A Facile Solid-Phase Strategy for the Synthesis of Oligonucleotide–Tetraphenylporphyrin Conjugates

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A convenient solid-phase synthesis of oligonucleotides conjugated at the 3′-end with a tetraphenylporphyrin residue, by means of a new polymeric support bearing as a linker a lysine derivative, has been developed. A porphyrin linked 17-mer, designed for antisense experiments, has been prepared in good yields, and its hybridization properties with a complementary DNA fragment evaluated by UV thermal analysis.

Introduction

Modified synthetic oligodeoxyribonucleotides (ODNs) are extremely powerful tools in molecular biology and in selective inhibition of gene expression. Their potential for analytical and therapeutical applications can be strongly increased by covalently linking of reporter groups at one end of the oligonucleotide. In particular, ODNs covalently attached to metallo-porphyrin or porphyrin derivatives have been studied by several research groups with the aim of obtaining new entities for a large number of analytical or therapeutical applications,[1–3] that basically depend on the oligonucleotide sequence and on the porphyrin or metallo-porphyrin moiety. For instance, potential drugs in anticancer or antiviral chemotherapy by inhibition of gene expression can be obtained either by using the antisense approach, targeting an *m*-RNA sequence, or in the antigene strategy, targeting a double stranded DNA sequence.[4–8]

Since porphyrins and metallo-porphyrins are hydrophobic cations, they show affinity for nucleic acids. The mode of binding of porphyrins to DNA is a function of their coordination state: unliganded, porphyrins are intercalating agents,[9] whereas in the presence of metal ions that bear axial substituents, they are minor groove binders.[10] The scission chemistry of porphyrins coordinated to metal ions (e.g. Co^{3+}, Fe^{3+} and Mn^{3+}) has also been extensively studied to shed light on the molecular basis of the DNA cleavage process mediated by metallo-porphyrins, and to possibly enhance the resulting cleavage yield. Manganese porphyrins have been widely investigated in the last decade,[11–14] because of their ability to perform oxidative DNA breaks when associated with potassium monopersulfate as an oxygen atom donor. The mechanism of DNA cleavage involves an oxidative attack of DNA deoxyribose units as the initial step, followed by a ß-elimination leading to strand scission.

The first oligonucleotide-porphyrin conjugate, containing a cationic manganese(III) tetraphenylporphyrin, was designed to target the region containing the initiation codon of the *tat* gene of HIV-1.[15] Other ODN-porphyrin conjugates have been studied as analytical non-radioactive DNA probes, by using redox active metallo-porphyrins as artificial peroxidase systems, therefore developing new immunoassay techniques based on luminol oxidation,[16] or by using the fluorescent nature of the porphyrin as free base. In fact, in the absence of metal ions, porphyrins can serve as photosensitizers in the scission of DNA, presumably *via* intermediacy of singlet oxygen. This metal-ion independent reactivity has generated interest because porphyrins spontaneously accumulate in malignant cells which then become vulnerable to irradiation.[17] On the other hand, ODN-porphyrin conjugates can be used in different applications which exploit specific recognition of target sequences, luminescence detection for diagnostic purposes, and intercalating activity for therapeutical applications.

Encouraged by the high activity of metallo-porphyrins as reactive DNA cleavers, a growing interest has been put in the use and the synthesis of ODN-porphyrin conjugates. We

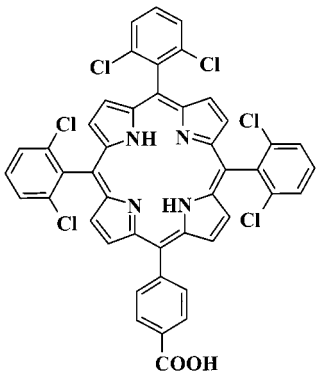

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Figure 1. Tetraphenylporphyrin (**1**)

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here describe a new and efficient solid phase strategy to covalently attach a tetraphenylporphyrin (**1**, Figure 1) at the 3′-end of ODNs. The binding affinity of a hybrid porphyrin-oligonucleotide 17-mer towards the complementary DNA fragment was also investigated by UV thermal denaturation analysis.

