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Fluorescent oligonucleotide incorporating 5-(1-ethynylpyrenyl)-2′-deoxyuridine: sequence-specific fluorescence changes upon duplex formation

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Abstract—We describe the design and properties of a pyrene-labeled deoxyuridine that can be inserted efficiently into oligodeoxynucleotides using phosphoramidite chemistry. An oligonucleotide incorporating the pyrene-labeled deoxyuridine is a sensitive fluorescence probe that can discriminate between perfect and single-base-mismatched pairing by changes in its fluorescence intensity.

The design and synthesis of fluorescent oligonucleotides have been subject to intensive effort because these oligonucleotide derivatives can be used as tools in molecular biology, diagnostics, and structural studies.[1] There is current interest in the development of fluorophore-labeled oligonucleotides that display a strongly enhanced signal upon hybridization with a target DNA. These types of oligonucleotide probes have become very attractive recently because they can be used as single-nucleotide polymorphism (SNP) probes.[2,3] Pyrene is one of the most attractive of several fluorophores for the development of fluorescent oligonucleotide probes because it possesses favorable photochemical properties, such as high stability and high quantum yields.[4] Various pyrene-labeled oligonucleotides have been developed as intercalators[5] and as probes that display monomer or excimer fluorescence upon hybridizations.[6] There has been little investigation, however, into fluorescent oligonucleotide probes that can discriminate between fully matched and one-base-mismatched sequences by using pyrene-monomer emission. A few research groups have used pyrene-labeled probes to distinguish between single-stranded oligonucleotides and their duplexes with complementary targets.[6c,d,e] In these cases, the intensity of the signal of the excimer increased when the probes hybridized to their complementary targets. Murakami et al. recently reported an RNA oligonucleotide[6a,b] containing a 2′-pyrenylmethyluridine unit whose fluorescence intensity is enhanced significantly upon hybridization to a target RNA. Little or no fluorescence changes were observed upon the binding of this probe to single-base-mismatched RNA. Herein we describe a 'smart' oligonucleotide that has enhanced fluorescence intensity upon fully matched hybridization and decreased intensity upon single-base-mismatched hybridization. Although there are several ways to attach a pyrene molecule to a nucleoside, we have chosen to covalently attach such a unit at the C-5 position of deoxyuridine,[7]which is a substitution that we expect will not perturb its DNA base pairing ability and so should have very little influence on the stability of duplex DNA. We have used palladium-catalyzed Sonogashira coupling[8] to incorporate a rigid ethynylpyrene moiety into the oligonucleotide.[9] Oligonucleotides containing 5-(1-ethynylpyrenyl)-2′-deoxyuridine have been reported by Berlin et al. and Wagenknecht et al.,[10] but the utility of these oligonucleotides as probes for detecting single-base changes of target sequences has not been explored.

The modified oligodeoxyribonucleotide (ODN) containing a 5-(1-ethynylpyrenyl)-2′-deoxyuridine unit at the central position, which is depicted in Figure 1, was prepared from the nucleoside **U*** using phosphoramidite chemistry.[11] The ODN was purified by reversed-phase HPLC and characterized by MALDI-TOF mass

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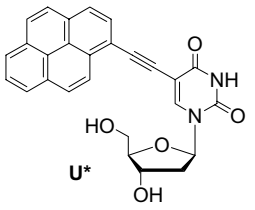

I: 5'-TGG ACA C**U***C GGA ATG-3'

II: 5'-CAT TCC G**T**G TGT CCA-3'
III: 5'-CAT TCC G**G**G TGT CCA-3'
IV: 5'-CAT TCC G**C**G TGT CCA-3'
V: 5'-CAT TCC G**A**G TGT CCA-3'
VI: 5'-TGG ACA C**T**C GGA ATG-3'

Figure 1. The synthesized oligonucleotides.

spectrometry and UV–vis absorption and emission spectra, which clearly suggest the presence of the 1-ethynylpyrenyl groups.

The single-stranded ODN **I** exhibits typical pyrene-monomer emission ($\lambda_{max} = 408$, 434 nm). Very interestingly, the emission intensities of single-base-pair-mismatched duplexes with ODNs **II**–**IV** are reduced considerably relative to that of single-stranded ODN **I** (Fig. 2). It is well established that nucleobases serve as efficient quenchers for pyrene fluorescence in nucleosides and single-stranded oligonucleotides.[12] In our nucleoside system, the fluorescence quantum yields of **U*** in $CHCl_3$ and MeOH solutions are 0.81 and 0.12, respectively.[13,14] Such fluorescence quenching in MeOH indicates that efficient electron injection take place from pyrene to uracil base. The injected electron is transferred to the flanking cytosine bases, which induces fluorescence quenching of ODN **I** upon excitation.

