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Oligonucleotide−Polyamide Hybrid Molecules Containing Multiple Pyrene Residues Exhibit Significant Excimer Fluorescence

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Abstract: Oligonucleotide−polyamide hybrid molecules bearing multiple pyrene residues in the polyamide moiety were prepared. These molecules were designed to promote pyrene excimer formation and avoid intercalation of the label into the DNA duplex. Significant excimer fluorescence was observed, and this was shown to increase with the number of pyrene residues present. Hence, there is potential for further increasing of the fluorescence intensity by incorporation of additional label residues. Excimer fluorescence intensity was also shown to be sensitive to duplex formation. Fluorescence measurements and melting temperature studies gave no evidence of intercalation of the pyrene residues into duplex DNA. The hybridization properties of these hybrid molecules are similar to those of unmodified oligonucleotides. It seems, then, likely that pyrenylated oligonucleotide−polyamide hybrids may be useful as oligonucleotide probes.

Introduction

Nonradioactive oligonucleotide probes have two advantages over conventional radiolabeled probes in that they are safer to handle and their stability renders them more suitable for commercial applications. Biotin is a widely-used label due to its highly specific and strong interaction with avidin and streptavidin as well as the large number of techniques available for its detection. A disadvantage of using biotin is the fact that a cascade of reactions is necessary for its detection. This makes its use prone to artifacts. The introduction of fluorescent labels would obviate these tedious and time-consuming procedures since it would allow direct detection of the probe. However, to achieve an adequate level of sensitivity, multiple labeling would probably be required. Our initial investigations into multiply-labeled fluorescent nucleic acid probes containing one to ten fluorescein moieties as the label showed that significant interlabel quenching occurred.[1] Manoharan *et al.*[2] have reported the synthesis of a difluoresceinylated oligonucleotide which had twice the fluorescence intensity of a singly fluoresceinylated analogue. However, the two fluoresceins in this case were separated by eight nucleotides, and hence introduction of more labels with the same spacing between them to avoid interlabel quenching would require a very large oligonucleotide. A smaller distance between the labels could result in significant quenching and consequent loss of sensitivity. These considerations led to our present study on the use of pyrene as a label. Pyrene is an attractive label because its simple polyaromatic hydrocarbon structure does not require protection for peptide/polyamide or oligonucleotide synthesis. It also has the ability to form bimolecular complexes called excimers, consisting of one ground state and one excited state species, which are themselves fluorescent [in the visible region (480 nm)].[3] Thus the interlabel interaction in this case is beneficial, and the problem of label−label quenching observed for fluorescein may be avoided. The aim of our present study is to ascertain whether our molecular design results in excimer formation (with an increase in fluorescence intensity with the number of pyrene residues present in the probe) and also whether the hybridization properties of the oligonucleotide are altered.

Design of the Pyrenylated Probes. Pyrene has been reported to interact with native[4] and denatured DNA[5] under certain conditions. Whether or not the hybridization properties of an oligonucleotide are altered depends critically on the placement of the pyrene residue(s). As expected, internal labeling of oligonucleotides with pyrene tends to facilitate intercalation of the pyrene into the DNA duplex as evidenced by decreased fluorescence emission and higher melting temperatures.[6−11] Attachment of the label to the 5′-position of the 5′-terminal nucleoside *generally* does not perturb the thermodynamics of the duplex to any significant degree[12] whereas label placement at other sites (including the 2′-position of ribonucleotides and various non-base-pairing positions on the heterocyclic bases)

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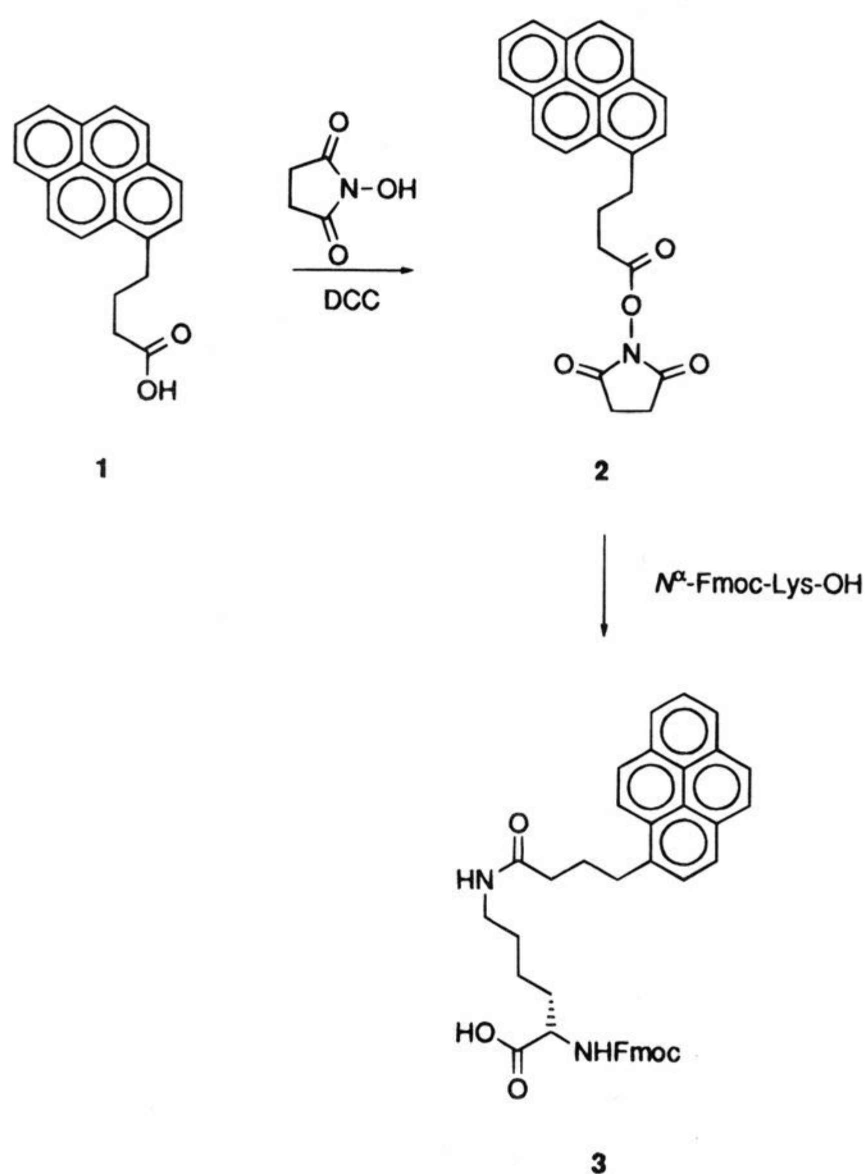
Scheme 1. Preparation of the Pyrenyl−Lysine Synthon



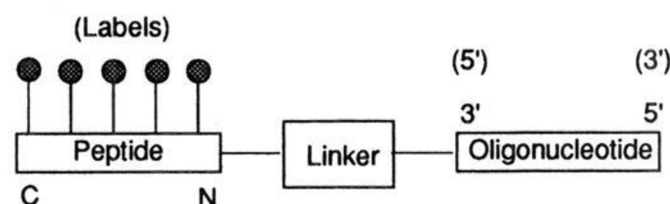


Figure 1. Schematic representation of labeled oligonucleotide−polyamide hybrid molecules.

