found in codon recognition patterns for the two types of wobble position uridines, U vs s^2U. For instance, in triplet-dependent binding to the ribosome, $tRNA^{Glu}$ with wobble position s^2mnm^5U recognized predominantly the codon GAA, whereas thio deficient $tRNA^{Glu}$ recognized GAG and GAU as well as GAA.[15] Those tRNA anticodons with non-thiolated (but 5-position modified) uridines at the wobble position exhibit recognition of codons ending in G as well as A (Yokoyama, S., personal communication). Thus, we conclude that for those tRNAs containing wobble position s^2U (vs U or 5-position modified U), the relatively more restricted dynamics and conformation of s^2U are responsible for the tRNAs preferential recognition of codons ending in adenosine.

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A Solid-Support Methodology for the Construction of Geometrical Objects from DNA

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Abstract: A solid-support procedure has been developed for the synthesis of geometrical stick figures from branched DNA. The method permits control over the synthesis of individual edges of an object. Control derives from the restriction endonuclease digestion of hairpin loops forming each side of the new edge. Restriction sites are destroyed when the edge forms. Each cycle of the procedure creates an object that is covalently closed and topologically bonded to itself. This features permits destruction of incompletely ligated edges by exonuclease digestion, thereby purifying the growing object while it is still on the support. The use of the solid support permits convenient removal of reagents and catalysts from the growing product. The solid support also isolates individual objects from each other, thereby eliminating a class of potential side products. The strategy permits the separate execution of steps involving additions and cyclizations, which are optimized under different conditions. A technique is presented to rescue those hairpin loops that fail to be digested by restriction endonucleases. The synthesis of a quadrilateral from three-arm junctions utilizing this protocol is reported. In principle, the methodology can be used to combine more complex components, in order to fabricate polyhedra and two-dimensional or three-dimensional arrays.

Introduction

There is considerable interest in the development of macromolecular chemical systems with well-defined structural properties for use as molecular scaffolding that orients and juxtaposes other molecules. The motivations for pursuing these constructions include the formation of "macromolecular zeolite" lattices to enable diffraction analysis of complex guest molecules that do not readily crystallize,[1-3] the caging of active biological macromolecules to form new multifunctional enzymes,[4] the development of drug delivery systems for therapeutic macromolecules,[5] the achievement of mechanical control on the nanometer scale,[6] and the assembly of molecular electronic components.[7,8] Branched nucleic acid molecules offer one of the most direct routes to these ends. DNA is particularly well-suited for use as a scaffolding medium, since it is a thick (2-nm diameter), stiff[9] molecule over the range of a few nanometers, a molecule whose structure is unlikely to be perturbed markedly by tethering to it smaller noninteractive molecules.

In living cells, DNA is found almost exclusively in the form of a linear duplex molecule; the molecule may be supercoiled,[10] stressed,[11] or even knotted,[12] but its helical axis is unbranched. Nevertheless, DNA branched junction structures may be observed transiently as intermediates in the process of recombination.[13] Naturally-occurring branched structures are inherently unstable because of their homologous (2-fold) sequence symmetry.[14,15] Since 1983, it has been possible to model these molecules in stable oligonucleotide systems[16] containing minimal sequence symmetry.[1,2,17] In principle, the attachment of a series of cohesive ("sticky") ends to a DNA branched junction converts it to a valence cluster whose ends are specifically available for binding only complementary cohesive ends.[1-3]

For several years, we have conducted experiments involving the ligation of branched DNA molecules to form larger structures. Stick figures are produced in which the vertices are the branch points of junctions and the edge are double helical DNA. We have oligomerized three-arm[18] and four-arm[19] branched junctions into macrocycles, and we have synthesized a specific quadrilateral from four separate junctions.[4] Recently, we have constructed a

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molecule whose helix axes have the connectivity of a cube, rhombohedron, or other equilateral parallelepiped.[20] Each edge contains two turns of DNA, and the entire molecule consists of six single-stranded cyclic molecules; each strand corresponds to a different face of the cube. This assembly is a hexacatenane, in which each of the strands is doubly linked to each of its four nearest neighbors.

