Ligation of DNA Triangles Containing Double Crossover Molecules

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Abstract: An antiparallel DNA double crossover molecule has been incorporated into one edge of a DNA triangle. The goal in this work is to combine a rigid geometrical motif, the triangle, with a rigid DNA motif, the double crossover, incorporating the properties of branched DNA. The triangle has been designed to contain 3 turns of DNA in each edge. The double crossover molecule produces an extra domain along its edge. The extra domain can be restricted by *Bbs I* to produce two complementary sticky ends separated by 4.5 turns of DNA. Ligation-closure experiments performed on the restricted triangle yield very long reporter strands and no indications of cyclization, suggesting that the double crossover molecule retains its stiffness when incorporated in the triangle. These experiments are evaluated through the use of denaturing gel electrophoresis. The ligation products have been visualized by atomic force microscopy, under nondenaturing conditions. Clear zigzag species can be identified as triangles ligated in one dimension. The images show no systematic distortions such as permanent bends. These visualizations have been confirmed by alternating triangles with simple double crossover molecules. When double crossover molecules containing 4.0 turns are alternated with triangles, the spacing of the vertexes is increased appropriately. When the double crossover molecules contain 4.5 turns, all the vertexes appear on the same side of the linear fragment, confirming the correlation between chemical input and mesoscopic images in the atomic force microscope. We conclude that double crossover molecules can be incorporated into at least one side of a triangle without any apparent effect on their rigidity.

Introduction

The control of the structure of matter on the finest possible scale is a key goal of chemistry, materials science, and nanotechnology.[1,2] We have pursued this goal by using building blocks derived from branched DNA molecules.[3] The combination of branched DNA and sticky-ended ligation, borrowed from biotechnology,[4] leads to a DNA nanotechnology capable of constructing multiply connected objects, networks, and devices.[5] To demonstrate this capability, we have constructed DNA molecules whose helix axes have the connectivities of a cube[6] and a truncated octahedron.[7] The latter molecule was built using a solid support-based methodology,[8] and its successful assembly suggests that it is possible to build most finite *N*-connected closed structures.

Prominent among the goals of DNA nanotechnology is the assembly of periodic matter. The structural requirements for these targets are more stringent than those for individual objects; each edge of a closed object can be programmed to be derived from the cohesion of two unique sticky ends. In the case of periodic matter, the sticky ends on one side of the unit cell must be complementary to those on the other side and the same as those in the next unit cell. Thus, there is an element of translational symmetry to be treated, and symmetry always decreases control over the behavior of a chemical system.[9]

Liu et al.[10] have enumerated the minimal requirements for periodic building blocks: (1) predictable intermolecular interactions; (2) predictable local product structures; (3) the structural integrity of the components. DNA sticky-ended associations clearly satisfy the first criterion very nicely. Indeed, the intermolecular interactions of DNA resulting from the complementarity of Watson−Crick base pairing are arguably the most simply predicted and readily programmed of all intermolecular interactions. Likewise, the second requirement is readily met by DNA, because cohesive ends associate to form B-DNA,[11] whose structure is well-known. However, simple branched DNA molecules do not meet the third need, structural integrity. Branched molecules produce a panoply of cyclic products in ligation-closure experiments,[12,13] suggesting that they are floppy rather than stiff.

To overcome this problem, we have explored other DNA motifs. The bulged DNA three-arm branched junction behaves more stiffly than the conventional three-arm branched junction,[10] but it does not appear to be stiff enough to be used in nanoconstruction.[14] Recently, we have reported that antiparallel DNA double crossover (DX) molecules[15] behave as inflexible

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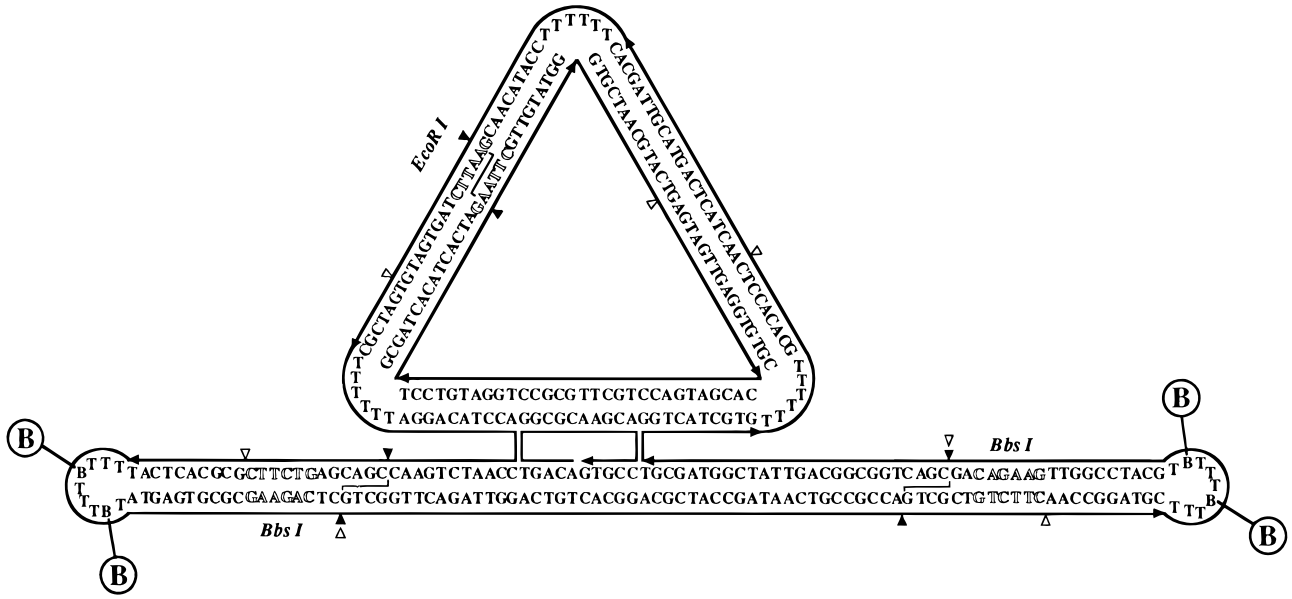