In general, both the intercalation of aromatic hydrocarbons, such as pyrene, into a duplex and the stacking onto a base quench the fluorescence of these hydrocarbons.[5c,15] The absorption maxima of single-base-mismatched duplexes are red-shifted relative to that of the fully matched duplex, from 397 to 402 nm, as shown in Figure 3. These results indicate that in the single-base-mismatched duplexes the pyrene units interact with one or more nucleobases at a site external to the helical base pairing sequence.[5c] Furthermore, the transition melting temperatures (T_m) of single-base-mismatched duplexes are similar to those of the fully matched duplex, as summarized in Table 1, because of the stabilization brought about by the pyrene units stacking with their

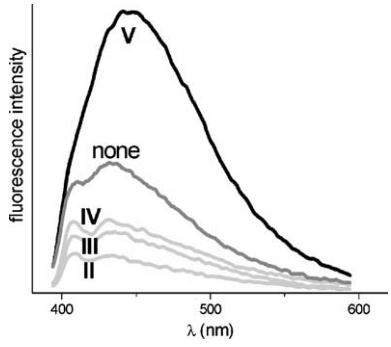

Figure 2. Emission spectra recorded at 25 °C of duplexes containing ODN **I** (1.5 M) in a buffer of 100 mM NaCl, 20 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.2). Fluorescence spectra were recorded with an excitation wavelength of 386 nm. The spectrum labeled 'none' is that of single-stranded **I**.

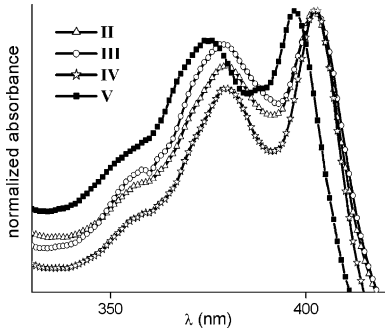

Figure 3. Normalized absorption spectra recorded at ambient temperature of duplexes containing ODN **I** in a buffer of 100 mM NaCl, 20 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.2).

Table 1. Thermal melting temperatures (T_m °C) of modified and unmodified ODN duplexes[a]

Modified duplexes	T_m	Unmodified duplexes	T_m
I·II	59	**VI·II**	57
I·III	61	**VI·III**	62
I·IV	62	**VI·IV**	56
I·V	60	**VI·V**	66

[a] Measured at 260 nm in 10 mM Tris–HCl buffer (pH 7.2) containing 100 mM NaCl and 20 mM $MgCl_2$. Estimated error is ±1 °C.

neighboring nucleobases. Therefore, the fluorescence intensities of the single-base-mismatched duplexes are decreased relative to that observed for the single-stranded ODN **I**. The pyrene unit in the fully matched duplex is likely to be located alongside the duplex and is, therefore, unable to intercalate or stack with the bases. This arrangement is probably responsible for the increased emission, which is also supported by circular dichroism (CD) spectra (Fig. 4a)[16] in which we observe little induced CD at 350–450 nm in perfect matched duplex **I·V** and single-base mismatched duplex **I·II**. Figure 4b shows the expanded CD spectra for the duplexes in the region between 300 and 450 nm. The perfect matched duplex **I·V** exhibits a peak (398 nm). The single-base mismatched duplexes **I·II**, **I·III**, and **I·IV**, however, show slight red-shifted peaks (402 nm), which are due to the interactions with neighboring nucleobases on the exterior of the ultimate base pairing in the single-base mismatched duplexes. The characteristic negative and positive absorptions at 240 and 280 nm, respectively, indicate B-form DNA for the secondary structure of the modified DNA duplexes.

The quenched intensity of the fluorescence emissions in the single-stranded ODN **I** and the single-base-mismatched duplexes is recovered in the fully matched duplex **I·V**; the corresponding emission maximum is shifted from 434 to 444 nm. The relative fluorescence intensities of **I·V**, **I**, **I·II**, **I·III**, and **I·IV** are 1.9, 1.0, 0.34, 0.46, and 0.69, respectively. Especially noteworthy is the observation that the fluorescence efficiency of **I·V** is 5.6 times stronger than that of **I·II**. Because of this difference, the naked eye can discriminate between perfect and mismatched base pairing of ODN **I**, as demonstrated in Figure 5.

