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DNA–Naphthyl Red conjugate as a visualizing probe of DNA hybridization†

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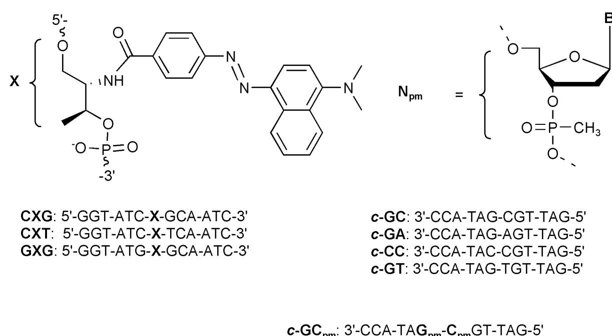
The Naphthyl Red moiety, conjugated to DNA, shows distinct chromism by hybridization with its complementary DNA. Single-stranded DNA involving the Naphthyl Red moiety exhibits an orange color and has λ_{max} at 466 nm at pH 7.0. The absorption maximum is shifted towards 545 nm by the presence of its complementary DNA, and the color of the solution changes from orange to magenta accordingly.

Various organic dyes have been developed as probes to detect or visualize oligonucleotides. A chromosome can be stained with quinacrine by its intercalation between the base-pairs, and thus is easily discriminated from other organelles in the cell.[1] Thiazole orange is available for the detection or purification of RNAs since it can stain RNAs.[2] Some cyanine dyes are known to be bound to the groove of a duplex by forming J- or H-aggregates.[3] However, there has been little investigation of the organic dye probe that changes its color by DNA hybridization.[4] If hybridization of oligonucleotides can be easily detected as a change of color, the scope of the application should be extended to areas such as SNP (single nucleotide polymorphism) detection by the naked eye.[5] Here, we report on the DNA–Naphthyl Red conjugate as a visualizing probe of DNA hybridization. A significant change of absorption maximum is induced when this conjugate is hybridized with its complementary DNA.

Modified DNAs carrying a Naphthyl Red moiety, listed in Scheme 1, were prepared from the corresponding phosphoramidite monomer synthesized in a similar manner to the method previously reported.[6,7] Single-stranded **CXG** involving a Naphthyl Red moiety exhibited an orange color and showed λ_{max} at 466 nm at pH 7.0 and 0 °C, as depicted by the dotted line in Fig. 1A. In contrast, addition of its complementary strand (***c*-GC**) to **CXG** significantly changed its color: the peak maximum at 466 nm disappeared and a new peak appeared at 545 nm as depicted by the solid line in Fig. 1A. The bathochromic shift induced by the hybridization was as large as 79 nm. Accordingly, the solution changed its color from orange to magenta (see graphical abstract). When the temperature of this solution was raised above the T_m of **CXG–*c*-GC** duplex (44.5 °C as estimated from the change of absorbance at 260 nm as a function of temperature), the magenta color (λ_{max} at 545 nm) disappeared and the solution became orange (λ_{max} at 455 nm) because the duplex was dissociated (see Fig. 1B). This color change was completely reversible. The chromism of Naphthyl Red did not significantly depend on the kind of nucleotide adjacent to the **X** residue (see Table 1). The melting temperature obtained from the change of absorbance at 550 nm (43.5 °C) coincided with the T_m (44.5 °C) within error, indicating that hybridization is responsible for this chromism.[8] Therefore, even a mismatch in oligonucleotide was detectable with this conjugate by the naked eye by choosing an appropriate temperature because of the notable decrease in T_m of the mismatched sequence (see Fig. 2).[9]

This chromism depended on the pH of the sample solution as depicted in Fig. 3. Below pH 5.0, since both single-stranded **CXG** and **CXG–*c*-GC** duplex had λ_{max} at around 550 nm due to the protonation of Naphthyl Red (see Scheme 2), chromism of Naphthyl Red was hardly observed. When the pH of the solution was above 6.5, distinct chromism could be induced by hybridization. It should be noted that this chromism was observed even when the pH was as high as 9.0, where Naphthyl



Scheme 1 Modified DNAs synthesized in this study.