Figure 1. Molecule used in this work. The molecule is composed of three strands, the outer strand, containing 280 nucleotides, the inner triangular strand, containing 90 nucleotides, and the 20-mer unsealed strand involved in the two crossover structures. Strand polarity is indicated by arrowheads along the strands pointing in the 3′ direction. The two restriction sites for *Bbs I*, in the extratriangular domain, and the site for *EcoR I*, in the triangle, are indicated by unfilled letters. The points of scission are indicated by filled triangles, and the resulting sticky ends are indicated by a line connecting the points of scission. The 5′ ends of the original synthetic strands (from which these large strands have been ligated) are indicated by unfilled triangles. Biotin groups are indicated by "B" in the sequence, and these are emphasized by the large B's inside circles on the ends of the extratriangular domain. The bends of the triangle are produced by dT_6 bulges in the outer strand.

units[16] in the same ligation-closure experiments failed by conventional branched junctions. Consequently, we have concentrated on using these DNA motifs to construct more elaborate systems. We have reported recently the use of DX molecules to tile the plane.[17] In that work, we have used sticky-ended hydrogen bonding associations to produce self-assembled 2-D networks. In that system, we have been able to produce predictable topographic features, visible on the mesoscopic scale in the atomic force microscope (AFM), by programming different sets of sticky-ended associations.

In addition to using DX molecules in hydrogen-bonded arrangements, it should be possible to use them to form the edges of triangles or deltahedra.[16] These molecules ought to be rigid species, because triangular and convex deltahedral structures have no conformational degrees of freedom;[18] one domain of the DX molecule could be used as an edge of the geometrical figure, and the second domain could be used to interact with a similar domain on another triangle or deltahedron. Here, we report a simple test of this approach, the incorporation of a DX molecule into an edge of a DNA triangle and the ligation of triangles into long linear molecules. Similar to DX molecules, we find by gel electrophoretic analysis that the ligation of these molecules results in no cyclic species. Furthermore, we are able to characterize the products by AFM, and we are able to produce predictable changes in the AFM structures seen when triangles are interspersed with DX molecules of specific lengths.

Materials and Methods

Synthesis and Purification of DNA. All DNA molecules in this study have been synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected using routine phosphoramidite procedures.[19] Biotin Virtual Nucleotide phosphoramidites were purchased from Clontech. DNA strands have been purified by electrophoresis; the bands are cut out of 12−20% denaturing gels and eluted in a solution containing 500 mM ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA.

Formation of Hydrogen-Bonded Complexes. Complexes are formed by mixing a stoichiometric quantity of each strand, as estimated by OD_{260}. This mixture is then heated to 90 °C for 5 min and cooled to the desired temperature by the following protocol: 20 min at 65 °C, 20 min at 45 °C, 30 min at 37 °C, 30 min at room temperature, and (if desired) 30 min at 4 °C. Exact stoichiometry is determined, if necessary, by titrating pairs of strands designed to hydrogen bond together and visualizing them by nondenaturing gel electrophoresis; the absence of monomer is taken to indicate the endpoint.

Polyacrylamide Gel Electrophoresis. A. Denaturing Gels. These gels contain 8.3 M urea and are run at 55 °C. Gels contain 10% acrylamide (19:1, acrylamide/bisacrylamide). The running buffer consists of 89 mM Tris-HCl (pH 8.0), 89 mM Boric acid, and 2 mM EDTA (TBE). The sample buffer consists of 10 mM NaOH, 1 mM EDTA, containing 0.1% Xylene Cyanol FF tracking dye. Gels are run on a Hoefer SE 600 electrophoresis unit at 60 °C (31 V/cm, constant voltage). They are then dried onto Whatman 3MM paper and exposed to X-ray film for up to 15 h.

B. Nondenaturing Gels. Gels contain 6% acrylamide (19:1, acrylamide/bisacrylamide) and a buffer consisting of 40 mM Tris-HCl (pH 8.0), 20 mM Boric acid, 2 mM EDTA, and 12.5 mM magnesium acetate (TAEMg). The DNA is dissolved in 10 μL of NEBuffer 2 (New England Biolabs). Tracking dye (1 μL) containing TAEMg, 50% glycerol, and 0.2% each of Bromophenol Blue and Xylene Cyanol FF, is added to the sample buffer. Gels are run on a Hoefer SE-600 gel electrophoresis unit at 4 V/cm at 37 °C and exposed to X-ray film for up to 15 h.

Enzymatic Reactions. A. Kinase Labeling. In 25 μL of a solution containing 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, and 10 mM dithiothreitol (DTT), 10 pmols of an individual strand of DNA are dissolved and mixed with 6 μL of 2.2 μM γ-^{32}P-ATP (10 mCi/mL) and 6 units of polynucleotide kinase (US Biochemical) for 80 min at

