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HOMOGENEOUS FLUORESCENCE ASSAYS FOR RNA DIAGNOSIS BY PYRENE-CONJUGATED 2′-*O*-METHYLOLIGORIBONUCLEOTIDES

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□ *We developed a bispyrene-conjugated 2′-O-methyloligoribonucleotide as an RNA-specific RNA-probe. The probe hybridized with the complementary RNA, greatly enhancing fluorescence and discriminating RNA from DNA. The assay was carried out in homogeneous aqueous media without removing the unbound probe from the detection solution. This homogeneous fluorescence assay also discriminated mismatch sequences in the target RNA. These pyrene probes could possess high potential to detect RNA in biological specimens simply.*

Keywords Pyrene-conjugated fluorescence probe; homogeneous fluorescence assay; RNA diagnosis; SNP

INTRODUCTION

Like proteome, transcriptome has become an important issue in the second generation of the genome project.[1,2] The profile of mRNA expression reveals the detailed features of the genetic cascade. Thus far, analyses have been carried out mainly by DNA chips after the amplification of target RNAs via an RT-PCR protocol. To elucidate the transcriptome from other viewpoints, it is desirable to directly analyze target RNAs as they are in the living cell. We have developed fluorescent RNA probes for such analysis.[3,4] These probes each contain one or two 2′-*O*-pyrenylmethyluridines in the middle of the oligonucleotides (mono- and bispyrene-conjugated 2′-*O*-methyloligoribonucleotide; OMUpy and OMUpy2, respectively). Both probes represent unique fluorescent characteristics.[3,4] The fluorescence intensity at 375 nm of OMUpy was drastically enhanced only when it was

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hybridized with its complementary oligoribonucleotides (cORN), and was scarcely enhanced when hybridized with its complementary DNA (cODN).[4] OMUpy2 showed broad fluorescence around 480 nm when hybridized with cORN, and barely showed fluorescence when hybridized with cODN. These results suggest that OMUpy2 could be used to visually detect specific RNAs in homogeneous media.[5] However, there is a limitation in the design of the probe sequences. That is, each target site should contain consecutive AA sites. In this proceeding, we report the development and characteristics of novel bispyrene-conjugated 2′-*O*-methyloligoribonucleotides that contain 2′-*O*-pyrenylmethylcytidine and/or 2′-*O*-pyrenylmethyluridine, denoted as OMCpy2, OMUpyCpy, and OMCpyUpy, respectively.

MATERIALS AND METHODS

Preparation of 2′-*O*-pyrenylmethylcytidine (Cpy)[6]

2′/3′-*O*-(pyrenylmethyl)cytidine (2). Sodium hydride (60%) (600 mg, 15 mmol) was added to a solution of cytidine (3.65 g, 15 mmol) in dry dimethylformamide (150 mL) at 5°C under N_2 atmosphere. After the mixture was stirred for 1 hour, chloromethylpyrene (1.25 g, 5 mmol) was added and the reaction was maintained at 70°C for 16 hours. Ice was then added and the reaction mixture was concentrated near dryness. After 50 mL of $CHCl_3$ and 50 mL of water were added to the residue, the insoluble solid was filtered, washed twice with cooled water and $CHCl_3$, and dried in vacuo. A mixture of 2′/3′-isomers (495 mg, 22%) was obtained as brown powder.

4-*N*-benzoyl-2′/3′-*O*-(pyrenylmethyl)cytidine(3)

A mixture of 2′/3′-isomers of 2′/3′*O*-pyrenylmethylcytidine (**2**) (228 mg, 0.5 mmol) was dried by repeated coevaporation with pyridine and suspended in 2.5 mL of dry pyridine. To the solution was added 317 μL (2.5 mmol) of trimethylchlorosilane. After the mixture was stirred for 15 minutes, 290 μL of benzoyl chloride (2.5 mmol) was added and the reaction was maintained at room temperature for 2 hours. The mixture was then cooled in an ice bath and 0.5 mL of water was added. After 5 minutes, 0.5 mL of 29% aqueous ammonia was added and the mixture was stirred at room temperature for 0.5 hour. The reaction was then evaporated to near dryness and the residue was dissolved in 50 mL of $CHCl_3$-MeOH (3:1, v/v). The solution was washed three times with 30 mL of water. The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give 2′/3′-isomers of **3**(127 mg, 45%) as brown powder.