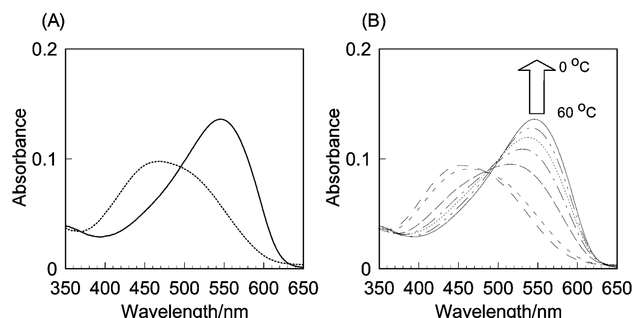


Fig. 1 UV–Vis spectra of (A) single-stranded **CXG** (dotted line) and **CXG–*c*-GC** duplex (solid line) at 0 °C, and (B) **CXG–*c*-GC** duplex at various temperatures (10 °C interval) at pH 7.0 (10 mM phosphate buffer) in the presence of 100 mM NaCl. [DNA] = 5 μM.

Table 1 Effect of the kinds of nucleobase adjacent to **X** residue on the chromism[a]

Duplex	T_m/°C	Absorption maximum/nm [b] at 60 °C	at 0 °C
CXG–*c*-GC	44.5	455	545
CXT–*c*-GA	37.5	457	540
GXG–*c*-CC	41.5	457	539

[a] Solution conditions: pH 7.0 (10 mM phosphate buffer), [NaCl] = 100 mM, [DNA] = 5 μM. [b] Since T_ms of all the duplexes are around 40 °C, modified DNAs exist as a single strand at 60 °C and as a duplex at 0 °C in solution.

† Electronic supplementary information (ESI) available: synthetic procedure (Scheme S1), quantitative data for the chromism in Fig. 2 (Table S1), and location of Naphthyl Red in the duplex (Fig. S1). See http://www.rsc.org/suppdata/cc/b3/b302875e/

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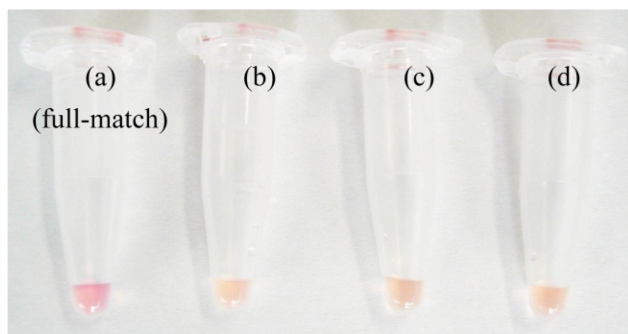


Fig. 2 Colour of Naphthyl Red moiety in **CXG** in the presence of (a) full match ***c*-GC**, (b) ***c*-GA**, (c) ***c*-GT**, and (d) ***c*-CC**. Solution conditions were pH 7.0 (10 mM phosphate buffer) in the presence of 100 mM NaCl at 40 °C, where only full-match ***c*-GC** (a) can sufficiently hybridize with **CXG** (see Table S1† for the quantitative data).

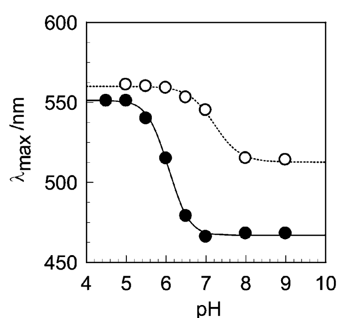
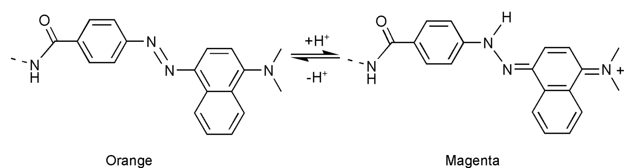


Fig. 3 Effect of pH on the absorption maximum of the Naphthyl Red moiety in **CXG** in the presence (open circles) or absence (closed circles) of ***c*-GC** at 10 °C in the presence of 100 mM NaCl.

Red was deprotonated (see Fig. 3).[10] Thus, duplex formation could be detected as chromism with this DNA–dye conjugate.

Naphthyl Red changes its color from orange to magenta by the protonation of the azo group at low pH as Methyl Red (4-[4-(dimethylamino)phenylazo]benzoic acid) does (Scheme 2).[11] But the present chromism is mainly caused by a large bathochromic shift of deprotonated Naphthyl Red by hybridization. Deprotonated Naphthyl Red in the single-strand and duplex gave λ_{max} at 460 and 510 nm, respectively. This large discrepancy of λ_{max} was the principal origin of this chromism. At around pH 6.5, an increase in the pK_a of Naphthyl Red by hybridization enhanced this chromism. The pK_a of single-stranded **CXG** was determined as 5.8, and was increased to 6.5 by hybridization with its complementary strand (***c*-GC**).[12] Accordingly, the increase in pK_a additionally contributed to the chromism of Naphthyl Red at pH 7 where the **CXG–*c*-GC** duplex was examined in Fig. 1: Naphthyl Red is partially protonated in the duplex and is deprotonated in the single strand.