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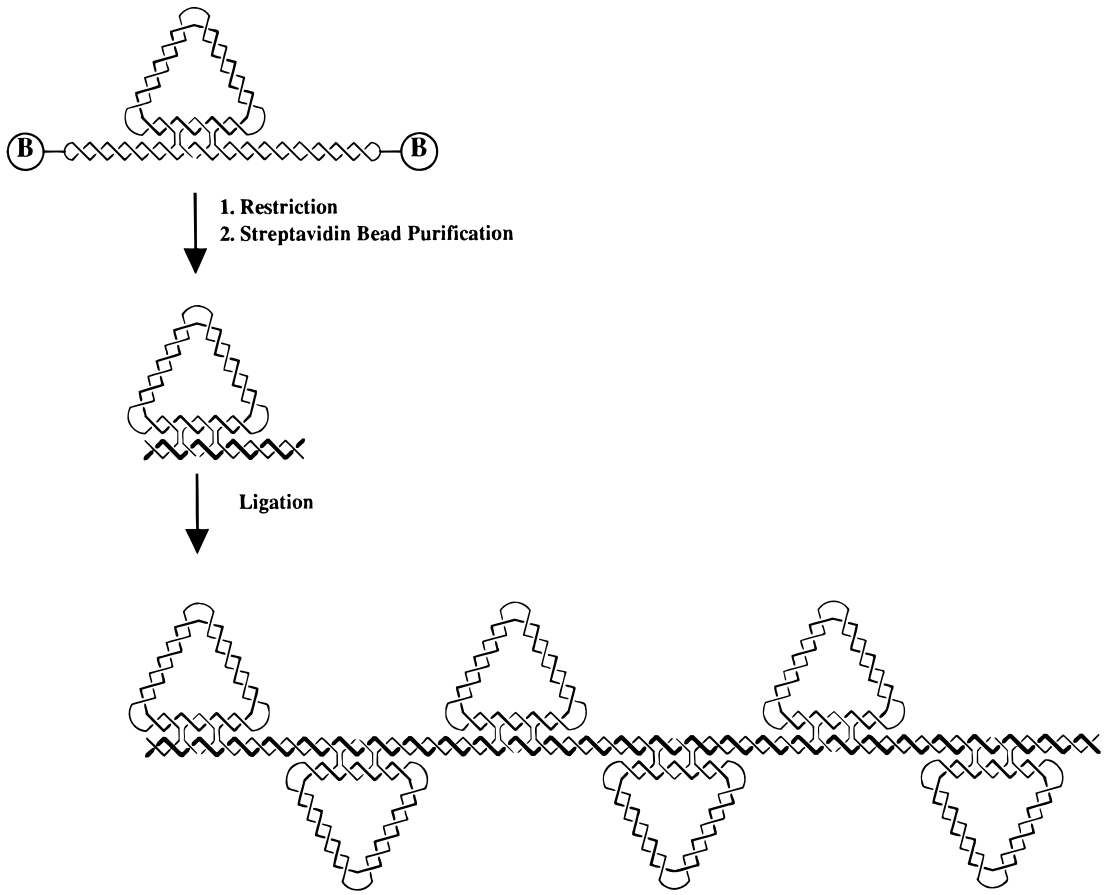

Figure 2. Ligation-closure experiment interpreted by denaturing gel electrophoresis. The top of the figure shows the same triangle illustrated in Figure 1, but now the helicity of the strands is shown. The biotin groups are indicated as a pair of B's inside two circles on the ends of the hairpins in the extratriangular domain. The long outside strand and the triangular strand are drawn with lines of different thicknesses. The first step involves restriction of the hairpins followed by streptavidin bead purification. This cuts the long outside strand into two segments, one of which is the 47-mer (4.5 turns) reporter strand, drawn with a thick line. Ligation of this molecule results in a long reporter strand shown at the bottom of the drawing. The product is expected to contain triangles pointing successively in alternate directions. If the molecule were to cyclize, the reporter strand would be catenated to the remainder of the outside strand; this problem is solved readily by restricting the product with *EcoR I* before analysis.

37 °C. Radioactive labeling is followed by the addition of 1 μL of unlabeled 10 mM ATP, and incubation proceeds for another 5 min. The reaction is stopped by heating the solution to 90 °C for 10 min followed by gel purification.

B. Ligations. Ligations are performed in the kination buffer, which has been brought to 1mM in ATP. All strands are mixed stoichiometrically, and the solution is then heated to 90 °C for 7 min and cooled slowly to room temperature. T4 Polynucleotide ligase (US Biomedical) in the amount of 30 units is added, and the reaction is allowed to proceed at 16 °C for 16 h. The reaction is stopped by phenol/chloroform extraction. Samples are then ethanol precipitated.

C. Restriction Endonuclease Digestions. Restriction enzymes are purchased from New England Biolabs and used in buffers suggested by the supplier. Digestion is performed at 37 °C for 2 h with 20 units of *EcoR I* or *Bbs I*. The reaction is stopped by two or three phenol extractions followed by ethanol precipitation. About 60 pmol of DNA triangles that is to be used in the ligation assay is restricted with 200 units of *Bbs I* in 250 μL at 37 °C for 10 h. The reaction is further treated with streptavidin-coated magnetic beads to remove biotinylated material. The remaining solution is then concentrated to 10−20 μL by a Microcon-10 column (Amicon).

D. Exonuclease Treatment. To the ligation mixture, 100 units of exonuclease III (exo III) (US Biochemical) is added directly, and the reaction is allowed to proceed for 0.5−2 h at 37 °C. The solution is heated to 90 °C for 5 min and cooled on ice for 2 min to generate single-stranded DNA. Then, 10 units of exonuclease I (exo I) (USB) are added and the digestion is continued for 0.5−2 h at 37 °C. The reaction is stopped by phenol extraction.

Streptavidin Bead Treatment. Streptavidin beads are purchased from Promega. They are supplied as 1 mg/mL magnetic particles in phosphate-buffered saline (PBS), 1 mg/mL BSA, and 0.02% Na azide. The binding capacity is 0.75−1.25 nmol/mg. Beads in the amount of 400−600 μL are added into a siliconized Eppendorf tube on a magnetic stand and allowed to sit for about 5 min. The buffer is removed, and the beads are washed three times with 400−600 μL of PBS buffer and then three times with 400−600 μL of NEBuffer 2. The DNA triangle restriction solution is then added to the beads, mixed well, and allowed to remain at room temperature for 20 min. The tube is then put on the magnetic stand to separate the beads from the solution, which now contains no biotinylated material.

AFM Imaging. A. Sample Preparation. Ligated DNA triangles are sequentially treated with phenol three or four times, phenol/chloroform/isoamyl alcohol once, chloroform alone once, and then water-saturated ethyl ether four or five times, to remove enzymes that have been introduced previously. Finally, a trace of ether is removed by evaporation. The sample solution is then passed through a NAP-10 desalting column (Pharmacia) three times.[20] The drop is suspended in 10 mM $MgCl_2$ for AFM imaging.

