Bispyrenyl Excimer Fluorescence: A Sensitive Oligonucleotide Probe

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The fluorescence of pyrene monomer has been widely investigated as a potential probe of DNA and RNA secondary and tertiary structure.[1] However, in most cases, its use as a probe suffers from the efficient quenching of its fluorescence by nucleobases.[2,3] Pyrene excimer fluorescence, on the other hand, is less subject to quenching by donors and acceptors than is pyrene monomer fluorescence,[4] and thus might prove to be a more selective probe of DNA base sequence or structure than pyrene monomer. Several attempts to employ pyrene excimer fluorescence as a probe for duplex or ternary complex formation between singly-labeled oligonucleotides have proven unsuccessful.[5] Mixtures of pyrene monomer and excimer fluorescence have been observed from single-stranded oligonucleotides with pyrene labels on adjacent bases,[6] from duplexes formed by multiply labeled poly(dG-dC) prepared by reaction with benzopyrenediol epoxide,[7] and from oligonucleotide−polyamide conjugates in which the polyamide bears multiple pyrenes.[8] However, the total emission is not sequence selective. We report here the synthesis of a bispyrenyl alcohol P_2OH and the oligonucleotide conjugates **I** and **II** in which the bispyrenyl label is attached to the 5′-phosphate (Chart 1). The pyrene monomer/excimer fluorescence ratio decreases upon duplex formation, and the pyrene fluorescence intensity is strongly quenched by an adjacent non-base-paired dT, both in single-stranded and duplex DNA.

The bispyrenyl alcohol P_2OH was synthesized in five steps from 1-pyrenecarboxaldehyde and 1-pyrenebutyric acid.[9] Reaction of P_2OH with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite provided the phosphoramidite in 37% yield. The bispyrenyl (P_2) labeled oligonucleotides **I** and **II** and unlabeled complementary sequences **III**−**VIII** shown in Chart 1 were prepared by using an automated DNA synthesizer with conventional phosphoramidite reagents and were purified by reversed-phase HPLC.

P_2OH displays a structured long wavelength absorption band similar to that of pyrene in methanol solution. Its fluorescence spectrum is dominated by structured monomer fluorescence (Figure 1a) for which the fluorescence quantum yield is 0.14 and the decay time is 181 ns.[10] The high monomer/excimer fluorescence ratio and long decay time indicate that neither static nor dynamic self-quenching of pyrene monomer occurs to an appreciable extent, as expected for a bispyrene with a seven-atom linker.[11] In aqueous solution, the absorption band of P_2-OH is broadened and its emission spectrum is dominated by excimer fluorescence (Figure 1a). Excitation in the red-edge of the absorption band (355 nm) results in a higher excimer/monomer ratio than excitation at the first absorption maximum (330 nm). These observations are indicative of ground state aggregation of P_2OH.[12] The excimer fluorescence quantum yield in aqueous solution is 0.49 and the fluorescence decay is biexponential (Table 1).[13] Biexponential fluorescence decay has been observed for other 1,*n*-bis(1-pyrenyl)alkanes and the long- and short-lived components attributed to fully and partially overlapping excimer geometries.[14]

The absorption spectra of the P_2-labeled oligonucleotides **I** and **II** exhibit long wavelength bands, similar to that of P_2OH

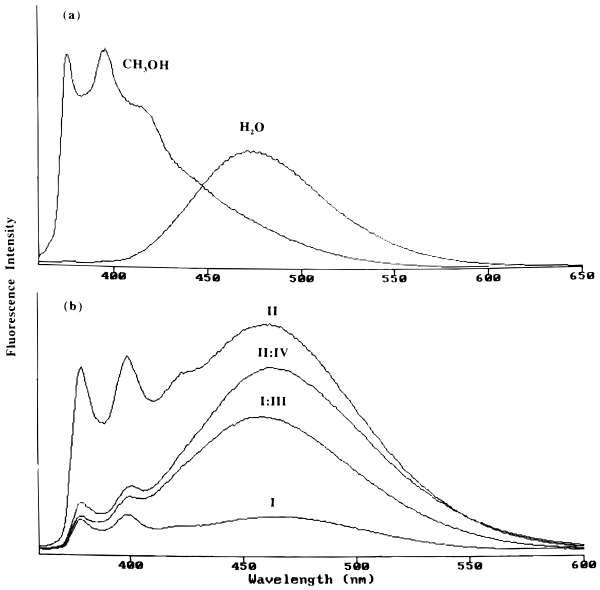
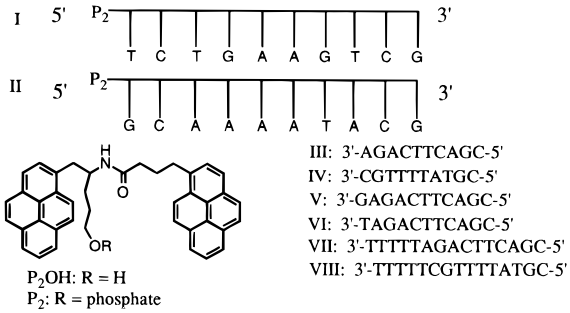


