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Distance Dependent Interhelical Couplings of Organic Rods Incorporated in DNA 4-Helix Bundles

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Abstract

The synthesis of a conjugated linear organic module containing terminal salicylaldehyde groups and a central activated ester, designed for conjugation to amino-modified oligonucleotides, is presented. The organic module has a phenylene-ethynylene backbone and is highly fluorescent. It is conjugated to oligonucleotides sequences and incorporated into specific locations in a well-defined DNA 4-helix bundle (4-HB). The DNA-nanostructure offers precise location control of the organic modules which allows for selective interhelical coupling reactions. In this study metal-salen formation as well as dihydrazone formation are used to covalently interlink the organic modules. Both coupling reactions are highly dependent on the distances between the organic modules in the 4-HB. Neighbouring modules dimerize easier whereas more distanced modules are less prone to react, even when the linkers are extended. The dimeric products are characterized by denaturing PAGE, HPLC and MALDI TOF mass spectrometry.

Keywords

DNA-directed chemistry; DNA-nanostructures; Organic synthesis; Hydrazone; Metal-salen

Introduction

The area of DNA-nanotechnology has evolved over the last 15 years and an increasing number of impressive 2D and 3D nanostructures formed by DNA self-assembly have been described (1–5). The majority of these self-assembled structures are made of DNA alone, however convincing results have been reported for the DNA controlled assembly of other materials (6). For example DNA-nanostructures have been used to arrange nanoparticles (7–11), proteins (12–14), and RNA (15). The use of DNA-programmed assembly of organic building blocks has also been reported (16–21), but in these cases only less complex DNA-scaffolds have been applied to template a chemical reaction.

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SUPPORTING INFORMATION AVAILABLE: HPLC chromatograms, UV-VIS spectre, MALDI-TOF MS, additional PAGE analysis and NMR spectre are available free of charge via the Internet at <http://www.pubs.acs.org>.

DNA-directed chemistry is a method that offers exclusive control of chemical reactions (22–23). By attachment of functional groups to oligodeoxynucleotides (ODN) the chemical reaction between two or more of such conjugates can be controlled by the ability of the ODNs to self-assemble. The functional groups of choice will typically spontaneously react with each other at millimolar concentrations, however at micro- to nanomolar concentration, reactions only happen when DNA-hybridization leads to the formation of a high local concentration i.e. a pseudo intramolecular reaction. Several classical chemical reactions have been applied in DNA-directed chemistry (22–28). Our interest in DNA-directed chemistry has mainly been to assemble conjugated organic rods into potentially conducting oligomers. We have reported on the direct self-assembly and coupling of ODN-rod conjugates (29–34) and recently demonstrated DNA-templated assembly of organic rods along the axis of a DNA helix (35). These methods were applied for the assembly and coupling of up to four modules, however, they could not be applied for preparing larger structures.

In our continued search for DNA structures that can serve as templates for directed assembly and coupling of organic building blocks, we have investigated interhelical couplings in a DNA 4-helix bundle (4-HB). To the best of our knowledge the only example of a DNA-directed interhelical coupling relies on a short distance amide formation (36), and the only two examples of longer distance couplings within the same helix are from Seeman et al (27) and our labs (35). Furthermore, Mao *et al.* has described a triplex controlled regioselective formation of an amide (37). Based on our previous reports on the DNA-directed assembly of organic rods into potentially conducting oligomers (38–41), we explore the incorporation of organic modules in a 4-HB as a template for organic reactions (42).

Experimental Details

General Methods

All organic solvents used in experiments were of HPLC grade purity. THF and triethylamine were distilled over sodium and CaH₂ respectively. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). Analytical thin layer chromatography (TLC) was performed using pre-coated aluminium-backed plates (Merck Kieselgel 60 F254) and visualized by ultraviolet irradiation. NMR spectra were acquired on a Varian AS 400 spectrometer, running at respectively 400, 100 and 376 MHz for ¹H, ¹³C and ¹⁹F. Chemical shifts (δ) are reported in ppm relative to solvent signals (CHCl₃ at 7.26 ppm for ¹H NMR and CDCl₃ at 77.0 ppm for ¹³C NMR). ¹³C NMR spectra were acquired on a broad band decoupled mode. High resolution mass spectra (HRMS) were recorded on a Micromass LCT spectrometer using electrospray (ES⁺) ionization techniques. Matrix assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry experiments were performed using a Bruker Daltonics Autoflex spectrometer, an AnchorChipTM target plate and one of the following matrices: Saturated dihydroxybenzoic acid (DHB) in methanol for organic compounds and a 4:1 ratio of 3-hydroxypicolinic acid (HPA) and diammoniumcitrate (DAC) 10 g/l in water for oligonucleotide containing compounds. The organic compounds were analysed using a reflective positive mode and oligonucleotide samples were analysed using linear negative mode. Water used in oligonucleotide containing experiments was triple distilled. UV-VIS absorption spectra were recorded on a Nanodrop ND 1000 spectrophotometer. HPLC analyses were accomplished on a Hewlett Packard Agilent 1100 series using a Phenomenex Clarity 3u oligo-RP (50 × 4.60 mm 3 micron) column. Oligonucleotides were purchased from DNA technology A/S, Risskov in Denmark and IDT, Coralville, USA.