Experience in synthesizing the cube-like object[20] has highlighted the differences between the ideal assembly of DNA objects originally envisioned for this system[1-3] and the reality of fabricating an object in a solution containing picomoles of reactants at concentrations of 10^{-7}–10^{-6} M. The published synthesis of this object entails the following steps:[20] (1) Two quadrilaterals with four exocyclic arms each are linked together at two adjacent corners to form a three-square ladder-like belt with four external arms; (2) the topologically bonded core of this belt is purified under denaturing conditions; (3) the ladder-like belt is reconstituted from the core and six starting strands; and (4) the belt folds up and is ligated to form the target molecule. Control over reactivity is derived only from the presence or absence of 5′ phosphates on particular exocyclic arms. Thus, only two logical stages of synthesis are possible if all strands are present throughout the synthesis: (i) Initial phosphorylation of certain strands followed by ligation and (ii) phosphorylation of the remaining strands in the intact molecule (or preceding reconstitution), followed by a second ligation. The intermediate steps (denaturation and reconstitution) are undesirable but necessary in this scheme, because it is not possible to purify side products and failure products from the target product of the first reaction under native conditions.

The synthesis of more complex structure from DNA requires greater control than that available in the synthesis of the cube-like object. Accordingly, we have developed a new methodology for the synthesis of DNA geometrical objects, with three improvements over the previous approach: (1) Each edge is formed individually, leading to greater control over intermediate products. (2) Every intermediate product is a covalently-closed and topologically-bonded molecule that is able to withstand vigorous treatments and exonucleolytic digestions designed to destroy failure products. (3) The entire procedure is performed on a solid support, which separates growing objects and simplifies the separation of products and reactants.

Individual Edge Formation. Even though it is possible to treat the sticky ends of each branched component as individually accessible, the formation of closed geometrical objects entails two fundamentally different types of reactions, intermolecular additions and intramolecular cyclizations. Additions are favored usually by high concentrations of reactants, but cyclizations are favored by low concentrations. Thus, unless one wishes to run all the additions in a single step and all the cyclizations in a second step (possible, but unwise), greater control over multistep synthesis is necessary. With hindsight, one of the key difficulties in the synthesis of the cube involves mixing an addition and a cyclization at high concentration on the first step of the synthesis; this strategy results in many side products that in turn necessitate denaturation and reconstitution of the key intermediate.

The new procedure liberates cohesive ends by the controlled removal of individual DNA hairpins, utilizing restriction endonuclease digestion. This is analogous to the deprotection of a functional group for use in a reaction. It is directly comparable to detritylation in solid-phase oligonucleotide synthesis,[21] a successful synthetic procedure we have tried to emulate within the constraints of this system. Stepwise restriction (deprotection) provides control over the formation of individual edges. We term the deprotecting restriction enzymes, "growth enzymes".

There are a number of restriction enzymes that recognize interrupted sequences, or sequences that are removed from the site of scission. AlwN I, Bgl I, BstX I, Dra III, PflM I, and Sfi I are

Figure 1. Elimination of restriction sites for enzymes that recognize interrupted sequences or that are distant from their scission site. (a) An interrupted site: The Bgl I site is underlined in this hairpin. After restriction, half of it is washed away. A new sequence, complementary to the sequence in the middle is ligated on, and the new DNA is immune to Bgl I. (b) A site distant from the site of scission. A Bbv II site is underlined in the hairpin. After restriction, the hairpin is washed away. Both schemes assume that the remaining part can be retained. In practice this is simple in a solid-state synthesis context and is even feasible in solution for special cases.

useful examples of the first group, which we call type A, and Bbv I, Bbv II, BsmA I, BspM I, Fok I, Hga I, and SfaN I are useful examples of the second group, termed type B. All of these enzymes expose 3–5 potentially *asymmetric* positions on the freed sticky end after restriction. By utilizing type A or type B growth enzymes, it is possible to destroy or eliminate the restriction site when a new molecule is ligated to the cohesive end. The principle is illustrated in Figure 1 for each of these two types of restriction enzymes. This feature of the new synthetic scheme makes the newly formed edge immune from traces of the restriction enzyme. In addition, elimination of the restriction site allows repeated use of the same restriction enzyme. This is a well-known strategy used in molecular cloning.[22]