B. Imaging. A drop containing 5 μL is spotted on freshly cleaved mica (Ted Pella, Inc.) and left to adsorb to the surface for 2 min. To remove buffer salts, 5−10 drops of doubly distilled water are placed on the mica, the drop is shaken off, and the sample is dried with compressed air. Imaging is performed under 2-propanol in a fluid cell

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on a NanoScope II using commercial 200 μm cantilevers with Si_3N_4 tips (Digital Instruments). The feedback setpoint is adjusted frequently to minimize the contact force to approximately 1−5 nN. Images are processed with a first- or third-order "flatten filter", which independently subtracts a first- or third-order polynomial fit from each scanline to remove tip artifacts.

Results

Design of the Molecule. Figure 1 illustrates the design of the triangular species, illustrating both its sequence and its key features. The molecule consists of a triangle, one of whose edges is a domain of a DAE-type DNA double crossover molecule. Each edge of the triangle contains 30 nucleotide pairs. The corners of the triangle are obtained by placing dT_6 loops on one strand of the molecule, drawn as the "outside" strand in Figure 1. The bottom edge forms a DAE double crossover with another helical domain drawn below it. The extratriangular (ET) helical domain contains two hairpin loops so that the molecule consists of two catenated strands, linked eight times and consisting of the outside strand, containing 280 nucleotides, and the inner strand containing 90 nucleotides. These parts of the molecule are designed intentionally to be topologically closed and bonded, because purification of the DNA species is much more effective under denaturing conditions.[6] In addition, DAE double crossover molecules contain a small cyclic strand between the two crossover points; this strand, 20 nucleotides long here, is shown unsealed, because it is very difficult to seal a significant amount of it. The 20-mer cyclic strand is hydrogen bonded to the catenane.

The sequence of the molecule has been designed using the program SEQUIN,[21] and it contains special sequence features that are necessary for the experiments. The left edge of the triangle in Figure 1 contains an *EcoR I* restriction site for analytical purposes. The ET domain contains two *Bbs I* sites. These sites permit the restriction of the closed molecule to produce a species that can participate in a ligation experiment. *Bbs I* has been chosen because the recognition site is unrelated to the sticky end so that two complementary, but not self-complementary, sticky ends may be produced upon restriction. Successful restriction by *Bbs I* results in a 47 nucleotide (4.5 turns of DNA) ET domain ready to oligomerize by ligation. We have employed a strategy used previously[14] to ensure the removal of restricted hairpins, as well as partially restricted species that could foul the ligation mixture; we have included biotin groups on the hairpins that can be bound to streptavidin beads.

The ligation experiment is illustrated in Figure 2, where the double helical nature of the molecule is shown. Restriction cleaves the outer strand into two species, one of which behaves as a reporter strand.[12−14,16,17] The reporter strand, drawn with a dark line in the central part of Figure 2, reports the fate of the complex, because its fate will mimic that of the complex; elongation of the complex results in elongation of the reporter strand, and cyclization of the complex results in cyclization of the reporter strand. In case of cyclization, the complex must be restricted at the *EcoR I* site, to isolate the reporter strand from molecules to which it would be catenated. The bottom portion of Figure 2 shows that the 4.5 turn unit results in an oligomer in which successive triangles point in opposite directions.

Assembly of the Molecule. The individual components of the outer strand are shown in Figure 1. These are ligated together in the presence of the 90-mer inner triangular strand and the small 20-mer unclosed circle to produce the catenane; the catenane is then purified on denaturing gels and reconstituted with the 20-mer unclosed circle. Figure 3 illustrates characterization of the catenated part of the molecule by enzymatic digestion. The three sections of this gel illustrate the results when the molecule is labeled at one of the *Bbs I* sites on the outer strand, on the inner strand, or on both. Lanes 2, 7, and 12 contain undigested material; lanes 3, 8, and 13 contain material digested by *Bbs I*. Lanes 3 and 13 show a small amount of linear 231-mer (the single *Bbs I* digestion product) and a substantial amount of 47-mer (the double *Bbs I* digestion product). Lanes 8 and 13 also contain the released inner circle, which is labeled in those experiments. The identities of the circular and linear species are made clear by the experiments shown in lanes 4, 9, and 14, in which the material in lanes 3, 8, and 13 is also digested with exo III. Lanes 5, 10, and 15 illustrate the products of *EcoR I* digestion, which are the 280-mer linear molecule in lane 5, the 90-mer linear in lane 10, and both in lane 15. The exonuclease digestions in lanes 6, 11, and 16 confirm these topologies.

