DNA Structures

Parallel, Double-Helix DNA Nanostructures Using Interstrand Cross-Linked Oligonucleotides with Bismaleimide Linkers**

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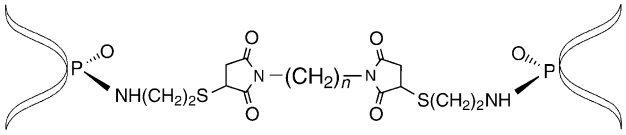
The creation of functional nanometer-scale wires and arrays has attracted increasing interest in the field of material sciences and molecular electronics.[1] For this purpose, molecular scaffolds, including polymers and supramolecules, with well-defined architectures are required for placing and arranging the desired functional molecules.[1] Nucleic acids provide a good scaffold for the following reasons: 1) The structure of DNA is well-defined and considered to be a periodic double-helical polymer; 2) association of DNA strands can be controlled by programming the sequence of DNA; 3) DNA molecules are easily modified chemically and biochemically for introduction of various functional molecules and nanoparticles;[2] and 4) DNA can be manipulated with modifying enzymes such as DNA ligase, kinase, polymerase, and restriction enzymes. Although DNA is an ideal scaffold, the flexibility of a long DNA chain makes the construction of desirable rigid structures difficult. Seeman and co-workers have built micrometer-scale DNA architectures by arranging previously prepared rigid DNA components.[3] The preparation and creation of wires and arrays would be much easier if such rigid DNA structures could be prepared simply by the addition of a chemical connector to the DNA strands.

Here we describe the design, synthesis, and properties of novel cross-linked oligonucleotides (XL-DNA) for the construction of a micrometer-scale rodlike DNA structure. Cross-linked oligonucleotides were designed to connect two double-helix DNA strands using a tether from a diastereochemically pure phosphoramidate (Scheme 1). Interstrand DNA cross-linking has been investigated in previous studies in which modification of a specific nucleic acid base was employed.[4] Our design is advantageous for connecting two duplex strands with less structural stress because the phosphate groups are located in the outer positions of the double-helical DNA. We employed a bifunctional cross-linker, alkyl bismaleimide, which can form a covalent bond with a thiol residue of a DNA strand under mild conditions. In addition, the distance between two DNA strands can be controlled using two different lengths of bismaleimide linkers.



Scheme 1. Interstrand cross-linked DNA having bismaleimidoethane (C_2) and bismaleimidohexane (C_6) linkers.

The diastereochemically pure oligonucleotides were prepared according to a literature method.[5] We used two DNA sequences, 5′-CGGCTpACTCC-3′ (**1XL**) and 5′-GTGCTpAGCGG-3′ (**2XL**; p denotes a phosphoramidate linkage where a cross-linker is introduced). Analysis by reversed-phase HPLC revealed two diastereomers, **A** (faster eluting) and **B** (slower eluting; see Supporting Information). The DNA was cross-linked according to Scheme 2. Oligonucleotides having a cystamine linker were treated with dithiothreitol (DTT) to reduce the disulfide linker. Thiol-tethered DNA (SH-DNA) was treated with excess 1,2-bismaleimidoethane (C_2) or 1,6-bismaleimidohexane (C_6) to produce a monocapped bismaleimide–DNA conjugate. This DNA conjugate and the SH-DNA were incubated in a 1:1 mol ratio, and the resulting XL-DNA was purified by HPLC. The extent of cross-linking was examined by 20% denaturing polyacrylamide gel electrophoresis (PAGE, Figure 1). All the cross-linked oligonucleotides migrated to a similar degree as a 20 mer single strand; no original 10 mer strand was observed, thus indicating that these oligonucleotides were cross-linked. The cross-linked oligonucleotides having a C_6 linker migrated slower than those with a C_2 linker.