Covalently-Closed Topologically-Bonded Intermediates and Products. There are several favorable consequences of the closed nature of the intermediate and final products. For one, the products may be subjected to vigorous treatment (e.g., heat denaturation or phenol extraction to remove enzymes), without irreversibly disrupting the structure. Furthermore, the covalently closed nature of the successfully ligated product means that failure products in addition steps can be treated with an exonuclease (e.g., exonuclease III), to destroy unsuccessfully ligated molecules (Figure 2). This treatment is analogous to "capping" in solid-phase oligonucleotide synthesis:[21] Destruction of unsuccessfully ligated material (failure products) is a great aid in purification of the target product.[4,20]

Solid-Support Based Synthesis. The complete removal of restricted hairpins, restriction enzymes, exonuclease III, or excess added DNA is difficult in solution. Therefore, we have adapted the synthesis to a solid-support scheme.[23] By using a solid support, the growing molecule is readily separated from reagents and cleavage products, particularly after restriction endonuclease digestion, when denaturing conditions can destroy the intermediate. Purification in a solid-support context entails washing, rather than inefficient and tedious gel purifications. In addition, growing

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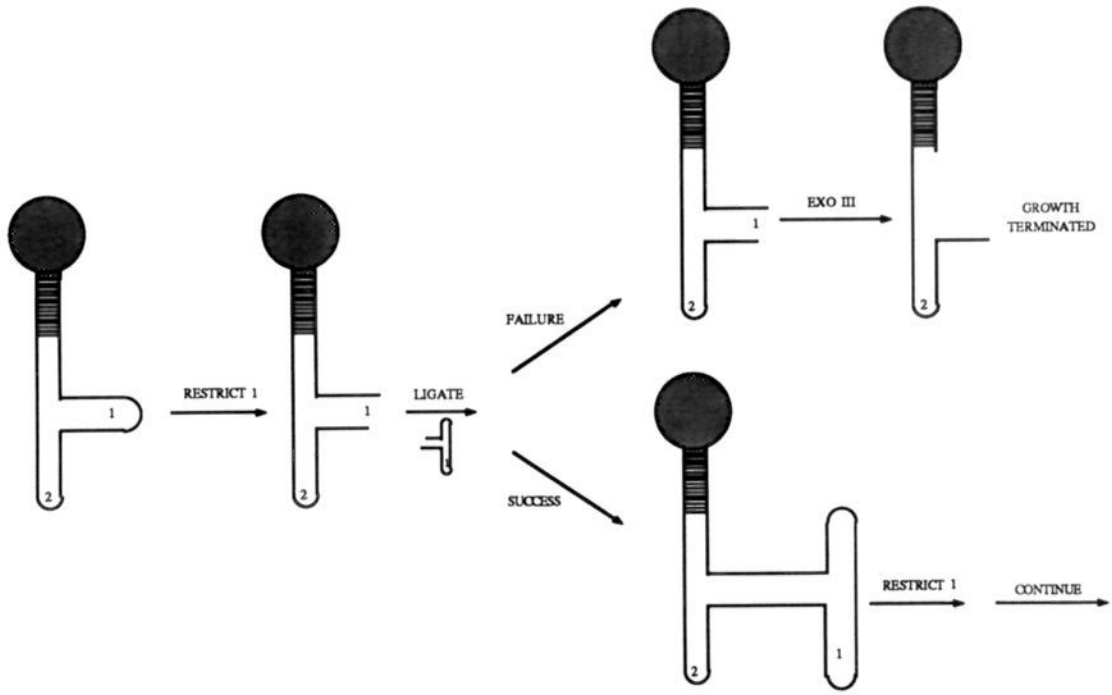

Figure 2. Solid-state synthesis with exonuclease III destruction of unligated material. The solid support is represented as the gray ball, and the cross-link is illustrated as crossbars. The portion below the cross-linked site has a restriction site for removal from the support. This diagram illustrates the use of solid-state synthesis in the first step of a synthesis. We emphasize the case of imperfect yield on a ligation step. Because the successful ligation results in a closed object, exonuclease III treatment only destroys the failure object, not the successful ligation, which can then continue. Thus, synthesis using this strategy destroys failure products, thereby effecting a purification on the support. In a complicated synthesis, the product may not be degraded all the way, but it should still be separable from the successful product.

