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Coumarin–fluorescein pair as a new donor–acceptor set for fluorescence energy transfer study of DNA

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Abstract

A method for introduction of the 2′-coumarin labeled nucleoside as a fluorescence energy donor into DNA duplexes has been described. Efficient FRET occurs between the coumarin–fluorescein pair in DNA owing to the high quantum yield of the donor. The present donor–acceptor pair may be useful as FRET indicator of DNA structures in solution. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: coumarin–fluorescein pair; nucleoside; fluorescence energy transfer; DNA.

There have been several reports concerned with applications of fluorescence energy transfer (FRET) in studies on nucleic acid structures such as helical four-way junctions in DNA topology,[1] geometry of bent DNA molecules,[2] and relative orientation of the helical segments in RNA hammerhead ribozyme.[3] In these applications, the 5′- or 3′-end or the phosphate backbone of oligonucleotides has been used for the attachment of the donor and acceptor molecules. Although FRET has been successfully applied to analysis of complex nucleic acid structures, there remained the difficulty in generating a donor–acceptor pair at appropriate positions of nucleic acids. We have recently shown that the sugar 2′-position of DNA is a suitable site for covalent attachment of 6-dimethylamino-2-naphthamide (DAN)[4] and fluorescein (F) moieties as a fluorescence energy donor–acceptor pair. Our approach to the synthesis of fluorescent labeled DNA is based on the combined use of pre- and post-labeling technique,[5] thus providing great flexibility for rapid construction of a donor–acceptor pair in DNA.

The DAN–F pair has been expected to be useful in FRET measurement of DNA.[5] However, the fluorescence of the donor (DAN) incorporated into DNA exhibited weak emission of ca. 1% quantum yield even in the absence of the acceptor. This low quantum yield causes difficulty or inaccuracy in determination of distance-dependent FRET by the usual fluorescence measurements. The donor quantum efficiency also affects the critical distance at which FRET efficiency is 0.5.[6] Higher quantum yield of the donor leads to longer critical distance of FRET. Efficient FRET at long distance (several tenth Å) should be necessary for analysis of complex nucleic acid structures in solution. We now describe the synthesis of DNA duplexes containing a coumarin–fluorescein pair at the sugar residues. This new fluorescence energy donor–acceptor set is promising as a FRET indicator of DNA.

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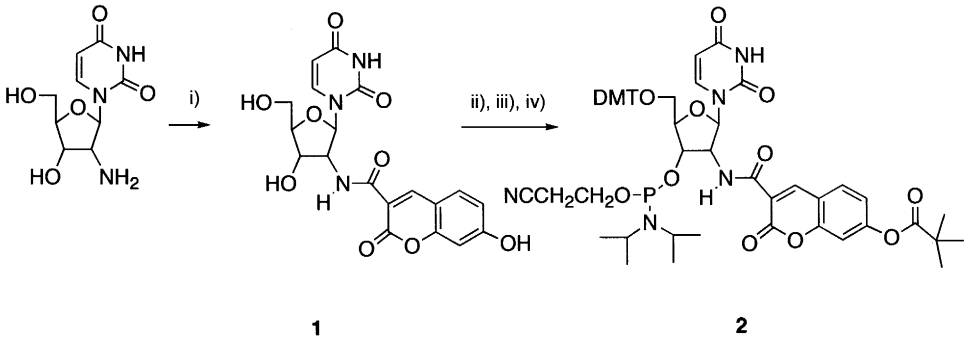
We have chosen the highly fluorescent coumarin derivative as the fluorescence energy donor, because coumarin and fluorescein fluorophores show suitably overlapping spectra for FRET.[7] For site-specific incorporation of coumarin fluorophore into oligonucleotides, the nucleoside phosphoramidite **2** was synthesized. As shown in Scheme 1, 2′-amino-2′-deoxyuridine was allowed to react with 7-hydroxy-3-coumarin carboxylic acid[8] using DCC (rt, overnight) giving modified uridine with coumarin at the 2′-position [U(HCC) **1**].[9] After protection at 5′-hydroxyl function by dimethoxytrityl and 7-OH of the coumarin ring by a trimethylacetyl group, nucleoside **1** was converted by the usual method to the phosphoramidite **2**.



