Hoechst 33258 Tethered by a Hexa(ethylene glycol) Linker to the 5′-Termini of Oligodeoxynucleotide 15-Mers: Duplex Stabilization and Fluorescence Properties

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A fluorescent Hoechst 33258 derivative has been prepared in which a hexa(ethylene glycol) linker is attached to the terminal phenol residue. Conjugation of this derivative to DNA sequences is accomplished by a reversed coupling protocol, one in which the 5′-terminal nucleoside residue of a fully protected DNA sequence is converted to a terminal phosphoramidite. In the presence of the Hoechst derivative and tetrazole the final coupling reaction is achieved to generate the conjugated nucleic acid. After deprotection and cleavage of the conjugate from the support, HPLC analysis indicates that the conjugation reaction proceeds with yields as high as 75%. The presence of the conjugated Hoechst derivative increases the stability of DNA duplexes typically by 10−16 °C. A variety of sequence variants indicate that the tether length is sufficient to reach beyond the terminus of the DNA duplex and bind to internal A-T rich target sequences as far away as four base pairs from the site of attachment. A four base pair binding site appears to be necessary for effective helix stabilization by the conjugate, but in some cases can include a G-C base pair, which is consistent with a previous X-ray diffraction study regarding the binding of Hoechst 33258 to duplex DNA. When A-T base pairs alternate with G-C base pairs, a small but discernible increase is T_m is observed (3.6 °C), indicating that binding to this sequence still occurs, but not in the same manner as to A-T rich sequences. Upon formation of the conjugated duplex, an enhanced quantum yield for the fluorescence emission spectrum of the tethered Hoechst derivative is observed. When an A-T rich binding site is present, the enhanced quantum yield increases by at least 16- and in some cases to nearly 30-fold relative to the value obtained for the single-stranded DNA−Hoechst conjugate.

Introduction

A number of well-characterized ligands are known to interact with double-stranded DNA by binding in the minor groove of the duplex structure. Among these agents are the fluorescent dyes Hoechst 33258 and Hoechst 33342 as well as the related benzindole DAPI. Crystal structures of DNA complexes containing Hoechst 33258 and DAPI have been reported,[1−4] confirming the minor groove binding nature of the fluorophores. Binding in the minor groove by these dyes results in helix stabilization and a dramatic enhancement in the observed fluorescence emission quantum yield.[5,6] The enhanced emission characteristics are largely due to the protection of the dye in the excited state when bound in the minor groove from nonradiative relaxation effects, presumably collisional processes involving water.[5] The fluorescence properties of the Hoechst dyes have previously been employed in a quantitative manner to automate DNA content assays,[7−9] to determine cell numbers,[10,11] and to sort chromosomes.[12] The quantum yield effects for these dyes are sensitive enough to permit the detection of one target cell per million in mixed cell populations.[13] Such highly sensitive applications suggest that an oligonucleotide tethering one of the Hoechst dyes could be valuable in the development of a sensitive nucleic acid hybridization diagnostic.

The ability to easily and rapidly prepare nucleic acid sequences tethered to fluorescent agents, or those possessing other unique properties, provides a vehicle for the development of a series of new hybridization probes, nucleic acid-based diagnostics, and ultimately nucleic acid-based therapeutics. The observation that Hoechst dyes can cross the cell membrane and nuclear membrane in order to stain chromosomes[12] also suggests that a DNA−Hoechst conjugate might be better able to cross cell membranes than the unconjugated naked DNA sequence. To prepare nucleic acid conjugates, one of the most common techniques involves the incorporation of a linker to the 5′-terminus of the oligonucleotide. When the linker contains an appropriate nucleophilic site, such as a sulfhydryl[14] or aliphatic amino group,[15] such linkers then permit postsynthetic conjugation by a variety of

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agents. An alternative approach simply converts the conjugation agent into a phosphoramidite building block to be used directly in the final coupling step during the assembly of the oligonucleotide conjugate.[16,17]

A variety of DNA conjugates have been prepared in order to enhance hybridization properties to complementary DNA or RNA target sequences. Intercalators,[18] polyamines,[19] and peptidelike agents related to distamycin[20] have been attached to DNA sequences and their hybridization properties subsequently examined. In previous work we described the preparation of DNA sequences tethering the Hoechst fluorescent dye 33258 to an internal site within the target binding site of the A-T rich sequence.[21] The preparation of these materials was complicated by aggregation of the Hoechst dye during the postsynthetic labeling procedures, and only small quantities of materials could be obtained. The helix-stabilizing and fluorescent properties of the double-stranded complexes prepared from such conjugates were also reported in that initial study. In the present work we describe the synthesis and properties of conjugates in which a Hoechst dye is tethered to the terminus of the DNA sequence through a hexa(ethylene glycol) linker, and conjugation of the oligonucleotide by the dye is accomplished during the assembly of the DNA sequence.