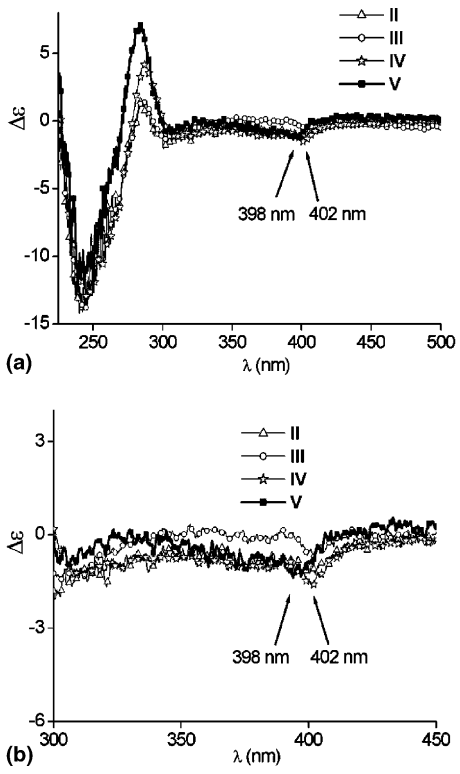

Figure 4. (a) CD spectra of DNA duplexes and (b) expanded CD spectra of DNA duplexes containing ODN **I** (4.5 μM) at 10 °C in a buffer of 100 mM NaCl, 20 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.2).

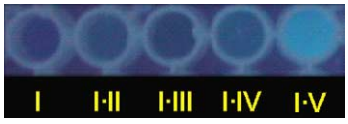

Figure 5. Photographs of the emission behavior at 25 °C of single-stranded ODN **I** alone and in duplexes with unmodified ODNs **II**–**V** (15 μM) upon irradiation with light at 365 nm in a buffer of 100 mM NaCl, 20 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.2).

Figure 6 presents the variable-temperature fluorescence spectra of single-stranded ODN **I** and duplex **I·V**. The temperature-dependent fluorescence of the latter is blue-shifted and its intensity decreases far more dramatically at ca. 60 °C, which is the melting point of duplex **I·V** (see inset in Fig. 6), than that of **I** alone as the temperature is increased.

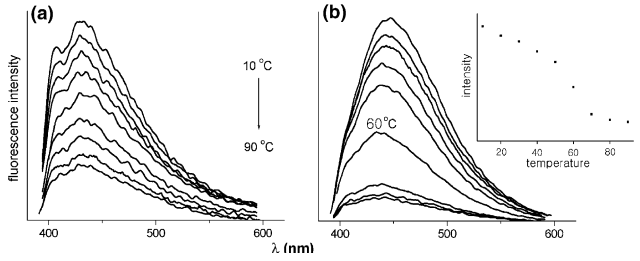

Figure 6. Emission spectra of (a) the single-stranded ODN **I** and (b) the duplex **I·V** (1.5 μM) recorded at temperatures from 10 to 90 °C (at 10 °C intervals). Conditions are the same as described in Figure 2. The inset displays the temperature-dependent fluorescence intensities of **I·V**.

In conclusion, pyrene-labeled nucleosides—substituted, using Sonogashira coupling, at the C-5 position of deoxyuridine—can be inserted efficiently into ODNs. The pyrene-labeled ODN **I** is a sensitive probe that discriminates between perfect and one-base-mismatched base pairing by changes in its fluorescence intensity. These findings suggest a way to design highly sensitive probes for SNP. These studies are now in progress; additionally, the structural basis for the different fluorescence properties between fully matched and single-base-mismatched duplexes requires further investigation.