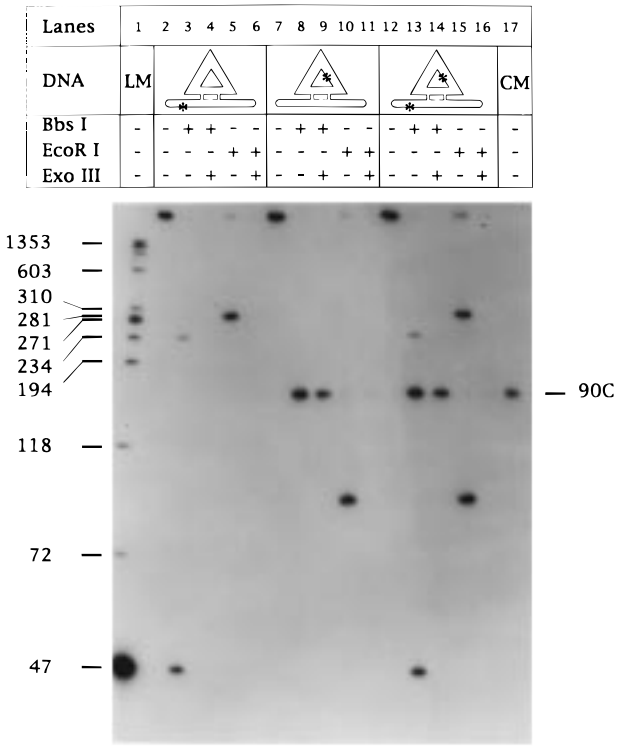

Figure 3. Construction of the molecule. This is an autoradiogram of a denaturing gel demonstrating the assembly and restriction of the catenane formed from the two large circles. Lane 1 contains linear markers whose lengths are indicated on the left; note that the crowding of bands has forced the length indicators away from the positions they label, so care should be exercised in noting assignments. Lane 17 contains a 90-mer single-stranded circular marker. The three drawings indicate that the material in lanes 2−6 is labeled in the outside strand, that in lanes 7−11 is labeled in the inside triangular strand, and that in 12−16 is labeled in both strands. Restriction with *Bbs I* or *EcoR I* or digestion with exo III is indicated above each lane, and the pattern is the same for each section. Note that restriction with *EcoR I* in the first section yields only a full-length linear 280-mer and that restriction with *Bbs I* and digestion with exo III in the second section produces a cyclic 90-mer.

Figure 4 contains a nondenaturing gel that illustrates the history of the molecule from initial ligation through reconstitution, digestion, and streptavidin bead treatment. The large drawing on the upper right shows the numbering scheme for four different labeling sites. These sites correspond to the

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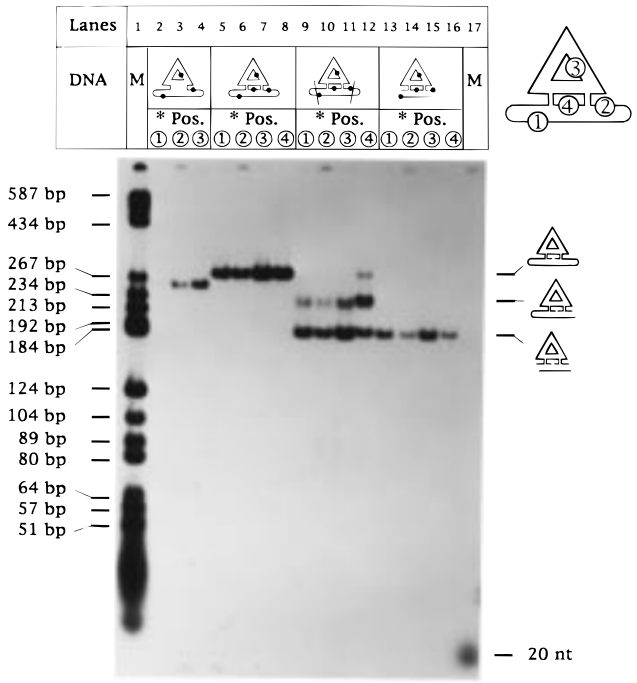

Figure 4. Preparation of the triangle for ligation. This is an autoradiogram of a nondenaturing gel demonstrating the hybridization, restriction, and purification of the triangle for ligation. The molecular schematic in the upper right portion of the figure indicates four numbered positions for strand labeling. The position of labeling above each lane corresponds to these numbers. The drawings in each of the sections indicate each of these positions by dots. Lanes 1 and 17 contain markers. The first section (lanes 2−4) contains only the catenane consisting of the inner triangular strand and the outer strand. The band in lane 2 is labeled faintly, but the same strand is labeled in lane 3. The second section (lanes 5−8) illustrates that regardless of the position of the label, the same band results when the 20-mer is added to the material in the first section. Note that the stoichiometry is correct in lane 8, because there is no overflow of 20-mer. The third section (lanes 9−12) contains the products of restricting the material in the second section with *Bbs I*. A variety of products are seen; completely restricted material migrates most rapidly, and partially restricted material migrates more slowly. The fourth section (lanes 13−16) contains the products of treating the material in the third section with streptavidin beads. All of the partially restricted material is seen to be removed, and the remaining material is ready for ligation.

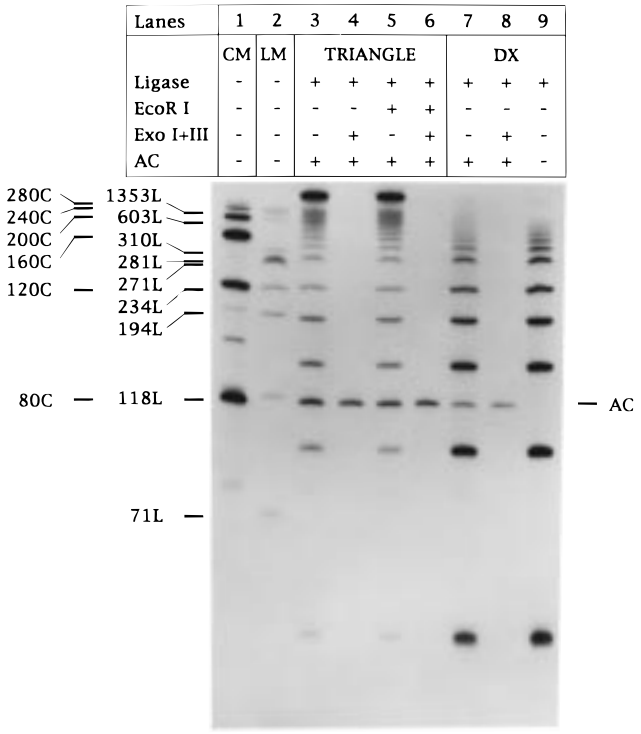

Figure 5. Ligation of the triangle. This is an autoradiogram of a denaturing gel showing the products of the ligation of the triangle. Only the reporter strand is labeled in this gel, so its fate is used to monitor the progress of the ligation. Lane 1 contains a series of circular markers prepared by treating the products of the ligation of branched junctions with exo I and exo III.[14] Their lengths are indicated on the far left; unlabeled bands are linear molecules incompletely digested by the exonucleases. Lane 2 contains linear markers, whose lengths are indicated to the right of the circular markers. An 80-mer single-stranded circle (AC) has been added to all experimental lanes except lane 9, as a positive control to demonstrate that roughly equal quantities of material were present during the preparation of the sample and that no endonuclease activity is present. Triangle ligation is contained in lanes 3−6, and a control double crossover (DX) ligation is shown in lanes 7 and 8. It is clear from inspection of both lanes 4 and 6 that no cyclic material has been produced in the triangle ligation; lane 8 illustrates the same fact about double crossover ligation. Lanes 3 and 5 demonstrate that extensive ligation has been achieved with the triangles, including the presence of a substantial amount of exo-sensitive material that does not penetrate the gel. This material is not seen in the double crossover experiment.