Experimental Section

Materials. HPLC grade solvents were either obtained from Aldrich (Milwaukee, WI) or from Fisher Scientific (Fair Lawn, NJ). 5′-Dimethoxytrityl nucleoside phosphoramidite monomers as well as all ancillary reagents for nucleic acid synthesis were obtained from BioGenex (San Ramon, CA) or from Applied Biosystems, Inc. (Foster City, CA). Oligonucleotides were synthesized using nucleoside phosphoramidite derivatives and an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC) was carried out on ODS-Hypersil column (0.94 × 25cm or 0.46 × 25 cm, Shandon Southern, England). Mass spectra were obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, Urbana, IL.

Methods. 1-(*p*-Cyanophenoxy)-2-penta(ethylene glycol)ethane (1). To an ice cooled solution of 0.105 g (0.88 mmol) of 4-cyanophenol, 0.5 g (1.77 mmol) of hexa(ethylene glycol), 0.464 g (1.77 mmol) of triphenylphosphine in anhydrous dioxane 15 mL was added dropwise 0.28 mL (1.77 mmol) of DEAD over a period of 30 min. The reaction mixture was left for stirring at 0 °C for 2 h. TLC analysis (ethyl acetate: methanol, 9:1) at this point indicated the completion of the reaction. Solvents were evaporated, and the residue was dissolved in ethyl acetate and washed with 5% sodium bicarbonate, washed with water, and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was purified by flash chromatography over silica gel using ethyl acetate and a gradient of methanol (1−10%) to yield 0.250 g (0.950 mmol, 54%) of an yellow oil. R_f in ethyl acetate: methanol 9:1 = 0.39. ^{1}H-NMR ($CDCl_3$) δ = 3.02 (bs, 1H), 3.52−3.73 (m, 20H), 3.84 (t, 2H), 4.14 (t, 2H), 6.91 (d, 2H), 7.53 (d, 2H). UV (H_2O): λ_{max} = 233 nm. IR 3465, 2873, 2219, 1747, 1602, 1507, 1451, 1350, 1300, 1262 cm^{-1}. HRMS: Calcd for $C_{19}H_3NO_7$ (M + H$^+$) 384.20222, found 384.20270.

2-{2-[4-[2-[Penta(ethylene glycol)]ethoxy]phenyl]-6-benzimidazolyl}-6-(1-methyl-4-piperazinyl)benzimidazole (4). To prepare the necessary imino methyl ester **2**, 0.5 g (1.27 mmol) of **1** was dissolved in 15 mL of dichloromethane, 0.14 g (4.2 mmol) of anhydrous methanol was added, and the reaction was cooled to 0 °C. The reaction mixture was saturated with HCl and maintained at 0 °C overnight. The resulting white precipitate was collected, washed thoroughly with diethyl ether, and dried to yield 0.52 g (1.21 mmol, 95%) of a white solid which was used in the following step without purification.

To 0.2 g (0.62 mmol) of 2-(3,4-diaminophenyl)-6-(1-methyl-4-piperazinyl)benzimidazole[22] (**3**) dissolved in anhydrous methanol (3 mL) was added 0.496 g (11.09 mmol) of imidate **2** followed by 0.66 mL (1.1 mmol) of glacial acetic acid. The mixture was stirred at 65−70 °C for 3 h. TLC analysis (dichloromethane:methanol, 7:3) containing trace of tri-*n*-butylamine indicated the completion of the reaction. Solvents were evaporated, and the residue was purified on a small column of silica gel using ethyl acetate with a trace of tri-*n*-butylamine and gradient of methanol to yield 0.198 g (0.287 mmol, 46%) of pure product. Mp = 115−120 °C dec. R_f: (dichloromethane:methanol 7:3 + trace of tri-*n*-butylamine) = 0.48. ^{1}H NMR (300 MHz, CD_3OD): δ = 2.57 (3H, s), 2.93 (4H, m), 3.30 (4H, m), 3.57 (2H, m), 3.87 (2H, t, J = 4.3 Hz), 4.20 (2H, t, J = 4.3 Hz), 7.06 (1H, dd, J = 2.1, 8.7 Hz), 7.10 (2H, d, J = 9.0 Hz), 7.15 (1H, d, J = 2.1 Hz), 7.52 (1H, d, J = 8.7 Hz), 7.69 (1H, d, J = 8.4 Hz), 7.94 (1H, dd, J = 8.4 Hz, 1.8 Hz), 8.05 (2H, d, J = 9.0 Hz), 8.25 (1H, d, J = 1.8 Hz). UV (H_2O): λ_{max} = 212, 255, 334 nm, λ_{min} = 238, 288 nm. IR: 3172, 2866, 23357, 1636, 1610, 1485, 1454, 1288, 1257, 1184, 1112 cm^{-1}. HRMS: Calcd for $C_{37}H_{49}N_6O_7$ (M + H$^+$) 689.36627, found 689.36660.