The stabilities of the DNA assemblies with one cross-linked oligonucleotide (Scheme 3a) were examined by measurement of the melting temperature (T_m) (Table 1). All the DNA assemblies containing one XL-DNA helix were destabilized relative to that of unmodified DNA, which indicates that the stabilities of the DNA assemblies are reduced by electrostatic repulsion of the two proximal double helixes connected by a cross-linker. However, the assemblies with one XL-DNA helix still form thermally stable complexes. We employed two different cross-linked oligonucleotides (**1XL, 2XL**) and one complementary strand (**1c, 2c**) for assembling two double-helix strands in a parallel orientation (Scheme 3b). The T_m values show that these parallel DNA assemblies are largely stabilized relative to those with a single XL-DNA helix, which indicates that the two cross-linked oligonucleotides cooperatively function to stabilize the DNA assemblies. The length of the bismaleimide linkers affected the stabilities of the DNA assemblies, and the cross-linked oligonucleotides with a C_2 linker formed more-stable assemblies than those with a C_6 linker. The stereochemical effects of the phosphoramidates were also observed,[5] and the assemblies containing the **B** diastereomers were thermally more stable than those with the **A** diastereomers.

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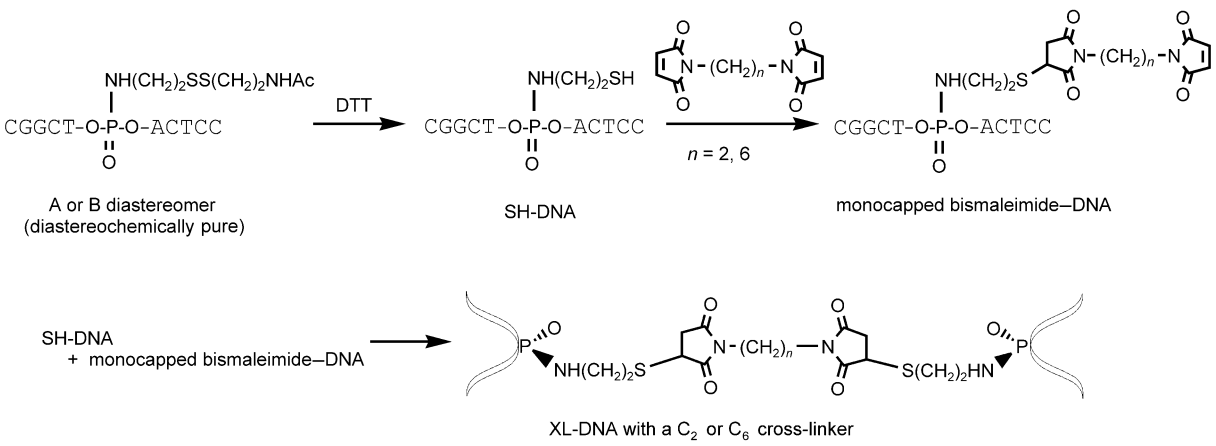


Scheme 2. Synthetic scheme for a diastereochemically pure cross-linked oligonucleotide having a C_2 and C_6 bismaleimide cross-linker.

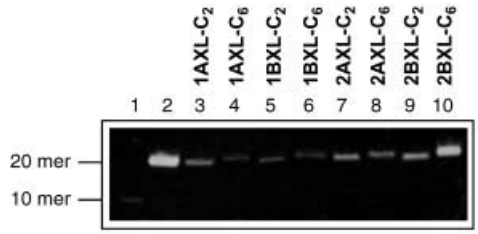
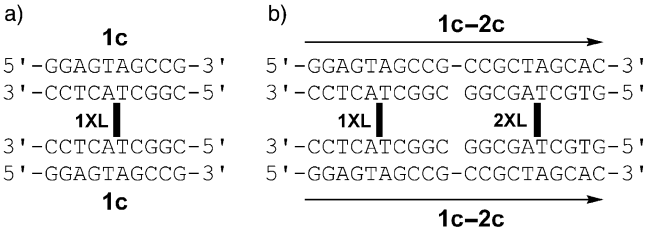


Figure 1. Polyacrylamide gel electrophoresis (PAGE) of cross-linked oligonucleotides having a C_2- and C_6-bismaleimide linker. **A** and **B** denote the diastereomers. **XL** represents XL-DNA.