Compound 4—A flame dried Schlenk flask charged with a magnet, 2-(1,3-dioxan-2-yl)-4-iodophenyl acetate (**3**) (691 mg, 2.79 mmol), Pd(PPh₃)₂Cl₂ (98 mg, 5 mol%) and CuI (26

mg, 5 mol%) was left under vacuum for 30 minutes. Upon turning to an argon atmosphere, THF (20 ml), NEt₃ (5 ml) and TMS-acetylene (820 mg, 1.16 ml, 8.36 mmol) was added and the mixture was stirred at rt over night. The crude product was concentrated by rotary evaporation and purified by flash chromatography on silica gel (1:1 pentane/CH₂Cl₂) to yield compound **2** as a clear oil (552 mg, 1.73 mmol, 62 %).

HRMS [M + Na]⁺ calc: 341.1185, found 341.1188. ¹H NMR (400 MHz, CDCl₃) δ 7.77 (s, 1H), 7.44 (d, *J* = 8.4, 1H), 7.02 (d, *J* = 8.4, 1H), 5.54 (s, 1H), 4.24 (dd, *J* = 4.8, 11.0, 2H), 3.93 (t, *J* = 11.3, 2H), 2.29 (s, 3H), 2.25 – 2.11 (m, 1H), 1.43 (d, *J* = 13.5, 1H), 0.22 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 168.8, 147.9, 133.0, 131.3, 130.5, 122.7, 121.1, 104.1, 97.3, 94.3, 67.4, 25.6, 20.8, –0.1.

Compound 5—A round-bottomed flask with compound **4** (550 mg, 2.57 mmol) in THF (5 ml) was cooled to 0°C and TBAF in THF (2.8 ml of 1 M solution) was added dropwise. After 10 minutes the mixture was poured into CH₂Cl₂ (40 ml) and the organic phase was washed twice with water (15 ml). The organic phase was dried with Na₂SO₄ (s) and evaporated to dryness by rotary evaporation of the solvent. The crude product was treated with acetyl chloride (0.5 ml) in THF (10 ml) and pyridine (1 ml) for 10 minutes at rt. The mixture was poured into CH₂Cl₂ (40 ml) and washed with NaHCO₃ (aq, sat., 15 ml) and twice with water (15 ml). The organic phase was dried with Na₂SO₄ and evaporated to dryness by rotary distillation. Compound **5** was obtained by flash chromatography on silica gel (1:1 Pentane/ CH₂Cl₂) as a light yellow crystalline compound (383 mg, 1.56 mmol, 61%).

Mp 93 – 96 °C, HRMS [M + Na]⁺ calc: 269.0790, found: 269.0786. ¹H NMR (400 MHz, CDCl₃) δ 7.80 (s, 1H), 7.47 (d, *J* = 8.2, 1H), 7.04 (d, *J* = 8.3, 1H), 5.54 (s, 1H), 4.22 (dd, *J* = 4.6, 11.2, 2H), 3.92 (t, *J* = 11.5, 2H), 3.05 (s, 1H), 2.29 (s, 3H), 2.25 – 2.11 (m, 1H), 1.40 (d, *J* = 13.5, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 168.65, 148.07, 133.15, 131.33, 130.62, 122.80, 119.86, 97.07, 82.56, 77.26, 67.27, 25.43, 20.67. Anal. Calcd for C₁₄H₁₄O₄: C, 68.28; H, 5.73; Found: C, 67.44; H 5.93. FTIR (KBr, cm^{–1}): 3271 s, 2849–2961 m, 2110 w, 1773 s, 1756 s, 1608 w, 1486 s.

Compound 1—A flame dried Schlenk flask containing a magnet, pentafluorophenyl-2,5-diiodobenzoate (**7**) (199 mg, 0.37 mmol), Pd(PPh₃)₂Cl₂ (8 mg, 2.5 mol%), CuI (2.2 mg, 2.5 mol%) and Cs₂CO₃ (240 mg, 0.74 mmol) was left under vacuum for 30 minutes. Upon turning to argon atmosphere, THF (10 ml) and compound **5** (200 mg, 0.81 mmol) was added and the mixture was stirred for 1 hour at rt. The solvent was removed by rotary distillation and the product **1** was obtained by flash chromatography on silica gel (1:50 Et₂O/CH₂Cl₂) as a white crystalline product (210 mg, 270 μmol, 73 %).

