# Fluorescence Detection of Hybrid Formation between Pyrrolidine-based Oxy-peptide Nucleic Acid and DNA

Mizuki Kitamatsu,* Masanori Shigeyasu, and Masahiko Sisido*
*Department of Bioscience and Biotechnology, Faculty of Engineering, Okayama University, 3-1-1 Tsushimanaka, Okayama 700-8530*

(Received June 1, 2005; CL-050705)

When an oxy-peptide nucleic acid that contains pyrrolidine rings (pyrrolidine-based oxy-PNA = POPNA) hybridized with its complementary DNA, a fluorophore, $DiSC_2(5)$, bound specifically to the hybrid in aqueous solution. Free $DiSC_2(5)$ did not fluoresce by itself, but the bound $DiSC_2(5)$ showed strong fluorescence. The fluorescence was observed only on a fully-matched POPNA–DNA hybrid, but not on hybrids with even single mismatches. This finding provides bases for highly sensitive and highly specific detection of single nucleotide polymorphisms.

Detection and analysis of single nucleotide polymorphisms (SNPs) are essential for tailor-made medical treatments. Several groups[1] reported the detection of SNPs using Nielsen-type peptide nucleic acids (PNAs) (Figure 1).[2] PNA is a DNA surrogate, that is a peptide of $\delta$-amino acids with pendant nucleobases. PNA is more advantageous than DNA, because it forms more stable hybrids with DNA without repulsive forces between negative charges. PNA also resists to proteases and nucleases and shows high specificity to its complementary DNA. Therefore, PNA is very suited for the SNP analysis. Recently, several researchers reported visible detection of PNA–DNA hybrids using a cyanine dye, $DiSC_2(5)$, and applied the method to SNP analyses.[1a,1b,3] However, because visible detection requires more than a trace amount of PNA and DNA, fluorescence detection is much more advantageous for sensitive SNP analyses. Although monomeric $DiSC_2(5)$ is highly fluorescent, the PNA–DNA–$DiSC_2(5)$ conjugate did not fluoresce, probably due to the aggregation of the dyes on the hybrids. Here, we report fluorescence detection of micromolar quantity of DNAs using $DiSC_2(5)$ and a new version of peptide nucleic acid, *cis*-L-configurated oxy-peptide nucleic acid that contains pyrrolidine rings of *cis*-L-configuration (POPNA) (Figure 1).[4] POPNA is more water-soluble than Nielsen-type PNA and shows a sharp melting curve when hybridized with complementary DNA. The fluorescence detection of the POPNA–DNA hybrids will open a way to a sensitive and highly specific DNA detection for successful SNP analyses.

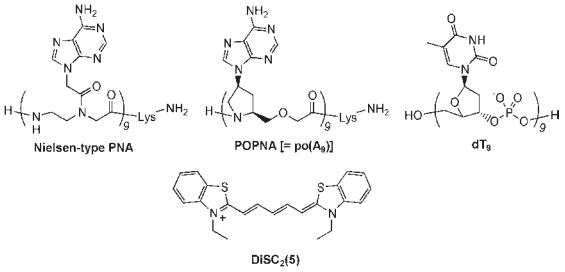


**Figure 1.** Chemical structures of Nielsen-type PNA, POPNA [=po($A_9$)], $dT_9$, and $DiSC_2(5)$.

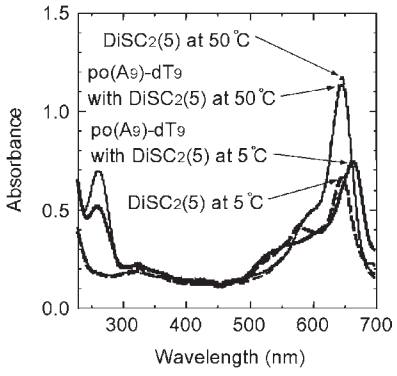


**Figure 2.** UV–vis spectra of $DiSC_2(5)$ in the 1:1 mixture of po($A_9$) and $dT_9$ at 5 °C (bold solid line) and at 50 °C (solid line). UV–vis spectra of $DiSC_2(5)$ alone at 5 °C (bold broken line) and at 50 °C (broken line). [po($A_9$)] = [$dT_9$] = [$DiSC_2(5)$] = 5 μM.[5]