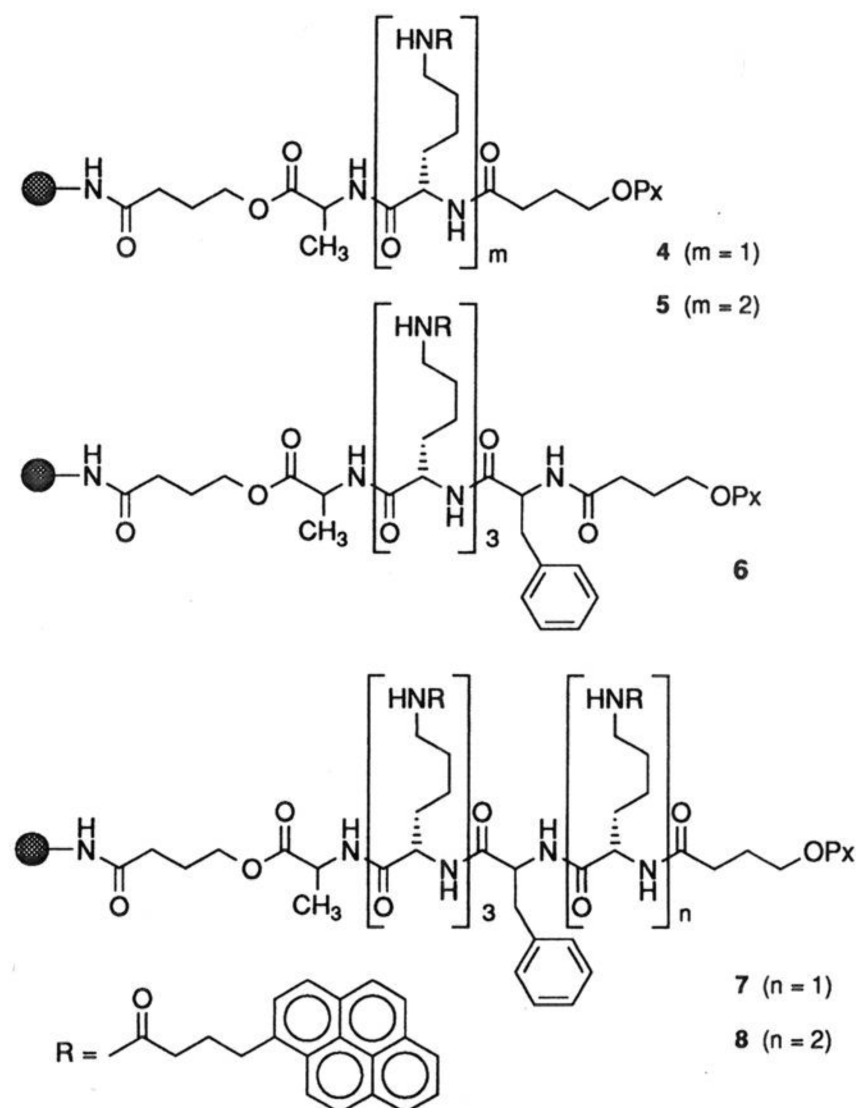


Figure 2. Solid supports derivatized with pyrenylated polyamides (**4**−**8**).

allows varying degrees of intercalation to occur.[9,13,14] However, even where the site of attachment is *via* a 5′-phosphate linkage, significant intercalation can occur.[15] The polyamide-derivatized solid supports we have developed[1,17] provide an efficient way of incorporating multiple pyrenes into an oligonucleotide. Once a polyamide bearing multiple pyrenes has been prepared on the solid support using a pyrenyl−lysine building block (**3** in Scheme 1), any oligonucleotide can then be synthesized. Depending on whether 3′- or 5′-phosphoramidites are used, the “string” of pyrenes can be placed at the 3′- or the 5′-terminus of an oligonucleotide, respectively (Figure 1). It is anticipated that this arrangement should promote strong excimer formation because the pyrenes are placed close together and have a minimal effect on the hybridization properties of the oligonucleotide because the pyrenes are attached to the polyamide moiety rather than directly to the oligonucleotide itself.

Results

Synthesis of N^{α}-Fmoc-Lys-N^{ϵ}-(4-pyrenylbutanoyl)-OH (3). For the purpose of incorporating multiple pyrene residues into an oligonucleotide−polyamide hybrid molecule in a highly specific manner, the pyrenyl−lysine synthon **3** was synthesized. This amino acid building block allows a solid support to be derivatized with pyrenes in an manner analogous to the way biotins were incorporated onto solid supports.[16] The desired compound **3** was accessible *via* a simple two-step procedure (Scheme 1). 4-Pyrenylbutanoic acid (**1**) was activated *via* DCC-mediated condensation with *N*-hydroxysuccinimide to give the active ester **2**. Reaction of **2** with N^{α}-Fmoc-Lys-OH then afforded the synthon **3** in 66% yield. Unlike the biotin analogue described elsewhere,[16] the pyrene synthon **3** was very soluble in DMF (the solvent most commonly used in Fmoc peptide synthesis) and there was no need to form an active ester of this compound. This compound was characterized by FABMS and ^{1}H and ^{13}C NMR spectroscopy. All spectral data were consistent with the proposed structure; the ^{1}H and ^{13}C NMR spectra were essentially the addition of the spectra of N^{α}-Fmoc-Lys-OH and 4-pyrenylbutanoic acid (**1**).

Preparation of Solid Supports Prelabeled with Pyrenes. Solid supports derivatized with pyrenes were prepared in an manner analogous to those we have previously described containing biotins.[16] In order to ascertain whether excimer formation occurs and whether this phenomenon enhances the fluorescence output of these hybrid molecules, solid supports **4** to **8** containing one to five pyrenes, respectively, were prepared (Figure 2) using standard Fmoc (*N*-fluoren-9-ylmethoxycarbonyl) solid phase peptide synthesis methodology.[18] Alanine and phenylalanine were included as internal reference amino acids, at the first and fourth residues, respectively, to facilitate characterization of the products by amino acid analysis.