1 **2**

Scheme 1. (i) 7-Hydroxy-3-coumarin carboxylic acid (1 equiv.), DCC (1.2 equiv.), HOBT (1.2 equiv.) in DMF; 89% yield; (ii) dimethoxytrityl chloride (1.2 equiv.) in pyridine; 80% yield; (iii) trimethylacetyl chloride (1 equiv.) in pyridine; 79% yield; (iv) 2-cyanoethyl-N,N′,N′′,N′′′-tetraisopropylphosphorodiamidite (1.5 equiv.), tetrazole (1 equiv.) in CH_2Cl_2; 59% yield

Oligonucleotides containing one coumarin fluorophore at the different sites, **3**–**5**, were synthesized by using **2** according to a fully automated procedure described earlier.[5] After deprotection, the oligomers were purified with 20% denaturing polyacrylamide gel electrophoresis. The integrity of each oligomer was verified by ion-spray MS.[10] The binding and fluorescence properties of oligomers **3**–**5** are summarized in Table 1. The oligonucleotide **3** that contains U(HCC) at the terminal fraying 5′-end retains normal binding affinity for DNA, whereas incorporation of U(HCC) into other sites as in oligonucleotides **4** and **5** causes destabilization of the modified duplexes. Similar duplex stability depending on the site of incorporation has been observed for the duplexes containing dansyl modified uridine[11] and U(DAN) modified uridine.[5] The fluorescence emission maximum of U(HCC) modified oligonucleotides appeared at 450 nm which is well overlapped with the UV–vis absorption of fluorescein. Fluorescence quantum yields of the modified duplexes were estimated to be ca. 10%. These observations indicate that the U(HCC) modified oligonucleotides may be a suitable donor to fluorescein labels in FRET study.

Table 1
Tm values and fluorescence properties of U(HCC) modified oligonucleotide duplexes

Sequence	Tm (℃)	λ emission (nm)	Q.Y. (%)
5′-TCTAGAGGTCAT	40.0		
3: 5′-U(HCC)CTAGAGGTCAT	39.4	451	9
4: 5′-TCU(HCC)AGAGGTCAT	35.4	450	10
5: 5′-TCTAGAGGU(HCC)CAT	30.6	451	8

UV melting at 260 nm and fluorescence measurements were carried out at single strand concentration of 2.0 x 10^{-5} M in 0.1 M NaCl, 0.01 M sodium phosphate (pH = 7.0) in which 5'-ATGCCTCTAGA was used for a complementary strand. Fluorescence quantum yields (Q.Y.) were estimated at 5 ℃ based on quinine sulfate in 1.0 N sulfuric acid as a standard.

To test the validity of the coumarin–fluorescein pair in distance-dependent FRET measurement, we prepared the DNA duplexes possessing different numbers of base-pairs between these fluorophores (**6**+**10**, **7**+**11**, **8**+**12**, and **9**+**13**).[12] Fig. 1 represents the fluorescence spectra observed in the typical FRET experiments. The duplex of oligomer **6** with unmodified oligonucleotide exhibited fluorescence at 450 nm whose quantum yield is 25%. When compared with this duplex, the fluorescence of the donor was largely weakened by introduction of the acceptor. With this fluorescence quenching, enhancement of the emission derived from the acceptor at 523 nm was observed. On the contrary, little or no fluorescence changes were observed in the presence of non-complementary oligonucleotides with fluorescein label. All the labeled duplexes displayed similar fluorescence properties. These observations indicate that the intramolecular FRET occurs between coumarin and fluorescein labels along the DNA duplex. The FRET efficiencies (Eapp) measured for the duplexes are shown graphically in Fig. 1 where the Eapp from the duplexes labeled with DAN–fluorescein pair[5] are also represented. It is seen that the Eapp is clearly dependent on the number of nucleotides in the DNA. The relatively large Eapp was observed between the coumarin–fluorescein pair when compared with the DAN–fluorescein pair in the DNA of the same length.[13] Since the spectral overlap integral is similar in DAN–fluorescein and coumarin–fluorescein pairs, the highly fluorescent coumarin is mainly responsible for the large Eapp.[6]