Figure 1. (a) Fluorescence spectra of P_2OH at room temperature in nitrogen-purged CH_3OH (1.05×10^{-6} M, $\lambda_{ex} = 340$ nm) and H_2O (1.16×10^{-6} M, $\lambda_{ex} = 348$ nm). (b) Fluorescence spectra of oligomers and duplexes at room temperature in nitrogen-purged aqueous solution (pH 7.2, 1 M NaCl, 30 mM sodium phosphate), $\lambda_{ex} = 350$ nm: oligomer **I** (5.4 μM), oligomer **II** (2.6 μM), duplex I:III (5.4 μM for each oligomer), and duplex II:IV (2.6 μM for each oligomer).

Chart 1



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(10) Fluorescence quantum yields were measured with use of 1-pyrenebutanoic acid ($\Phi_f = 0.6$)[3] as an actinometer. Decay times were determined with use of a PTI-LS1 photon counting apparatus.

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Table 1. Fluorescence Quantum Yields, Monomer/Excimer Ratios, and Decay Times for Bispyrenyl-Labeled Oligomers and Duplexes

bispyrenyl label	Φ_f[a]	I_m/I_e[b]	τ, ns (%)[c]
P_2OH	0.49	0.04	22.4 (58), 66.6 (42)
I	0.008	0.88	4.5 (88), 36.8 (12)
I:III	0.04	0.27	4.7 (96), 21.1 (4)
II	0.10	0.81	14.4 (51), 42.9 (49)
II:IV	0.08	0.27	13.6 (96), 46.0 (4)

[a] All samples were in air-saturated aqueous solution (pH 7.2, 1 M NaCl, 30 mM sodium phosphate) at room temperature; 1-pyrenebutanoic acid ($\Phi_f = 0.6$[3]) was used as an actinometer. [b] Ratio of emission maxima for monomer (379 nm) and excimer (462 nm) fluorescence. [c] Aqueous solutions ($\sim 10^{-6}$ M, pH 7.2, 1 M NaCl, 30 mM sodium phosphate) were purged with nitrogen. Decay times were measured at room temperature by means of single photon counting with $\lambda_{ex} =$ 350 nm and $\lambda_{em} =$ 530 nm. Values in parentheses are the preexponentials for the decay components.

in aqueous solution, and overlapping pyrene and nucleobase absorption bands below 300 nm. The unlabeled oligonucleotides **III**−**VIII** have no absorption bands above 300 nm. The fluorescence spectra of **I** and **II** are shown in Figure 1b. Monomer/excimer fluorescence ratios and excimer fluorescence quantum yields and decay times for **I** and **II** are reported in Table 1. Comparison of the data to that for P_2OH reveals that the reduction in fluorescence quantum yield is significantly greater than the reduction in lifetime. We have observed a similar disparity in intensity vs lifetime quenching of singlet stilbene in oligonucleototide conjugates.[15] This disparity might reflect the polymorphic nature of single-stranded oligonucleotides, which could result in highly efficient fluorescence quenching by neighboring nucleobases in some conformations but not in others.[13] The higher monomer/excimer ratio for **I** and **II** vs P_2OH is consistent with the absence of aggregation of the labeled oligonucleotides.