4-*N*-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(pyrenylmethyl) cytidine (4)

A mixture of 2′/3′-isomers of 4-*N*-benzoyl-2′/3′-*O*-(pyrenylmethyl) cytidine(**3**) (400 mg, 0.71 mmol) was dried by repeated coevaporation with pyridine and suspended in 10 mL of dry pyridine. To the solution was added 4,4'-dimethoxytritylchloride (290 mg, 0.86 mmol), and the mixture was stirred at room temperature for 12 hours. The mixture was concentrated to half volume, diluted with $CHCl_3$, and washed three times with 5% $NaHCO_3$(aq). The organic layer was dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified by flash chromatography to give **4** (515 mg, 84%) as white foam.

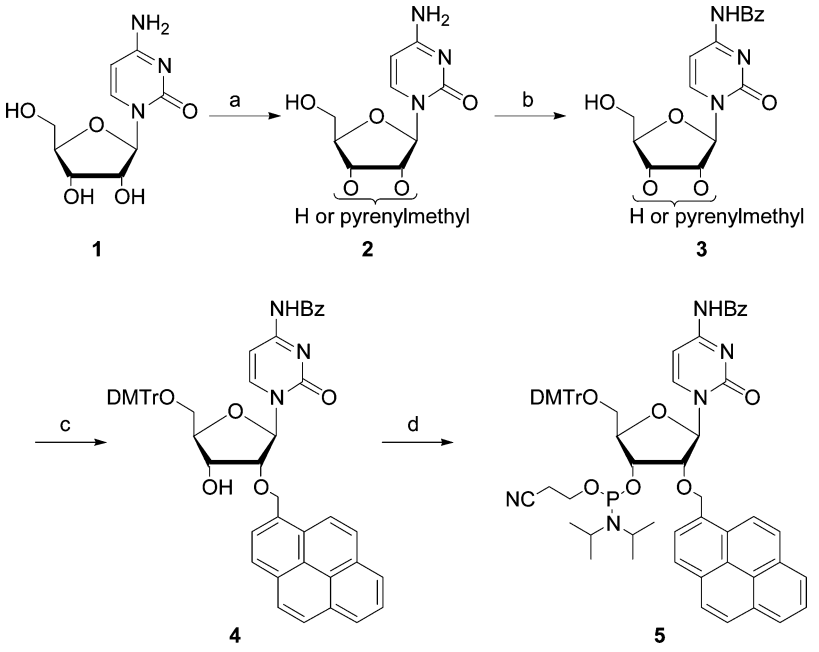
Preparation of Bispyrene-Conjugated 2′-*O*-methyloligoribonucleotides

Bispyrene-conjugated 2′-*O*-methyloligoribonucleotides (OMCpy2, OMUpyCpy, OMCpyUpy) were synthesized according to the reported procedure.[3] Fluorescence spectra were obtained by a spectrophotometer (RF-5300PC, Shimadzu, Japan) equipped with a thermal controller. Equimolar solutions of OMUpy2 (0.75 μM) and corresponding ORN (0.75 μM) were denatured at 75°C for 5 minutes and slowly cooled to 11°C prior to measurements in 10 mM sodium phosphate (pH 7.0), 0.1 M NaCl.

RESULTS AND DISCUSSION

The synthesis of the Cpy phosphoramidite unit is shown in Scheme 1. The synthetic route begins with 2′-*O*-alkylation of cytidine using chloromethylpyrene. A mixture of the 2′,3′-isomers was obtained, and the pure 2′-product was not isolated until the DMT stage. The ratio of the 2′-isomer to the 3′-isomer, estimated from the intensity of the 1'-H proton of 2′/3′-*O*-(pyrenylmethyl)cytidine using ^{1}H NMR, was 5:1. Subsequent protection of the amino group of cytosine with the benzoyl group, followed by the addition of a dimethoxytrityl group at the 5′-*O*-position, gave compound **4**. Conversion to phosphoramidite using chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine provided a Cpy phosphoramidite unit.

