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Assembly of DNA Nanostructures with Branched Tris-DNA

Takahiro Kuroda, Yoshimasa Sakurai, Yukinao Suzuki, Akiko O. Nakamura, Masayasu Kuwahara, Hiroaki Ozaki, and Hiroaki Sawai*[a]

Abstract: Branched tris-DNA, in which two oligonucleotides of the same sequence and one other oligonucleotide of a different sequence are connected with a rigid central linker, was prepared chemically by using a DNA synthesizer. Two branched tris-DNA molecules with complementary DNA sequences form dimer and tetramer as well as linear and spherical oligomer complexes. The complex formation was studied by UV/thermal denaturation, enzyme digestion, gel electrophoresis, and AFM imaging.

Keywords: branched tris-DNA · DNA assembly · nanostructures · oligomerization · oligonucleotides

Introduction

DNA or DNA-like molecules provide suitable building blocks for the construction of special geometric DNA objects, as the base sequences of DNA associate through special complementary base pairing with high specificity.[1–3] Thus, assembly of special-sequence DNA units result in special supramolecular nanostructures that include cubes,[4] octahedrons,[5,6] cages,[7] and two-dimensional arrays.[8] Since the pioneering work by Seeman and co-workers,[1–5] combinations of several single-stranded DNA units with unique sequences have been used as building blocks for DNA-based nanostructures, which may be applied in molecular devices.[8–14]

In another strategy for the formation of DNA nanostructures by the assembly of DNA units, linear and branched DNA units, in which two, three, or four oligonucleotides are connected to a central linker molecule, were recently devised by several groups. Four example, bi- or trifunctional organic molecules have been used as branched linker molecules for the programed assembly of DNA nanostructures.[15–17] Alternatively, metal complexes of Ru^{II},[18,19] Fe^{II},[20] and Ni^{II}[21] have been employed as central linker molecules for the assembly of DNA-based supermolecules. The combination of a Mn^{II} complex with a linear or branched DNA unit was also reported for the formation of DNA-based superstructures.[22] Shi and Bergstrom devised a V-shaped molecule in which the 3′-ends of two complementary oligonucleotides are attached to a bifunctional linker.[15] The V-shaped DNA molecule forms several nanosized macrocycles by self-assembly. Von Kiedrowski and co-workers reported a tris-DNA module in which three oligonucleotides of the same sequence are connected to a trifunctional linker at the 3′-end.[16] The two complementary trifunctional DNA units associate with each other to form dimeric nanoacetylene and tetrameric nanocyclobutadiene structures. The assembly of the DNA modules was analyzed by gel electrophoresis. Shchepinov et al. reported a UV/thermal denaturation study of complex formation from complementary DNA dendrimers.[23] Although they estimated the structure of the DNA supramolecules from gel electrophoresis or UV melting studies, further experiments are needed to obtain more information on the structure of such DNA association complexes. We describe herein the synthesis of a branched tris-DNA module in which two identical and one different oligonucleotide are connected by a rigid linker molecule at the 5′-ends. We studied the association of the two complementary tris-DNA modules by AFM, gel electrophoresis, and UV melting studies to gain more detailed insight into the assembled structure.

[a] T. Kuroda, Y. Sakurai, Y. Suzuki, A. O. Nakamura, Dr. M. Kuwahara, Prof. H. Ozaki, Prof. H. Sawai
Department of Applied Chemistry
Faculty of Engineering, Gunma University
Kiryu, Gunma 376-8515 (Japan)
Fax: (+81) 277-30-1224
E-mail: sawai@chem.gunma-u.ac.jp