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Table 1. Amino Acid Analysis of Solid Supports **4**–**8**

resin	expected ratio of Lys:Ala:Phe	ratio of Lys:Ala:Phe found[a]
4	1.0:1.0:0.0	1.1:1.0:0.0
5	2.0:1.0:0.0	2.0:1.0:0.0
6	3.0:1.0:1.0	2.0:1.0:1.3
		(2.6):(1.0):(0.7)
7	4.0:1.0:1.0	3.3:1.0:1.2
		(3.1):(1.0):(0.5)
8	5.0:1.0:1.0	3.3:1.0:0.6
		(3.6):(1.0):(0.5)

[a] Figures in parentheses were obtained after 72 h hydrolysis; remaining data is after 24 h hydrolysis.

Both Fmoc-Lys-N^{ϵ}-(4-pyrenylbutanoyl)-OH (**3**) and Fmoc-Phe-OH were activated by HBTU (*O*-benzotriazolyl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate),[19] and two successive 0.5 h couplings were used. A TNBSA (trinitrobenzenesulfonic acid) assay[20] was performed on the resin after each coupling; the results were always negative (indicating no residual amino groups) after the first coupling. However, due to difficulties experienced in our initial attempts to prepare these solid supports, the degree of amino acid incorporation was also determined quantitatively after selected couplings using the Fmoc assay.[16] To determine whether a double coupling had any advantage over a single coupling, Fmoc assays were performed after the first and second couplings. For the first pyrene residue, a significant increase in loading (from 36 ± 5 to 49 ± 6 μmol/g) was observed after the second coupling. However, for the second pyrene residue, no increase (from 39 ± 1 to 42 ± 5 μmol/g) was observed. Double couplings were used for the rest of the synthesis. In addition, a capping step (Ac_2O/DMAP) was performed after the second coupling of each residue to ensure that any residual amino groups did not give rise to hybrid molecules with polyamide moieties missing certain amino acids (deletion peptides). The peptide loading as determined by the Fmoc assay remained in the vicinity of ~30 μmol/g after incorporation of the other pyrene residues. The loadings were confirmed by the pixyl assay[16] after the incorporation of the 4-hydroxybutyric acid linker at the *N*-terminii in the usual manner[16,17] (data not shown). The resin-bound polyamides were further characterized by amino acid analysis (Table 1); the solid supports **4** and **5** showed the expected ratio of amino acids. The tri-, tetra-, and pentapyrenylated solid supports **6** to **8** had low ratios of lysine to alanine. Taking into account the hydrophobic nature of these polyamides, it was deemed desirable to try a 72 h hydrolysis in the amino acid analysis rather than the usual 24 h to ascertain whether the anomalous results were due to incomplete hydrolysis. A significant difference was observed, although the values still did not match the theoretical values. Since amino acid analysis results obtained for support-bound polyamides and peptides can differ significantly from those of the purified products, it was decided to continue on to the oligonucleotide synthesis and analyze the hybrid molecules after purification.

Synthesis and Characterization of Hybrid Molecules Containing Multiple Pyrene Residues. A series of hybrid molecules (**9**–**13** in Figure 3) containing one to five pyrene residues were synthesized from the solid supports **4** to **8** using conventional nucleoside 3′-*O*-phosphoramidites. This places the string of pyrene residues at the 3′-terminii of the hybrid molecules. The sequence chosen for the oligonucleotide moiety is a 30-mer test sequence we have employed in previous studies.[17]

The coupling yields as assessed by the trityl assay were the same as for oligonucleotide synthesis on normal solid supports (data not shown). The cleavage of the oligonucleotide from the solid support and base and phosphate deprotection were effected by treatment with a 0.1 M NaOH solution (rather than concentrated ammonia) to avoid the possibility of forming C-terminal amides.[21]

The crude oligomers were purified by polyacrylamide gel electrophoresis (PAGE). One major highly fluorescent band was observed for each oligomer by UV shadowing (data not shown). The purified yields and amino acid analysis results for each oligomer are shown in Table 2. With the exception of the monopyrenylated conjugate **9**, the amino acid analysis results of this series of conjugates did not match the expected values. The amount of phenylalanine present in these conjugates was underestimated due to the proximity of the phenylalanine peak to an artifact in the amino acid analysis chromatogram.

Ion spray mass spectrometry gave the expected molecular ions for the mono- to tripyrenylated conjugates **9**–**11** (Table 2). Under the experimental conditions attempted, no molecular ions could be obtained for the tetra- and pentapyrenylated conjugates **12** and **13** due to very high backgrounds. However, the mass spectrometry results for **10** and **11** clearly show that even though the amino acid analysis results were significantly different from the expected values, this does not necessarily mean that the polyamide moiety is not of the correct composition.

The capillary electrophoresis (CE) electropherograms of **9**–**13** are shown in Figure 4. The mono- to tripyrenylated conjugates **9**–**11** showed up as one major peak with a minor peak of earlier retention time. The tetra- and pentapyrenylated conjugates **12** and **13** also consisted of single major peaks, but there also seemed to be some minor peaks, suggesting that there may be a small degree of heterogeneity in these samples. For the mono- to tripyrenylated conjugates **9**–**11**, there is a retardation of electrophoretic mobility with an increasing number of pyrene residues. However, the tetra- and pentapyrenylated conjugates **12** and **13** had shorter retention times than **11**, but they were still slower running than **9** and **10**. Although this breaks up the trend, it is not altogether surprising since the electrophoretic mobility of a molecule during gel electrophoresis is dependent not only on its molecular weight but also on its shape. It is conceivable that the tetra- and pentapyrenylated conjugates adopt a conformation which renders them more electrophoretically mobile than the tripyrenylated conjugate. Nevertheless, it is clear that all five hybrid molecules (three of which have been characterized by mass spectrometry) have distinct retention times.

The UV spectra of the pyrenylated conjugates are shown in Figure 5. The monopyrenylated conjugate has three absorption maxima at 259, 335, and 350 nm. As the number of pyrenes in the conjugate increases, a fourth peak at 247 nm appears. The presence of pyrene residues in the conjugate does not perturb the UV spectrum in the 260 nm region to any significant degree, and hence accurate quantitation of these hybrid molecules can still be achieved *via* spectrophotometry.

The fluorescence excitation spectra of **9**–**13** were very similar, with maxima at 240 and 350 nm (data not shown). The fluorescence emission spectra of **9**–**13** are shown in Figure 6. In the spectrum of the monopyrenylated conjugate **9**, only two

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(21) We observed no difference (by CE analysis) in the crude product resulting from either 0.1 M NaOH or 35% aqueous NH_4OH cleavage of analogous oligonucleotide–polyamide conjugates bearing biotin residues.