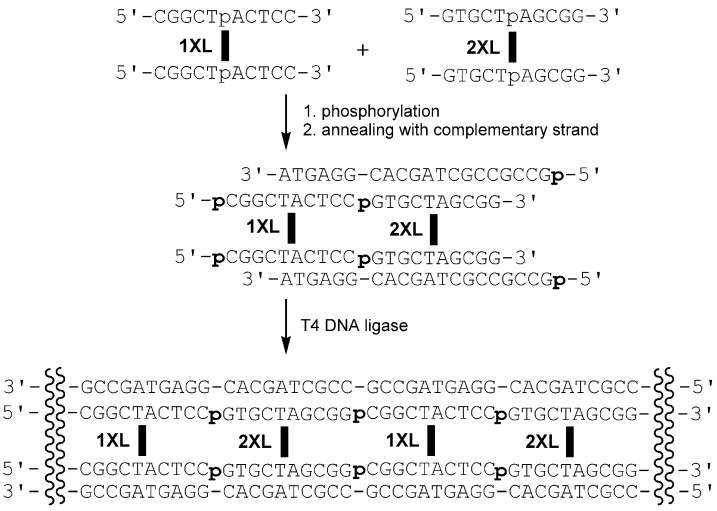
Scheme 3. DNA assemblies with cross-linked oligonucleotides and complementary strands for the T_m measurements. The bold bars represent cross-linkers. The arrows indicate the orientations of the double helixes.

Table 1: Melting temperatures (T_m) of the DNA assemblies with **1XL** and 10 mer complementary strand **1c**, and those with two cross-linked oligonucleotides **1XL, 2XL**, and 20 mer complementary strand **1c-2c**.[a]

XL-DNA	T_m [°C]	ΔT_m [°C]
1AXL-C_2	44.1	−6.2
1AXL-C_6	42.0	−8.3
1BXL-C_2	44.7	−5.6
1BXL-C_6	42.6	−7.7
1 (native)	50.3	–
1AXL-C_2 + 2AXL-C_2	55.2	+2.2
1AXL-C_6 + 2AXL-C_6	53.6	+0.6
1BXL-C_2 + 2BXL-C_2	58.3	+5.3
1BXL-C_6 + 2BXL-C_6	56.5	+3.5
1 + 2 (native)	53.0	–

[a] The measurement conditions are described in the Experimental Section.

Micrometer-scale parallel duplex DNA constructs (300 base pairs, 0.1 μm) were prepared by molecular assembly (first step) and DNA ligation (second step) from these cross-linked oligonucleotides. The two phosphorylated cross-linked oligonucleotides **1XL** and **2XL** were annealed with a phosphorylated complementary strand to form a DNA assembly (Scheme 4). The assembly was then treated with



Scheme 4. Schematic representation of the procedure for the construction of the DNA nanostructures with cross-linked oligonucleotides by self-assembly of the DNA and subsequent DNA ligation. The bold bars represent cross-linkers.

T4 DNA ligase to enable extension and formation of the tandem double-helix constructs. The DNA assemblies containing cross-linked oligonucleotides were extended in the presence of T4 DNA ligase, as shown by agarose gel electrophoresis (Figure 2). Interestingly, differences in the distribution of the ligated products were observed. The products with the cross-linked oligonucleotides having a C_2 linker contained 200–400 base pairs (lanes 4 and 8) and those with **1BXL-C_6** and **2BXL-C_6** contained 200–800 base pairs (lane 10). On the other hand, the reactions with **1AXL-C_6** and **2AXL-C_6** showed broad distributions of products with