An adenine 9-mer of POPNA (po($A_9$)) was mixed with $dT_9$ at 5 °C ($T_m$ = 27.3 °C, [po($A_9$)] = [$dT_9$] = 1.0 μM), and then $DiSC_2(5)$ was added to the hybrid in aqueous solution. UV spectrum of the mixture after annealing for 1 h at 5 °C is shown in Figure 2. The absorption peak of $DiSC_2(5)$ in the presence of po($A_9$)–$dT_9$ hybrid is red-shifted by 20 nm (668 nm), compared with the absorption peak of monomeric $DiSC_2(5)$ in aqueous solution (648 nm). It is well known that absorption of $DiSC_2(5)$ monomer shows a red-shift in hydrophobic environment.[3a] When the hybrid dissociated at 50 °C, the absorption of $DiSC_2(5)$ in the po($A_9$)–$dT_9$ mixture coincided with that of free $DiSC_2(5)$. These results indicate that $DiSC_2(5)$ binds to a po($A_9$)–$dT_9$ hybrid and becomes free when the hybrid dissociated.

The binding of $DiSC_2(5)$ to po($A_9$)–$dT_9$ hybrid is also evident from induced CD spectra in Figure 3. With a gradual addition of $DiSC_2(5)$ to the hybrid, induced CD peak of $DiSC_2(5)$ at 668 nm emerged, indicating that the bound $DiSC_2(5)$ exists in chiral environment, probably in the minor groove of the double-stranded hybrid. The CD titration curve for $DiSC_2(5)$ with the hybrid suggests a 1:1 stoichiometry between the $DiSC_2(5)$ and the hybrid (inset in Figure 3). The 1:1 stoichiometry is in contrast to the Nielsen-type PNA–DNA–$DiSC_2(5)$ system.[3a] In the latter system, the $DiSC_2(5)$ molecules bind to the hybrid as aggregates that can be observed visibly as the color changes. No fluorescence can be detected from the aggregates of $DiSC_2(5)$. For the fluorescence detection of hybrid formation, it is essential that the chromophores bind to the hybrids as monomeric forms.

The monomeric $DiSC_2(5)$ bound to po($A_9$)–$dT_9$ hybrid may induce strong fluorescence, because the chromophore is isolated

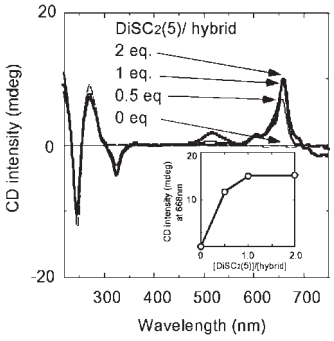


**Figure 3.** CD spectra of various concentrations of $DiSC_2(5)$ in a po($A_9$)–$dT_9$ hybrid. Molar ratios of $DiSC_2(5)$ to the hybrid are: 0, 0.5, 1, and 2. [po($A_9$)] = [$dT_9$] = 5 μM. Inset: CD intensity at 668 nm vs [$DiSC_2(5)$]/[po($A_9$)–$dT_9$ hybrid].[5]

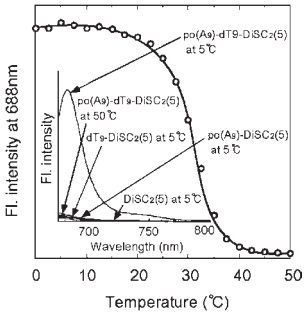


**Figure 4.** Temperature dependence of fluorescence intensity from $DiSC_2(5)$ in the po($A_9$)–$dT_9$ 1:1 mixture at 688 nm, $\lambda_{ex}$ = 668 nm. Inset: fluorescence spectra of $DiSC_2(5)$ in the presence of po($A_9$) and/or $dT_9$ and in the absence of po($A_9$)–$dT_9$ at 5 °C. [po($A_9$)] = [$dT_9$] = [$DiSC_2(5)$] = 1 μM.[5]