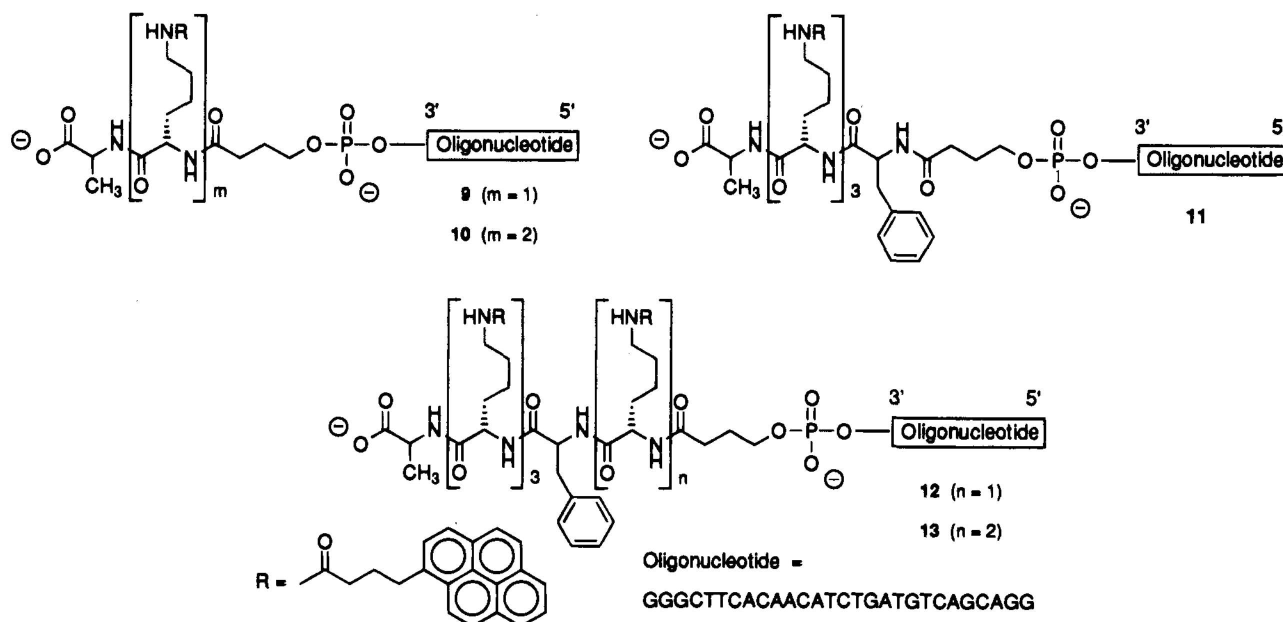

Figure 3. Pyrenylated oligonucleotide–polyamide hybrid molecules (**9**–**13**).

Table 2. Purified Yields, Amino Acid Analyses, and Ion–Spray Mass Spectrometry of Conjugates **9**–**13**

hybrid molecule	purified yield (%)	expected ratio of Lys:Ala:Phe	ratio of Lys:Ala:Phe found[a]	calcd mol wt	mol wt found
9	15	1.0:1.0:0.0	1.0:1.0:0.0	9882.7	9881.0
10	15	2.0:1.0:0.0	1.5:1.0:0.0	10281.2	10278.8
11	8	3.0:1.0:1.0	2.7:1.0:0.6	10826.9	10827.0
12	5	4.0:1.0:1.0	3.1:1.0:0.4		
13	3	5.0:1.0:1.0	2.2:1.0:0.4		

[a] 24 h hydrolysis.

peaks, at 381 and 401 nm, were observed, corresponding to pyrene monomer emission. In the di- and tripyrenylated conjugates **10** and **11**, excimer fluorescence at 488 nm became significant. The excimer and monomer fluorescence emissions for **9**–**13** were compared by integration of the appropriate areas in their fluorescence emission spectra (Table 3). As expected, the ratio of excimer to monomer fluorescence increased considerably with the number of pyrenes in the conjugate. There was a dramatic increase in excimer fluorescence for the tetra- and pentapyrenylated conjugates **12** and **13**; these conjugates have the same order of magnitude of fluorescence output as an oligonucleotide probe bearing a single fluorescein label.[1]

The fluorescence emissions of these hybrid molecules at 480 nm (excimer emission) were assessed before and after duplex formation (Table 4). The monopyrenylated compound **9** showed very little fluorescence emission at 480 nm; the small amount of emission could be due to intermolecular excimer formation, which at such low concentrations (0.22 μM) would be minor. There was no significant change in fluorescence output on binding with the complementary strand. As expected, there was an increase in excimer fluorescence of the single strand with the number of pyrene residues present except in the case of the tri- and tetrapyrenylated conjugates **11** and **12** which had the same emission intensity. Although Figure 5 confirms that the excimer emission looks similar for these two conjugates, it is clear from Table 3 that the tetrapyrenylated conjugate **12** has a significantly higher excimer to monomer fluorescence ratio than the tripyrenylated conjugate **11**. The multiply-pyrenylated hybrids **10**–**13** showed an increase (up to 100%) in fluorescence intensity on duplex formation. Hence, not only was there an absence of interlabel quenching in the single-stranded species but the lack of quenching in the duplex suggested that there was no intercalation of the pyrene residues. The pentapyrenylated hybrid **13** was ~28 times more fluorescent than the monopyrenylated conjugate **9** (at emission λ = 480 nm) and had the same order of magnitude of fluorescence as free carboxyfluorescein. Although the fluorescence of **9** was half that of 4-pyrenylbutanoic acid, the fluorescence emission of the multiply-labeled species **10**–**13** was 4 to 14 times that of the free label.

The hybridization properties of the pyrenylated conjugates were assessed by UV melting temperature studies (Table 5). All melting curves suggested that these conjugates participate in cooperative binding (data not shown). There is little difference in the T_m values of the conjugates and the unmodified oligonucleotide of the same sequence. This, together with the fluorescence emission data previously outlined, strongly indicates that introduction of multiple pyrene residues did not perturb the hybridization properties of the oligonucleotide to any significant extent.