DNA Synthesis. The 15-mers were synthesized using standard phosphoramidite protocols.[23] To attach the Hoechst derivative to the 15-mers through the hexa(ethylene glycol) linker, the following procedure was employed: After the final nucleoside coupling on the solid support, the DMT group was removed under standard conditions using trichloroacetic acid. After washing the support with anhydrous acetonitrile, equal volumes of 1 M (2-cyanoethoxy)bis(diisopropylamino)phosphine and 0.5 M tetrazole, both dissolved in anhydrous acetonitrile, were added to the support. After a reaction period of 30 min, the support was washed with anhydrous acetonitrile, and this phosphitylation step was repeated a second time. Alternatively, a solution of 1.0 M of (2-cyanoethoxy)(diisopropylamino)-chlorophosphine and 1.2 M diisopropylethylamine in acetonitrile was employed. To complete the coupling, equal volumes of 0.5 M tetrazole/acetonitrile and a 0.1 M solution of the Hoechst derivative **4** dissolved in anhydrous DMF were added to the support, and the reaction was continued for 16 h. After washing the support with acetonitrile, the acetylation and oxidation steps were performed in the usual manner. The conjugate was deprotected and removed from the support by treatment with concentrated aqueous ammonia at 50 °C for 16 h.

HPLC using a 9.4 × 250 mm column of ODS-Hypersil was used to purify the conjugated oligonucleotides using a linear gradient of 10−40% buffer B over 30 min (buffer A: 50 mM triethylammonium acetate, pH 7.0, buffer B: 50 mM triethylammonium acetate, pH 7.0 containing 70% acetonitrile). An estimation of the efficiency of the final coupling step could be determined by comparing the ratio of the free 15-mer (failed conjugation product) to the conjugated 15-mer obtained in the HPLC isolation procedures. This final coupling step varied somewhat in yield between 50 and 75%.

Nucleoside Analyses. To 0.5 A_{260} units of oligonucleotide in 100 μL of 50 mM Tris·HCl, pH 8.0, containing 100 mM

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$MgCl_2$ were added 0.003 units of snake venom phoshodiesterase and 1 unit of alkaline phosphatase and the mixtures incubated 2−6 h at 37 °C. The digestion products (approximately 0.2 A_{260} units) were analyzed by reversed phase HPLC using a 4.6 × 250 mm column of ODS-Hypersil and a gradient of 5% buffer B for 10 min, followed by a linear gradient from 5−100% buffer B over 10 min (buffer A and buffer B were as described above). Under these conditions, the following retention times were observed (min): dC 3.1, dm^5C, 5.2, dG 7.5, T 8.0, dA 9.2, and Hoechst derivative **4** (see Scheme 1) 17.5.

Thermal Denaturation Studies. Thermal denaturation studies were performed in 21 mM HEPES pH 7.5, 100 mM NaCl and 20 mM $MgCl_2$ at duplex concentrations of ~1 μM. Absorbance (260 nm) and temperature values were measured with an AVIV 14DS UV/visible spectrophotometer equipped with digital temperature control. The temperature of the cell compartment was increased in 1.0 °C steps (from 0 to 95 °C), and when thermal equilibrium was reached, temperature and absorbance data were collected. T_m values were determined both from first-order derivatives and by graphical analysis of the absorbance vs temperature plots.

