtained were confirmed by reverse-phase high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), respectively. We selected Eo, TR and TMR as the photo-energy donor, acceptor and mediator, respectively, due to the overlapping excitation and emission wavelength ranges. The λ_{max} values of excitation/emission spectra for Eo/oligo-DNA, TMR/oligo-DNA, TR/oligo-DNA were 525/547, 554/578, and 597/609 nm, respectively. The T_m value of stoichiometric mixtures of the three chromophore/oligo-DNA conjugates with matrix oligo-DNA (30 mer) was determined to be 52°C in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) (Fig. 2).

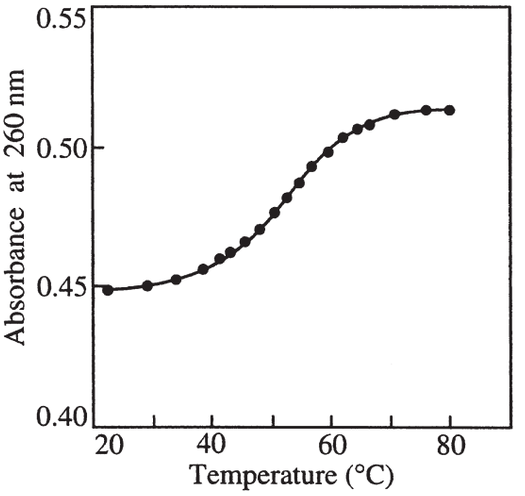


FIGURE 2 Thermal dissociation curve for equivalent mixture of Eo/oligo-DNA, TMR/oligo-DNA, TR/oligo-DNA and matrix oligo-DNA (30 mer) monitored by UV absorbance at 260 nm in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5, 1.7×10^{-7} mol dm^{-3}).

The matrix oligo-DNA was composed of 3 units of 10 residues. Each of the 3 units was complementary with a different conjugate. The melting curve seemed to display a single transition point, and no obvious double or triple transition points. This means each of the 10 residue units had almost the same T_m. The T_m value of the stoichiometric mixture of matrix oligo-DNA and three kinds of oligo-DNA having the same sequences as the conjugates was 55°C. The mixture of conjugates and matrix oligo-DNA revealed a T_m 3°C lower than the corresponding oligo-DNAs assembly without chromophores. The chromophores attached

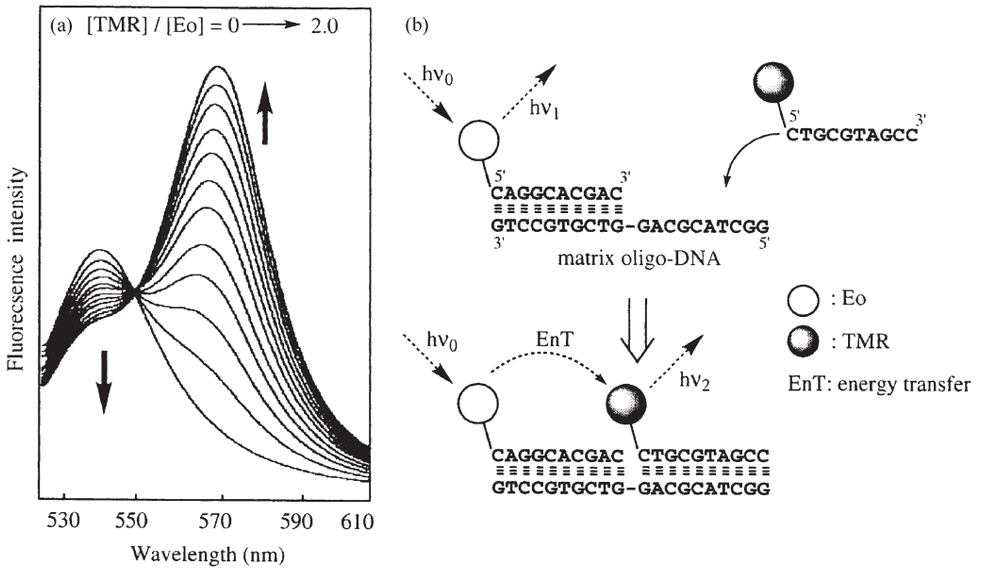


FIGURE 3 (a) Fluorescence spectra for titration of complex of Eo/CAGGCACGAC and GGCTACGCAGGTCGTGCCTG by TMR/CTGCGTAGCC in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C and (b) schematic representation of experiment. [GGCTACGCAGGTCGTGCCTG] = [Eo/CAGGCACGAC] = 1.0×10^{-6} mol dm^{-3}, [TMR/CTGCGTAGCC]/[Eo/CAGGCACGAC] = 0–2.0. Excitation wavelength: 525 nm.