Mp 147 – 152 °C. HRMS [M + Na]⁺ calc: 799.1579, found 799.1591. ¹H NMR (400 MHz, CDCl₃) δ 8.32 (dd, *J* = 0.5, 1.6, 1H), 7.88 (d, *J* = 2.1, 1H), 7.81 (d, *J* = 2.1, 1H), 7.72 (dd, *J* = 1.7, 8.1, 1H), 7.68 (dd, *J* = 0.5, 8.1, 1H), 7.57 – 7.50 (m, 2H), 7.10 (d, *J* = 8.4, 1H), 7.07 (d, *J* = 8.4, 1H), 5.59 (s, 1H), 5.55 (s, 1H), 4.32 – 4.15 (m, 5H), 4.03 – 3.86 (m, 5H), 2.32 (s, 3H), 2.30 (s, 4H), 2.29 – 2.13 (m, 3H), 1.51 – 1.37 (m, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 168.75, 168.73, 161.04, 148.38, 148.26, 135.78, 134.53, 134.40, 133.05, 132.78, 131.17, 131.12, 130.87, 130.78, 128.21, 124.48, 123.35, 123.06, 123.03, 120.51, 120.28, 97.25, 97.17, 96.93, 91.88, 89.13, 87.64, 87.28, 67.43, 67.40, 25.58, 25.55, 20.79. ¹⁹F NMR (376 MHz, CDCl₃) δ –152.11 – –152.27 (m, 1H), –158.36 (t, *J* = 21.8, 0H), –162.61 (dt, *J* = 11.1, 22.1, 1H). Anal.: Calcd for C₄₁H₂₉F₅O₁₀: C, 63.41; H, 3.76; Found: C, 63.39; H, 3.58. FTIR (KBr, cm^{–1}): 2850–2924 m, 2849 m, 2225 w, 1762 s, 1653 w, 1521 s, 1505 s.

Compound 8—Compound 8 was synthesized according to a modified literature procedure as follows (43). Hydrazine dihydrochloride (5 g, 48 mmol), 1,6-dichlorohexane (1.47 g, 9 mmol), NaOH (4.2 g, 2.2 eq) was dissolved in ethanol (40 ml) and heated to reflux for 3 hours. The product was obtained upon extraction into EtOAc (40 ml), washing twice with water (20 ml), dried with Na₂SO₄ and evaporated to dryness by rotary evaporation of the solvent. The product was analysed with ¹H-NMR which showed identical signals as literature procedure.

General procedure for ODNs O1, O2, O3, O4, O7a, O7b, O8a and O8b—In an Eppendorf tube a solution of compound 1 in DMF (10 µl, 25 mM) and an aqueous solution of the amino-modified oligonucleotide (10 µl, 0.5 mM) were mixed. Acetonitrile (20 – 25 µl) was added to produce a clear solution and finally NEt₃ (1 µl) was added and the mixture was left for 2 hours at room temperature. The solution was treated with an aqueous solution of ammonia (50 µl, 35 % NH₃ in H₂O) for 30 minutes and then all solvents were removed on a Speed Vac. A buffer (100 µl, 10 % acetic acid adjusted with NEt₃ to pH 4) was added to the dry sample and the mixture was vortexed carefully and was left at 60 °C for 2 hours. Upon centrifugation (13000 rpm) for one minute the aqueous phase was removed carefully and injected into a RP-HPLC column. The column was eluted with TEAA (100 mM, pH 7) and an increasing percentage of acetonitrile. The pure conjugate were eluted from the column with approximately 35 % acetonitrile.

Formation of the 4-HBs—Each ODN was dissolved in water and diluted to a final concentration of 10 µM. The 9 ODN strands, needed for the 4-HB, were mixed equimolar. To the resulting solution was added MES-buffer (pH 6.5, 100 mM) and NaCl (aq, 4 M) to a final concentration of 10 mM and 400 mM, respectively. The resulting solution was annealed in a PCR tube cooling from 90°C to 25 °C over two hours and they are then ready for the coupling conditions.

General procedure for Ni-salen coupling—The coupling was achieved by addition of NiCl₂ and EDA to the 4-HBs obtaining a final concentration of 1 mM and 2 mM respectively. The mixtures were left for reaction in 4 hours at room temperature. The crude reaction was analysed by PAGE without further purification.

General procedure for dihydrazone coupling—The coupling was achieved by addition of dihydrazine (60 mM in a MES buffer (100 mM, pH 5)) to the 4-HBs to obtain a final concentration of 6 mM. The mixtures were left for reaction in 3 hours at room temperature. The crude reaction was analysed by PAGE without further purification.