Discussion

Multiply-pyrenylated hybrid molecules were efficiently synthesized. These conjugates were characterized by amino acid analysis, ion-spray mass spectrometry, CE, UV, and fluorescence spectroscopy, and melting temperature studies. The amino acid analysis results for the monopyrenylated conjugate **9** confirmed the expected composition of the polyamide moiety; the results for the rest of the conjugates (**10**–**13**) did not. However, it is important to note that the correct molecular ions were nevertheless obtained (by ion-spray mass spectrometry) for the mono-, di-, and tripyrenylated conjugates **9**–**11**. Hence, amino acid analysis is not necessarily a reliable method of characterizing this type of conjugate. CE analysis showed that each of the five conjugates has a distinct retention time, although the tetra- and pentapyrenylated conjugates had higher than expected electrophoretic mobility, which may be due to conformational effects. For example, the tendency for the highly hydrophobic pyrenyl moieties to aggregate could increase with a larger number of pyrene residues. The UV spectra of these conjugates confirmed the presence of the pyrene residues. As expected, there was an increase in the intensity of the absorption bands

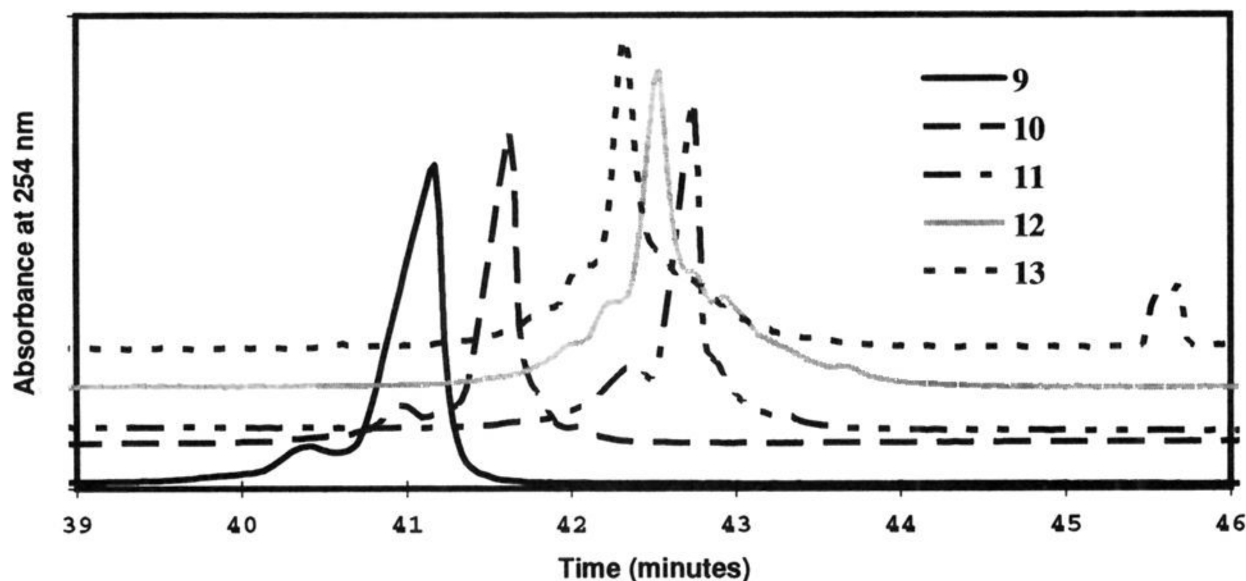

Figure 4. CE electropherograms of PAGE-purified **9–13**.

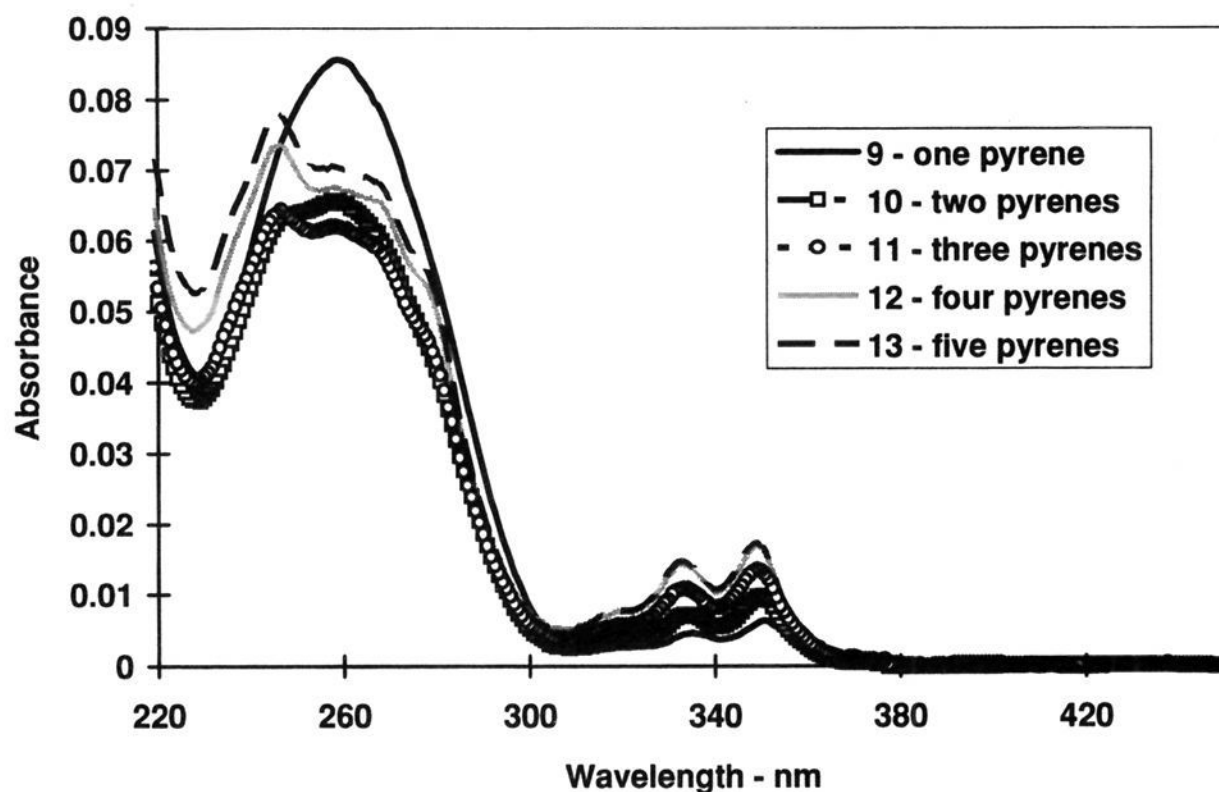

Figure 5. Normalized UV spectra of PAGE-purified **9–13**.