labeling schemes for the lanes in the gel. In the first section (lanes 2−4), the catenane is labeled at two sites in the outside strand and one site in the inside strand; although not visible in this reproduction, the band in lane 2 is seen to comigrate with the band in lane 3 in the original. The second section (lanes 5−8) contains the reconstitution with the small circular strand; material with the same mobility is seen in each lane, regardless of whether the label is in the small strand or one of the others; this fact evinces the success of the reconstitution. The third section (lanes 9−12) contains the products of digesting the triangle with *Bbs I*. Several bands are seen, demonstrating that some of the hairpins remain; on this nondenaturing gel, the presence of the hairpins could be due to covalent or hydrogen-bonded attachment. The final section (lanes 13−16) shows that the undenatured products of the same mobility are seen in each lane after the restricted material has been treated with streptavidin beads. Partially restricted molecules and those to which the hairpins are cohering noncovalently are all removed, and the final product is ready to be used in ligation experiments.

Ligation Experiment. The key question addressed here is whether a DX molecule can retain its structural integrity when incorporated into the edge of a geometrical figure, such as a triangle. The stiffness of the DX motif was established by means on the ligation-closure experiment, so it is appropriate to utilize that experiment to ascertain the flexibility of the DX when it is incorporated into the edge of a triangle. Figure 5 contains a gel that illustrates the results of this experiment. Lanes 3 and 5 contain the products of ligating the triangle with sticky ends that has been prepared as described above. The bands result from the radioactivity present in the reporter strand. A substantial ladder of species is visible, extending at least to 27 bands and perhaps further. Lanes 4 and 6 contain the results of adding exonuclease to the ligation products, indicating that no circular material can be detected. The material in lanes 5 and 6 differs from that in lanes 3 and 4 in that it has been treated by *EcoR I* so that any catenanes involving possibly cyclic reporter strands have been cleaved. In fact, there is no material visible in either of the lanes. This result is similar to the result seen in lanes 7 and 8 for the ligation of an isolated DX molecule. Thus, ligation-closure experiments suggest that it is possible to