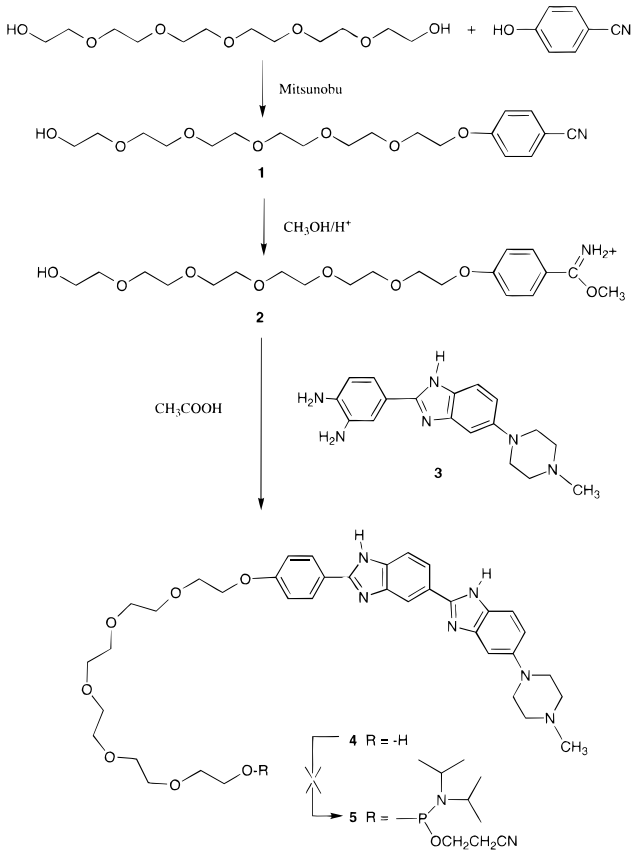
Fluorescence Studies. Fluorescence emission spectra were collected on a Shimadzu RF5000U fluorescence spectrophotometer containing a Shimadzu DR-15 microprocessor and graphics display terminal. Spectra were obtained in 21 mM HEPES pH 7.5, 100 mM NaCl and 20 mM $MgCl_2$ at duplex concentrations of ~1 μM. All measurements were performed with the following list of parameters: slit width Ex/Em = 10 nm/10 nm, low sensitivity, medium speed. Samples were introduced into a 1.25 mL cell thermally isolated with a water jacket. Temperature was controlled with a recirculating water bath. Excitation wavelength = 342 nm, emission range = 380−600 nm.

Results and Discussion

In our initial attempts at preparing a DNA−Hoechst 33258 conjugate, we wanted to synthesize a bis-benzimidazole derivative tethering an appropriate linker and then functionalize the linker as a phosphoramidite. We chose hexa(ethylene glycol) for the linker. The 18 atom length of this ethylene glycol oligomer should provide adequate flexibility in order for the Hoechst derivative to reach an appropriate A-T rich minor-groove binding site, while the polyether character results in potentially advantageous hydrophilic characteristics for complexes formed in aqueous solutions. This linker has also functioned well for conjugates used to study DNA triplexes.[24] Hexa(ethylene glycol) and similar glycol-based linkers have been employed in other studies involving both DNA[25] and RNA[26−28] complexes.

Synthesis. Our attempts to attach the hexa(ethylene glycol) linker directly to the parent Hoechst 33258 fluorophore failed under a variety of conditions. The alternative strategy involved incorporating the linker into the bis-benzimidazole chromophore during a complete synthesis of a Hoechst 33258 derivative.[22] In this latter approach, we could take advantage of the described synthetic procedures for the Hoechst fluorophore and simply alter the components of the final cyclization step to provide a derivative containing the desired hexa(ethylene glycol) linker. With this design, relatively few new steps were required; *p*-cyanophenol could be alkylated by hexa(ethylene glycol) using a Mitsunobu protocol[29] to generate **1** (Scheme 1). The cyano group was then converted to the imidate **2**, and without purification, the imidate was reacted with the diamine **3** obtained using the described synthetic procedures for Hoechst 33258.[22] The product of this cyclization (**4**) was simply the Hoechst 33258 fluorophore with a covalently attached hexa(ethylene glycol) linker.

Scheme 1



The final step in this process required that the bis-benzimidazole derivative with the hexa(ethylene glycol) linker be converted into a phosphoramidite. This reaction proved to be problematic. Regardless of the conditions used, we were unable to isolate any significant quantities of the phosphoramidite derivative **5** (Scheme 1). The most likely explanation for this difficulty is that the relatively nucleophilic imidazole nitrogens competed for the reactive chlorophosphine derivative. After standard aqueous workup, the phosphorus imidazole bond would hydrolyze regenerating the starting material—other side reactions are of course also possible. Although we considered the possibility of introducing protecting groups at these sites, we opted for a more pragmatic approach. The alternative strategy for the preparation of the DNA−Hoechst conjugate involved coupling the linker to the 5′-terminus of the oligonucleotide using what might be termed a "reverse" protocol. That is, the phosphoramidite would be incorporated into the support-bound oligonucleotide, and the coupling would be facilitated by adding **4** in the presence of tetrazole. Similar approaches to coupling at the 5′-terminus have been described in earlier studies in which phosphotriester chemistry was