Results and Discussion

The incorporation of organic modules into DNA-nanostructures requires a rational geometrical design to enable the coupling reactions. As illustrated in Figure 1, we aim for a setup where the termini of a 4-HB is functionalized with organic modules. The four DNA helices in the bundle are arranged in parallel and each helix is coupled to its neighbour with a double crossover giving the 4-HB a well-defined shape. The attachment of organic modules in specific positions allows the 4-HB to serve as a scaffold at which the organic modules are positioned. The organic modules are linked to the 4-HB via spacers that allow the modules to rotate and define the space in which the modules can move.

The organic module 1 (Scheme 1) is related to the module applied in a recent report by our laboratories (35), however, in a shorter version that fits with an interhelical coupling. It is based on a phenylene-ethynylene backbone with diprotected terminal salicylaldehydes and a central activated pentafluorophenyl ester. The latter functional group module allows for the

conjugation to amino-modified ODNs. The module has the required solubility in a polar media which is needed for the conjugation to ODNs.

The synthesis of **1**, shown in Scheme 1, is based on 5-iodosalicylaldehyde (**2**) and 2,5-diiodobenzoic acid (**6**).

The aldehyde in **2** was acetal-protected followed by acetyl-protection of the phenol moiety (30). The resulting diprotected 5-iodosalicylaldehyde (**3**) was then ethynylated in a Sonogashira coupling (44) by reaction with trimethylsilyl-acetylene to obtain compound **4**. After removal of the TMS group, **5** was obtained in a moderate overall yield. The central aryl moiety (**7**) was prepared from 2,5-diiodobenzoic acid (**6**) by activation of the acid with pentafluorophenol in the presence of dicyclohexylcarbodiimide (DCC) (35). To prevent hydrolysis or cleavage of the labile pentafluoroester (**7**), the final Sonogashira coupling between **5** and **7** was performed using anhydrous Cs_2CO_3 as a base instead of the more common use of secondary or tertiary amines. The coupling reaction gave the desired product **1** in 73 % yield.

The excitation and emission spectra of the product **1** were measured in ethanol at a concentration of 1.66×10^{-4} mg/ml (2.1×10^{-7} M). The module has two excitation peaks at 321 nm and 348 nm (Figure 2). After conjugation to an ODN and removal of the protecting groups the peak at 321 nm disappears (see supporting information, Figure S1). When the compound is excited at 348 nm an emission maximum at 412 nm is obtained. It is clear that the organic module is highly fluorescent and hence it can be used to track ODNs conjugated to the organic module **1**.

The reactive pentafluoroester in **1** serves as a handle for attachment of amino-modified ODNs. As illustrated in Scheme 2 we attached compound **1** to 8 different amino-modified ODNs by mixing the components in a mixture of 25% water, 25% DMF and 1% triethylamine in acetonitrile. The desired amides were formed within 2 hours. It is important that the ratio of water and organic solvent is well balanced since too high water content causes the organic module to precipitate, whereas too low water content makes the ODN insoluble. After conjugation the acetyl protecting groups were removed by treatment with aqueous ammonia for 30 minutes. After evaporation of all solvents the conjugate could be dissolved in a 10 % acetic acid buffer at pH 4. This treatment has a dual purpose, it removes the acetal protecting group and it causes unreacted organic modules to precipitate which makes it easy to remove by simple centrifugation.

The conjugates were purified by preparative reverse phase (RP) HPLC. The RP-HPLC chromatograms for the conjugation reaction are shown in the supporting information (Figure S1). The product is easily identified because the organic module has a unique absorption peak at 348 nm. Furthermore the relative height between the calculated extension coefficient of the ODN at 260 nm and the measured extension coefficient of the organic module at 348 nm can verify that exactly one organic module per ODN is present. For all eight conjugates the product were further characterised by MALDI-TOF mass spectroscopy (See supporting information, Figure S2).

The 4-HB applied in this study is build up by 9 DNA strands and is a modification of a 4-HB structure designed by Yan and co-workers (42). In the original layout the 4-HB contained sticky ends for wire formation however in this study the sticky ends are omitted. We have furthermore applied eight T1-C6 or T3-C6 amino-modified linkers to the organic module, which in different combinations were incorporated into the 4HB bundle. A list of all ODN sequences can be seen in Table 1. Permutations of the oligonucleotides in the 4-HB

allow for the construction of 144 possible 4-HBs of which a representative number was prepared.

We have focussed on three different designs. The first design has only 3'-modified ODNs, the second has two 3'- and two 5'-modified ODNs and the third design has alternating 5'- and 3' modified ODNs (see Figure 3).