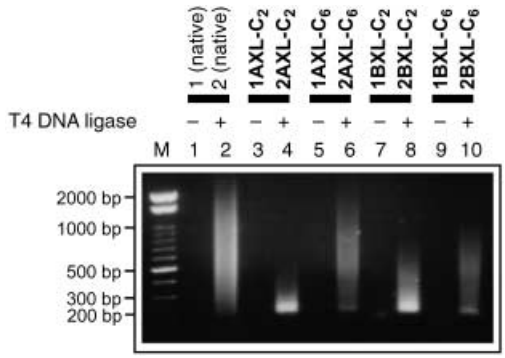


Figure 2. Agarose gel electrophoresis of DNA ligation products containing cross-linked oligonucleotides having a C_2-and C_6-bismaleimide linker. The cross-linked oligonucleotides used for the ligation reaction, with (+) and without (−) addition of T4 DNA ligase, are indicated above the lane number.

200 to over 2000 base pairs after ligation, a result similar to that found with the unmodified DNA (lanes 2 and 6). These results indicate that the C_2 linker is not long enough for efficient ligation because of some steric hindrance between the XL-DNA and T4 DNA ligase.[6] Clearly, the length of the cross-linker is the determinant for the ligation efficiency. In the cases of the C_6 linker, the cross-linked oligonucleotides of the **A** diastereomers do not seem to inhibit the ligation reaction relative to those of the **B** diastereomers.

The structures of the ligated products containing **1AXL-C_6** and **2AXL-C_6** were analyzed by atomic force microscopy (AFM; Figure 3).[7] The products can be observed at low concentrations as straight structures up to 0.6 μm in length (Figure 3a). This observation is consistent with the results obtained by gel electrophoresis. On the other hand, the ligated products seem rather flexible at a high concentration, but they are not aggregated (Figure 3b). Only aggregated products were detected by AFM analysis in the case of the ligated products generated from the other cross-linked oligonucleotides. The long DNA double strand can usually be observed as a flexible chain by AFM,[7] but the parallel double helixes in these cross-linked oligonucleotides suppress the mobility of the flexible DNA structures and form rigid structures.

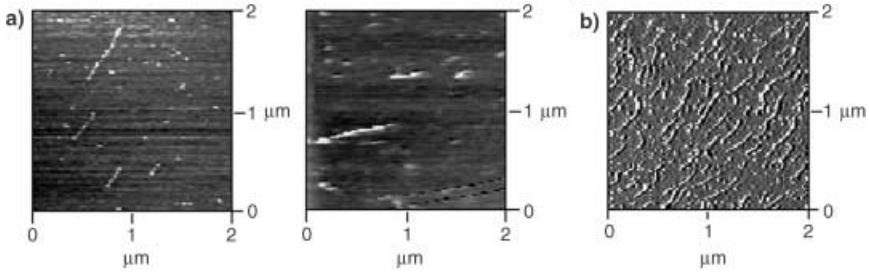


Figure 3. AFM images of the ligated products containing **1AXL-C_6** and **2AXL-C_6**. The samples containing low (a) and high (b) concentrations of DNA were adsorbed onto a mica plate.

In conclusion, we have designed and synthesized cross-linked oligonucleotides to form potentially rigid DNA assemblies. We have demonstrated the construction of a micrometer-scale rodlike DNA architecture, which can be achieved by a one-step molecular assembly using these cross-linked oligonucleotides and subsequent DNA ligation. The DNA assemblies can be extended into two-dimensional structures simply by changing the direction of the connection. These DNA structures can act as a scaffold for functionalized wires and arrays through modification of the DNA strands with reactive and small molecules followed by introduction of nanoparticles and biomolecules such as proteins and enzymes.[2b,c]

Experimental Section

Preparation of cross-linked oligonucleotides: A disulfide tether was introduced through a phosphoramidate linkage in the center of a 10 mer single-strand DNA according to a previously reported method.[5] Two diastereomers were separated by reversed-phase HPLC (see Supporting Information).