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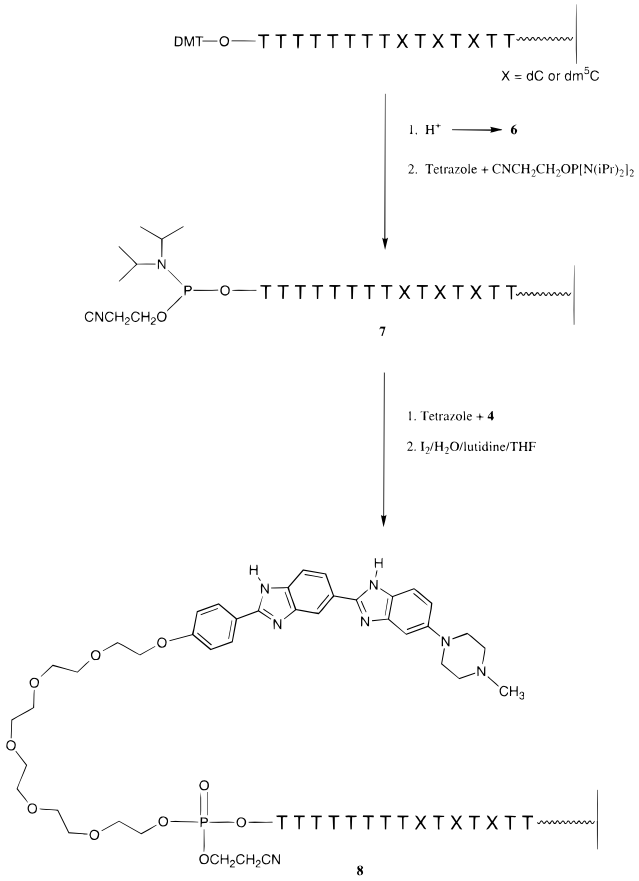
Scheme 2



employed.[30] Using this approach, a fully protected support-bound 15-mer was initially assembled using standard protocols. The terminal DMT protecting group was removed using trichloroacetic acid to generate the support-bound oligomer such as **6**, containing a 5′-terminal hydroxyl (Scheme 2). Conversion of **6** to the corresponding phosphoramidite **7** was accomplished by two rounds of phosphitylation using (2-cyanoethoxy)bis(diisopropylamino)phosphine and 0.5 equiv of tetrazole (or using the corresponding chlorophosphine derivative in the presence of base). Two treatments may not be required to generate **7**, but since we could not confirm the extent of the reaction, two cycles of phosphitylation seemed a prudent course of action. The coupling reaction between **4** and **7** proceeded by adding sufficient solution (0.1 M of **4** and 0.5 M of tetrazole) to immerse the solid support. We could detect significant, but less than optimal quantities of conjugate after a 1−2 h reaction period (<50%), but since the extent of conjugation can only be judged after complete workup of the crude product followed by HPLC analysis (see below), it was generally more efficient to simply allow the reaction to proceed for an extended period of time, typically overnight. After a reaction period of approximately 16 h, the support was washed and oxidized in the normal fashion to complete the coupling protocol and generate conjugates of varying sequence composition such as **8** (Scheme 2).

The extent of the conjugation reaction could only be judged after treatment of the coupling product with aqueous ammonia to effect deprotection of the conjugate and its removal from the solid support. HPLC analysis

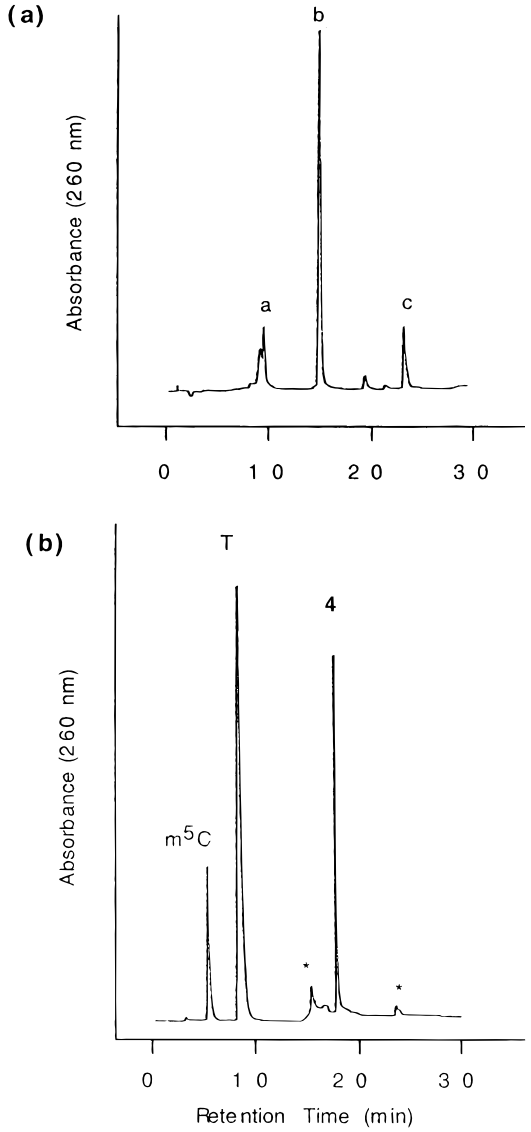


Figure 1. (a) HPLC analysis of the crude reaction mixture obtained after ammonia deprotection of the conjugate **8**. a: 15-Mer polypyrimidine DNA sequence, b: Hoechst−15-mer conjugate, c: Hoechst−hexa(ethylene glycol) derivative **4**. (b) HPLC analysis of the snake venom phosphodiesterase/bacterial alkaline phosphatase digest of a DNA 15-mer conjugated to the Hoechst derivative **4**. * marks unidentified contaminants in the buffers.