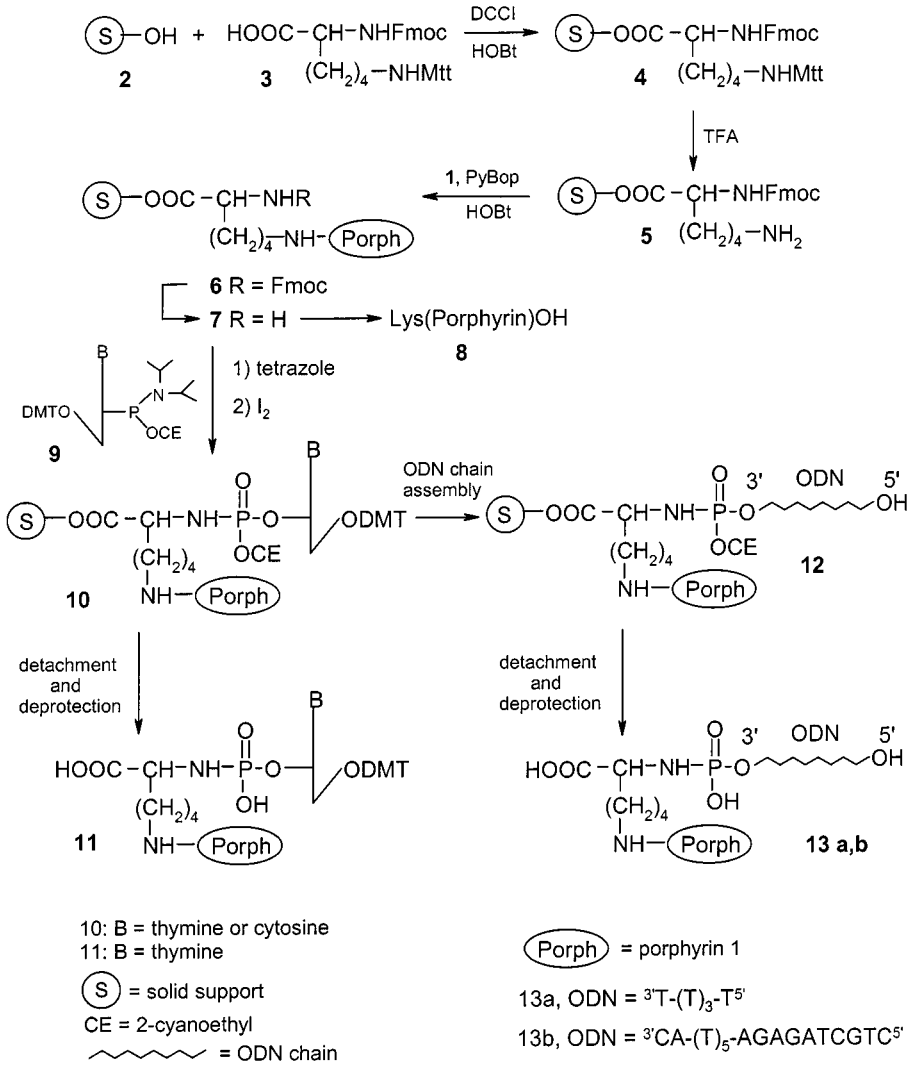
Results and Discussion

Notwithstanding the relevance of porphyrin-oligonucleotide conjugates, to the best of our knowledge, a fully automated solid phase method to covalently link synthetic porphyrins to ODNs or DNA is still lacking. All the reported ODN-porphyrin conjugates have been prepared by solution coupling of preformed ODN fragments with the porphyrin moiety bearing different organic linkers. Only recently were ODNs conjugated with expanded porphyrins (sapphyrin and texaphyrin) prepared[18–19] on a DNA synthesizer in which the phosphoramidite or H-phosphonate derivatives of the macrocycles were coupled to the appropriate growing ODN chain in place of either the first or the last nucleoside to give 3′ or 5′ conjugates, respectively.[18]