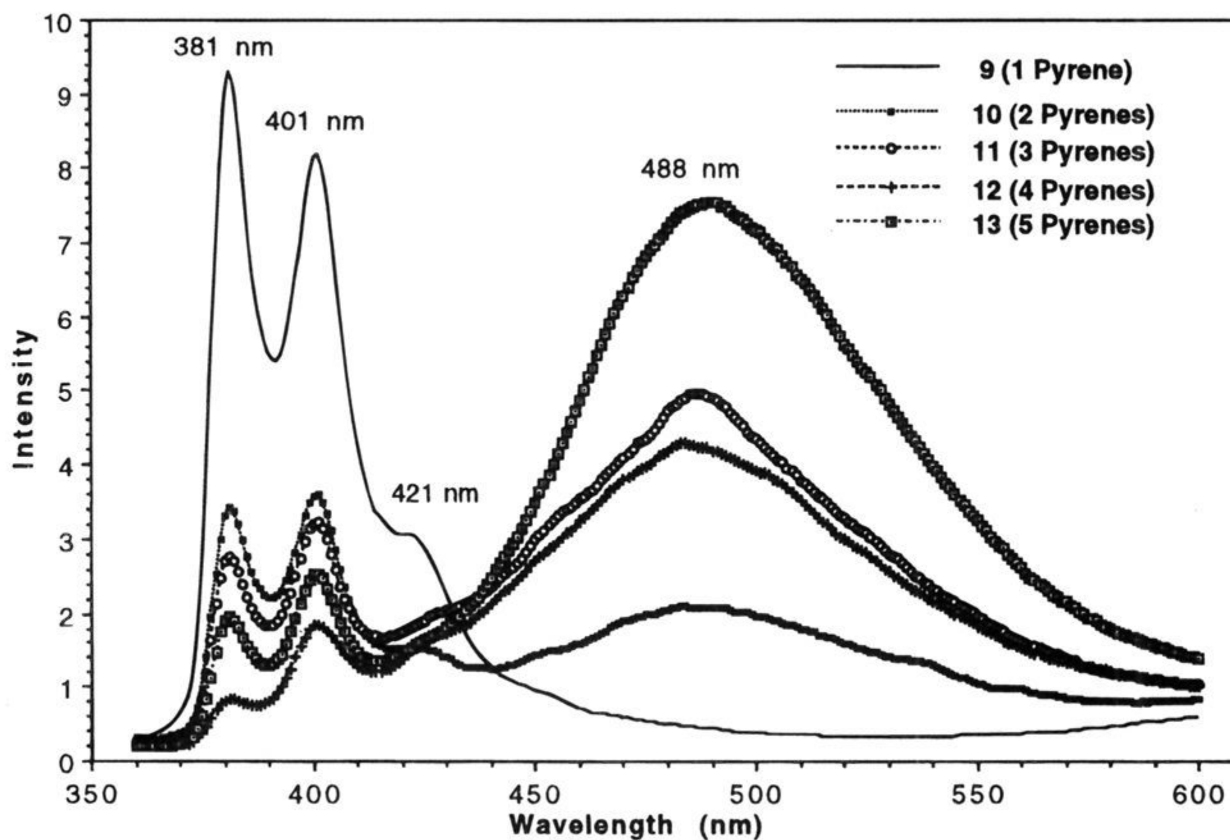

Figure 6. Fluorescence emission spectra of PAGE-purified **9–13**.

(at 335 and 350 nm) due to pyrene with increasing number of pyrene residues. The emission spectra of **9–13** (in H_2O) were consistent with the proposed structures—the ratio of the intensity of excimer emission (in the 430 to 600 nm region) to monomer emission (in the 370 to 420 nm region) increased as the number of pyrene residues increased. UV melting studies on **9–13** and

Table 3. Ratio of Excimer Fluorescence (A) vs Monomer Fluorescence (B) for **9**−**13**[a]

compd	integration A (370−420 nm)	integration B (430−600 nm)	ratio B/A
9	274	96	0.4
10	115	240	2.1
11	102	481	4.7
12	55	439	8.1
13	78	732	9.4

[a] The concentration of the oligonucleotide was 0.22 μM in H_2O.

Table 4. Fluorescence Emission of **9**−**13** before and after Duplex Formation

compd	fluorescence emission of single strand[a,b,e] (luminescence units)	fluorescence emission of duplex[a,c,e] (luminescence units)
4-pyrenylbutanoic acid[d]	60	
9	31	25
10	255	425
11	600	1260
12	600	835
13	860	1240

[a] Excitation slit width = emission slit width = 2.5 nm; excitation wavelength = 350 nm; emission wavelength = 480 nm. [b] Concentration of oligonucleotide = 0.22 μM (aqueous solutions). [c] Concentration of each oligonucleotide = 0.22 μM (aqueous solutions); the complementary strand was ACCTGCTGACATCACAGATGTTGTGAAGCCC (unmodified). [d] Dissolved in 0.1 M NaOH (same conditions as A and B). [e] Fluorescence emission of 5- and 6-carboxyfluorescein in 0.1 M NH_4OAc (same conditions as in A and B except that excitation wavelength = 495 nm and emission wavelength = 520 nm) was 1690 luminescence units.

Table 5. T_ms of **9**−**13** with the Complementary Strand

compd	no. of pyrenes present	T_m (°C)
normal oligonucleotide	0	67.7 ± 0.3
9	1	69.9 ± 0.3
10	2	70.2 ± 0.5
11	3	70.0 ± 0.6
12	4	66.9 ± 0.2
13	5	66.1 ± 0.4

their complementary strands showed that each conjugate has a distinct T_m and these were very similar. Hence, although the amino acid analysis results were not as expected, there is a considerable body of additional evidence which is consistent with the expected structures.