of the crude material indicated the presence of three species (Figure 1a). The earliest eluting peak corresponds to the unconjugated 15-mer. The peak multiplicity for this first product likely reflects some variation in the end group character (with or without a terminal phosphorus species). The latest eluting peak corresponds to **4**. We believe that its presence in the crude material is mostly likely the result of nonspecific binding by **4** to the solid support during the final coupling reaction. Traces of **4** may remain nonspecifically bound to the support after the various washings, and only under extended treatment with hot ammonia are they removed. Alternatively, the presence of **4** might represent some minor cleavage of the conjugate **8** during ammonia deprotection. The major peak of the analysis in Figure 1a corresponds to the fully deprotected derivative corresponding to **8**. Its identity could be confirmed by digestion with snake venom phosphodiesterase and alkaline phosphatase. Analysis of the digestion mixture indicated

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Table 1. Thermal and Fluorescence Properties of Double-Stranded DNA Complexes Tethering a Hoechst 33258 Derivative

		T_m values[a]			
entry	sequence[c]	duplex[d] X = C/M, °C	conjugate[e] X = M, °C	ΔT_m,[a] °C	ΔF_{max}[b]
1	(H)-eg$_6$-TTTTTTTTXTXTXTT AAAAAAAAGAGAGAA	49.2/57.5	62.1/70.2[f]	12.9/12.7	29
2	(H)-eg$_6$-XTTTTTTTXTXTXTT GAAAAAAAGAGAGAA	53.0/56.5	72.4	5.9	19
3	(H)-eg$_6$-XXTTTTTTXTXTXTT GGAAAAAAGAGAGAA	52.8/57.6	71.4	13.8	25
4	(H)-eg$_6$-XTXTTTTTXTXTXTT GAGAAAAAGAGAGAA	53.1/58.1	71.1	13.0	20
5	(H)-eg$_6$-TTGGTTTTXTXTXTT AACCAAAAGAGAGAA	57.0/59.4	72.8	13.4	22
6	(H)-eg$_3$-TTGGTTTTXTXTXTT AACCAAAAGAGAGAA	53.8/59.4	72.5	13.1	16
7	(H)-eg$_6$-TTTGGTTTXTXTXTT AAACCAAAGAGAGAA	52.2/57.6	68.1	10.5	16
8	(H)-eg$_6$-XTXTXTXTXTXTXTT GAGAGAGAGAGAGAA	56.5/64.1	67.7	3.6	5

[a] Values are the average of at least two determinations (+0.5 °C). ΔT_m values represent the change in T_m when the conjugate was incorporated into the M-containing duplex. [b] Fluorescence enhancement measured as the ratio of the emission maxima for the double-stranded conjugate relative to the single-stranded conjugate. [c] (H)-eg$_6$- represents Hoechst 33258 tethered to the 5′-terminus of the DNA sequence through a hexa(ethylene glycol) linker. (H)-eg$_3$- is a similar conjugate, but the tether is a tri(ethylene glycol) linker. X = C (2′-deoxycytidine), or M (2′-deoxy-5-methylcytidine). [d] T_m values for the parent 15-mer duplex lacking the Hoechst 33258 conjugate. [e] T_m values for the conjugated 15-mer duplex. [f] T_m values for this conjugate are reported for both the C- and M-containing complexes (C/M).

the correct ratio of nucleosides and one equivalent of **4** (Figure 1b). A comparison of the early eluting peak(s), representing the unconjugated 15-mer, with the major peak, representing the conjugate (see Figure 1a), indicated that the conjugation reaction (both phosphitylation and coupling) proceeded with yields varying from 50 to 75%.