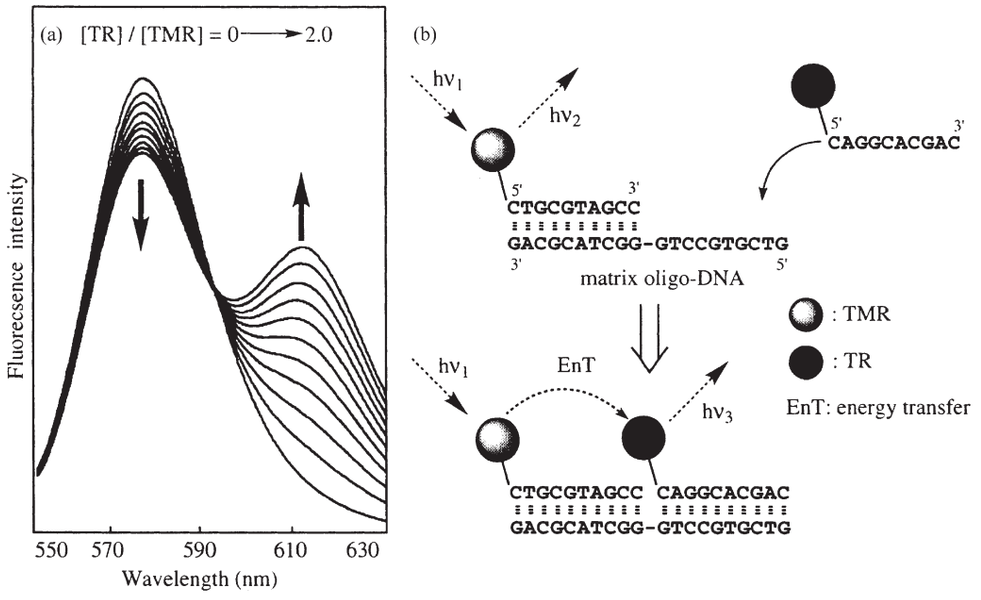


FIGURE 4 (a) Fluorescence spectra for titration of complex of TMR/CTGCGTAGCC and GTCGTGCTGGGCTACGCAG by TR/CAG GCACGAC in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C and (b) schematic representation of experiment. [GTCGTGCTGGGCTACGCAG] = [TMR/CTGCGTAGCC] = 1.0×10^{-6} mol dm^{-3}, [TR/CAGGCACGAC]/[TMR/CTGCGTAGCC] = 0–2.0. Excitation wavelength: 554 nm.

at the 5′-terminals of oligo-DNA exerted slight inhibitory effects on the formation of the duplex, but the difference was minimal. According to the results, the following fluorescence measurements were performed at 10°C, which is sufficiently lower than the T_m. In addition, specific and non-specific interactions of chromophores with single- and double-strand DNA, such as intercalation, were not observed in either UV or fluorescence spectra measurements.

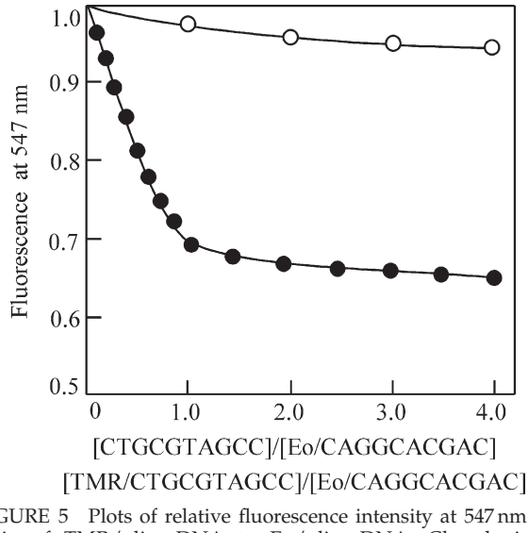


FIGURE 5 Plots of relative fluorescence intensity at 547 nm vs. ratio of TMR/oligo-DNA to Eo/oligo-DNA. Closed circle, TMR/oligo-DNA; open circle, 1:1 mixture of complementary oligo-DNA without chromophores and free TMR.

Before studying multi-step energy transfer, we investigated single-step photo-induced energy transfer behavior between two chromophores. Experiments were performed using 20 mer matrix oligo-DNA, donor/oligo-DNA displaying complementarity with the 3′-terminal 10 residues of the matrix oligo-DNA and acceptor/oligo-DNA displaying complementarity with 5′-terminal 10 residues of the matrix oligo-DNA. Figure 3 shows the fluorescence spectra for titration of the Eo/CAGG-CAAGAC conjugate (donor, Eo/oligo-DNA) and a 20 mer matrix oligo-DNA (5′-GGCTACGCAGGTC-GTGCCTG-3′) by the TMR/CTGCGTAGCC conjugate (acceptor, TMR/oligo-DNA). Fluorescence spectra were measured with an excitation wavelength of 525 nm (λ_{max} of Eo) in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C. A schematic illustration of the experiment is also shown. The quenching of fluorescence from donor chromophores (Eo) and increased fluorescence from acceptor chromophores (TMR) were observed on the addition of TMR/oligo-DNA. Effective quenching and increased fluorescence of TMR were not observed when a mixture of free TMR and oligo-DNA (5′-CTGCGTAGCC-3′) was used instead of TMR/oligo-DNA. The same experiment was performed for the combination of TMR/oligo-DNA and TR/oligo-DNA. Figure 4 shows the fluorescence spectra for titration of TMR/oligo-DNA conjugate (donor) and a 20 mer matrix oligo-DNA (5′-GACT-CACCCGGGCTACGCAG-3′) by the TR/CGGGTG-AGTC conjugate (acceptor, TR/oligo-DNA). Fluorescence spectra were measured with an