Table I. DNA Sequences[a]

strand name	sequence
S-Strand-1	PO_4-C-C-T-A-A-C-G-C-G-T_6-T_{16}-SS
X-Strand-1	A-A-A-A-A-A-C-G-C-G-T-T-A-G-G-T-G-A-C-A-T-A-T-A-T-C-T-A-T-A-G-A-T-A-T-A-T-T-T-C-G-G-A-C-C
X-Strand-2	PO_4-C-G-G-C-G-G-T-C-C-G-A-A-A-T-A-T-A-T-C-T-A-T-A-G-A-T-A-T-A-T-G-T-C-A
J-Strand-1	G-C-C-G-A-A-G-C-C-A-C-A-C-C-*A-T-C-C*-A-C-G-T-A-C-A-A-T-G-A-T-C-T-*C-C-A-T-C-A-C*-C-T-G-G-A-G-C-T-T-T-T-G-C-T-*C-C-A-G-G-T-G-A-T-G-G*-A-G-A-T-T-A-A-C-G-*C-A-G-G-T-G*-C-T-G-C-T-C-C-T-T-T-T-G-G-A-G-*C-A-G-C-A-C*-C-*T-G*-C-G-T-T-A-C-A-T-T-G-T-A-C-G-T-*G-G-A-T-G*-G-T-G-T-G-G-C-T-T
J-Strand-2	A-G-A-T-G-A-G-T-A-T-T-A-A-C-G-*C-A-G-G-T-G*-C-T-G-C-T-C-C-T-T-T-T-G-G-A-G-*C-A-G-C-A-C*-C-T-*G*-C-G-T-T-A-G-A-C-C-T-A-C-G-A-C-T-T-T-T-G-T-C-G-T-A-G-G-T-C-A-T-A-C-T-C-A-T-C-T-C-A-C
J-Strand-3	A-G-A-T-G-A-G-T-A-T-T-A-A-C-*G-C-C-G-G-T-G*-C-G-G-C-T-C-C-T-T-T-T-G-G-A-G-*C-C-G-C-A-C*-C-*G-G-C*-G-T-T-A-G-A-C-C-T-A-C-G-A-C-T-T-T-T-G-T-C-G-T-A-G-G-T-C-A-T-A-C-T-C-A-T-C-T-C-A-C
L-Strand-1	G-C-C-G-T-T-G-A-C-G-A-C-T-*G-T-C-T-C*-G
L-Strand-2	C-G-G-C-C-*G-A-G-A-C*-A-G-T-C-G-T-C-A-A
A-Strand-1	C-G-G-C-G-A-C-G-C-G-G-T-T-T-T-T-T-A-C-C-G-C-G-T-C

[a] Sequences are presented 5′ → 3′. 'PO_4' at the 5′ end indicates chemical phosphorylation. 'SS' indicates the Teflon-based solid support. Restriction sites are in *italics*. Excisable hairpins embedded in longer strands are underlined. Exposed or exposable sticky ends are in **bold**.

objects are isolated from each other, preventing cross-reactions between them; this feature permits the use of symmetric or blunt-ended restriction sites in cyclization reactions. The covalently-closed nature of the growing object is maintained on the support by interposing a covalently cross-linked duplex linker between the support and the object. In principle, reagents and enzymes may be recovered from washes, although we have not done so in this work. Solid-phase gene assembly is already being used to make long linear DNA molecules.[24,25]

Overview of the Methodology and Its Application. The procedure contains the following steps: (1) attachment of the first unit to a solid support, (2) restriction of one or two hairpins to expose sticky ends, (3) additive or cyclizing ligation to form the next edge, (4) exonuclease destruction of failure products, (5) removal from the support, and (6) annealing the released product to covalency. Steps (2), (3), and (4) are repeated as many times as necessary, in order to build up the connectivity of the object. We demonstrate the effectiveness of this procedure by using it to synthesize a quadrilateral molecule. Scheme I illustrates the synthetic strategy. Two growth enzymes are employed in the demonstration synthesis detailed here; the same synthesis can be done with a single growth enzyme.