All 5'-modified ODNs have one thymine (T) nucleotide (nt) and a C6 linker between the ODN and the organic module. If it is assumed that distance from the 4-HB to the terminal of the organic module is 2.3 nm (measured in ChemDraw 3D) the module can occupy a volume of 26 nm³ and the concentration in this volume will be 130 mM (two salicylaldehydes in 26 nm³). The 3'-modified conjugates have three Ts and one C6 linker and the distance will from the last hybridised nucleotide and to the terminal position of the organic module is estimated to be 3.5 nm. The volume is calculated to be 90 nm³ and the concentration of aldehydes 36 mM. The maximum bridging distance will then theoretically be 4.6 nm and 7.0 nm respectively. Relating this to the dimensions of the 4-HB dimension reveals that when at least one 3'-modified ODN are incorporated, it should be possible to bridge all the way from the first to the fourth helix and form a dimer. On the other hand, if we use the 5'-modified ODNs we have a large actual concentration but a smaller overlapping volume where the two organic compounds can react. Using the long spacer gives a smaller actual concentration but a larger overlapping volume (see Figure 4). However, it should be taken in consideration the calculated distances are based on the assumption that the 4-HB is rigid and DNA hybridization irreversible. This is of course not the correct picture since DNA is a soft and flexible material that can breathe. Nevertheless, the 4-HB will induce a great increase in the local concentration of the organic module, and the position of the organic modules in the 4-HB highly affects the plausibility for coupling reactions to happen.

The 4-HBs were assembled by heating the nine ODNs of choice to 90 °C followed by cooling to room temperature over two hours in a MES-buffer (pH 6.5, 10 mM) containing 400 mM NaCl. The resulting 4-HB was analysed by native polyacrylamide gel electrophoresis (PAGE). As mentioned above the organic module is highly fluorescent. This enables imaging of the gel without staining with ethidium bromide or other DNA interchelators. Only structures containing the labelled DNA strand are visible and this greatly simplifies the gel analysis. Figure 5a illustrates the stepwise assembly of a 4-HB containing **ODN O1** and Figure 5b illustrates fully assembled 4-HBs with one or more organic modules incorporated. The fully assembled 4-HBs with one or more organic modules incorporated migrate similarly since the modules constitute a very small fraction of the total molecular mass. The migration of the 4-HBs containing 189 base pairs corresponds to the migration of a linear dsDNA strand of 250 base pairs. The bulk and rigidity of the bundle retards its mobility compared to a linear DNA helix.

In this study we have used two different approaches to perform the interhelical chemical coupling reactions of the terminal salicylaldehydes of the modules. In the first approach a Ni(II)-salen complex is formed by reaction of Ni(II) chloride and ethylenediamine with two salicylaldehydes (Scheme 3) (35). In the other approach the dihydrazine **8**, synthesized according to modified literature procedure (43), reacts with the two salicylaldehydes aldehyde to form a hydrazone bridge.

In the first approach the 4-HBs containing organic modules were treated with 1 mM Ni(II) chloride (NiCl₂) and 2 mM ethylenediamine (EDA) for 4 hours. The products were analysed with denaturing PAGE. Since two oligonucleotides are covalently coupled the product of the chemical reactions migrates corresponding to the additive number of nucleotide in the

conjugates that react *i.e.* as a slower migrating band. We find that product is only observed in cases where the organic modules are incorporated at the right positions in the 4-HB and if structures have been treated with NiCl₂ and EDA. A typical denaturing 9% PAGE analysis for the reaction is shown in Figure 6. As Ni-salen complexes have been reported quench fluorescence (45–47) the gel has to be stained with ethidium bromide before imaging by UV-light irradiation. Lane 3 is a control, containing a 4-HB with four different organic modified ODNs but without coupling reagents and as expected no higher-order structures are observed. In lane 2 and 5 products arising from coupling of **ODN O2** and **ODN O3** are observed. The band corresponds well with the 86 nt's of the dimeric product. In lane 2 it is also possible to form other products, but the dimer between helix 2 and 3 seems to be favored. Lane 1 and 4 illustrate experiments where two shorter dimers containing two organic modules and 59 nt's should be formed. However this product is not observed in the denaturing PAGE gel although we do observe disappearance of starting material. We believe that the reason for this is fluorescence quenching by the Ni-salen complex (45–47). The existence of the small dimer was however observed by a number of other techniques. Accumulating the 4-HBs on filter cups before loading to the polyacrylamide gel gave a weak and smeared dimer band according to PAGE analysis. We also analyzed the reaction mixture by RP-HPLC. The HPLC fractions were analyzed with UV-VIS, PAGE analysis and MALDI-TOF mass spectrometry and the existence of the short dimeric product was confirmed (see supporting information, Figure S3). We attribute, in part, the weakness of the bands containing the Ni complexes to be caused by quenching of the ethidium bromide emission (45–47).