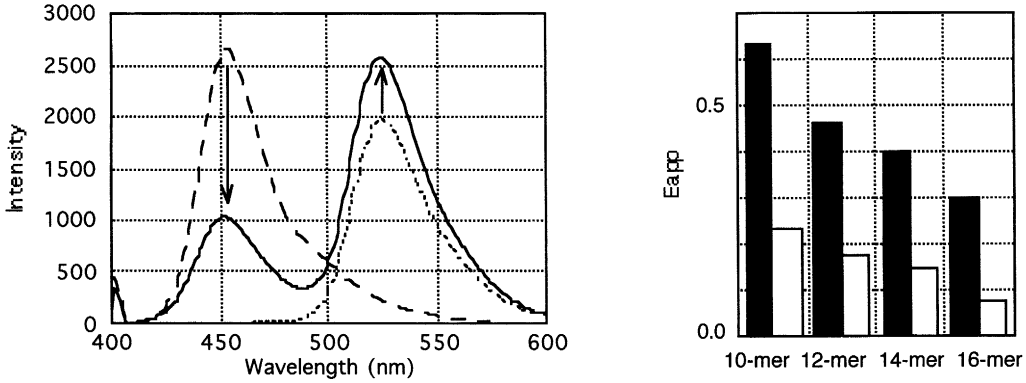


Fig. 1. *Left panel*: Fluorescence spectra for typical FRET measurement of DNA duplex (10-mer) at a total strand concentration of 4.0×10^{-5} M. The measurements were carried out at 3°C in a buffer containing 1.0 M NaCl and 0.01 sodium phosphate, adjusted to pH 7.0. Oligomer **6**+5′-TA_9 (- - - -), 5′-T_9A+oligomer **10** (· · · · ·), oligomer **6**+**10** (solid line). *Right panel*: Apparent FRET efficiency (Eapp) in DNA duplexes containing various numbers of base pairs in which the coumarin–fluorescein pair is shown by black bars and the DAN–fluorescein pair by white bars. The sequences of oligomers used for FRET study are shown below. For the study of the DAN–F pair, U(DAN) was incorporated into the donor strand in place of U(HCC)[5]

Donor strand	Acceptor strand
6: 5′-U(HCC)T_8A	**10:** 5′-U(F)A_9
7: 5′-U(HCC)T_{10}A	**11:** 5′-U(F)A_{11}
8: 5′-U(HCC)T_{12}A	**12:** 5′-U(F)A_{13}
9: 5′-U(HCC)T_{14}A	**13:** 5′-U(F)A_{15}

We have described a method for introduction of the 2′-coumarin labeled nucleoside as a FRET donor in DNA duplexes. Efficient FRET occurs between the coumarin–fluorescein pair in DNA owing to the high quantum yield of the donor. The present FRET donor–acceptor pair may thus be useful for analysis of DNA structures in solution.

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6. For energy transfer occurring via the Forster mechanism, the distance in Å between donor and acceptor fluorophores are deduced from the relationship, $R=R_0[(1-E)/E]^{1/6}$. R_0 is the critical distance at which transfer efficiency E is 0.5, calculated according to $R_0^6=8.8\times10^{-25}\times\kappa^2$ F J n^{-4}, where κ^2 is the orientation factor, J is the spectral overlap integral, n is the refractive index of the medium, and F is the fluorescence quantum yield of the donor in the absence of the acceptor.
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9. Nucleoside **1**, TLC (silica, CH_2Cl_2:MeOH=1:1, v/v) R_f 0.61; ^{1}H NMR (500 MHz, DMSO-d_6): δ: 3.62 (m, 2, $H_{5',5''}$), 4.00 (m, 1, $H_{4'}$), 4.17 (m, 1, $H_{3'}$), 4.58 (m, 1, $H_{2'}$), 5.22 (t, 1, 5′-OH, D_2O exchange), 5.71 (d, 1, uracil H_5), 5.96 (d, 1, $H_{1'}$), 6.15 (d, 1, 3′-OH, D_2O exchange), 6.79 (d, 1, coumarin H_4), 6.86 (dd, 1, coumarin H_5), 7.80 (d, 1, coumarin H_6), 7.96 (d, 1, uracil H_6), 8.83 (s, 1, coumarin H_8), 9.18 (d, 1, C2′-amide), 11.22 (s, 1, uracil NH, D_2O exchange).
10. Ion-spray mass data for oligomers **3**–**5** were 3849.5 (calcd 3848.6), 3848.9 (calcd 3848.6), and 3849.0 (calcd 3848.6), respectively.
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12. The fluorescein labeled oligonucleotides were synthesized by the published procedure (Ref. 5).
13. The distances between coumarin and fluorescein fluorophores estimated from the theory (Ref. 6) were in fairly good agreement with the values based on the molecular model of DNA.