Our synthetic approach for the preparation of ODNs bearing the porphyrin ring at the 3′-end is based on the use of a new solid support (**4**, Scheme 1) linking a lysine residue having the α and ε amino functions orthogonally protected with Fmoc and 4-methyltrityl (Mtt) groups, respectively. In this way, the ε-NH_2 could be used to attach the porphyrin moiety, whereas the α-NH_2 was used to anchor the first nucleotide unit on which the ODN chain was elongated by an automated solid phase synthesis following a classical phosphoramidite protocol.

In **4**, the ester bond connecting the lysine residue to the solid support was completely stable under the reaction conditions of ε-Mtt protecting group removal and ODN chain assembly.[20] Moreover, its final cleavage could be performed in relatively mild basic media, which did not affect the amide bond linking the porphyrin to the ODN chain.

Support **4** was prepared by reaction of the hydroxy functionalized Tentagel resin **2** (a commercially available polystyrene-polyethylene glycol copolymer, 0.22 mmol/g) with Fmoc-Lys(Mtt)-OH in the presence of DCCI/HOBt in DCM/DMF. These conditions led to a typical loading of 0.15 meq/g, which was the best result obtained. The use of



Scheme 1

an alternative coupling agent, such as PyBop, or of different solvents did not result in any significant yield improvement. Support **4**, after a treatment with acetic anhydride/DMAP in DMF to cap the unreacted hydroxy functions, was left in contact with 1% TFA/5% TIS in DCM to remove the Mtt protecting group, yielding **5**. This support was then reacted with the carboxy-functionalized porphyrin **1**[21] in the presence of PyBop/HOBt in DMF/DIEA, affording support **6**. The incorporation in the solid matrix of the porphyrin residue was almost quantitative, as determined by evaluating the unreacted amino functions by quantitative Kaiser test[22] on resin **6**. After deprotection of the α-amino group by piperidine treatment (20% in DMF, v/v), obtained support **7** was treated in a standard automated procedure with the chosen 5′-*O*-DMT-2′-deoxyribonucleoside-3′-phosphoramidite **9** to give support **10** (0.12 mmol/g of nucleotide), which was treated with acetic anhydride to cap the unreacted amino functions. The incorporation of the first nucleotide unit was found in the range 90–92%, as calculated by spectroscopic measurement of the DMT cation released by acidic treatments of weighed amounts of **10**.

To test the efficiency of this strategy we first prepared 5-mer porphyrin-$^{3'}$T(T)$_3$T$^{5'}$ (**13a**) and successively 17-mer porphyrin-$^{3'}$CA-TTT-TTA-GAG-ATC-GTC$^{5'}$, complementary to a tract of the PBS region of genomic RNA of HIV-1 (**13b**). For both oligomers, coupling efficiency, checked by DMT cation quantitation, was always found to be greater than 98%.

Detachment and deprotection of the ODNs from supports **7**, **10**, and **12** were achieved by addition of hydrazine/ethanol solution (1:1, v/v). The structure of released materials **8** and **11**, after HPLC purification, was confirmed by ^{1}H NMR analysis. The crude detached ODN porphyrin conjugates were purified by HPLC. Due to the different length of the ODN chain in **13a** and **13b**, which imparted different lipophilic characters to the resulting porphyrin conjugates, different columns were used. For **13a** an RP18 column was adopted, whereas **13b** was purified on an anionic exchange column and desalted by gel filtration chromatography on a Sephadex G25 column. Crude and purified **13b** HPLC patterns are shown for comparison in Figure 2.