For the further analysis of interhelical Ni-salen couplings we used 4-HBs containing **ODN O1 – O4** (42 – 44 nt) in order to obtain dimers containing at least 84 nt's. We performed PAGE analysis of 8 different 4-HBs, treated with Ni(II) chloride and ethylenediamine, testing the possibility to form interhelical couplings between neighbouring helices, across one helix, across two helices and attempting to form trimers and tetramers. The results are shown in Figure 7. Lane 1 shows the formation of dimer product between helix 1 and 2 (**ODN O1** and **O2**). Lane 2, 3 and 4 shows attempts to form trimer and tetramers, but we only observe increasing amount of dimer. Lane 5 and 6 shows dimer bands formed between helix 2 and 3 and between helix 3 and 4. Lane 7 shows an attempt to form a dimer across one helix from helix 1 to helix 3. The linker size has only little influence on this result.

Since quenching of ethidium bromide fluorescence by the Ni-salen and other Ni complexes seems to be a serious problem for determining the yields of the experiments, alternative strategies had to be considered. Aldehydes are known to react with hydrazines to form stable hydrazones and therefore we developed an approach which is based on this. First we synthesized the dihydrazine (**8**, scheme 3) by reacting 1,6-dichlorohexane with hydrazine according to a modified literature procedure (42). The modified 4-HB was then treated with 5 mM of the dihydrazine (**8**) which allows formation of interhelical couplings. Figure 8 illustrates a representative gel, denaturing 9% PAGE, for this approach. One great advantage of this approach is that it allows imaging the gel without staining which implies that only ODNs with conjugated organic modules are observed whereas bare ODNs not are visible.

From the PAGE analysis in Figure 8, both the small dimer (with ca 59 nt) and the large dimer (with 86 nt) are observed in lanes 2, 3 and 5 – 9. A weak trimeric product is observed in lane 2. Furthermore, it can be seen that no dimer or higher ordered structures are formed in absence of the dihydrazine (lanes 1 and 4). Important information about the distance dependence can also be derived from this gel image. When the distance between organic modules is increased, the product yield decreases. We do observe a weak band for the coupling between modules attached to helix 1 and helix 3 (across one helix, lane 9).

It is notable that the interhelical couplings are highly specific and rely on the 4-HB template. Both for the Ni-salen and the dihydrazone coupling reactions were tested for unspecific couplings where modified ODNs were treated with coupling reagents without the ODNs required for the formation of 4-HB. Here PAGE-analysis (see supporting information, Figure S5) revealed that no coupling product was formed which implies that the interhelical couplings exclusively happen in the 4-HB.

To compare the Ni-salen and the hydrazone couplings we have repeated the Ni-salen experiments shown in Figure 7, using the same 4-HB and conjugates but applying the dihydrazone coupling instead (see Figure S4). It is assumed that the results obtained by the Ni-salen couplings shown in Figure 7, are better than they appear from the gel image since Ni-complexes quench the emission from ethidium bromide (45–47). The conjugates which have not participated in formation of a dimer will also react with the diamine and form a complex with nickel (48). If it is assumed that the quenching of dimer and the other salicylaldehyde containing sequences is comparable the relative intensities of the **ODN O1 – O4** and dimer bands in Figure 7 indicate the formation in up to or above 50% yields. For the results obtained for the dihydrazone coupling reactions shown in Figure S4, we assume that all aldehyde groups are converted to hydrazones and therefore potential quenching of the starting materials and the dimer is the same. The yields obtained for the dihydrazone couplings appears to be lower than the corresponding Ni-salen couplings, but the relative intensity distribution for the different setups are almost identical in the two experiments. The highest dimer yield is obtained when all four organic modules are present and the lowest yields are obtained when the organic modules are placed with larger distance in between. It was also attempted to form trimers and tetramers by placing 3 or 4 modules on 4 neighbouring helices. The results from these attempts were in general disappointing since no tetramer was identified. It was possible to form the trimer as verified by PAGE in the dihydrazine reactions, but yields were low.

Based on the results presented in Figure 6–Figure 8 a consistent picture of the dimerization reactions can be drawn. The efficiency of the coupling reactions is moderate with a maximal yield of around 50% as estimated from relative intensities in the gels. We and others have previously observed that DNA-directed metal-salen couplings can proceed in high yields when the salicylaldehyde moieties are positioned in close proximity (30,49). On the other hand we have also previously observed that increased distance, between the attachment points of the organic module at the DNA-structure, in this case 3.5 nm corresponding to one helical turn in a B-DNA helix, gives rise to significantly lower yields. In the 4-HB the length of the organic module is 1.9 nm and the length distance between neighbouring helices around 2 nm, depending on the position of the attachment point in the helix. It is clearly demonstrated that the most favourable positioning of modules for the formation of dimers is where the organic modules are placed closest to a module at a neighbouring DNA helices (between helix 2 and 3).