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Results and Discussion

For the key building block of the branched tris-DNA, we designed a branched linker molecule with a central 1,3,5-trisubstituted benzene molecule connected to rigid phenylacetylene and benzene linkers to keep the three oligonucleotide units apart (Figure 1). Thus, 1,3,5-tribromobenzene was treated with trimethylsilylacetylene and palladium(II) complex/copper(I) iodide catalyst, followed by alkaline hydrolysis of the trimethylsilyl group to give 1,3,5-triethynylbenzene (**1**). Compound **1** was then treated with ethyl 4-iodobenzoate in the presence of palladium(II) complex/copper(I) iodide catalyst to yield the 1,3,5-trisubstituted benzene **2** with terminal ester groups, which were hydrolyzed by lithium hydroxide to form the corresponding tricarboxyl-substituted benzene derivative **3**. Treatment of **3** with thionyl chloride followed by reaction with ethanolamine gave **4**, which bears three terminal hydroxy groups and a rigid linker. Compound **4** was treated with dimethoxytrityl chloride (DMTrCl; 2.1 mol equiv) to form mono-, bis-, and tris-DMTr-substituted compounds along with the unsubstituted one. Bis-DMTr-substituted benzene derivative **5** was obtained in 34% yield after silica-gel chromatography. Phosphitylation of **5** with 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosphoramidite gave the amidite reagent **6**, which was used to synthesize the branched tris-DNA by solid-phase phosphoramidite chemistry with a conventional DNA synthesizer (see Experimental Section). Denatured polyacrylamide (8%) gel electrophoresis (PAGE) analysis of the branched tris-DNA showed the formation of the target tris-DNA A along with two main oligonucleotide byproducts A′ and A″ (Figure 2). The latter is likely to be truncated at the branched part, whereas the former is a bis-oligonucleotide in which extension of one oligonucleotide (ODN) unit from the branched linker was unsuccessful. The resulting tris-DNA A was purified by disc-preparative gel electrophoresis. From half of the crude mixture, we obtained 9.6 ODU_{260} of A. The structure of A was confirmed by enzyme digestion with nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase, followed by HPLC analysis. The nucleoside composition A/G/C/T, analyzed by HPLC, was 12.7:17.6:18.8:23.1, which is nearly consistent with the desired composition of the A. The structure of A was further confirmed by MALDI-TOF MS. The branched tris-DNA B was synthesized and purified by the same method, and 23 ODU_{260} of B was obtained after preparative PAGE. The sequences of A and B are shown in Figure 2. A has one ODN-1 and two ODN-2 strands, and B has one ODN-1′ and