The ^{31}P NMR spectrum of **13a** showed two signals at δ = 0.96 and 7.53, which were attributed to four phosphodiester groups and one phosphoramidate, respectively. The ^{1}H NMR spectrum ([D$_6$]DMSO) of **13a** confirmed the expected structure in which the porphyrin protons (21 H) are in the correct ratio with respect to the thymine protons (5 imidic protons, 5 H-6 and 5 H-1′, Figure 3). Efforts to obtain a better resolution in this spectrum, including increasing temperature, were unsuccessful. This behaviour could be ascribed to an intrinsic propensity of this molecule to form aggregates.

Thermal denaturation experiments have been carried out to establish the influence of the porphyrin moiety conjugated at the 3′-end of 17-mer **13b** on the binding properties of the oligonucleotide with the complementary DNA fragment ($^{5'}$GT-AAA-AAT-CTC-TAG-CAG$^{3'}$). We found a

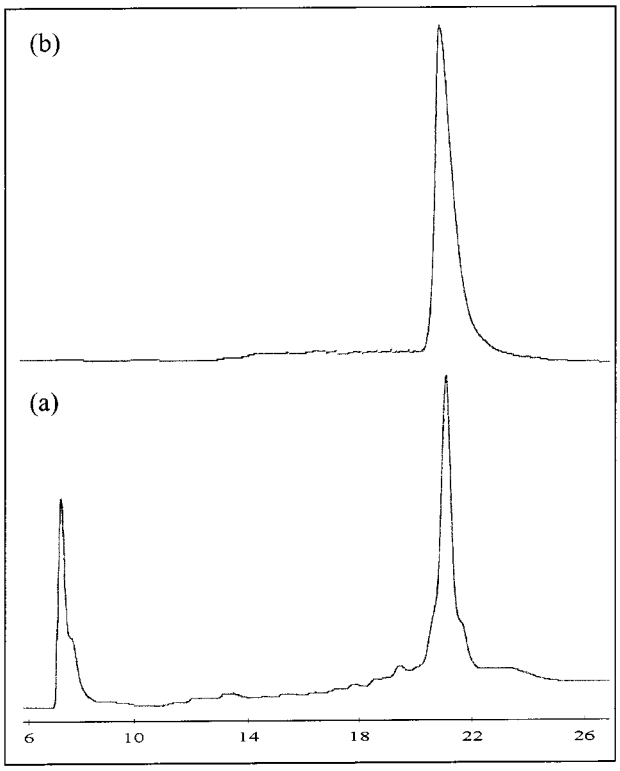


Figure 2. HPLC profiles of (a) crude **13b**; (b) purified **13b** on a Nucleogen DEAE column; buffer A: 20 mM K_2HPO_4 aq. solution, pH = 7.0, containing 20% (v/v) CH_3CN; buffer B: 1 M KCl, 20 mM K_2HPO_4 aq. solution, pH = 7.0, containing 20% (v/v) CH_3CN; elution: linear gradient from 0 to 100% B in 30 min; flow 0.07 mL/min

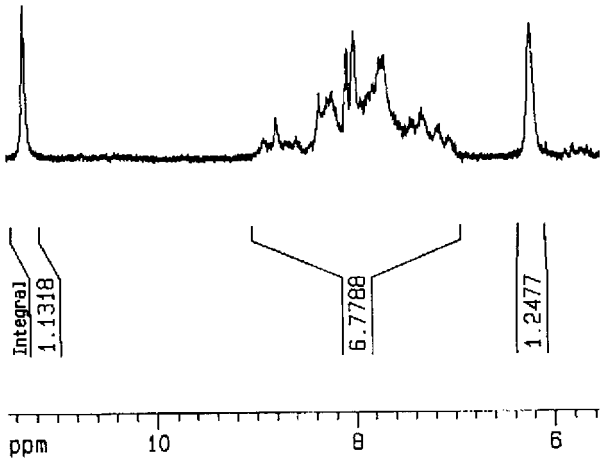


Figure 3. Low field region of the ^{1}H NMR spectrum ([D$_6$]DMSO, 400 MHz) of **13a**