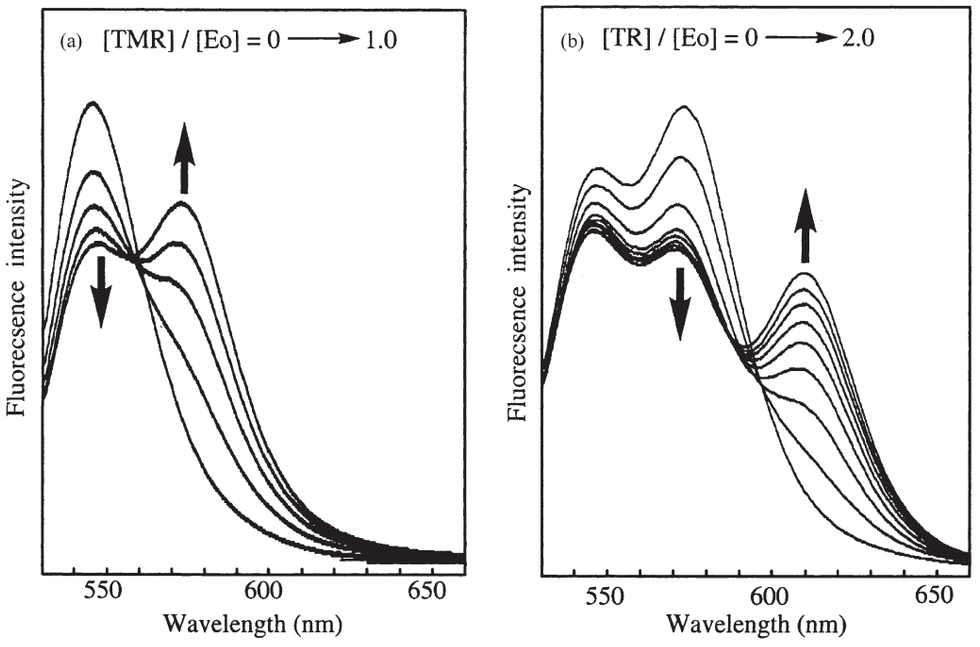


FIGURE 6 (a) Fluorescence spectra for titration of complex of Eo/CAGGCACGAC and GACTCACCCGGGCTACGCAGG TCGTGCCTG by TMR/CTGCGTAGCC, and (b) titration of equivalent complex of Eo/CAGGCACGAC, TMR/CTGCGTAGCC and GACTCACCCGGGCTACGCAGGTCGTGCCTG by TR/CGGGTGAGTC in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C. [GACTCACCCGGGCTACGCAGGTCGTGCCTG] = [Eo/CAGGCACGAC] = 1.0×10^{-6} mol dm^{-3}, [TMR/CTGCGTAGCC]/[Eo/CAG GCACGAC] = 0–1.0, [TR/CGGGTGAGTC]/[Eo/CAGGCACGAC] = 0–2.0. Excitation wavelength: 525 nm.

excitation wavelength of 554 nm (λ_{max} of TMR) in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C. A schematic illustration of the experiment is also shown. The quenching of fluorescence from donor chromophores (TMR) and increased fluorescence from acceptor chromophores (TR) was observed on the addition of TR/oligo-DNA, too. Effective quenching and increased fluorescence of TR were not observed when a mixture of free TMR and oligo-DNA (5′-CGGGTGAGTC-3′) was used instead of TR/oligo-DNA. Figure 5 shows relative fluorescence intensity of Eo at 547 nm vs. the molar ratio of TMR/oligo-DNA to Eo/oligo-DNA as a typical example. The quenching of donor (Eo) was almost saturated when the molar ratio of acceptor(TMR)/oligo-DNA to donor(Eo)/oligo-DNA was 1.0. Energy transfer efficiency based on quenching of the donor chromophore was calculated using the following equation:

$$\mathrm{EnT}(\%) = (q - q_0)/(1 - q_0)$$

$$q = 1 - I/I_0$$

where q is the quenching with acceptor/oligo-DNA conjugate, q_0 the quenching with free chromophore and oligo-DNA without chromophore, I the fluorescence intensity, and I_0 the initial fluorescence intensity of donor/oligo-DNA conjugate

Energy transfer efficiencies for Eo–TMR system and TMR–TR system were 31 and 19%, respectively, when the molar ratio of acceptor/oligo-DNA to donor/oligo-DNA was 1.0. These results indicate that effective fluorescence resonance energy transfer (FRET) can occur between these chromophores arranged along a DNA duplex with one pitch distance (ca. 34 Å).