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References and notes

1. Wojczewski, C.; Stolze, K.; Engels, J. W. *Synlett* **1999**, 1667–1678.
2. (a) Brookes, A. J. *Gene* **1999**, *234*, 177–186; (b) Collins, F. S.; Brooks, L. D.; Chakravarti, A. *Genome Res.* **1998**, *8*, 1229–1231.
3. For recent applications of modified oligonucleotides as SNP probes see: (a) Okamoto, A.; Tanaka, K.; Fukuta, T.; Saito, I. *J. Am. Chem. Soc.* **2003**, *125*, 9296–9297; (b) Asanuma, H.; Kashida, H.; Liang, X.; Komiyama, M. *Chem. Commun.* **2003**, 1536–1537.
4. Winnik, F. M. *Chem. Rev.* **1993**, *93*, 587–614.
5. (a) Filichev, V. V.; Pedersen, E. B. *Org. Biomol. Chem.* **2003**, *1*, 100–103; (b) Christensen, U. B.; Pedersen, E. B. *Nucleic Acids Res.* **2002**, *30*, 4918–4925; (c) Mann, J. S.; Shibata, Y.; Meehan, T. *Bioconjugate Chem.* **1992**, *3*, 554–558.
6. (a) Mahara, A.; Iwase, R.; Sakamoto, T.; Yamana, K.; Yamaoka, T.; Murakami, A. *Angew. Chem., Int. Ed.* **2002**, *41*, 3648–3650; (b) Yamana, K.; Zako, H.; Asazuma, K.; Iwase, R.; Nakano, H.; Murakami, A. *Angew. Chem., Int. Ed.* **2001**, *40*, 1104–1106; (c) Yamana, K.; Iwai, T.; Ohtani, Y.; Sato, S.; Nakamura, M.; Nakano, H. *Bioconjugate Chem.* **2002**, *13*, 1266–1273; (d) Paris, P. L.; Langenhan, J. M.; Kool, E. T. *Nucleic Acids Res.* **1998**, *26*, 3789–3793; (e) Lewis, F. D.; Zhang, Y.; Letsinger, R. L. *J. Am. Chem. Soc.* **1997**, *119*, 5451–5452; (f) Kierzek, R.; Li, Y.; Turner, D. H.; Bevilacqua, P. C. *J. Am. Chem. Soc.* **1993**, *115*, 4985–4992; (g) Okamoto, A.; Tainaka, K.; Saito, I. *J. Am. Chem. Soc.* **2003**, *125*, 4972–4973; (h) Komiyama, M.; Ye, S.; Liang, X.; Yamamoto, Y.; Tomita, T.; Zhou, Z.-M.; Aburatani, H. *J. Am. Chem. Soc.* **2003**, *125*, 2217–2223.
7. For recent syntheses of C5-modified fluorescent uridines see: Hurley, D. J.; Tor, Y. *J. Am. Chem. Soc.* **2002**, *124*, 3749–3762, and references cited therein.
8. (a) Sonogashira, K. In *Metal-Catalyzed Cross-Coupling Reactions*; Diederich, F., Stang, P. J., Eds.; Wiley-VCH:

Weinheim, 1998; (b) Sonogashira, K.; Tohda, Y.; Hagihara, N. *Tetrahedron Lett.* **1975**, *16*, 4467–4470.
9. We have previously synthesized fluorophores using Sonogashira coupling methods. See: (a) Hwang, G. T.; Son, H. S.; Ku, J. K.; Kim, B. H. *J. Am. Chem. Soc.* **2003**, *125*, 11241–11248; (b) Hwang, G. T.; Son, H. S.; Ku, J. K.; Kim, B. H. *Org. Lett.* **2001**, *3*, 2469–2471.
10. (a) Malakhov, A. D.; Malakhova, E. V.; Kuznitsova, S. V.; Grechishnikova, I. V.; Prokhorenko, I. A.; Skorobogatyi, M. V.; Korshun, V. A.; Berlin, Y. A. *Russ. J. Bioorg. Chem. (Engl. Transl.)* **2000**, *26*, 34–44; (b) Rist, M.; Amann, N.; Wagenknecht, H.-A. *Eur. J. Org. Chem.* **2003**, 2498–2504.
11. Gait, M. J. *Oligonucleotide Synthesis: A Practical Approach*; IRL: Washington, DC, 1984.
12. (a) Wagenknecht, H.-A. *Angew. Chem., Int. Ed.* **2003**, *42*, 2454–2460; (b) Amann, N.; Pandurski, E.; Fiebig, T.; Wagenknecht, H.-A. *Chem. Eur. J.* **2002**, *8*, 4877–4883; (c) Amann, N.; Pandurski, E.; Fiebig, T.; Wagenknecht, H.-A. *Angew. Chem., Int. Ed.* **2002**, *41*, 2978–2980; (d) Telser, J.; Cruickshank, K. A.; Morrison, L. E.; Netzel, T. L.; Chan, C. *J. Am. Chem. Soc.* **1989**, *111*, 7226–7232; (e) Telser, J.; Cruickshank, K. A.; Morrison, L. E.; Netzel, T. L. *J. Am. Chem. Soc.* **1989**, *111*, 6966–6976.
13. Quantum yields of nucleosides were determined using 9,10-diphenylanthracene in EtOH as a standard.
14. Quantum yield of ODN **I** is 0.08 determined using 9,10-diphenylanthracene in EtOH as a standard. The quantum yields of ODNs 5′-CATTCCGU*GTGTCCA-3′ and 5′-CATTCCAU*ATGTCCA-3′, which were synthesized for other study are 0.11 and 0.14, respectively.
15. Masuko, M.; Ohtani, H.; Ebata, K.; Shimadzu, A. *Nucleic Acids Res.* **1998**, *26*, 5409–5416.
16. Berova, N.; Nakanishi, K.; Woody, R. W. *Circular Dichroism. Principles and Applications*; Wiley-VCH: New York, 2000.