dramatic destabilization (Δ*T*m = –14.6 °C) of the duplex containing the porphyrin residue (*T*m natural = 52.6 °C, *T*m duplex-3′-porphyrin = 38.0 °C, Figure 4) when compared with the corresponding non-conjugated 17-mer (Figure 4). In renaturation experiments, the absorbance *vs.* temperature profile was almost superimposable with the melting one, indicating that the process is, as expected, quasi-reversible.

The denaturation process of the porphyrin-containing duplex showed almost the same hypercromic effect as the

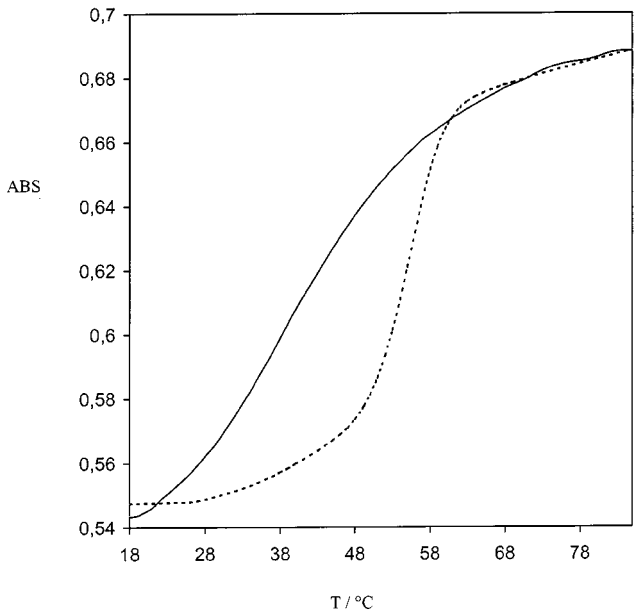


Figure 4. UV melting profiles of porphyrin-containing duplex (continuous line, *T*m = 38.0° C) and natural duplex (dotted line, *T*m = 52.6° C). 1 μM for each strand in 100 mM NaCl, 10 mM NaH_2PO_4, aq. solution at pH = 7.0

unconjugated duplex, but involved a much wider temperature range, which could be explained by a loss of cooperativity of binding due to the porphyrin residue or to the presence of a number of porphyrin induced structures melting at different temperatures.

Conclusions

A profitable solid-phase strategy for the synthesis of oligonucleotides 3′-conjugated with a porphyrin residue, a class of modified oligonucleotides not easily synthesized, whose properties have not yet been completely explored, has been developed. The proposed method uses new, valuable solid support **4** functionalized with a lysine residue orthogonally protected with Fmoc and Mtt groups on the α− and ε−amino functions, respectively. The ε-amino function was used to attach the porphyrin molecule, whereas the oligonucleotide chain was grown on the α-amino group by formation of a stable phosphoramidate bond. A 17-mer, designed for antisense experiments against HIV-1, linking the porphyrin moiety at the 3′-terminus, was prepared in good yields. Duplex formation experiments carried out by mixing porphyrin-17-mer **13b** with the complementary DNA fragment in a 1:1 ratio, showed, as evaluated by comparing *T*m values in UV thermal analysis, a notable decrease of the binding affinity towards the target oligonucleotide with respect to the unconjugated 17-mer.

As described above, matrix **4** proved to be a convenient solid support for the fully automated synthesis of ODNs 3′-conjugated with porphyrin residues, and, in our opinion, its use could in principle be easily extended to the insertion of a large number of conjugating molecules at the 3′ terminus of ODNs. Studies towards the preparation of ODNs conjugated with Mn^{III}-porphyrin **1** using this synthetic strategy are currently underway.