Since single-step energy transfer behavior was successfully observed between two chromophores, we then investigated two-step energy transfer from Eo (donor) through TMR (mediator) to TR (acceptor) on a duplex with a 30 mer matrix oligo-DNA. In the first step, Eo/oligo-DNA displaying complementarity with the 3′-terminal 10 residues of the matrix oligo-DNA was mixed with matrix oligo-DNA in a 1:1 ratio. The complex formed was then titrated by TMR/oligo-DNA displaying complementarity with the central 10 residues of the matrix oligo-DNA to an equivalent amount. In the second step, the complex of Eo/oligo-DNA and TMR/oligo-DNA with matrix oligo-DNA (1:1:1 ratio) was titrated by TR/oligo-DNA displaying complementarity with the 5′-terminal 10 residues of the matrix oligo-DNA. Figure 6 shows the fluorescence spectra on the two-step titration for the complex of 30 mer matrix oligo-DNA and Eo/oligo-DNA by TMR/oligo-DNA, and subsequent titration by TR/oligo-DNA. Fluorescence spectra were measured with an excitation wavelength of 525 nm (λ_{max} of Eo) in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C. The schematic illustration of the experiment is shown in Fig. 7. In the first step, quenching of Eo and increased

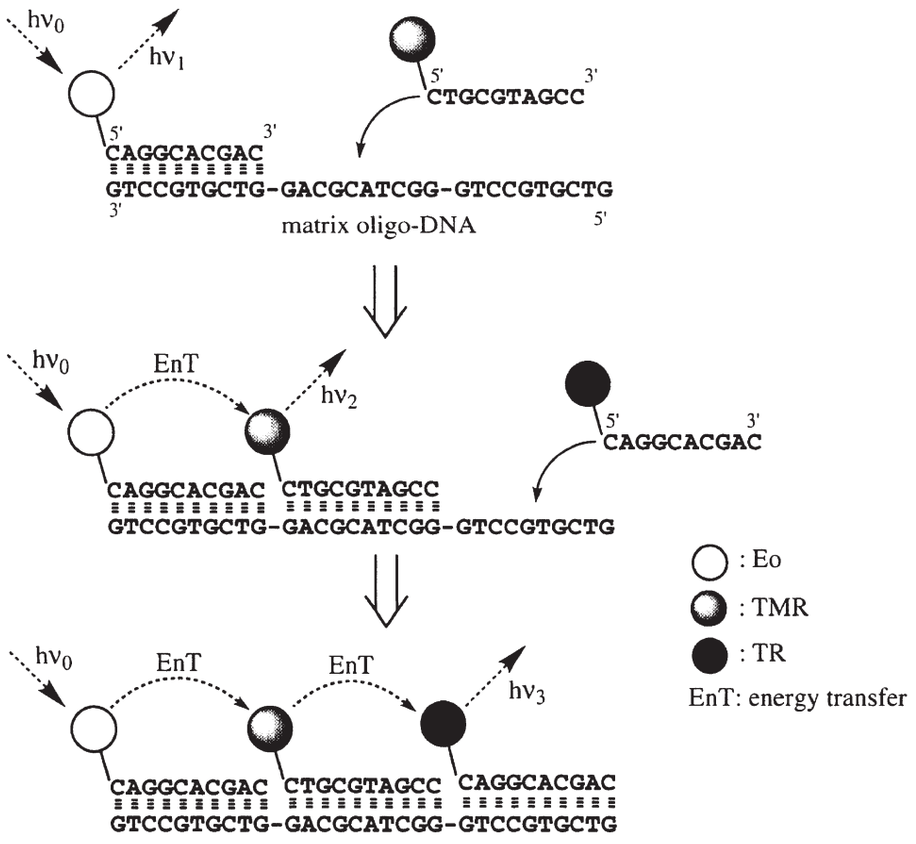


FIGURE 7 Schematic illustration for the experiment shown in Fig. 6.