Materials and Methods

DNA Synthesis and Sequences. DNA molecules are synthesized and deprotected by routine phosphoramidite procedures,[21] on an Applied Biosystems 380B automated DNA synthesizer. Conventional phosphoramidites are purchased from Applied Biosystems (Foster City, CA). Chemical 5′ phosphorylation is done by the method of Horn and Urdea.[26] The 5′ phosphorylation reagent and the Teflon-based solid support[27] are obtained from Glen Research (Sterling VA). All strands greater than 30 nucleotides in length are purified by polyacrylamide gel electrophoresis. Shorter strands are purified by preparative HPLC as described previously.[28] Molecules are prepared for use under native conditions by

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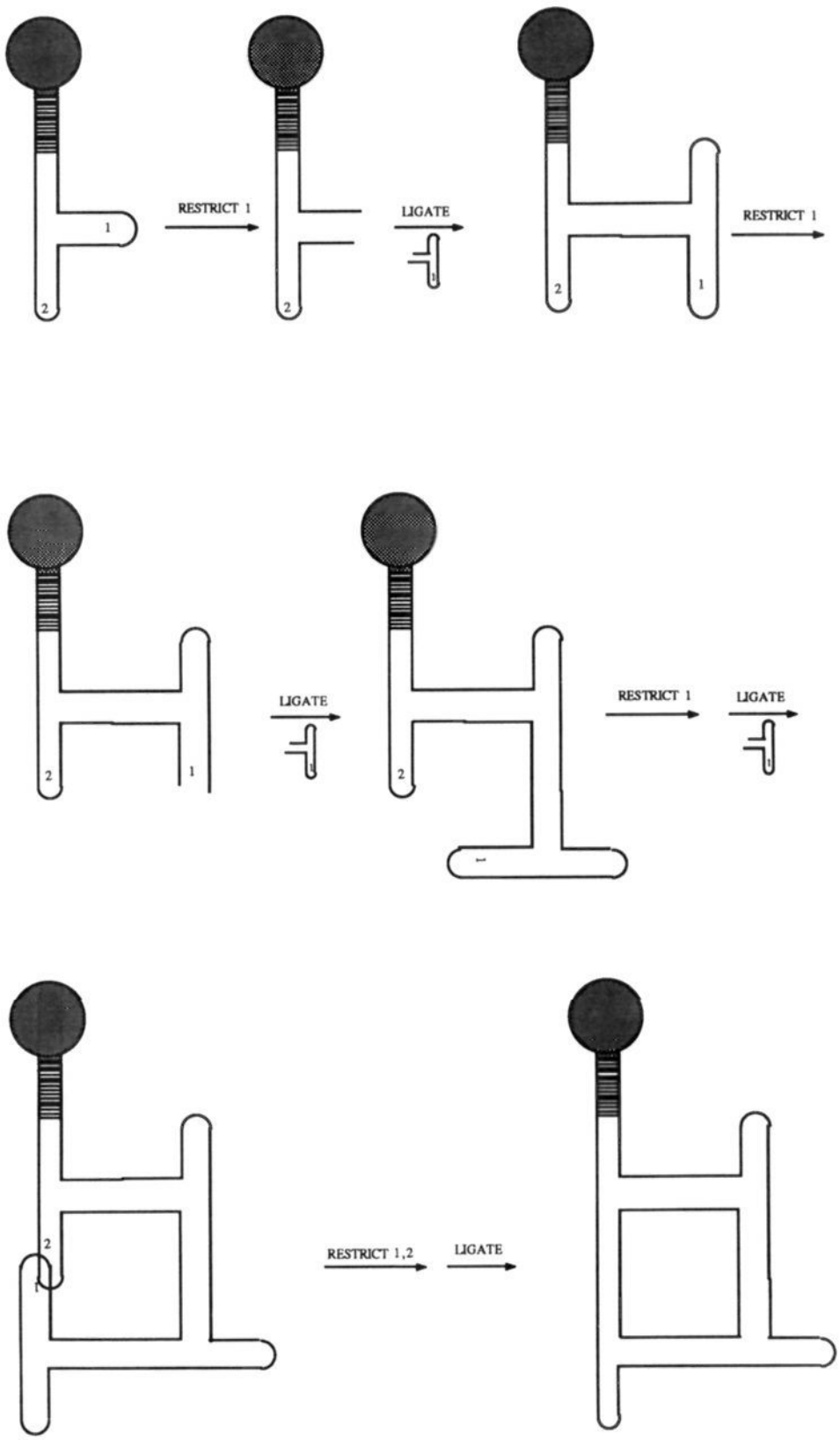
Scheme I. Protocol for the Synthesis of a Quadrilateral[a]