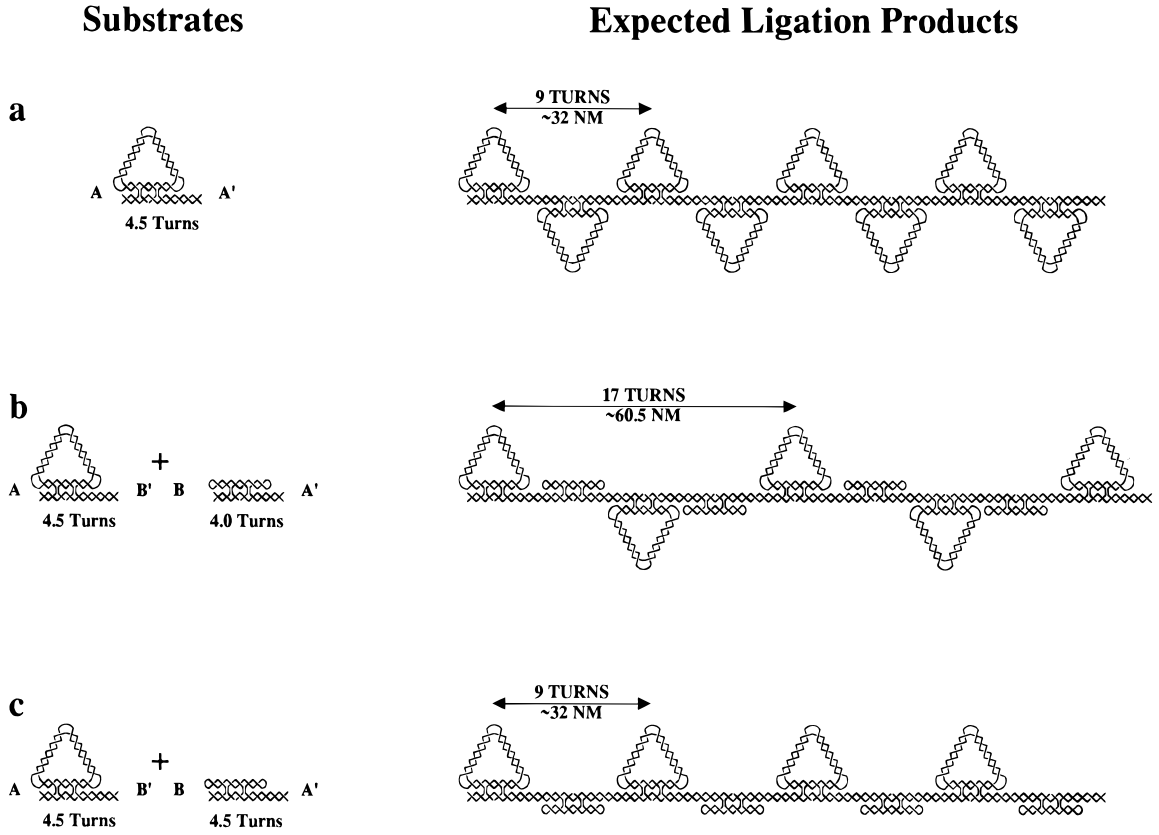

Figure 6. Atomic force microscopy ligation experiments. Three ligation experiments are shown in this diagram. The substrates are shown on the left, and the expected ligation products are shown on the right. The experiment in panel a is identical to the ligation experiment analyzed in Figure 5. The triangle is prepared with complementary sticky ends and ligated to produce a linear array in which the vertex opposite the DX points alternately in opposite directions, because the length of the ligated helix is 4.5 turns of DNA; successive vertexes on the same side of the central helix are expected to be separated by about 32 nm. The experiment in panel b illustrates the ligation of the same triangle, but this time alternating with a DX molecule whose ligation domain is 4.0 turns. One domain on the DX molecule is sealed with hairpin loops. The sticky ends on the triangle (represented as A and B′) and on the DX (represented as B and A′) enforce this strict alternation. This structure is expected to spread out the separation of successive vertexes on the same side of the central helix to about 60.5 nm. The experiment in panel c is similar to the middle experiment, except that the ligation domain of the DX molecule is 4.5 turns. This is expected to produce a product in which all of the triangles are on the same side of the central helix.

generate a noncyclic species by ligating triangles that have incorporated DX motifs along one edge.

AFM Imaging. Direct visualization of the ligation products provides the strongest evidence for the inferences drawn from the denaturing gel described in the previous section. Fortunately, the triangle we have produced is sufficiently large that its ligation products can be seen in a conventional AFM imaging experiment. We have performed three different ligation experiments to prepare samples for AFM imaging; these experiments are illustrated in Figure 6, which shows both the substrates of ligation and the expected products. The experiment in Figure 6a is the same as the experiment described in the previous section: The substrate triangle is ligated to itself, because its sticky ends, indicated schematically as A and A′ are complementary. The length of the ligation unit is 4.5 turns of DNA, so the expected products of this experiment are linear arrays of triangles pointing in opposite directions around a central linear helix. These products may be compared with the AFM images shown in Figure 7a−c. Figure 7a shows very long lines of ligated triangles, visible as zigzag chains. The most likely interpretation of this pattern is that the extrema of the zigzag correspond to the vertexes of the triangles opposite the edges containing DX motifs. Figure 6 indicates that the separation of the vertexes on the same side of the chain should be about 32 nm. Figure 7b, which is a zoom of Figure 7a, shows that this is approximately the distance seen between vertexes. Figure 7c superimposes in Figure 7b a scaled version of the products drawn in Figure 6a; the relationship between the image and the molecular model is evident.

This is the first time that an attempt has been made to visualize DNA geometrical objects by AFM, and there is only a single previous example of AFM characterization of a complex DNA motif.[17] Borrowing the approach of the preceding work, we have made alterations to the ligation substrates that should lead to predictable changes in the AFM images. The first of these experiments is illustrated in Figure 6b. Here, we have alternated the 4.5 turn triangles with a simple DX molecule containing 4.0 turns of DNA. We are able to control this structure by changing the sticky ends so that the triangle contains sticky ends A and B′ and the DX contains sticky ends B and A′. The structure of the ligated material should be similar to that seen in the previous experiment, with successive vertexes oriented in opposite directions. However, vertexes on the same side should now be separated by about 60.5 nm, rather than 32 nm. Figure 7d illustrates a product of this ligation, and Figure 7e is a closeup of this molecule that shows that the spacing is approximately correct. Figure 7f superimposes the scaled molecular model from Figure 6b in Figure 7e, illustrating the relationship between the image and expected molecular structure.

A similar experiment should yield a strikingly different result. Figure 6c shows the consequences of changing the length of