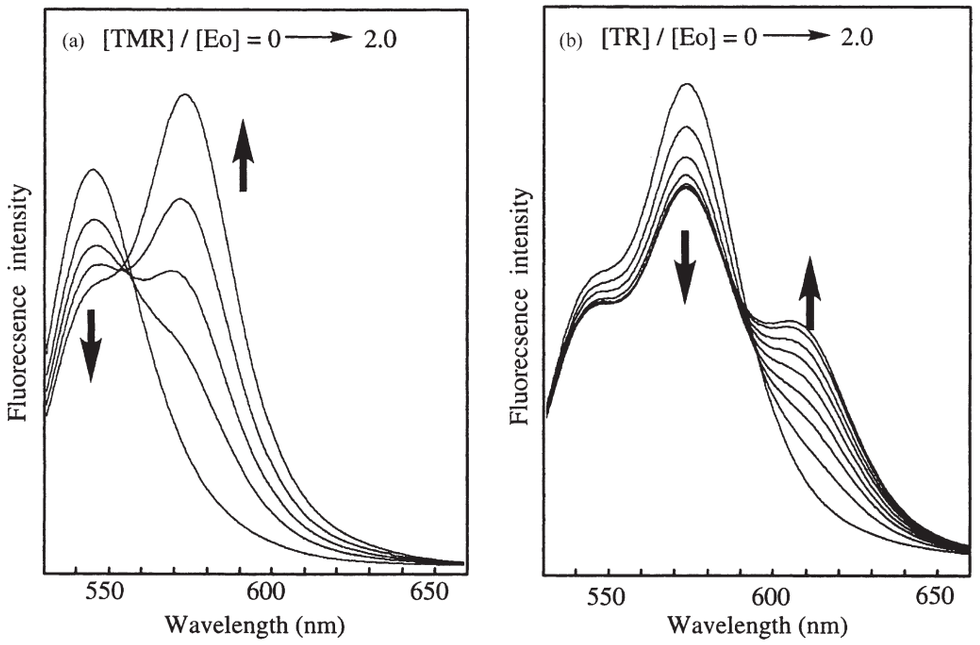


FIGURE 8 (a) Fluorescence spectra for titration of complex of Eo/CAGGCACGAC and GACTCACCCG(GGCTACGCAG)$_2$GTC GTGCCTG by two equivalent of TMR/CTGCGTAGCC, and (b) titration of 1:2:1 complex of Eo/CAGGCACGAC, TMR/CTGCGTAGCC and GACTCACCCG(GGCTACGCAG)$_2$GTCGTGCCTG by TR/CGGGTGAGTC in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C. [GACTCACCCG(GGCTACGCAG)$_2$GTCGTGCCTG] = [Eo/CAGGCACGAC] = 1.0×10^{-6} mol dm^{-3}, [TMR/CTGCGTAGCC]/[Eo/CAG GCACGAC] = 0–2.0, [TR/CGGGTGAGTC]/[Eo/CAGGCACGAC] = 0–2.0. Excitation wavelength: 525 nm.

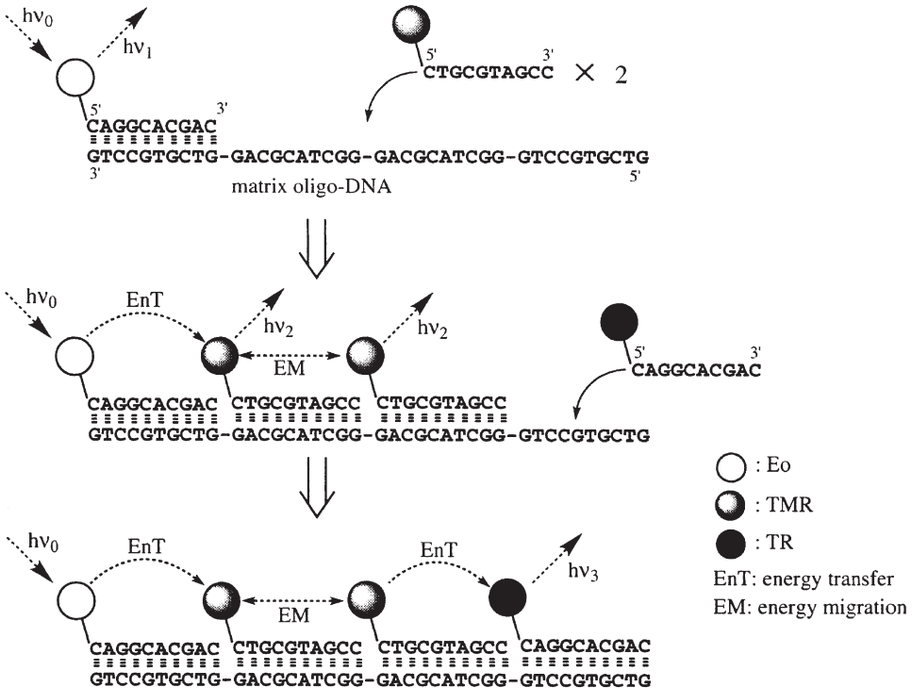


FIGURE 9 Schematic illustration for the experiment shown in Fig. 8.

fluorescence from TMR were observed on the addition of TMR/oligo-DNA, as shown in Fig. 3. In the second step, quenching of TMR and increased fluorescence from TR were observed on the addition of TR/oligo-DNA, as shown in Fig. 4. The fact that obvious fluorescence from TR was observed with the excitation wavelength at 525 nm indicates that the photo-energy transferred from Eo to TMR was further transferred to TR by two-step FRET. As a control experiment, we also measured the direct energy transfer from Eo to TR using oligo-DNA (5′-CTGCGTAGCC-3′) without the TMR chromophore instead of TMR/oligo-DNA. The energy transfer efficiency calculated from quenching of Eo was only 9% due to 20 residues separation (two pitches) and less overlapping excitation and emission wavelength ranges.