The purified diastereochemically pure oligonucleotides (10 nmol) were treated with DTT (10 mM) in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; pH 8.0, 50 mM) at 50 °C for 30 min, and thiol-tethered DNA (SH-DNA) was purified by reversed-phase HPLC (HPLC conditions are described in the Supporting Information). SH-DNA (5 nmol) was treated with 1,2-bismaleimidoethane (C_2, 1 μmol) or 1,6-bismaleimidohexane (C_6) in a 10% DMF/water solution at 50 °C for 2 h, and the monocapped bismaleimide–DNA conjugate was purified by HPLC. This DNA conjugate and the SH-DNA were then reacted in a 1:1 mol ratio at 37 °C overnight. After treatment with DTT (10 mM) at 37 °C for 2 h to remove any trace of an unwanted disulfide XL-DNA,[8] the bismaleimide XL-DNA was purified by HPLC. All the reaction steps were quantitative. The cross-linking was confirmed by 20% denatured PAGE analysis and the gel was visualized by staining with ethidium bromide.

Melting temperature measurements: Thermal denaturation profiles were obtained on a JASCO V-530 UV/VIS spectrophotometer equipped with a JASCO ETC-505T temperature controller. A solution containing XL-DNA (0.5 μM), complementary DNA (1.0 μM), Tris-HCl (pH 7.6; 10 mM), and 1.0 M NaCl was heated at 80 °C, then gradually cooled down to 10 °C. Measurements were carried out by heating from 10 to 80 °C at a rate of 1.0 °C min^{-1}, and monitored at 260 nm. The first derivative calculated from a melting profile was used to determine the T_m value. Complementary strands used here are **1c**: 5′-GGAG-TAGCCG-3′ (10 mer) and **1c**–**2c**: 5′-GGAG-TAGCCGCCGCTAGCAC-3′ (20 mer).

Enzymatic reactions: Phosphorylation of XL-DNA was carried out at 37 °C for 2 h in a solution (30 μL) containing XL-DNA (0.5 nmol), Tris-HCl (pH 7.5, 50 mM), $MgCl_2$ (5 mM), DTT (1 mM), ATP (1 mM), and T4 polynucleotide kinase (10 units). After the reaction, the solutions were heated at 65 °C for 20 min to inactivate the kinase. The ligation of a DNA assembly was carried out in a solution (20 μL) containing phosphorylated cross-linked oligonucleotides (0.2 nmol each), phosphorylated complementary DNA (0.4 nmol), Tris-HCl (pH 7.5; 50 mM), 0.1 M NaCl, $MgCl_2$ (10 mM), DTT (10 mM), ATP (1 mM), bovine serum albumin (BSA, 0.5 μg), and T4 DNA ligase (4 units). Mixtures (10 μL) were loaded onto a 1% agarose gel in a 1 × tris-borate-edta (tris = tris(hydroxymethyl)aminomethane, edta = ethylenediaminetetraacetate) buffer. The ligated products were visualized by staining with ethidium bromide.

AFM: The ligated DNA samples (0.5 pmol or 10 pmol) were placed on a freshly cleaved mica plate pretreated with a 0.01%

aminopropyltriethoxysilane/95% ethanol/water solution.[7b] After incubating the plate at RT for 5 min, it was rinsed with MilliQ water twice and dried under reduced pressure. The samples were observed by SP400-AFM (Seiko Instruments, Inc., Tokyo, Japan) with a silicon nitrite cantilever (SN-AF01, Olympus Optical Co., Ltd., Tokyo, Japan), and the collected data were processed on a Spise132 software (Seiko Instruments, Inc.).

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[8] A disulfide linkage of an interstrand cross-linked oligonucleotide was completely cleaved at 37 °C after 30 min in a solution containing Tris-HCl (pH 8.0; 50 mM) and DTT (10 mM).