These yields do not compare with the high yield (~98%) phosphoramidite couplings typically observed with standard DNA assembly protocols, but they resulted in material sufficient for the subsequent studies. In fact this procedure produced much more material than could be obtained using the previously described approach with an internally placed linker conjugated by a postsynthetic alkylation reaction.[21] It should be noted that with standard DNA assembly protocols,[23] the reactive phosphoramidite species is present in solution at a concentration far in excess of the support-bound oligonucleotide. With such protocols, trace amounts of water only marginally affect the quantity of the reactive species present and do not impact the coupling efficiency to any great extent. When this process is reversed, that is the reactive phosphoramidite species is now attached to the support bound oligonucleotide, there is no excess in the reactive species present. In these cases, even minute quantities of water will have a significant and detrimental effect on the coupling efficiency by reacting with the support-bound oligonucleotide. It is therefore necessary to ensure that these conjugation reactions are performed under anhydrous conditions.

***T*$_m$ Values for Hoechst−DNA Conjugates.** We have examined the thermal characteristics of double-stranded DNA complexes of varying sequence composition tethering the Hoechst 33258 fluorophore (Table 1). We began with a 15-mer sequence (entry 1, Table 1) in which the terminal portion of the duplex (that nearest the conjugated Hoechst derivative) contained eight A-T base pairs. Initially we examined a homopurine-homopyrimidine duplex in which the C-residues of the pyrimidine strand were either 2′-deoxycytidine (C) or the corresponding 5-methyl derivative (M). In all cases examined, the nonconjugated duplexes containing the methylated C-derivative exhibited higher T_m values than those observed for the C-containing sequences (Table 1). This observation is consistent with those described previously[31] in which DNA duplexes containing the C-5 methylated pyrimidine T always exhibited higher T_m values than those containing the corresponding non-methylated 2′-deoxy derivative U.

Tethering the Hoechst derivative through the hexa(ethylene glycol) linker increased the T_m value for both duplexes (e.g., C-containing and M-containing) duplex by approximately 13 °C. This observation suggests that the tethered groove-binder can fold back into the minor groove as designed and provide additional binding interactions to stabilize the DNA duplex. We cannot at this time exclude the possibility that some intermolecular ligand−helix binding occurs. That is, the ligand from one conjugated duplex binds to the minor groove of a second duplex. But at the concentrations employed, this process seems unlikely. The combination of the tethered ligand and the use of 2′-deoxy-5-methylcytidine (M) residues results in T_m values that are increased by as much as 20 °C relative to the simple non-conjugated C-containing hybridization complex (see entry 1, Table 1). With the exception of this initial sequence, the remaining Hoechst conjugates have been prepared from the more stable M-containing sequences to take advantage of the enhanced stability provided by the C-5 methyl group in these complexes. In order to judge the available flexibility for minor-groove binding by the tethered Hoechst ligand, we examined a series of duplexes formed from various sequences in which the extended (A-T)$_8$ minor groove binding site was disrupted by the presence of various G-C (C-G) base pairs. For example, the introduction of a terminal G-C base pair into the duplex (entry 2, Table 1) at the terminus of the duplex resulted in only a minor increase in the T_m value for the native duplex. The T_m for the corresponding Hoechst conjugate increased by nearly 16 °C. Although the Hoechst deriva-

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tives do not bind well to sites containing GC base pairs, the length of the glycol linker presumably permits the groove-binding agent to "reach" beyond the G-C base pair and bind within that portion of the minor groove containing seven A-T base pairs.

The addition of a second adjacent G-C base pair to the end of the duplex has only a moderate effect on the T_m value for the conjugated complex. Its presence both reduces the size of the A-T rich binding site, and shifts it to a position that is now two base pairs from the end of the duplex. The minimum required binding site for Hoechst 33258 is generally considered to be four A-T (T-A) base pairs, and the six A-T base pairs present in the conjugate of entry 3 provide an effective target for the tethered ligand. Replacement of positions 1 and 3 by G residues (entry 4, Table 1) also results in a conjugated complex with essentially the same T_m value as observed for the previous entries (Table 1). The minor groove binding site in this duplex is reduced to five A-T base pairs, but is still sufficient for effective binding the Hoechst dye. Placing two C-G base pairs at positions 3 and 4 of the duplex still results in a binding site of four A-T base pairs (entry 5, Table 1), but the binding site is shifted four residues from the end of the helix. The T_m value for this complex is virtually indistinguishable from those complexes containing longer runs of A-T base pairs (entries 1−4, Table 1).