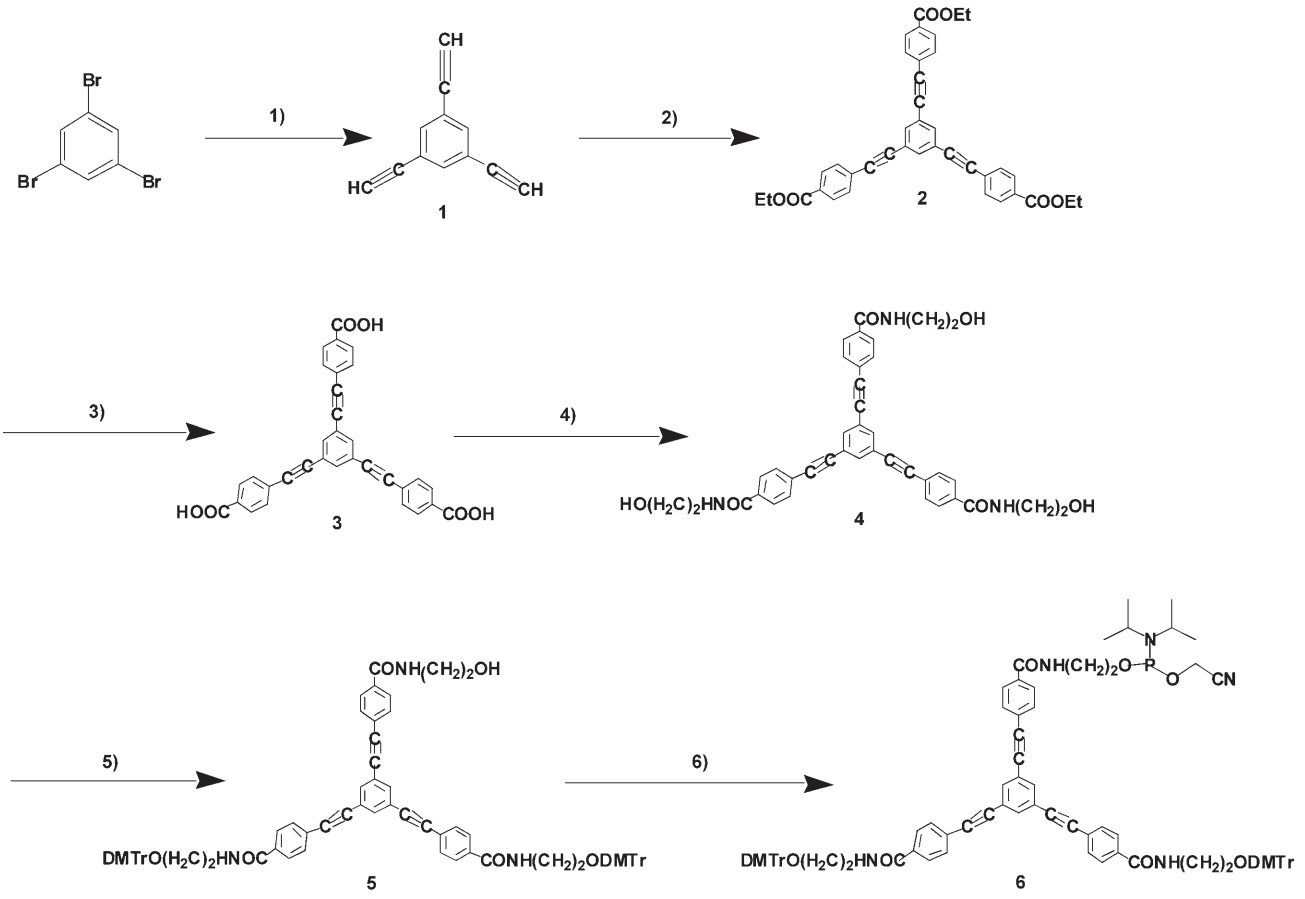

Figure 1. Synthesis of branched phosphoramidite **6**. 1) $HC{\equiv}C{-}Si(CH_3)_3/[Pd(PPh_3)_2Cl_2]$, CuI, Et_2NH. 2) NaOH (aq)/EtOH. 3) Ethyl 4-iodobenzoate/$[Pd(PPh_3)_2Cl_2]$, CuI, Et_2NH. 4) LiOH (aq)/THF. 5) a) $SOCl_2$; b) $H_2N(CH_2)_2OH/CH_2Cl_2$; c) DMTrCl/DMAP, pyridine, TEA. 6) $(iPr)_2NP(Cl)OCH_2CH_2CN$/DIPEA, CH_2Cl_2. DIPEA = diisopropylethylamine, DMAP = 4-dimethylaminopyridine, TEA = triethylamine.

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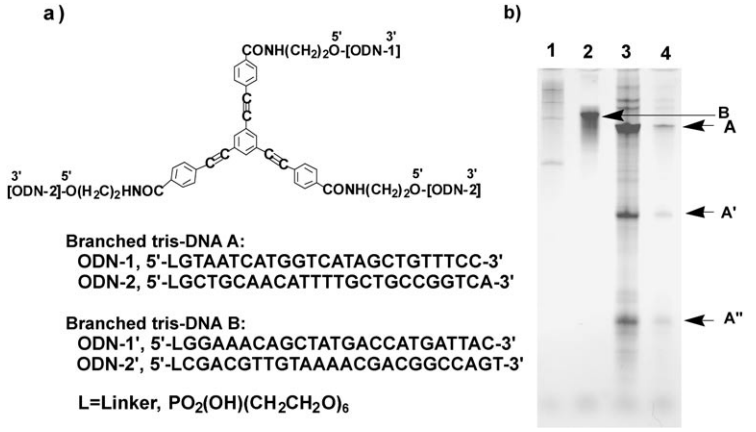

Figure 2. a) Structure of tris-DNA A and B. b) Results of PAGE of A and B. Lane 1: size-marker DNA (100–400 bp); lane 2: purified B; lane 3: unpurified A (large amount); lane 4: unpurified A (small amount). A′ and A″ are the two truncated byproducts of the synthesis of A.

two ODN-2′ strands. The sequence of ODN-1 is complementary to that of ODN-1′, and that of ODN-2 is complementary to that of ODN-2′. Thus, A and B are complementary to one another.

Figure 3 shows a UV thermal denaturation curve for a combination of A and B, along with five different combinations of component ODNs under the same conditions: A and ODN-1′, A and ODN-2′, A, ODN-1′, and ODN-2′,

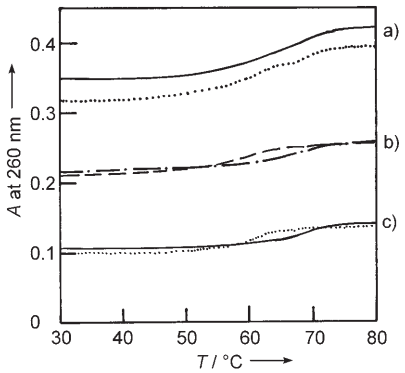

Figure 3. Thermal denaturation curves of the complexes between tris-DNA A and B and their component oligonucleotides. UV T_m measurements were carried out for the complex between the complementary oligonucleotides (0.25 μM each) in phosphate buffer (10 mM, pH 7.0) containing NaCl (0.1 M) at 260 nm with a rate of temperature increase of 0.2 °C min^{-1}. a) ——= A and B, •••••= A and ODN-1′ (0.25 μM) + ODN-2′ (0.5 μM). b) – – – = A and ODN-1′, –•–•– = A and ODN-2′. c) •••••= ODN-1 and ODN-1′, ——= ODN-2 and ODN-2′.