OMCpy2, OMUpyCpy, and OMCpyUpy, denoted as RNA probes (Figure 1), each contained one or two 2′-*O*-pyrenylmethylcytidines inside the ORN sequence. The fluorescence intensity of each probe around 375 nm was less than 5% that of 1-pyrenemethanol, showing that the fluorescence due to the pyrenes was greatly quenched. In contrast, the hybrid of the probe with cORN showed eminent and structureless fluorescence around 480 nm, as shown in Figure 2, as well as slight fluorescence appearing around



SCHEME 1 Reagents and conditions : a) (1) NaH (3 equiv.) DMF, 5°C, 1 hour; (2) chloromethylpyrene (1 equiv.), 70°C, 16 hours; b) (1) TMSCL (5 equiv.), pyridine, (2) BzCl, (5 equiv.), pyridine, (3) NH_3, c DMTrCl (1.2 equiv.), pyridine, d chloro(2-cyanoethoxy)-(N,N-diisopropylamino)phosphine (2 equiv.) diisopropylethylamine (5 equiv.), $CHCl_2$, 1.5 hours.

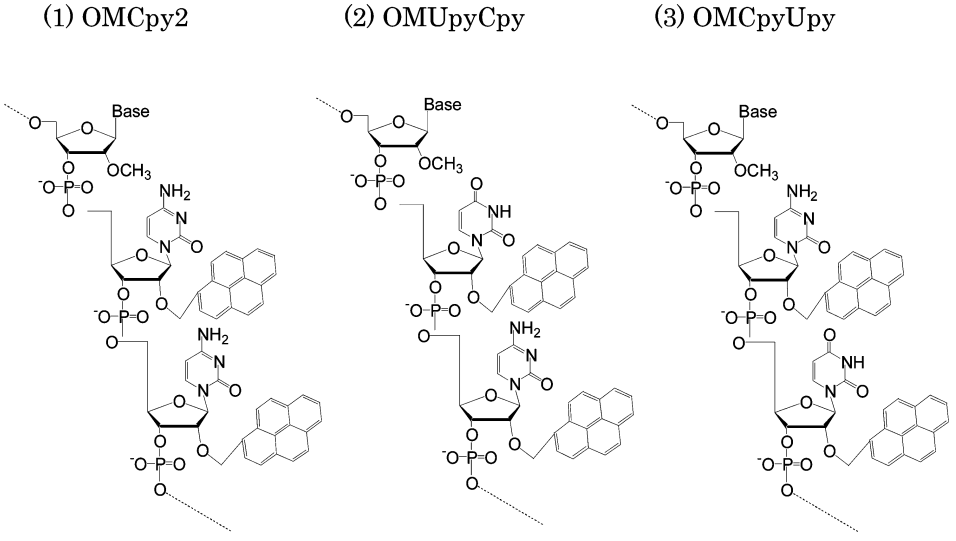


FIGURE 1 Bispyrene-conjugated 2′-*O*-methyloligoribonucleotide.