Furthermore, we investigated multi-step energy transfer from Eo (donor) through two TMRs (mediators) to TR (acceptor) on a duplex with a 40 mer matrix oligo-DNA containing two repetitions of the 10 residues complementary with TMR/oligo-DNA in the central region. As a first step, Eo/oligo-DNA displaying complementarity with the 3′-terminal 10 residues of the matrix oligo-DNA was mixed with the matrix oligo-DNA in a 1:1 ratio. The complex formed was then titrated by TMR/oligo-DNA displaying complementarity with the central 2×10 residues of the matrix oligo-DNA, up to an equivalent amount with the matrix oligo-DNA. In the second step, the complex of Eo/oligo-DNA and TMR/oligo-DNA with matrix oligo-DNA (1:2:1 ratio) was titrated by TR/oligo-DNA displaying complementarity with the 5′-terminal 10 residues of the matrix oligo-DNA. Figure 8 shows the fluorescence spectra for the two-step titration for the complex of 40 mer matrix oligo-DNA with Eo/oligo-DNA by TMR/oligo-DNA, and subsequent titration by TR/oligo-DNA. Fluorescence spectra were measured with an excitation wavelength of 525 nm (λ_{max} of Eo) in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 10°C. The schematic illustration of the experiment is shown in Fig. 9. In the first step, quenching of Eo and increased fluorescence from TMR were observed on addition of TMR/oligo-DNA, as shown in Figs. 3 and 6. In the second step, quenching of TMR and increased fluorescence from TR were observed on the addition of TR/oligo-DNA, as shown in Fig. 6. The fact that obvious fluorescence from TR was observed with the excitation at 525 nm indicates that the photo-energy transferred from Eo through the paired TMRs was further transferred to TR. These results suggest that energy migration between two TMRs occurred.

The energy transfer efficiencies observed on the molecular assemblies of chromophore/oligo-DNA conjugates with matrix oligo-DNAs based on quenching of the nearest chromophore to acceptor chromophore considering quenching by the complementary oligo-DNAs were summarized in Table I. The values of EnT from Eo to TMR were similar

TABLE I Energy transfer efficiency (EnT) observed on the molecular assembly using chromophore/oligo-DNA conjugates

Chromophore combination	Single step	Eo–TMR–TR system	Eo–TMR–TMR–TR system
Eo → TMR*	31	30	29
TMR → TR†	19	29	20
Eo → (blank) → TR*	–	9‡	–

EnT(%) = $(q - q_0)/(1 - q_0)$, $q = 1 - I/I_0$; where q is the quenching with acceptor/oligo-DNA conjugate, q_0 the quenching with oligo-DNA without chromophore and free chromophore, I the fluorescence intensity and I_0 the initial fluorescence intensity of donor/oligo-DNA conjuagate. The fluorescence intensity of each chromophores in the assembly system was calculated by decomposition of the obtained spectra to the emission spectra from the each chromophores. *Calculated based on the quenching of Eo. †Calculated based on the quenching of TMR. ‡Observed in Eo-(bank)-TR system by using oligo-DNA having the same sequence with TMR/oligo-DNA.

(ca. 30%) in all the experiments. These results mean that the distances between Eo and TMR were consistent in all systems. Although the values of EnT from TMR to TR for TMR–TR and Eo–TMR–TMR–TR systems were similar (ca. 20%), higher EnT from TMR to TR (29%) was observed in the Eo–TMR–TR system. This is because this value includes the direct energy transfer from Eo to TR (9%) over a distance of 20 residues (two pitches of duplex, 68 Å) as observed in the control experiment using oligo-DNA (5′-CTGCGTAGCC-3′) without the TMR chromophore instead of TMR/oligo-DNA.

To obtain information about the conformation of the assembly, circular dichroism (CD) spectra were measured for equivalent mixtures of chromophore/oligo-DNA conjugates and matrix oligo-DNA at 15°C. Figure 10 shows a typical example for the equivalent mixtures of Eo/oligo-DNA, TMR/oligo-DNA, TR/oligo-DNA and 30 mer matrix oligo-DNA, which were used in the experiment shown in Fig. 6. Strong negative and positive cotton effects were observed around 245 and 280 nm, respectively. This pattern displays the typical characteristics of a B-form DNA duplex. The assembly system of the three kinds of chromophore/oligo-DNA conjugates with the matrix oligo-DNA thus displayed B-form double helical conformation. Figure 11 shows geometrical illustrations for the assemblies of the three kinds of chromophore/oligo-DNAs and matrix oligo-DNA (30 or 40 mer) when assuming B-form double helical conformation. As the B-form DNA duplex has 10 residues per turn (34 Å), the system was expected to have all chromophores on the same side of the duplex at one-pitch distance (34 Å), as shown in Fig. 11. From the results shown in Fig. 9 and Table I, it was suggested that the multi-step photo-induced energy transfer occurred from Eo to TR separated by three helical pitches distance (104 Å)