ODN-1 and ODN 1′, and ODN-2 and ODN-2′. The UV melting temperatures (T_m) and hypochromicities of A, B, and the component ODNs are listed in Table 1. A and B could only hybridize with one-armed components ODN-1 and ODN-1′ or ODN-2 and ODN-2′. However, this possibility was excluded by enzyme digestion of the complex from A and B with mung bean nuclease (see below), which cleaves single-stranded but not double-stranded DNA. The enzyme digestion shows that the complex is resistant to the enzyme (Figure 4d) and has no single-stranded region. The results show that A and B hybridize simultaneously with three-armed component ODN units. Shchepinov et al. reported that complementary branched oligonucleotide dendrimers show an increase in T_m values relative to the component complementary linear oligonucleotides.[23] Their branched oligonucleotide dendrimers have a flexible linker as a branching unit. The hypochromicity of the complex formed from A and B was smaller than those from the corresponding component ODNs. The cooperativity of duplex formation could be affected by the formation of a special structure from the complementary branched tris-DNA molecules. Our study indicates that the hybridization ability between the branched tris-DNA molecules with a rigid linker is likely to be reduced relative to that of the component oligonucleotides by the steric hindrance of the central linker.

Solutions of equimolar amounts of A and B were denatured at 95 °C and then cooled slowly to room temperature. The formation of the complex between A and B was monitored by agarose gel electrophoresis. The mobility data of the gel indicates formation of the dimer and tetramer along with small amounts of higher complexes up to the decamer (Figure 4b). The branched tris-DNA molecules and their complex showed lower mobility relative to linear DNA of similar chain length because the branched DNA has a large linker molecule and cannot form a compact structure. At lower tris-DNA concentrations, formation of higher complexes was decreased relative to the dimer and tetramer (Figure 4b, lanes 4–8). When the mixture of A and B was

Table 1. UV melting temperatures and hypochromicities of A, B, and the component ODNs.[a]

DNA	T [°C]	Hypochromicity [%]	DNA	T [°C]	Hypochromicity [%]
A + B	64.4	15	ODN-1 + ODN-1′	61.7	26
A + ODN-1′ + 2 × ODN-2′	60.9, 68.0	19	ODN-2 + ODN-2′	68.0	23
A + ODN-1′	59.4	19	A		
A + ODN-2′	68.5	16	B		

[a] 0.25 μM of each DNA compound was used for the UV melting study for both A and B.