Experimental Section

Abbreviations: DMF = *N*,*N*′-dimethylformamide; DCM = dichloromethane; DCCI = *N*,*N*-dicyclohexylcarbodiimide; DIEA = diisopropylethylamine; DMAP = 4-dimethylaminopyridine; HOBt = *N*-hydroxybenzotriazole; PyBop = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; TFA = trifluoroacetic acid; TIS = triisopropylsilane.

Materials and Methods: NMR spectra were recorded on Bruker WM-400 and WM-250 spectrometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. The solid support functionalizations (**4**–**7**) were carried out in a short glass column (5 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock and a cap. The oligonucleotides were assembled on a Millipore Cyclone Plus DNA synthesizer, using commercially available 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite 2′-deoxyribonucleosides as building blocks. HPLC analyses and purifications were performed on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. Thermal denaturation experiments were carried out on a Cary 1E Varian spectrophotometer equipped with a Haake PG20 thermoprogrammer with detection at λ = 260 nm. Tentagel resin was purchased from Rapp Polymere, Tübingen, Germany. Fmoc-Lys(Mtt)OH was purchased from NovaBiochem. Porphyrin **1** was obtained as previously described[21] by hydrolysis of the corresponding methyl ester which was obtained by reacting pyrrole and the appropriate benzaldehyde according to the Lyndsey method.[23]

Functionalization of Tentagel Resin. – Support 4: Lysine derivative **3** (274 mg, 0.44 mmol) and 50 mg (0.24 mmol) of DCCI were dissolved in 3.0 mL of DCM/DMF (95:5, v/v). After 10 min at room temp. the resulting mixture and 60 mg (0.44 mmol) of HOBt were added to 200 mg of Tentagel resin **2** (0.044 mmol of hydroxy groups) previously washed with DMF. The mixture was kept at room temp. for 16 h under shaking. Resulting support **4** was washed with DMF and treated with a solution of acetic anhydride (80 μL, 0.85 mmol) and DMAP (50 mg, 0.41 mmol) in 1 mL of DMF for 1 h at room temp. under shaking. The support was filtered and washed with DMF and Et_2O and then dried under reduced pressure. This resulted in the incorporation of the lysine residue at 0.15 mmol/g, as estimated by spectroscopic measurements (λ = 453 nm, ε = 46250 cm^{-1} M^{-1}) of the Mtt cation released by acidic treatment (TFA, 1% in DCM) on a weighed amount of dried support **4**.

Support 6 and Product 8: Support **4** (200 mg, 0.030 mmol) was washed with DCM and then treated with 3 mL of a DCM solution of TFA (1%) and TIS (5%) for 15 min. This treatment was repeated three times, and resulting support **5** was exhaustively washed with DCM and DMF. Support **5** was then treated with a DMF solution (1.2 mL) containing porphyrin **1** (180 mg, 0.21 mmol), PyBop (109 mg, 0.21 mmol), DIEA (72 μL, 0.42 mmol) and HOBt (28 mg, 0.21 mmol), and the mixture was kept at room temp. for 24 h under shaking. Obtained support **6** was washed with DMF and DCM and then dried under reduced pressure. The incorporation of the porphyrin moiety, calculated by performing a quantitative Kaiser test[22] on the unreacted amino functions of support **6**, was found to be 0.13 mmol/g. Support **6** (50 mg) was treated with a solution

of aq. hydrazine (65%)/EtOH (1:1, v/v) at room temp. for 2 h. The filtered solution and EtOH washings were dried under reduced pressure and purified on silica gel plates, eluted with $CHCl_3$/MeOH/H_2O (14/6/1, v/v). The band at R_f = 0.33 was scratched from the plates and eluted with $CHCl_3$/MeOH/H_2O (14/14/1, v/v) to afford pure **8**. UV (CH_3OH) λ_{max} = 515 and 420 nm. ^1H NMR ([D_6]DMSO), δ = 9.10–8.10 (22 H, complex signals, porphyrin protons and N*H*CO); 3.34 (1 H, t, CH-α); 1.78 (2 H, m, CH_2-β); 1.70 (2 H, m, CH_2-δ); 1.60 (2 H, m, CH_2-γ); –2.62 (2 H, s, 2 pyrrolic NH). ε CH_2 was submerged by the residual solvent signal at δ = 2.55.