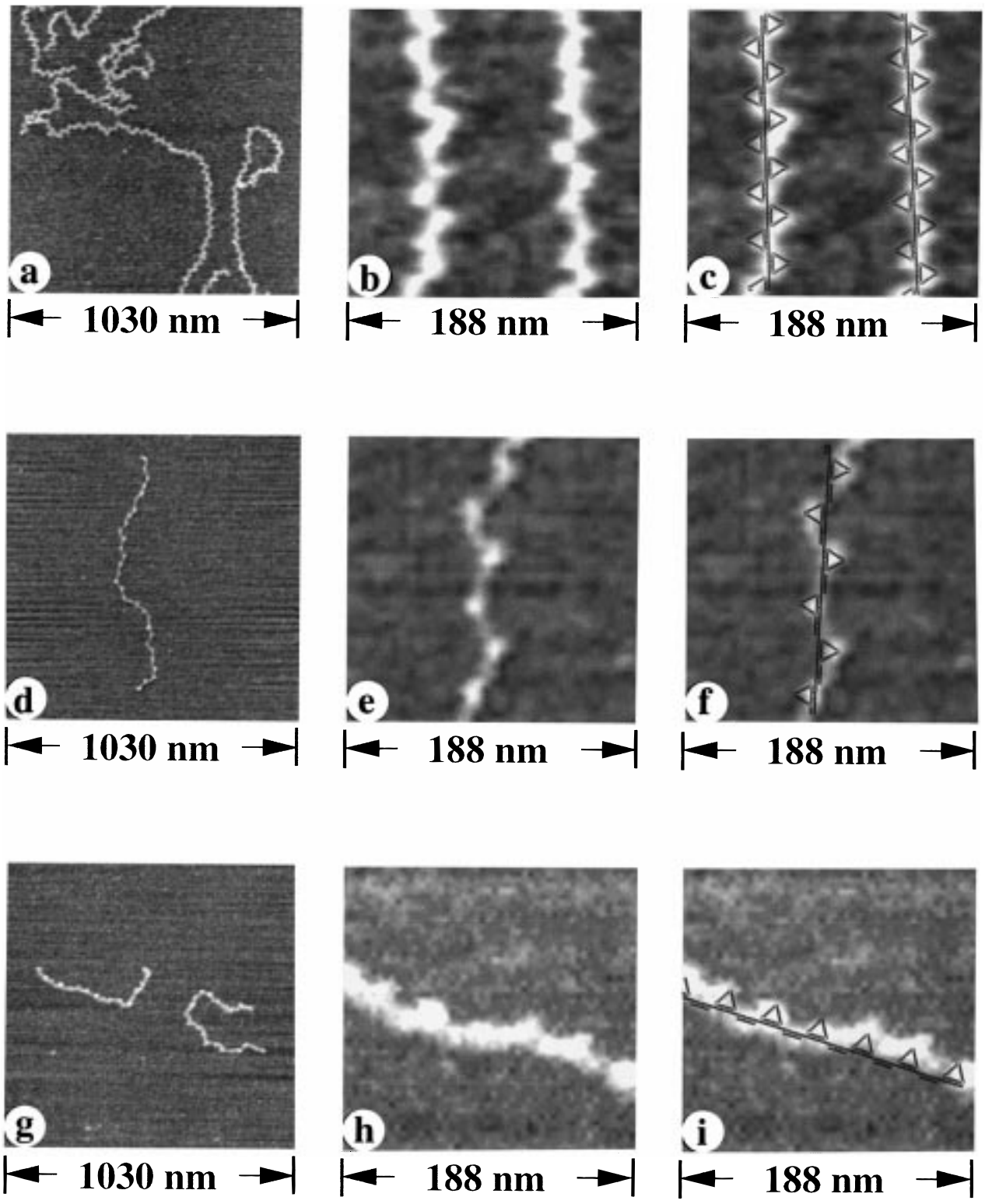

Figure 7. Atomic force microscopic visualization of triangular ligation products. The three sets of images here correspond to the experiments illustrated in Figure 6. Each experiment is illustrated in three square images, one of edge 1030 nm (a, d, g), a zoom of that image (b, e, h) with an edge of 188 nm, and the superposition of the scaled molecular products from Figure 6 upon the zoomed image (c, f, i). The top three panels (a−c) show the products of the ligation experiment of Figure 6a. The zigzag character of the strands in (a) is similar to the expected ligation products. The zoom in (b) shows that points on the same side of the central strand are roughly 32 nm apart; the superposition in (c) illustrates the relationship between the molecular model and the image. The middle panels (d−f) show the products of the experiment in Figure 6b. Panel d shows a zigzag chain, again, but this time the zoom in (e) illustrates that the separation of peaks is roughly 60 nm, again in line with the prediction; (f) shows that the molecular model fits the image well. Panels g, h, and i display the products of the experiment in Figure 6c. It is clear from the two strands visible in (g) that one side of each strand appears smooth and the other is ragged. The spacing apparent in the zoomed panel g shows that the prominences on the same side are separated by roughly 31 nm, similar to the prediction. The image shows a slightly curved molecule, and the molecular model in Figure 6c is straight, but the close relationship between the model and the image can be seen nevertheless, in panel i. The models and the images agree remarkably well.

the interspersed DX motif by a half of a turn of DNA. If it contains 4.5 turns of DNA, rather than 4.0 turns of DNA, all of the vertexes should lie on the same side of the central helix and their separation should be about 32 nm. Parts g and h of Figure 7 are AFM scans showing that this indeed occurs. It is clear from Figure 7g that all of the vertexes lie on one side of the object. Figure 7g emphasizes the fact that the spacings between vertexes are roughly those predicted. Figure 7i superimposes the scaled molecular model from Figure 6c on the image of Figure 7h. The image shows a slightly curved molecule, but the relationship between it and the model is clear. These two control experiments demonstrate that changes made on the molecular level have direct and predicted structural consequences on the mesoscopic scale, thereby validating our assignments of molecular species.

Discussion

Lack of Cyclization. The object of the work performed here was to determine whether it was possible to incorporate a DX motif as an edge of a DNA geometrical object without impairing its stiffness. We have incorporated the DX into an inherently rigid motif, the triangle. If lack of cyclization is used as the criterion for rigidity, this experiment has been successful. We have demonstrated by ligation-closure methods employing reporter strands that little if any cyclization occurs. These findings are similar to those seen previously for isolated DX molecules. In addition, we have confirmed this finding by AFM observation of the products. Cyclic species are not seen in the samples examined. Indeed, few bends are visible, suggesting that these molecules are surprisingly inflexible. As in our previous study involving the ligation of isolated DX molecules,[16] ligation is quite extensive, far more extensive than in samples involving single crossover branched molecules. This suggests that the shorter distribution of products seen in the ligation of single crossover molecules may result from the lack of linear species available for extension.

AFM Imaging of Products. This is the first time that ligation products of complex DNA motifs have been visualized. This has been a great aid in the characterization of the molecules and has confirmed the conclusions drawn from the less direct method of analyzing reporter strands by denaturing gel electrophoresis. Previously, we have imaged 2-dimensional hydrogen-bonded networks of DX molecules,[17] but AFM imaging of those arrays when ligated proved intractable. This work represents the first imaging of synthetic DNA geometrical objects. It is clear from the features seen that this technique would need far better resolution[22] to yield valuable information about individual molecules. Nevertheless, useful data have been obtained by AFM observation of a 1-dimensional array of these species. In particular, it is evident from the images obtained, particularly Figure 7a, that there is no structural bias in the individual units, arguing against the presence of permanent bends in the molecules examined. Although the molecules are not perfectly straight, the large bends seen do not appear to have a systematic basis.

Nanoconstructions. We have suggested previously that DX molecules could be combined with inherently rigid triangular motifs to produce complex and useful arrays.[16] We have not yet achieved this goal, but we have made a good start in that direction by demonstrating that the incorporation of a DX molecule into a triangle does not appear to lessen the stiffness of either component. We hope to be able to utilize this finding to produce 2-dimensional and 3-dimensional periodic arrays from DNA geometrical objects. These constructions are dependent on the rigidity of their component parts,[10] and we have demonstrated here that at least in one dimension, a DX-sided triangle has the requisite properties.

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