and cannot be quenched by collisions with other molecules. Furthermore, the nonradiative decay of the excited state of $DiSC_2(5)$ will be more or less retarded by the constraints.[3d] Fluorescence spectra of $DiSC_2(5)$ complexed onto po($A_9$)–$dT_9$ hybrid is shown in the inset of Figure 4. When the complex was exited at 668 nm at 5 °C where po($A_9$) was completely hybridized with $dT_9$, strong fluorescence of $DiSC_2(5)$ was observed at 688 nm. At 50 °C, where the po($A_9$)–$dT_9$ mixture was in the dissociated state, little fluorescence of $DiSC_2(5)$ was detected. $DiSC_2(5)$ in the absence of the hybrid or with only po($A_9$) or only $dT_9$ did not fluoresce even at 5 °C. Temperature dependence of the fluorescence intensity in the presence of po($A_9$)–$dT_9$ 1:1 mixture was measured and plotted in Figure 4. At low temperatures where po($A_9$) hybridized with $dT_9$, strong fluorescence was observed. The fluorescence gradually reduced its intensity and then sharply decreased around $T_m$ (27.3 °C) of the po($A_9$)–$dT_9$ hybrid. At high temperatures where the hybrid dissociated, no fluorescence was detected. The profile of the temperature dependence of fluorescence intensity is consistent with that of UV melting curve of the po($A_9$)–$dT_9$ hybrid. Accordingly, the temperature dependence of fluorescence intensity reflects the melting curve of the hybrid.

Very interestingly, the enhanced fluorescence of $DiSC_2(5)$ bound to the POPNA–DNA hybrid was observed only on a fully-matched pair. The $DiSC_2(5)$ in the presence of po($A_9$)–$dT_4AT_4$ hybrid ($T_m$ = 16.4 °C) and po($A_9$)–$dT_7AT$ hybrid

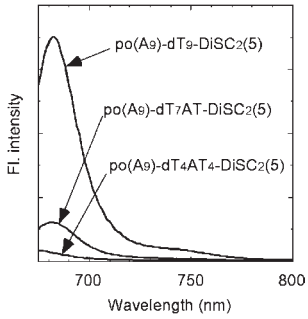


**Figure 5.** Fluorescence spectra of $DiSC_2(5)$ in the presence of po($A_9$)–$dT_9$ hybrid, po($A_9$)–$dT_4AT_4$ hybrid, or po($A_9$)–$dT_7AT$ hybrid at 5 °C. [po($A_9$)] = [$dT_9$] = [$DiSC_2(5)$] = 1 μM.[5]

($T_m$ = 17.0 °C) showed much weaker fluorescence, even at 5 °C where the two mixtures are in the hybrid state (Figure 5). Furthermore, the fluorescence of the mismatched hybrids did not show marked intensity change with the hybrid formation. The $DiSC_2(5)$ in the presence of po($A_9$)–$dT_4AT_4$ hybrid showed only 1.6-fold fluorescent enhancement with lowering the temperature from 50 to 5 °C. In the case of po($A_9$)–$dT_7AT$ hybrid, a somewhat larger enhancement was observed (13.1-fold). These enhancement ratios are, however, much smaller than that observed for po($A_9$)–$dT_9$ hybrid (44.6-fold). The somewhat large enhancement for po($A_9$)–$dT_7AT$ hybrid may be interpreted in terms of a partial hybridization between the seven base pairs.

The above results indicate that the $DiSC_2(5)$ can discriminate not only dissociated/hybridized forms of POPNA–DNA mixture but also full-match/single-mismatch sequences in the hybrids, at least for 9-mer sequences of DNAs with high population of T units. Furthermore, the fluorescence detection of fully-matched POPNA–DNA hybrids does not require washing process to remove free $DiSC_2(5)$ from the mixture.

To conclude, the $DiSC_2(5)$–POPNA–DNA mixture provides a simple, sensitive, and specific detection system for fully-matched POPNA–DNA hybrids, at least for 9-mer DNAs with high population of T units.

## References and Notes

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5 These experiments were measured in aqueous buffer (100 mM of NaCl, 10 mM of $NaH_2PO_4$, and 0.1 mM of EDTA, pH 7.0).

Published on the web (Advance View) July 30, 2005; DOI 10.1246/cl.2005.1216