By comparison, placement of two G-C base pairs into the center of the $(A\text{-}T)_8$ double-stranded sequence results in two $(A\text{-}T)_3$ binding sites. In this case, the stability of the double-stranded conjugate is reduced somewhat from those containing longer A-T rich binding sites, but a 10 °C increase in T_m suggests that the Hoechst ligand is still able to bind reasonably effectively to this target sequence (entry 7, Table 1). One of the crystal structure analyses[1] of a DNA duplex containing a bound Hoechst ligand indicates that the bis-benzimidazole binds across three of the A-T base pairs with the *N*-methylpiperazine ring located within the minor groove of the adjacent G-C base pair. In this case, the four-base pair binding site contains three A-T base pairs and one G-C base pair. A similar sequence target is present with the complex illustrated by entry 7. We have not determined whether the tethered ligand binds to the terminal or internal $(A\text{-}T)_3$ target site. However the noted crystal structure analysis indicates that the Hoechst ligand binds to a GAAA sequence (internal site) rather than a CAAA sequence (terminal site) (see entry 7). The final duplex of this series is that represented by entry 8 (Table 1) which is composed of alternating A-T and G-C base pairs. In principle the Hoechst ligand binds poorly to such a sequence, but the 3.6 °C increase in T_m for this conjugate suggests that some form of binding to duplex is present when the Hoechst ligand is conjugated to one of the strands of the duplex, and this binding provides at least a measurable increase is duplex stability.

The hexa(ethylene glycol) linker provides sufficient flexibility that the ligand can reach a binding site at least four to five base pairs from the end of the helix (entry 5, Table 1). We took the conjugate represented by entry 5 of Table 1 and reduced the length of the linker from the 18 atoms present in hexa(ethylene glycol) to only 9 atoms by using tri(ethylene glycol) as the linker. The subsequent conjugate was capable of stabilizing the duplex to the same extent as did the conjugate containing the longer linker (compare entries 5 and 6 of Table 1). That the shorter 9-atom linker permits the tethered ligand to "reach" a binding site four base pairs removed from the end of the duplex suggests that the longer linker will be effective in permitting binding to sites even further removed from the terminus of the hybridization complex.

Fluorescence Characteristics. Upon hybridization to the target sequence, the fluorescence signal from the tethered Hoechst derivative is enhanced, as the result of binding in the minor groove. The enhanced quantum yield observed for these tethered Hoechst conjugates varies somewhat with target sites, but as long as a four base pair binding site (with a minimum of three A-T base pairs) is present, the emission quantum yield is observed to increase by at least 16-fold and in some cases nearly 30-fold. These values are generally in the same range as those reported previously for the free ligand[6] and also similar to previous work in which the ligand was tethered to an internal site within the DNA duplex.[21] The strong fluorescence signals observed for all the complexes containing an appropriate minor groove binding site suggests that the tethered ligand is capable of penetrating deeply within the minor groove even when the target binding site is removed from the site of the linker by four or even five base pairs. In this respect, the tethered ligand functions well to report upon the success of the hybridization event. Recognition of the target sequence by the single-stranded conjugate results in the formation of the minor groove which triggers a ligand binding event which results in a complex with a strong fluorescence emission signal.

Six of the duplexes containing a four base pair A-T rich binding site all result in T_m values of at least 71 + 1 °C; the enhancement in quantum yield varies by as much as a factor of nearly two—from 16-fold (entry 6) to nearly thirty-fold (entry 1). These variations in fluorescence characteristics likely reflect some polymorphism in ligand binding to the sequences. With a four base pair A-T binding site and the hexa(ethylene glycol) linker, fluorescence enhancements of at least 20-fold are observed and suggest similar modes of binding. Reducing the size of the binding site from four to three A-T base pairs, or reducing the length of the linker to tri(ethylene glycol) (with the binding site removed by four base pairs) results in a moderate decrease in fluorescent signal, presumably as a result of some modulation in the nature of the ligand binding. It is noteworthy that even with the alternating A-T/G-C sequence, a 5-fold enhancement in quantum yield was measured. This signal suggests that some groove binding occurs even to sequences high in G-C content. However, the presence of the guanine N^2-amino groups likely prevents deep penetration into the groove structure, and in such locations, relaxation of the excited state by radiative mechanisms is not likely to predominate. This latter complex (entry 8) results in only a 5-fold enhancement in quantum yield.

Conclusions

Tethering a Hoechst chromophore to the terminus of a DNA sequence through a polyethylene glycol linker can be accomplished during the solid-phase based assembly of DNA sequences. The presence of the ligand enhances duplex stabilization, providing that a four base pair binding site is present (containing at least three A-T base pairs). Owing to the length of the tether, it is not necessary that this binding site be at the terminus of the hybridization complex, that is, near the site at which the linker is attached. The binding site can be at least four

to five base pairs removed from the end of the duplex and the minor groove binding ligand is still able "reach" the binding site. The use of the tethered Hoechst derivative, and the substitution of 5-methylcytosine in place of C, results in T_m values that are increased by as much as 20 °C relative to the nonconjugated C-containing duplexes. Upon binding, a dramatic enhancement in the fluorescence emission signal results, and these enhancements, as much as 30-fold over the value observed for the single-stranded conjugate, can be used to report upon the success of the hybridization event.

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