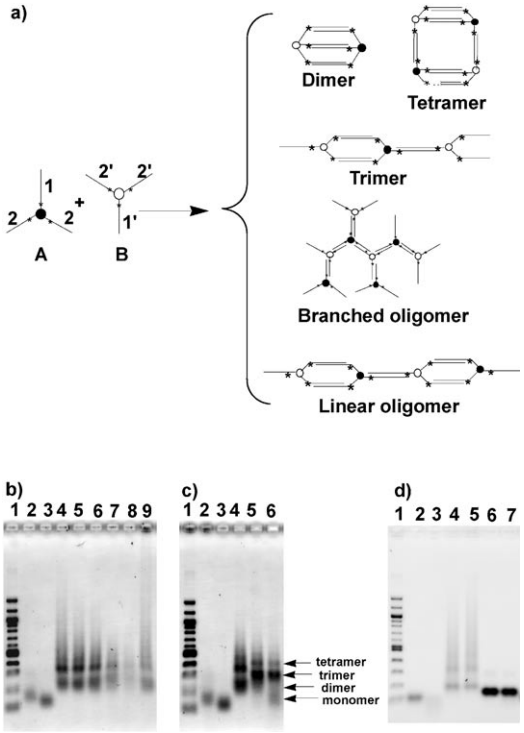

Figure 4. The association of branched tris-DNA A and B studied by agarose gel electrophoresis. a) Possible structure of complexes from A and B. b) Association complex from A and B (1:1) after annealing in NaCl (100 mM) and phosphate buffer (10 mM, pH 7.0) with slow cooling. Lane 1: DNA marker (100–1500 bp); lane 2: B (0.5 μM); lane 3: A; lane 4: A and B (2 μM); lane 5: A and B (1.0 μM); lane 6: A and B (0.5 μM); lane 7: A and B (0.25 μM); lane 8: A and B (0.1 μM); lane 9: A and B (0.5 μM) with quick cooling. c) Complex from A and B in different molar ratios after annealing in NaCl (100 mM) and phosphate buffer (10 mM, pH 7.0) with slow cooling. Lane 4: A (0.5 μM) and B (0.5 μM); lane 5: A (0.33 μM) and B (0.67 μM); lane 7: A (0.2 μM) and B (0.8 μM). d) Complex from A and B before and after digestion with mung bean nuclease. Lanes 2, 4, and 6: before enzyme digestion; lanes 3, 5, and 7: after enzyme digestion; lanes 2 and 3: A (0.5 μM); lanes 4 and 5: A and B (1.0 μM); lanes 6 and 7: A (1.0 μM), ODN-1′ (1.0 μM) and ODN-2′ (2.0 μM).

cooled quickly, formation of higher oligomer complexes was increased, probably because hybridization between A and B took place randomly (Figure 4b, lane 9). The dimer and oligomeric complexes may contain one, two, or three double-stranded oligonucleotide regions. We conducted enzyme digestion of the complex with mung bean nuclease to characterize the structure of the complexes (Figure 4d). Mung bean nuclease cleaves single-stranded regions of DNA, and thus degrades A completely (Figure 4d, lane 3). On the other hand, the complex between A and B was found to be completely resistant to the enzyme (Figure 4d, lane 5). The complex formed from A and its branch components ODN-1′ (1 mol equiv) and ODN-2′ (2 mol equiv) also showed resistance to the enzyme (Figure 4d, lane 7). The fact that the complex formed between A and B is resistant to this enzyme indicates that the complex has three double-stranded oligonucleotide regions. Similar bimolecular association was also observed in the case of the self-assembly of tris-oligonucleotides.[16] When A and B are mixed in a 1:2 or 1:4 molar ratio to form the complex, the band ascribed to the trimer is observed in substantial amounts (Figure 4c, lanes 5 and 6). A possible structure of the trimer is shown in Figure 4a.

We conducted an AFM study to characterize the structure of the complex formed from equimolar amounts of A and B. The concentration of the tris-DNA molecules was decreased to 0.1 μM in the AFM study. The measuring space was 1×1 or $0.5\times0.5\ \mu m^2$. Figure 5 shows a typical AFM image of the complexes observed at one portion. There are many spots approximately 10 nm in size, three of which are shown at the tip of the arrow on the upper left. These are images of the dimer or tetramer complexes. The length of a double strand of DNA with chain length of 24 mer is estimated to be 8–9 nm, because one base-pair unit is 0.34 nm long. The tris-DNA unit has three 24-mer DNA arms and long linkers. If A and B, each with three 24-mer DNA arms, hybridize together to form the dimer shown in Figure 5, it will be an ovallike structure approximately 10 nm long. Besides the dimer images, some linear and spherical images are also observed, as shown at the tip of the thin arrow and in the circle, respectively. The linear complexes are composed of four to eight branched DNA units, as estimated from the length of the images. The spherical complexes are also likely to be composed of four to eight units (Figure 5). The AFM image of the other observation point also displays the presence of dimeric, linear, and spherical oligomeric complexes (Supporting Information). The sizes of the complexes in the AFM images are roughly consistent with those observed with gel electrophoresis. This study indicates that the branched tris-DNA unit could serve as a module for the assembly of various types of highly ordered structures, although our attempt at forming a single discrete DNA nano-

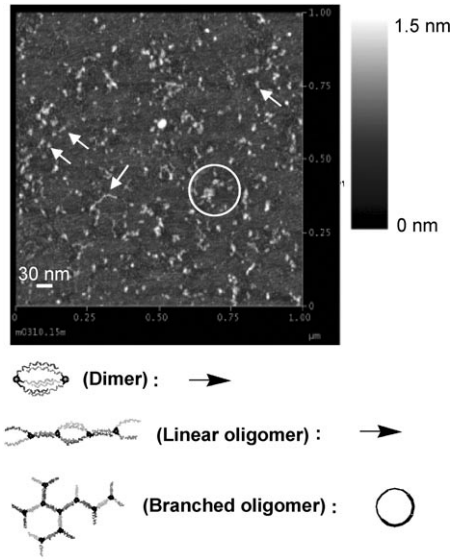

Figure 5. AFM image of the association complex from tris-DNA A and B and the assigned structure.

structure by hybridization of the branched DNA was unsuccessful. The branched DNA units of this type could form dimer, trimer, tetramer, linear, spherical, and dendrimer complexes with various DNA units by hybridization. Further studies on the type of linker molecule and hybridization procedure will be required to provide information to allow us to form a single, special complex structure among the many possible structures.