It is assumed that the negatively charged environment provided by the deprotonated phosphodiester linkages in the duplex caused this large bathochromic shift and the change of pK_a by hybridization. These anions on phosphodiesters would decrease the electron density on the dimethylamino group and raise the density on the azo group, which contributed to both the large bathochromic shift and the increase in pK_a.[13] NMR analysis revealed that the dimethylamino group on the naphthalene ring was located near the phosphodiesters on the



Scheme 2 Protonation and deprotonation of Naphthyl Red.

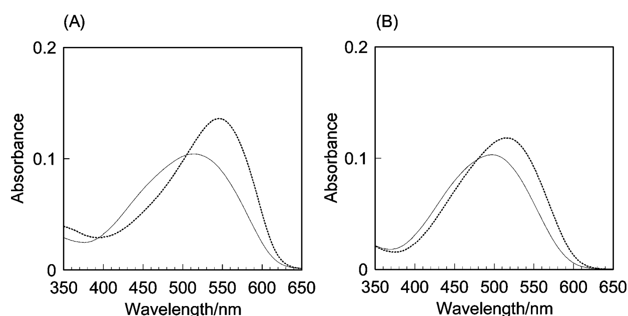


Fig. 4 UV–Vis spectra of **CXG–*c*-GC$_{pm}$** (solid line) and **CXG–*c*-GC** (dotted line) at 0 °C in the presence of 100 mM NaCl, (A) pH 7.0 and (B) pH 9.0. Note that the T_ms of **CXG–*c*-GC$_{pm}$** and **CXG–*c*-GC** are 40.9 and 44.5 °C at pH 7.0, and 41.3 and 42.0 °C at pH 9.0, respectively.

complementary strand due to the intercalation of the Naphthyl Red moiety.[14] Significantly, hybridization of **CXG** with ***c*-GC$_{pm}$** in which two phosphodiesters nearest to the dimethylamino group are replaced with non-charged methylphosphonate (see Scheme 1 and Fig. S1) induced much smaller chromism both at pH 7.0 and 9.0 (compare the solid line with the dotted line in Fig. 4).[15] These results demonstrate that negatively charged phosphodiesters on the complementary strand are mainly responsible for this chromism.

In conclusion, Naphthyl Red conjugated to DNA shows distinct chromism by duplex formation with its complementary strand. By using this phenomenon, hybridization can be easily detected by the naked eye.

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7 See Scheme S1 for details of the synthetic procedure†.
8 Introduction of a dye residue does not destabilize the duplex: see ref. 13*b*.
9 The minimum concentration of the sample solution required for mismatch detection by the naked eye is around 100 nM.
10 The difference in λ_{max} between single-stranded **CXG** and **CXG–*c*-GC** duplex was as large as 50 nm even at pH 9.0. This difference is sufficient for discrimination of its color by the naked eye.
11 A. Buvári and L. Barcza, *J. Inclusion Phenom. Mol. Recognit. Chem.*, 1989, **7**, 313–319.
12 The pH titration was carried out by monitoring the change of absorbance at 550 nm as a function of pH.
13 The large bathochromic shift from 460 to 510 nm by hybridization, observed at pH higher than 8, is not only attributable to the intercalation of chromophore, because it is usually at most 10 nm; (*a*) R. L. Letsinger and M. E. Schott, *J. Am. Chem. Soc.*, 1981, **103**, 7394–7396; (*b*) H. Asanuma, T. Takarada, T. Yoshida, D. Tamaru, X. Liang and M. Komiyama, *Angew. Chem., Int. Ed.*, 2001, **40**, 2671–2673.
14 The location of the Naphthyl Red moiety in the duplex is depicted in Fig. S1†.
15 Note that ***c*-GC$_{pm}$** is a mixture of four diastereomers due to the chirality of the methylphosphonate group. Introduction of two methylphosphonates does not significantly affect the duplex structure because the CD spectra of **CXG–*c*-GC** and **CXG–*c*-GC$_{pm}$** are almost the same.