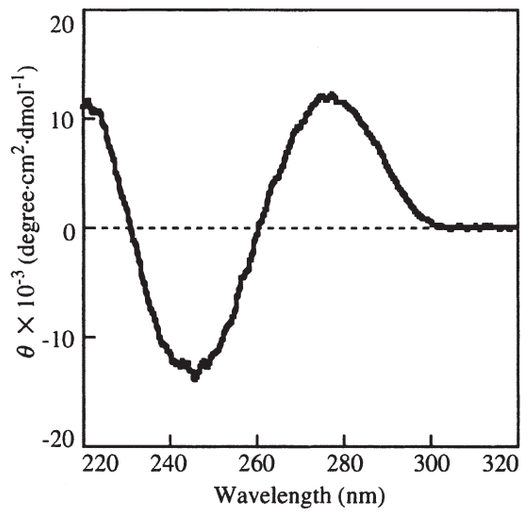


FIGURE 10 CD spectra for equivalent mixture of Eo/CAGGC ACGAC, TMR/CTGCGTAGCC, TR/CGGGTGAGTC and GAC TCACCCGGGCTACGCAGGTCGTGCCTG in 0.05 M Tris HCl–0.5 M NaCl buffer (pH 7.5) at 15°C. Concentration of each conjugate and matrix oligo-DNA = 1.0×10^{-5} mol dm^{-3}.

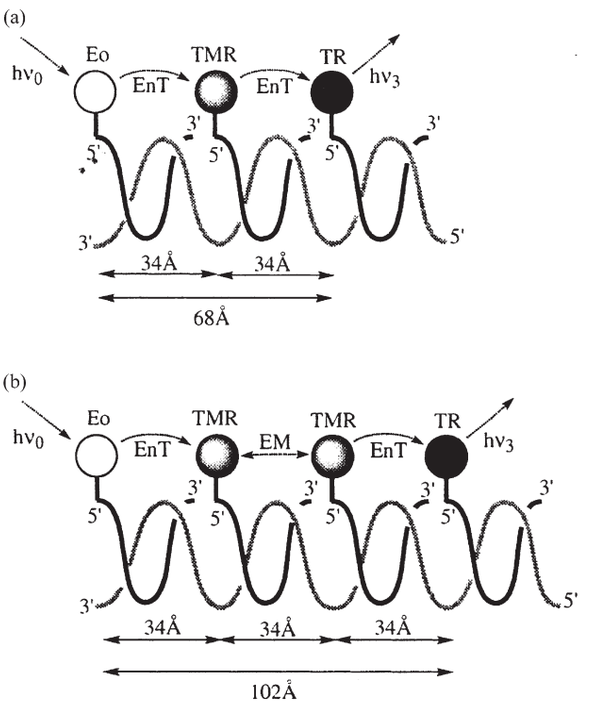


FIGURE 11 Schematic illustration for geometry of chromophore arrays constructed on oligo-DNA assemblies. (a) 1:1:1:1 mixture of Eo/CAGGCACGAC, TMR/CTGCGTAGCC, TR/CGGGTGAGTC and GACTCACCCGGGCTACGCAGGTCGTGCCTG and (b) 1:2:1:1 mixture of Eo/CAGGCACGAC, TMR/CTGCGTAGCC, TR/CGGGTGAGTC and GACTCACCCG(GGCTACGCAG)$_2$GTC GTGCCTG.

through TMR mediators on the molecular assemblies.

Details of the fluorescence resonance energy transfer behaviors of chromophores on oligo-DNA assemblies should be discussed using measurements of fluorescence lifetime and transition state absorption spectra of the chromophores. Such data should be investigated in subsequent research.

CONCLUSIONS

Three kinds of chromophores, Eo, TMR and TR, were attached to the 5′-terminals of 10 mer oligo-DNAs to produce chromophore/oligo-DNA conjugates. Programmed sequential arrangements of chromophores separated by regulated distances of about one pitch of DNA duplex (34 Å) on non-covalent molecular assembly systems in aqueous media were constructed by mixing conjugates with longer matrix oligo-DNAs. Multi-step photo-induced energy transfer from Eo to TR through one or two TMR mediators was observed on the molecular assemblies. A long distance energy transfer along the oligo-DNA duplex was suggested. The information obtained in these systems will be useful in constructing a photon-collecting antenna for an artificial photosynthetic system, in addition to enhancing our understanding of the mechanisms of energy transfer behavior in non-covalent molecular assembly system such as natural photosynthetic systems.

MATERIALS AND METHODS

General Methods

The UV–Vis. absorption spectra and fluorescence spectra were recorded on UV-2500PC (Shimadzu, Japan) and F4010 (Hitachi, Japan) spectrophotometers, respectively. CD spectra were measured using a J-600 (JASCO, Japan). Matrix-assisted laser desorption ionization time of flight mass spectrometry experiments for chromophore/oligo-DNA conjugates were performed on a MAT Vision 2000 [negative mode, matrix: 3-hydroxypicolic acid (H_2O/acetonitrile = 7/3)] (Finnigan, USA) after samples were treated with 0.1 M diammonium hydrogen citrate solution. Reverse-phase HPLC was carried using a Toso-8020 system with a TSKgel ODS-120T column.