Conclusions

We have prepared a branched tris-DNA molecule in which two different sequences of oligonucleotides are linked in a 1:2 ratio by a rigid and long linker molecule. Two complementary branched tris-DNA molecules can hybridize to form primarily a dimer complex in addition to tetramer, linear, and spherical oligomeric complexes.

Experimental Section

Materials

The phosphoramidite reagents for DNA synthesis 5′-DMT-deoxynucleoside, 3′-β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite, 3′-DMT-deoxynucleoside, 5′-β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite, and DMT-hexaethyloxyglycol- β-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite (DMT = 4,4′-dimethoxytrityl) were purchased from ChemGenes Co. Oligonucleotides 5′-GTAATCATGGTCATAGCTGTTTCC-3′ (ODN-1), 5′-GCTGCAACATTTTGCTGCCGGTCA-3′ (ODN-2), 5′-GGAAACAGCTATGACCATGATTAC-3′ (ODN-1′), and 5′-CGACGTTGTAAAACGACGGCCAGT-3′ (ODN-2′) were obtained from Hokkaido System Science Co. All other chemicals were reagent-grade and were used without further purification.

Analytical Methods

^{1}H and ^{31}P NMR spectra were obtained with a JEOL α-500 or a JEOL AL-300 spectrometer. Tetramethylsilane (TMS) and phosphoric acid (85%) were used as the internal standards for ^{1}H and ^{31}P NMR spectroscopy, respectively. UV/Vis spectra were obtained with a Shimadzu 1200 spectrometer. ESI mass spectra were recorded on a PE-Sciex API-100 mass spectrometer. MALDI-TOF mass spectra were recorded on a Shimadzu AXIMA-CFR instrument with 3-hydroxypicolinic acid/ammonium citrate/ethanol as matrix. The UV/thermal denaturation behavior of the association complex of the DNA was recorded with a Shimadzu UV-2550 instrument with TMSPC-8 from 30 to 80 °C at a rate of 0.2 °C min^{-1}. The T_m value of the association complex was estimated from the first differential of the thermal denaturation curve. The AFM image was taken at the Research Institute for Biomolecular Studies (Tsukuba, Japan) with a Digital Instruments NanoScope IIIa. HPLC was carried out with a Wakosil-ODS/silica-gel column (4 mm × 250 mm) and a linear-gradient elution of acetonitrile in triethylammonium acetate (50 mM) at a flow rate of 1.0 mL min^{-1}. Gel electrophoresis of the association complex from the branched-DNA units was carried out with agarose (2%) at 50 V for 1.5 or 2 h, and visualization was obtained by staining with ethidium bromide.

Syntheses

1: Trimethylsilyl acetylene (13.0 mL, 94.0 mmol) was added to a solution of 1,3,5-tribromobenzene (8.0 g, 25.4 mmol), cuprous iodide (0.09 g, 0.45 mmol), and dichlorobis(triphenylphosphine)palladium(II) (0.338 g, 0.47 mmol) in diethylamine (230 mL) with stirring, and the reaction mixture was stirred at 50 °C for 12 h. The mixture was filtered through a glass filter, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in diethyl ether and washed with water. The organic layer was dried with anhydrous magnesium sulfate, evaporated, and subjected to chromatography on silica gel with ethyl acetate/hexane as eluent. Tris(trimethylsilylethynyl)benzene was obtained as a white solid (8.3 g, 89%). Aqueous NaOH (1 N, 100 mL) was then added to a solution of the tris(trimethylsilylethynyl)benzene (8.3 g, 22.7 mmol) in ethanol (80 mL), and the reaction mixture was heated with stirring at 40 °C for 12 h. The solvent was evaporated, and the residue was extracted with diethyl ether. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness to yield **1** (3.34 g, 98%). ^{1}H NMR ($CDCl_3$): $\delta = 7.58$ (s, 3H), 3.10 ppm (s, 3H).