Conclusion

The organic module **1** has been synthesized from 5-iodosalicylaldehyde (**2**) and 2,5-diiodobenzoic acid (**6**) in six synthetic steps closely related to a previously developed compound (**35**). The module **1** was conjugated to 8 different amino-modified oligonucleotides which were applied for the formation of 4-HBs. The stability of the modified DNA-nanostructure has been proved by native PAGE analysis. It is shown for the first time, that a more complex DNA-nanostructure (4-HB) is applied to control the formation of a variety of different dimers formed by interhelical coupling of the modules. The bis(salicylaldehyde) terminated molecular rods in the 4-HBs were coupled either by Ni-salen formation or by a dihydrazone linkage, developed for this study. The products were

analysed with denaturing PAGE analysis and a single dimer has also been analysed with MALDI-TOF analysis (additional gels and MALDI-TOF spectrum are shown in supporting information). The yields of the Ni-salen dimers are highly distance dependent. Even though we have designed the modules to be able to couple across one or two helices, we did not observe these long distance coupling. Even at neighbouring helices we observe a significant higher yield when the modules are placed in close proximity. We did not see full conversion of the modified ODNs but on the other hand no non-templated coupling is observed. The dihydrazone coupling is less distance dependent and the products are much easier to characterize since the fluorescence of the organic modules can be used to image the gel bands. We accomplished to couple across one helix and to produce a trimer by this method. In future we will aim at improving the yields of interhelical couplings of organic modules and thereby enable the formation of monodisperse macromolecular networks templated by 2D DNA structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
A schematic representation of a 4-HB with four organic modules (yellow) incorporated.

Figure 2. Excitation (blue) and emission (red) spectrum of the organic module **1**. The spectra are measured with a solution of 1.66×10^{-4} mg/ml of **1** in 96% ethanol.

Figure 3.

A) 4-HB with **ODN O7b, O2, O8b** and **O4** (all 3'-modified ODNs). B) 4-HB with **ODN O7b, O2, O3** and **O8a** (two 3'- and two 5'-modified ODNs). C) 4-HB with **ODN O1, O2, O3, O4** (alternating 5'- and a 3'-modified ODNs). D) Model of the 4-HB illustrates the position of the eight different organic modified ODNs.

Figure 4.

The top of the 4-HB is shown. The yellow spheres placed at helix 1 and 4 indicate the actual volume occupied by the organic modules. The small spheres refer to the short spacer (1 T) and the large spheres refer to the long spacer (3 T). The overlap indicates the chance for coupling.

Figure 5.

A: Stepwise assembly of 4-HB. The conjugated DNA strand **ODN 01** is used as fluorescent probe and no staining is performed. PAGE (75 V, 2.5 hours, 7.5 % acrylamide). Lane 1 (**ODN 5 + ODN 01**), Lane 2 (Lane 1+ **ODN 7**), Lane 3 (Lane 2+ **ODN 9**), Lane 4 (Lane 3+ **ODN 2**), Lane 5 (Lane 4+ **ODN 3**), Lane 6 (Lane 5+ **ODN 8**), Lane 7 (Lane 6+ **ODN 6**), Lane 8 (Lane 7+ **ODN 4**). The gel is imaged by UV-irradiation. B: 4-HBs with one or more organic modules incorporated. PAGE (75 V, 2.5 hours, 7.5 % acrylamide). The gel is stained with ethidium bromide before imaging. Lane 1 (marker). Lane 2 (4-HB with **O1** and **O7a**). Lane 3 (**O1** and **O3**). Lane 4 (**O1** and **O8a**). Lane 5 (**O1**, **O7a** and **O3**). Lane 6 (**O1**, **O7a**, **O3** and **O8a**).

Figure 6.

Ethidium bromide stained 9% denaturing PAGE. The 4-HBs with the incorporated organic modules are illustrated on the top of each well. Lane 1 (4-HB with **ODN O2** and **O8b**) treated with Ni^{2+} and ethylene diamine (EDA), lane 2 (4-HB with **ODN O7b**, **O2**, **O3** and **O8a**) treated with Ni^{2+} and EDA, lane 3 (4-HB with **ODN O7b**, **O2**, **O3** and **O8b**) not treated with Ni^{2+} and EDA, lane 4 (4-HB with **ODN O7b** and **O2**) treated with Ni^{2+} and EDA, lane 5 (4-HB with **ODN O2** and **O3**) treated with Ni^{2+} and EDA. The dimer products of the Ni-salen dimer between **ODN O2** and **ODN O3** (organic dimer + 86 nt) are observed. The smaller dimer as well as trimers and tetramer are not observed.

Figure 7.