Our objective was to develop a method for the synthesis of multiply-pyrenylated oligonucleotide probes which are more sensitive and fluoresce at a higher wavelength than singly-pyrenylated probes. This has been achieved. Using a prelabeled solid support, we were able to prepare hybrid molecules containing one to five pyrene residues in the polyamide moiety. Hybrid molecules containing two or more pyrenes showed significant excimer fluorescence at 480 nm. The singly-pyrenylated conjugate **9** had half the fluorescence emission of free 4-pyrenebutanoic acid at the same concentration while the multiply-pyrenylated conjugates were 4 to 14 times more fluorescent than the free label, indicating an absence of interlabel quenching in the single-stranded species. The lack of fluorescence quenching in the duplex formed with the complementary strand and the fact that the UV melting characteristics of the labeled probes were not significantly different from that of the underivatized probe strongly suggest that there was no intercalation of the pyrene residues. This indicates that we have succeeded in finding a design for these molecules which avoids intercalation and promotes excimer formation. The fluorescence emission of the hybrid molecules containing multiple pyrene residues (**10**−**13**) was sensitive to the conformation of the oligonucleotide: up to a 2-fold increase in fluorescence was observed on formation of the duplex. This increase may suggest that the pyrene residues in the single-stranded hybrids participate in some stacking interaction with the heterocyclic bases of the oligonucleotide moiety, which is removed when the bases engage their complementary strand in the duplex. The single-stranded pentapyrenylated probe **13** had half the fluorescence intensity of an oligonucleotide labeled with one fluorescein.[1] On duplex formation, the fluorescence intensity of this multiply-pyrenylated probe increased to 73% that of the probe containing one fluorescein label.[1] However, in contrast to fluorescein which undergoes interlabel quenching,[1] we have now shown that the fluorescence intensity of pyrene-labeled oligonucleotide probes can be increased by increasing the number of pyrene residues. Hence there is potential for increasing the fluorescence output of these pyrenylated probes by incorporating even more pyrene residues in the polyamide moiety. Korshun *et al.*[10] have described a methodology based on the use of long (18 atom) chains to separate multiple pyrene residues to promote monomer fluorescence, and they assert that this would provide a higher level of sensitivity due to the additivity of monomer fluorescence. The coupling yield of the pyrenylated phosphoramidite monomer used was only 93%, which renders synthesis of oligonucleotides containing more than a few pyrene residues impractical, although these workers have reported the synthesis of an octapyrenylated PCR (polymerase chain reaction) primer by this method. Another method, reported by Berlin *et al.*,[22] places multiple pyrenes on the 5′-terminus of an oligonucleotide in a branched arrangement, but no physical properties were reported. We feel that it would be far more desirable for a multiply-pyrenylated probe to have strong excimer fluorescence because the monomer fluorescence is in the UV region whereas excimer fluorescence is in the visible region. Since the vast majority of microscopes and microtiter plate readers have visible detectors, pyrenylated probes which produce excimer fluorescence would be far more useful than those producing monomer fluorescence. The dramatic difference in fluorescence emission between the single-stranded and double-stranded species could lead to other important applications. For example, these conjugates could potentially be used in solution-phase hybridization techniques (in an analogous manner to ethidium bromide[23]). Another potential application of these conjugates is as probes of DNA/protein interactions.

This class of multiply-pyrenylated hybrid molecules provides a solution to a number of previous problems with probes containing multiple fluorophores. There is no interlabel or intercalation-induced quenching so potentially any number of labels can be incorporated to increase the sensitivity of these probes. Pyrene is much more resistant to photochemical bleaching than fluorescein. In fact, we have found that these pyrenylated conjugates can be kept for extended periods (~12 months) at −20 °C as frozen aqueous solutions without any special precautions to protect them from light and still remain highly fluorescent and intact.

The 3′-blocked conjugates synthesized here can be used as hybridization probes and also in PCR-based assays. With the many methods available for immobilization of PCR-amplified

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DNA,[24] the pyrenylated hybridization probes can be used to hybridize onto amplified DNA captured on a solid support (*e.g.*, microtiter well) or, as already mentioned, in a solution-phase hybridization assay. These PCR products can then be directly detected using a variety of fluorescence detectors available (*e.g.*, a microtiter plate reader equipped for fluorescence measurements). Alternatively, multiple pyrene labels can be incorporated into the PCR amplification products by using conjugates with a free 3′-terminus as primers. These may be prepared either by using nucleoside 5′-*O*-phosphoramidites to synthesize the oligonucleotide moiety in the 5′→3′ orientation or by adapting our previously described methodology, in which the peptide is attached to the base of the 3′-terminal nucleoside, leaving it with a free 3′-hydroxyl.[16]

Conclusion

A series of oligonucleotide–polyamide hybrid molecules bearing multiple pyrene labels on the polyamide moiety has been synthesized. This design produced strong excimer fluorescence in the products which increased with the number of pyrene residues present. In addition, the hybridization properties of the oligonucleotide were not significantly perturbed. The excimer fluorescence intensity of these molecules was highly sensitive to duplex formation, which points to the exciting possibility of using them as solution-phase hybridization probes, normal hybridization probes, and also as fluorescent PCR primers.

Experimental Section

General Remarks. N^{α}-Fmoc-L-lysine and Fmoc-Ala-OPfp were obtained from Auspep, Melbourne. 4-Pyrenylbutanoic acid (obtained from Aldrich) was purified by recrystallization from ethyl acetate. *N*-Hydroxysuccinimide was obtained from Pierce. Controlled pore glass (200–400 mesh, 500 Å pore size, cat. no. 27720), 4-(dimethylamino)-pyridine, DCC, and diisopropylcarbodiimide (DIC) were purchased from Fluka. DMF was distilled under reduced pressure and used within 14 days or was purchased from Auspep, Melbourne, already distilled and stored over 3 Å sieves. All other reagents were used without further purification. Thin layer chromatography (TLC) was performed on Merck SG-60 precoated plastic plates. 5-(and 6-)-Carboxyfluorescein was purchased from Molecular Probes, Eugene, OR. *N*-Fmoc-ϵAhx-OPfp was prepared according to the literature method.[17] NMR spectra were recorded at 399.9 MHz (^{1}H) and at 99.98 MHz (^{13}C). DEPT experiments were performed with a 135° ^{1}H selection pulse. The internal reference was residual DMSO-d_5 in DMSO-d_6. UV spectra were recorded in 0.1 mM EDTA solutions on a Varian Cary 1 spectrophotometer. Fluorescence spectra were obtained from a Hitachi F-4010 spectrometer; single wavelength readings were obtained on a Perkin-Elmer LS5B fluorimeter. Amino acid analysis was performed on a Beckman 6300 high performance analyzer; both 24 and 72 h hydrolyses in concentrated hydrochloric acid were performed. Samples for high-resolution FAB–MS measurements were suspended in a polyethylene glycol (600)/thiolglycerol/glycerol/DMSO matrix, low-resolution samples in a thioglycerol matrix. The ionization gas was Xe. Ion-spray mass spectrometry measurements were performed on a Perkin-Elmer PE SCIEX LC/MS in the negative ionization mode; the samples were prepared according to the method of Reddy and Iden.[25]