4: 1,3,5-Triethynylbenzene (3.19 g, 21 mmol) was added to a solution of ethyl 4-iodobenzoate (14.3 mL, 86 mmol), cuprous iodide (0.06 g, 0.30 mmol), and dichlorobis(triphenylphosphine)palladium(II) (0.31 g, 0.44 mmol) in diethylamine (230 mL) with stirring. The reaction mixture was stirred for 24 h and then evaporated. The residue was dissolved in dichloromethane, washed with water, and the organic layer was dried with anhydrous magnesium sulfate. The solvent was removed by evaporation, and the residue was subjected to chromatography on silica gel with ethyl acetate/dichloromethane as eluent to yield **2** (11.4 g, 93%). A solution of LiOH–H_2O (5.14 g, 122 mmol) in 50 mL water was added to a solution of **2** (7.3 g, 12.2 mmol) in THF (150 mL) containing water (15 mL), and the reaction mixture was stirred for 12 h at room temperature. THF was removed from the reaction mixture by evaporation, and the pH of the aqueous solution was adjusted to pH 1.0 by addition of HCl (4.0 M). The resulting white precipitate was collected by filtration, washed with ethanol, and dried in vacuo to give **3** (5.4 g, 86%). Thionyl chloride (11.4 mL, 0.16 mol) was then added to a solution of **3** (5.3 g, 10.5 mmol) in dry dichloromethane (200 mL), and the mixture was heated under reflux with stirring overnight. The reaction mixture was evaporated, and the residue was dissolved in dichloromethane (170 mL), followed by the addition of ethanolamine (8.3 mL, 0.14 mol). The solution was stirred overnight at room temperature and then evaporated; the resulting residue was dissolved in a small quantity of methanol, which was then added dropwise to distilled water with stirring. The resulting white precipitate was collected by centrifuge, washed with water, and dried in vacuo to give **4** (3.4 g, 51%). ^{1}H NMR ([D_6]DMSO): $\delta = 8.57$ (t, $J = 5.1$ Hz, 3H, NH), 7.82 (d, $J = 8.1$ Hz, 6H, ArH), 7.73 (s, 3H, ArH), 7.62 (d, $J = 8.1$ Hz, 6H, ArH), 3.52 (t, $J = 5.1$ Hz, 6H, CH_2), 3.32 ppm (t, 6H, CH_2); MS (ESI) (positive mode): m/z calcd: 640.7 [M+H], 662.7 [M+Na]$^+$; found: 640.4, 662.4.

6: 4-Dimethylamino pyridine (0.05 g), dry pyridine (6 mL), and dry triethylamine (1.4 mL) were added to **4** (0.8 g, 1.25 mmol), which was then co-evaporated with pyridine three times to remove trace amounts of water. A solution of DMTrCl (0.89 g, 2.63 mmol) in dry pyridine (3 mL) was added to the above solution and kept overnight, both with stirring. The reaction mixture was treated with saturated sodium hydrogen carbonate solution and extracted with dichloromethane. The organic layer was dried with anhydrous magnesium sulfate and evaporated, and the residue was subjected to chromatography on silica gel. The appropriate fractions were collected and evaporated to dryness. The residue was dissolved in a small quantity of dichloromethane, and the solution was poured into hexane with stirring to precipitate **5** as a white powder (0.34 g, 33%). Compound **5** (0.385 g, 0.31 mmol) was then co-evaporated with pyridine three times and dissolved in dichloromethane (5 mL). After the addition of diisopropylethylamine (0.135 mL, 0.78 mmol) to the solution, 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosphoramidite (152 mL, 0.68 mmol) was added dropwise, and the mixture was stirred for 1 h at room temperature. Dry methanol (0.2 mL) was added to the reaction mixture, and the solution was poured into ethyl acetate (50 mL) and washed with saturated sodium hydrogen carbonate solution. The organic layer was dried with anhydrous sodium sulfate and evaporated. The residue was subjected to chromatography on silica gel with ethyl acetate/dichloromethane as eluent. The appropriate fractions were collected and evaporated to dryness. The residue was dissolved in a small quantity of dichloromethane and poured into hexane with stirring to precipitate **6** as a white powder (0.32 g, 72%). ^{1}H NMR ($CDCl_3$): $\delta = 7.72$ (d, $J = 5.1$ Hz, 6H, ArH), 7.70 (s, 3 h, ArH) 7.60 (d, $J = 5.1$ Hz, 6H, ArH), 7.43 (d, $J = 4.2$ Hz, 4H, ArH), 7.32 (d, $J = 5.4$ Hz, 8H, ArH), 7.28 (t, $J = 4.2$ Hz, 4H, ArH), 7.22 (d, $J = 4.2$ Hz, 2H, ArH), 3.78 (s, 12H, OCH_3), 3.71 (t, 2H, OCH_2), 3.65 (t, $J = 3.3$ Hz, 6H, OCH_2), 3.38 (t, $J = 3.3$ Hz, 6H, CH_2N),

2.63 (t, 2H, CH_2CN), 1.20 (dd, 12H, CH_3), 0.90 ppm (m, 2H, CH); ^{31}P NMR ($CDCl_3$): $\delta = 149.2$ ppm (s).