Support 10 and Product 11: Support **6** (100 mg) was washed with DMF and then left in contact with a 20% piperidine solution in DMF at room temp. for 45 min. Resulting support **7**, after separate washings with DMF, CH_2Cl_2, and CH_3CN, was dried under reduced pressure. The coupling with the first 5′-*O*-DMT-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite nucleoside building block **9** was performed with an automated DNA synthesizer by following the phosphoramidite procedure.[24] This coupling step was carried out using a solution of **9** (45 mg/mL) in CH_3CN with longer reaction times (15 min) than in the standard automated procedure. The support was washed with CH_3CN, and Et_2O, and dried under reduced pressure. The incorporation of nucleotidic material was found to be 0.12 mmol/g, as estimated by DMT cation quantitation (λ = 498 nm, ε = 71700 cm^{-1} M^{-1}) released upon acidic treatment (70% $HClO_4$/EtOH, 3:2, v/v) of a weighed amount of the dried support. Solid support **10** (50 mg) was treated with hydrazine/EtOH as indicated above for **6**, and the released material was purified by TLC (as described for **8**). The band at R_f = 0.5 afforded pure **11**: UV (CH_3OH) λ_{max} = 514, 423, 276, 242 nm. ^1H NMR ([D_6]DMSO), δ = 11.5 (1 H, broad s, imidic thymine proton); 9.91 (1 H, s, COOH); 9.30–8.10 (23 H, complex signals, porphyrin protons, N*H*CO and N*H*PO); 7.71 (1 H, s, H-6 thymine); 7.60–6.95 (13 H, complex signals, DMT protons); 6.41 (1 H, dd, H-1′); 4.95 (1 H, m, H-3′); 4.25 (1 H, m, H-4′); 3.80 (2 H, m, H_2-5′); 3.51 (6 H, s, OCH_3); 3.30 (1 H, d, CH-α); 2.50 (2 H, m, H_2-2′); 1.85, 1.75, 1.62 (2 H each, m′s, CH_2-β,δ,γ, respectively); 1.39 (3 H, s, CH_3-5 thymine); –2.65 (2 H, s, 2 pyrrolic NH); ε CH_2 was submerged beneath the residual solvent signal at δ = 2.55.

ODN Chain Assembly and Porphyrin-Conjugated Oligomers 13a,b: ODN chain assembly was performed on 50 mg (0.006 mmol) of support **10** on an automated DNA synthesizer by following the standard phosphoramidite procedure, including capping and detritylation steps, with final DMT removal. In the coupling cycles (10 min) a concentration of 40 mg/mL of nucleoside-3′-phosphoramidite building blocks was used. Two sequences were assembled: in **12a** ODN chain = $^{3'}$T-$(T)_3$-T$^{5'}$, in **12b** ODN chain = $^{3'}$CA-TTT-TTA-GAG-ATC-GTC$^{5'}$. Coupling efficiencies (by DMT test) were always greater than 98%. Oligomers **13a** and **b** were detached from the solid matrix and deprotected by hydrazine/EtOH treatment (2 h at room temp. for **13a** and 12 h for **13b**); the released material, dissolved in water, was analysed and purified. Crude **13a** was purified on a Maxi-Clean Solid Phase Extraction Cartridges column (Alltech, C18) using a linear gradient (from 0 to 100%) of CH_3OH in H_2O (flow 0.30 mL/min); the fractions eluted with H_2O/CH_3OH 1:1 (v/v) were collected and, after lyophilization, afforded 18.0 A_{260} units of **13a**,[25] with a total yield, measured by UV absorbance at 260 nm and 90 °C, of 7.5%. UV (H_2O) λ_{max} = 516, 416, 267 nm (A_{267}/A_{416} = 0.62, A_{416}/A_{516} = 8.2). ^1H NMR ([D_6]DMSO), δ = 11.32 (5 H, broad s, imidic thymine protons); 8.98–7.03 (28 H, complex signals, porphyrin, H-6 thymine and NHCO protons); 6.24 (5 H, broad s, H-1′); 4.83 (5 H, broad signal, H-3′); 4.26–3.96 (15 H, complex signals, H-4′ and H_2-5′); 3.22 (1 H, broad signal, Hα); 2.32 (12 H, complex signals, H_2-2′ and CH_2-ε); 1.83 (15 H, broad s, CH_3-5); 1.88–1.10 (6 H, complex signals, CH_2-β, CH_2-γ, CH_2-δ); –2.74 (2 H, s, exchangeable, 2 pyrrolic NH). ^{31}P NMR (CD_3OD), δ = 7.53 and 0.96.