Materials

The EITC, TRITC and TRS-Cl were purchased from Molecular Probes, Inc. (USA). 5′-End free fully protected oligo-DNAs on solid support (control pore glass, CPG) and non-modified oligo-DNAs were obtained from Toagosei Co., Ltd. (Japan) or Hokkaido System Science, Co. (Japan). Aminoethanol, ethyl trifluoroacetate, 1*H*-tetrazole/acetonitrile solution and anhydrous acetonitrile were purchased from Wako Pure Chemical Industries Ltd. (Japan). 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite was purchased from Aldrich Chemical Co. Inc. (USA). Water was purified using a reverse-osmotic membrane. *N*,*N*-Dimethylformamide and other organic solvents were purified by usual distillation methods. Other materials were of commercial grade and used without further purification.

Synthesis of Chromophore/oligo-DNA Conjugates

Synthesis of chromophore/oligo-DNA conjugates was performed using the same methods reported previously [18]. Structures and sequences of the chromophore/oligo-DNA conjugates synthesized are shown in Fig. 1. A typical example for the preparation of an Eo/oligo-DNA conjugate is as follows. 5′-Aminoethyl-oligoDNA prepared by the methods reported previously [18] was dissolved in 200 ml of sodium carbonate/bicarbonate buffer (1 M, pH 9, 200 ml) and added to EITC solution in DMF (50 ml, 10 mg ml^{-1}). The reaction mixture was kept in the dark at room temperature for 5 h. The resulting Eo/oligo-DNA conjugate was purified by gel-filtration chromatography (Sephadex G-25, water) and reverse-phase HPLC (column: TSKgel ODS-120T, eluent: 0.1 M aqueous triethylammonium acetate/acetonitrile, 90/10 to 60/40 gradient) until a single sharp peak was obtained. Identification of conjugates was performed using MALDI-TOF-MS. Attachment of TMR or TR to oligo-DNAs was performed under the same procedures using TRITC or TRS-Cl, respectively, instead of EITC. Matrix-assisted laser desorption ionization time of flight mass spectrometry (negative mode, $[M - H]^-$): for Eo/CAGGCAAGAC calc. 3850.0, found 3852.8; for TMR/CTGCGTAGCC calc. 3571.6, found 3570.0; for TR/CGGGTGAGTC calc. 3795.8, found 3793.2.

Spectroscopic Studies

The concentration of unmodified oligo-DNA was calculated from the UV absorbance at 260 nm (A260), and extinction coefficients were determined using the nearest-neighbor approximation. The concentration of each chromophore/oligo-DNA conjugate was determined using UV–Vis. absorbance at 525, 554 or 597 nm for Eo/oligo-DNA conjugate, TMR/oligo-DNA conjugate or TR/oligo-DNA conjugate, respectively. The concentration of the conjugate in solution was confirmed by the fact that their hypochromicity with complementary unmodified oligo-DNAs was maximal at a 1/1 mole ratio (data not shown). Hypochromicity measurement was

performed by monitoring A_{260} of the solution containing the conjugate and complementary oligo-DNA in various ratios. Melting curves were recorded by starting at a temperature sufficiently above T_m and reducing temperature at a rate of 10°C h^{-1} to a temperature sufficiently below T_m. Absorbance values were continuously recorded at intervals of 5°C. The CD spectra were measured on a JASCO J-600 using quartz cells with 0.5 cm light-path length at 15°C. Concentrations of each conjugate and matrix oligo-DNA were 1.0×10^{-5} mol dm^{-3}. Fluorescence measurements were performed on a Hitachi F4010, using a 1 cm × 1 cm quartz cell. Excitation wavelengths used were 525, 554 and 597 nm for Eo, TMR and TR, respectively. Fluorescence spectra for the mixture of several chromophore/oligo-DNA conjugates and complementary matrix oligo-DNA were obtained by varying the mixing ratios. The following procedure shows a typical example for titration of the complex of Eo/CAGGCACGAC and GGCTACGCAGGTCGTGCCTG by TMR/CTGCGTAGCC. "Solution A" containing Eo/CAGGCACGAC (1.0×10^{-6} mol dm^{-3}) and GGCTACGCAGGTCGTGCCTG (1.0×10^{-6} mol dm^{-3}), and "Solution B" containing Eo/CAGGCACGAC (1.0×10^{-6} mol dm^{-3}), GGCTACGCAGGTCGTGCCTG (1.0×10^{-6} mol dm^{-3}) and TMR/CTGCGTAGCC (8.0×10^{-6} mol dm^{-3}) were prepared. The fluorescence spectra of Solution A were measured under titration by "Solution B". The final ratio of TMR/CTGCGTAGCC to Eo/CAGGCACGAC was 4.0. All fluorescence experiments were performed in 50 mM Tris–HCl buffer, pH 7.5, containing 500 mM NaCl at 10°C. The fluorescence intensity of each chromophore in mixed systems with two or more chromophores was calculated by decomposition of obtained spectra to the emission spectra from each chromophore.

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