Branched tris-DNA: Branched tris-DNA was synthesized by solid-phase phosphoroamidite chemistry with an ABI-392 DNA synthesizer on the 1-μmol scale. The synthesis of A was carried out in the direction 3′→5′ with 3′-phosphoramidite reagents, followed by hexaethyloxylglycol spacer amidite, then **6**. The time and number of couplings of **6** was extended to 360 s and two, respectively, to improve the coupling yield. Subsequently, removal of the two DMTr groups from **6** was also performed twice to increase its efficiency. The hexaethyloxyglycol spacer amidite was then introduced, and the two identical oligonucleotides were extended in the direction 5′→3′ with 5′-phosporamidite. The efficiency for the introduction of **6** was 60%, and that for the introduction of hexaethyloxylglycol spacer after **6**was 72%, as estimated from a trityl coloring assay. On the other hand, the mean coupling efficiencies of DNA elongation before and after **6** was introduced were 99.2 and 96.1%, respectively. After the extension and removal of DMTr by acid treatment, removal of the protecting groups and cleavage from the solid support were carried out by the usual method with aqueous ammonia (25%) at 55°C for 12 h. The formation of the branched tris-DNA was checked by PAGE followed by staining with ethidium bromide. The resulting oligonucleotide was purified by disc-preparative gel electrophoresis by using polyacrylamide (12%) with urea (7.0 M), and its structure was confirmed by enzyme digestion and MALDI-TOF MS. A: MS (MALDI-TOF): *m*/*z* calcd: 23829 [*M*−1]; found: 23822 (23000–24800). B: MS (MALDI-TOF): *m*/*z* calcd: 23999 [*M*−1]; found: 24369 (22000–27000).

Enzyme digestion of the branched-DNA was carried out in a solution (25 μL) containing the DNA (0.5 ODU_{260}), snake venom phosphodiesterase (0.25 units), alkaline phosphatase (1 unit) in $MgCl_2$ (10 mM), and Tris/HCl buffer (50 mM, pH 8.0) at 37°C overnight, followed by digestion with nuclease P1 (5 ng) in sodium acetate buffer (100 mM, pH 4.75) at 37°C for 3 h. The resulting solution was heated at 95°C for 1 min to deactivate the enzyme and analyzed by HPLC.

Complex formation between A and B: UV T_m measurements were carried out for the complex between A and B (0.25 mM each) in phosphate buffer (10 mM, pH 7.0) containing NaCl (0.1 M) at 260 nm with a rate of temperature increase of 0.2°C min^{-1}. UV/thermal denaturation of A and the component complementary oligonucleotide units was also measured under the same conditions.

Agarose gel electrophoresis was carried out for the complex formed from A and B as described below. A mixture of equimolar amounts of A and B (both 2.0, 1.0, 0.5, 0.25 and 0.1 μM) in NaCl (100 mM) and phosphate buffer (10 mM, pH 7.0) was heated at 95°C on a hot-water bath and then cooled. To investigate the effects of the cooling rate, a mixture of A and B (0.5 mM) was heated at 95°C and then cooled quickly on an ice bath. Complex formation between A and B in 0.5 and 0.5 μM, 0.33 and 0.67 μM, and 0.2 and 0.8 μM proportions, respectively, occurred under the same conditions described above. The complex from A (1.0 μM) and B (1.0 μM) was subjected to digestion with mung bean nuclease, as were A only and the complex from A (1.0 μM), ODN-1′ (1.0 μM), and ODN-2′ (2.0 μM). Mung bean nuclease digestion was carried out in a solution (10 μL) containing the DNA, the enzyme (0.05 unit mL^{-1}) in $NaCl_2$ (100 mM), $Zn(OAc)_2$ (1 mM), and AcONa buffer (30 mM, pH 5.0) at 37°C for 30 min.

AFM was carried out with an atomic pressure-tapping mode microscope and a silicone single-crystal probe (sssNCH, Nanoworld Co.). The sample solution (1 μL) was mixed with fixation buffer (49 μL) and put on the mica support, kept for 5 min, washed five times with milli-Q-treated water, and dried with nitrogen gas.

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