Crude **13b** was purified by HPLC on an anion exchange column (Nucleogen DEAE 60–7 Macherey–Nagel, 125 × 4 mm, 7 μm); buffer A: 20 mM KH_2PO_4 aq. solution, pH 7.0, containing 20% (v/v) CH_3CN; buffer B 1M KCl, 20 mM KH_2PO_4 aq. solution, pH 7.0, containing 20% (v/v) CH_3CN; a linear gradient from 0 to 100% B in 30 min, flow rate 0.7 mL/min, was used; the main peak with retention time 21.0 min, accounting for more than 70% of the crude material, was collected. The isolated compound was then desalted by gel filtration on a Sephadex G25 column eluted with H_2O to afford 16.0 A_{260} units of **13b**,[25] therefore obtained in a yield of 6.6%, calculated after purification by UV absorbance at 264 nm and 90 °C. UV (H_2O) λ_{max} = 514, 422, 264 nm (A_{264}/A_{422} = 2.7, A_{422}/A_{514} = 7.5).

Thermal Denaturation Experiments: The concentration of the synthesized ODNs was determined spectrophotometrically at λ = 260 nm and at 90 °C, using the molar extinction coefficient calculated for the unstacked oligonucleotide using the following extinction coefficients: 15400 (A); 11700 (G); 7300 (C); 8800 (T) $cm^{-1}M^{-1}$.[25,26] A 100 mM NaCl, 10 mM NaH_2PO_4, aq solution at pH = 7.0 was used for the melting experiments. Melting curves were recorded using a concentration of approximately 1 μM for each strand in 1 mL of the tested solution in Teflon stoppered quartz cuvettes of 1 cm optical path length. The resulting solutions were then allowed to heat at 80 °C for 15 min, then slowly cooled and kept at 5 °C for 20 min. After thermal equilibration at 10 °C, UV absorption at λ = 260 nm was monitored as a function of the temperature, increased at a rate of 0.5 °C/min, typically in the range 15–80 °C. The melting temperatures were determined as the maxima of the first derivative of absorbance *vs.* temperature plots.

Acknowledgments

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[25] Yield calculations for purified hybrids **13a** and **13b** have been based on the absorbance values at 260 nm and at 90 °C (temperature at which stacking interactions are normally absent); in the Lambert–Beer equations, the ε values of the corresponding non-porphyrinated oligomers have been introduced, assuming that the contribution of the porphyrin residue to the total absorbance of the hybrid molecules at this wavelength and temperature is negligible. Even if the UV spectrum of porphyrin **1** in H_2O could not be directly obtained due to its extremely scarce solubility in this solvent, the above assumption is supported by spectra of **1** dissolved in other solvents, such as methanol or DMF, which all showed a close to zero absorbance in the 260-nm region.
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