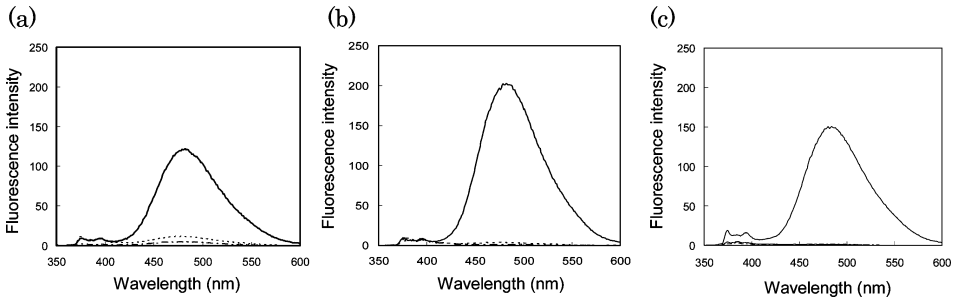


FIGURE 2 Fluorescence spectra of OMCpy2, OMUpyCpy, and OMCpyUpy in the presence of the complementary oligoribonucleotide. (a) OMCpy2, (b) OMUpyCpy, (c) OMCpyUpy Dashed line: OMCpy2; OMUpyCpy; OMCpyUpy, Solid line: OMCpy2 + cORN; OMUpyCpy + cORN; OMCpyUpy + cORN. [OMCpy2] = [OMUpyCpy] = [OMCpyUpy] = [ORN] = 0.75 μM Temperature: 11°C.

375 nm, suggesting that the pyrenes relocated from the regions between nucleobases to the outside of the duplex and consequently interacted with each other. Interestingly, the fluorescence intensity of the hybrid of the probe with cODN was scarcely changed, suggesting that the pyrenes in the duplex of OMUpy2 and cODN remained intercalated between nucleobases. These results were substantially similar to the characteristics of OMUpy2, while the fluorescence intensities varied depending on the kind of pyrene-conjugated nucleoside. The difference could be attributed to the local duplex structure near the pyrene moieties. It should be noted that these RNA probes are RNA-specific fluorescence probes and that they are applicable to gene diagnosis.

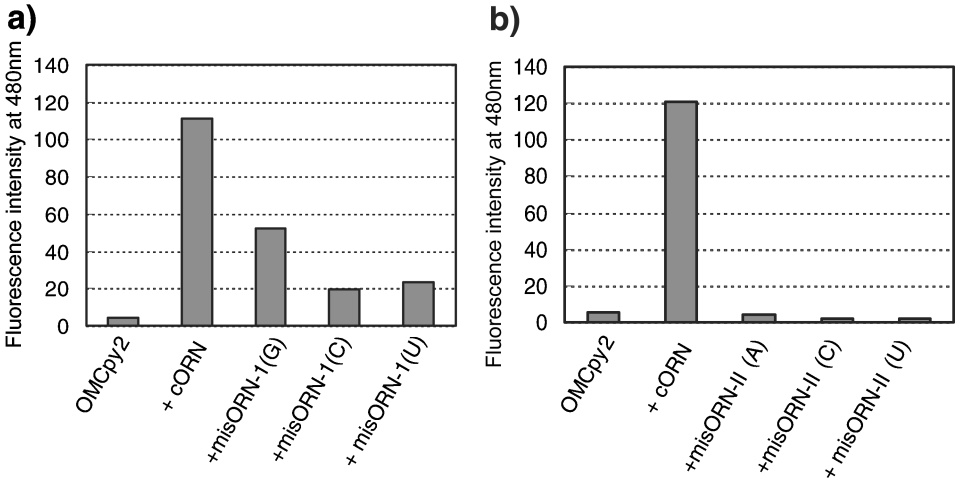


FIGURE 3 Fluorescence intensity at 480 nm of OMCpy2 in the presence of the oligoribonucleotides. ORNs sequence: r (GGUGGUGAAGC**X**GGCG); ORNs sequence: r (GGUGGUGAAGCA**X**GCG).

The characteristics of the probes were then applied to discriminate mismatch sequences in ORNs. Figure 3 shows the results of the case of OMCpy2. Fluorescence was scarcely observed when the mismatch site was located in the vicinity of the Cpy residue, and increased as the mismatch site grew apart from the Cpy residue. These results suggest that the pyrene excimer (or pyrene dimer) formed only when the pyrene-conjugated nucleoside was incorporated in the firm duplex, and that these bispyrene-conjugated oligonucleotides could be unique and prominent RNA probes to detect SNPs. The probe was then immobilized on solid matrix such as a glass plate to fabricate an RNA chip, which was able to detect the complementary ORN without a washing protocol. Further study is underway.

CONCLUSION

We have developed a novel fluorescent oligonucleotide probe that can detect specific RNA and can discriminate a singly altered sequence of RNA. Future development of the probe should enable its use in living cells. For this purpose, we have chosen phosphorothioate linkages to construct the probes, and this work is under way.

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