Quenching of pyrene monomer fluorescence has been extensively studied in both covalent and non-covalent complexes of nucleosides and single-stranded oligonucleotides.[2,3,16] Geacintov and co-workers[16,17] have proposed an electron transfer mechanism for fluorescence quenching in which the base serves either as an electron donor or acceptor, thymine being the best acceptor and guanine the best donor. Faster rates are observed for quenching of pyrene monomer by dT vs dG, in accord with the larger calculated driving force for quenching by dT. Faster quenching of pyrene monomer by the adjacent 5′-dT in **I** vs the 5′-dG in **II** can account for the lower yield of both monomer and excimer fluorescence for **I** vs **II** (Figure 1 and Table 1). Once formed, the pyrene excimer may be less sensitive to quenching by nucleobases than is pyrene monomer, in accord with the lower singlet state oxidation and reduction potentials of pyrene excimer vs monomer.[4] Comparison of the excimer fluorescence decay times for **I** and **II** vs P_2OH suggests that dT is a more effective quencher than is dG for pyrene excimer as well as pyrene monomer fluorescence.

Hybridization of **I** and **II** with their unlabeled complements **III** and **IV**, respectively, results in the formation of duplexes which have higher melting temperatures ($T_m = 56$ °C for both **I:III** and **II:IV**) than those of the duplexes formed by **III** and **IV** with their unlabled complements (49 and 51 °C, respectively). Similar thermal dissociation profiles are obtained for both duplexes **I:III** and **II:IV**, monitoring absorption at 260 (nucleobase band) or 350 nm (pyrene band). The fluorescence spectra of the duplexes **I:III** and **II:IV** are shown in Figure 1b. The monomer/excimer fluorescence intensity ratio decreases upon duplex formation. In addition, the excimer fluorescence intensity of **I:III** is 5-fold larger than that of **I**, whereas the intensity of **II:IV** is slightly less than that of **II**. Addition of the mismatched oligomer **IV** to **I** results in neither hypochromicity nor a change in fluorescence spectra of **I**. Thus the increase in fluorescence intensity observed upon addition of **III** to **I** must be a consequence of duplex formation. The fluorescence decays of both **I:III** and **II:IV** can be best fit by a dual exponential, which is dominated by the shorter-lived component. The increase in excimer fluorescence intensity of **I** upon duplex formation is not accompanied by a proportional increase in lifetime. A decrease in static quenching upon duplex formation could account for this disparity.

The effect of hybridization of **I** and **II** with oligonucleotides having the same sequence as **III** and **IV** but possessing one or more extra bases at the 3′ terminus has also been investigated. The excimer fluorescence intensity of the hybrid of **I** with **V**, which possesses an extra G, is similar to that of **I:III**. However, the hybrid of **I** with **VI**, which possess an extra T, is more weakly fluorescent ($\Phi_f = 0.01$). Very weak excimer fluorescence is observed for the hybrids of **I** and **II** with the oligonucleotides **VII** and **VIII**, respectively ($\Phi_f < 0.005$ for **I:VII** and $\Phi_f = 0.02$ for **II:VIII**).

The increase in T_m observed for the duplexes **I:III** and **II:IV** compared to duplexes lacking the P_2 label indicates that the label perturbs the equilibrium between the single strand and duplex. A similar increase in T_m has been reported for the duplex formed between a 5′-pyrene labeled dT 15-mer and the complementary dA 15-mer,[2a] and a larger increase in T_m has recently been reported for a self-complementary DNA duplex with "dangling" pyrenes at both ends.[18] These observations have been attributed to π-stacking of the pyrene with the exterior of the ultimate base pair. Model building studies indicate that P_2 label is capable of end stacking, grove binding, or intercalation of one pyrene between the terminal base pair and its neighbor. Grove binding of pyrene seems incompatible with the significant increase in T_m[1a] and the observation of excimer quenching by dangling dT's in the duplexes **I:VI**, **I:VII**, and **II:VIII**. Intercalation is incompatible with the observation of strong excimer and little monomer fluorescence and excimer fluorescence quenching by dangling dT's. An end-stacked structure is consistent with the observation of hypochromism for the pyrene absorption band, the increase in T_m, the low monomer/excimer fluorescence ratio, and quenching by dangling dT's.

In the absence of proximate non-base-paired dT's, the P_2-labeled oligonucleotides **I** and **II** and their duplexes have moderately high quantum yields for excimer fluorescence. Based on the pronounced solvent dependence of the monomer/excimer fluorescence ratio for P_2OH (Figure 1a), P_2-labeled oligonucleotides should also be sensitive to local solvation effects. As such they have potential applications in probing the environment of specific segments of DNA or RNA both in vitro and in biological systems. Further studies of oligonucleotide conjugates with multiple fluorescent labels are in progress in our laboratories.

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