***N*-Hydroxysuccinimidyl Ester of 4-Pyrenebutanoic Acid (2).** A solution of *N*-hydroxysuccinimide (1.91 g, 16.6 mmol) and DCC (2.58 g, 12.5 mmol) in DMF (5 mL) was added to a solution of 4-pyrenylbutanoic acid (2.40 g, 8.32 mmol) in DMF (25 mL). The reaction was monitored by TLC (25% ethyl acetate/toluene) and was found to be complete after 16 h. The precipitated DCU (dicyclohexylurea) was removed by filtration and washed with ethyl acetate (4 × 25 mL). Ethyl acetate (300 mL) was added to the filtrate, and residual precipitated DCU was removed by gravity filtration. The filtrate was washed with 10% $NaHCO_3$ (2 × 200 mL) and H_2O (1 × 200 mL) to remove excess *N*-hydroxysuccinimide (NHS). After the solution had been dried (Na_2SO_4) and the solvent removed *in vacuo*, recrystallization from ethyl acetate (30 mL) gave **2** as a fawn-colored powder (1.48 g, 50%), mp 127–128 °C. ^{13}C NMR (DMSO-d_6): δ 25.5 (2 × NHS CH_2), 26.8 (C3), 30.1 (C4), 31.4 (C2), 123.3 (pyrene CH), 124.1, 124.2 (pyrene C_{quat}), 124.9 (pyrene CH), 125.0 (2 × pyrene CH), 126.2, 126.6 (pyrene CH), 127.4 (2 × pyrene CH), 127.5 (pyrene CH), 128.1, 129.5, 130.4, 130.9, 135.6 (pyrene C_{quat}), 169.0 (C1), 170.3 (2 × NHS CO).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{ϵ}-(4-pyrenylbutanoyl)-lysine (Fmoc-Lys(Pyrene)-OH, 3). A solution of Et_3N (1.26 mL, 9.04 mmol) in DMF (53 mL) was added to a mixture of the *N*-hydroxysuccinimidyl ester of pyrenebutanoic acid (**2**) (3.45 g, 8.95 mmol) and N^{α}-Fmoc-Lys-OH (3.33 g, 9.04 mmol). The reaction was monitored by TLC (25% ethyl acetate/petroleum spirit and 30% MeOH/CH_2Cl_2) and was found to be complete after 3 h. The solvent was removed *in vacuo*, the resulting syrup was redissolved in 50% MeOH/CH_2Cl_2 (70 mL), Dowex 50 (H^+) ion exchange resin was added, and the mixture was stirred for 10 min. After the resin had been removed by filtration and washed with 50% MeOH/CH_2Cl_2 (2 × 25 mL), the filtrate was concentrated and the crude residue recrystallized from acetone (325 mL)/petroleum spirit (200 mL) to give **3** as a light green powder (3.55 g, 66%), mp 118–120 °C dec. ^{1}H NMR (DMSO-d_6): δ 1.35–1.81 (m, 6H, Lys Hα, Hβ and Hγ), 2.10 (m, 2H, butanoate H3), 2.24 (t, 2H, butanoate H2, J = 7.1 Hz), 3.31 (t, 2H, butanoate H4, J = 7.7 Hz), 3.40 (m, 2H, Lys Hϵ), 3.70 (m, 1H, Lys Hα), 4.20 (t, 1H, Fmoc H9, J = 6.6 Hz), 4.27 (d, 2H, Fmoc CH_2, J = 6.6 Hz), 7.32 (dd, 2H, Fmoc H3 and H6, J = 7.3, 7.3 Hz), 7.41 (dd, 2H, Fmoc H3 and H6, J = 7.2, 7.2 Hz), 7.66 (d, 1H, Lys Hα, J = 8.1 Hz), 7.72 (d, 2H, Fmoc H1 and H8, J = 7.4 Hz), 7.88 (d, 2H, Fmoc H4 and H5, J = 7.4 Hz), 7.91–8.39 (m, 9H, pyrene H). ^{13}C NMR (DMSO-d_6): δ 23.1 (Lys Cγ), 27.6 (butanoate C3), 28.8 (Lys Cδ), 30.4 (Lys Cβ), 32.3 (butanoate C2), 35.0 (butanoate C4), 38.2 (Lys Cϵ), 46.6 (Fmoc C9), 53.8 (Lys Cα), 65.6 (Fmoc CH_2), 120.1 (Fmoc C3 and C6), 123.5 (pyrene CH), 124.1, 124.2 (pyrene C_{quat})124.8 (pyrene CH), 124.9 (2 × pyrene CH), 125.2 (Fmoc C2 and C7), 126.1, 126.5 (pyrene CH), 127.0 (Fmoc C4 and C5), 127.2, 127.4, 127.5 (pyrene CH), 127.6 (Fmoc C1 and C8), 128.1, 129.3, 130.4, 130.9, 16.6 (pyrene C_{quat}), 140.7 (Fmoc C4a and C4b), 143.77, 143.81 (Fmoc C8a and C9a), 156.2 (Fmoc CO), 171.7 (C1), 174.0 (Lys αCO). HR-FABMS: exact mass found 639.2845 (M + H), calcd for $C_{41}H_{39}N_2O_5$ 639.2859.

Synthesis of Multiply-Pyrenylated CPG Resins 4–8. The series of derivatized CPG (controlled pore glass) resins was prepared as described previously;[17] the loading of the internal reference amino acid Ala was 55 ± 7 μmol/g. The general procedure for peptide synthesis was as follows. Amine deprotection was *via* treatment with 20% piperidine/DMF (12 min), and amino acids were activated by *O*-benzotriazolyl-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU). For example, in the case of the pyrene synthon **3**, the following conditions were used: the resin was treated with **3** (3 equiv), HBTU (2.9 equiv), and *N*-methylmorpholine (4.83 equiv) in DMF in two successive 0.5 h couplings. The peptide loading after incorporation of each residue was assessed by Fmoc assay.[16] Incorporation of the Phe residue for resins **6–8** was achieved by single 0.5 h couplings under the same conditions as described above. The 4-hydroxybutanoic acid linker was incorporated under identical conditions to those we have described elsewhere.[17] Prior to removal of the Fmoc group and also after the final coupling of the 4-hydroxybutanoic acid linker, the resin was capped by reaction with Ac_2O/DMAP (250 μL) and DMAP (0.05 g) in dry pyridine (1 mL). The resins **4–8** were characterized by pixyl assay[16] and amino acid analysis to determine the final *N*-terminal hydroxyl loading (data not shown) and the integrity of the resin-bound peptide, respectively (results are shown in Table 1).

Oligonucleotide Synthesis. Synthesis conditions and purification procedures were identical to those previously described.[16] Synthesis scale was 1 μmol in all cases.

Melting Temperature Studies. The samples were prepared by dissolving 5 μg of each oligonucleotide (primer plus the complementary

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sequence) in 1.3 mL of degassed (He) PCR buffer (10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3) to give a solution with a concentration of 0.42 μM with respect to each oligonucleotide. The T_m experiments were carried out on a Varian Cary 1 spectrophotometer equipped with a temperature controller and a multicell compartment. The temperature was increased from 25 to 85 °C at a rate of 2 °C/min, and a data point was taken every 0.1 °C. The temperature readings were taken from a temperature probe in a similar cell adjacent to the sample cell. The experiments were performed in triplicate, and the T_m was determined by the hyperchromicity method[26] using software provided by Varian.

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