Ethidium bromide stained 9 % denaturing PAGE. The 4-HBs with the incorporated organic modules are illustrated on the top of each well. Lane 1 (4-HB with **ODN O1** and **O2**) treated with Ni²⁺ and EDA, lane 2 (4-HB with **ODN O1**, **O2** and **O3**) treated with Ni²⁺ and EDA, lane 3 (4-HB with **ODN O2**, **O3** and **O4**) treated with Ni²⁺ and EDA, lane 4 (4-HB with **ODN O1**, **O2**, **O3** and **O4**) treated with Ni²⁺ and EDA, lane 5 (4-HB with **ODN O2** and **O3**) treated with Ni²⁺ and EDA, lane 6 (4-HB with **ODN O3** and **O4**) treated with Ni²⁺ and EDA, lane 7 (4-HB with **ODN O1** and **O3**) treated with Ni²⁺ and EDA, lane 8 (4-HB with **ODN O1** and **O4**) treated with Ni²⁺ and EDA. The Ni-salen dimer products of **ODN O1**, **ODN O2**, **ODN O3** or **ODN O4** (organic dimer + 84–88 nt) are clearly observed.

Figure 8.

Unstained 9% denaturing PAGE. The 4-HBs with the incorporated organic modules are illustrated on the top of each well. Lane 1 (4-HB with **ODN O7b, O2, O8b** and **O4**) with no dihydrazine treatment, lane 2 (4-HB with **ODN O7b, O2, O8b** and **O4**) with dihydrazine, lane 3 (4-HB with **ODN O7b, O2, O3** and **O8a**) with dihydrazine, lane 4 (4-HB with **ODN O7b, O2** and **O8b**) with no dihydrazine, lane 5 (4-HB with **ODN O7b, O2** and **O8b**) with dihydrazine, lane 6 (4-HB with **ODN O7b, O2** and **O3**) with dihydrazine, lane 7 (4-HB with **ODN O3** and **O8a**) with dihydrazine, lane 8 (4-HB with **ODN O7b** and **O2**) with dihydrazine, lane 8 (4-HB with **ODN O7b** and **O3**) with dihydrazine, lane 9 (4-HB with **ODN O7b** and **O4**) with dihydrazine. We observe clear dimer bands of both 59–61 nt (dimer 1) and 86 nt (dimer 2) and in lane 2 we observe a trimer with 78 nt.

Scheme 1.

Synthesis of the organic module **1**. TMS: Trimethylsilyl, NEt_3 : triethylamine, THF: tetrahydrofuran, TBAF: tetrabutylammonium-fluoride.

Scheme 2. Attachment of organic module **1** to amino-modified ODNs followed by deprotection.

Scheme 3.

Two different approaches for interhelical couplings. Top: Aldehyde reacts with dihydrazine to form a dihydrazone. Bottom: Salicylaldehydes react with ethylenediamine and Ni^{2+} to form a Ni-salen complex.

Table 1

Sequences of all 17 different ODNs used in the study. Org: conjugated organic module, C6: C₆H₁₂ spacer.

Oligo name	Sequence
ODN 1	TGT TCG GAC TAC TAA TCG TCT GAA CGG GAC AAC ACT CCA AC
ODN 2	CGA GCC TGC TAC CTG TAA CCG AGA TCC TGA TCC CTG AGT CG
ODN 3	CTA GCG GAC CAG GGA CGG AAC GAG TAG GAC TAT GGA CAT CG
ODN 4	GAT GGG AAA TGC CTG TGT CAT ACC GTA AGA ACC CTG TCC TC
ODN 5	GTT GCG TGG ATC TCG GTT ACA CCC GTT CAG ACG ATT AGT AGT GGG ATC ACC CTG GTG GGT TCT TAC GGT ATG ACA CAC CTA CTC
ODN 6	CGA TGT GGC TCG
ODN 7	CGA CTC ACC GAA CA
ODN 8	GAG GAC ACC GCT AG
ODN 9	GTT GGA GTG TTG TGG TAG CAC CAT AGT GGC ATT TCC CAT C
ODN O1	Org-CONH-C6-T TGT TCG GAC TAC TAA TCG TCT GAA CGG GAC AAC ACT CCA AC
ODN O2	CGA GCC TGC TAC CTG TAA CCG AGA TCC TGA TCC CTG AGT CGT TT-C6-HNCO-Org
ODN O3	Org-CONH-C6-TCTA GCG GAC CAG GGA CGG AAC GAG TAG GAC TAT GGA CAT CG
ODN O4	GAT GGG AAA TGC CTG TGT CAT ACC GTA AGA ACC CTG TCC TCT TT-C6-NHCO-Org
ODN O7a	Org-CONH-C6-TCGA CTC ACC GAA CA
ODN O7b	CGA CTC ACC GAA CAT TT-C6-NHCO-Org
ODN O8a	Org-CONH-C6-TGAG GAC ACC GCT AG
ODN O8b	GAG GAC ACC GCT AGT TT-C6- NHCO-Org