[a] Beginning with the support containing J-Strand-1, alternate cycles of restriction and ligation are performed, always at the position indicated as '1'. The demonstration synthesis uses two different enzymes to liberate the same sticky end at site 1, but the synthesis has also been performed with a single enzyme. Selection of the target product (triangle, quadrilateral, pentalateral, ...) is determined by the point at which one chooses to restrict at site 2, exposing a sticky end complementary to that exposed by restriction at site 1. Previous work with three-arm junctions indicates that a large number of different cyclic products can be produced from such components.[18]

heating to 90 °C, followed by slow cooling to equilibrate them to optimally hybridized structures.

The sequences of the branched DNA molecules used in this work (Table I) have been assigned by the program SEQUIN,[17] according to previously described symmetry-minimization algorithms.[1,2,17] S-Strand-1 is synthesized directly on the Teflon-based solid support. The partially complementary molecules used in a psoralen-cross-linked linker between the solid support and the growing DNA object are X-Strand-1 and X-Strand-2. The three-arm junction attached to the cross-linked duplex is J-Strand-1; it contains AlwN I (CAGNNN|CTG) and PflM I (CCANNNN|NTGG) restriction sites for excision of hairpins and a Fok I (GGATG $^{9}/_{13}$) site for removal from the support. The two three-arm junctions with a single restriction site are J-Strand-2, containing an AlwN I site, and J-Strand-3, containing a Bgl I (GCCNNNN|NGGC) site, which exposes the same GTG sticky end as the AlwN I site of J-Strand-2. In order to anneal the final closed quadrilateral, it is necessary to put a double-stranded linker, composed of L-Strand-1 and L-Strand-2, containing a BsmA I (GTCTC $^{1}/_{5}$) site between the psoralen-cross-linked linker and J-Strand-1. The strand that anneals the quadrilateral is A-Strand-1.

Psoralen Cross-Linking. The protocol of Sinden and Cole[29] is modified as follows: 10 μg of X-Strand-1 and 7 μg of X-Strand-2 are mixed in 100 μL of a solution containing 40 mM Tris acetate, pH 8.1, 2.0 mM Na acetate, 2 mM EDTA, 12.5 Mg acetate (TAEMg), and 1 mM 4,5′,8-trimethylpsoralen (Sigma) at 4 °C for 30 min. The solution is exposed to 360-nm irradiation produced by a UVGL-25 Mineralite lamp (Ultra-Violet Products, San Gabriel, CA) for 3–5 h. The cross-linked material is separated from the uncross-linked material by denaturing polyacrylamide gel electrophoresis.

Denaturing Polyacrylamide Gel Electrophoresis. Gels contain 8% acrylamide (19:1, acrylamide/bisacrylamide) and 8.3 M urea and are run at 55 °C. They are prepared and treated as described previously.[28]

Enzymatic Reactions: A. General Considerations Involving the Solid Support. Restrictions and ligations are done on the solid support. At the end of the reaction, unwanted enzymes, unreacted ligation materials, or freed digestion products are separated from the solid support by washing. Five cycles of washing prove sufficient for the purposes of the next stage. Closed molecules on the support are denatured by heating to 90 °C and renatured by slow cooling.

B. Kinase Labeling. Two micrograms of an individual strand of DNA is dissolved in 20 μL of a solution containing 66 mM Tris·HCl, pH 7.6, 1 mM spermidine, 100 mM $MgCl_2$, 15 mM dithiothreitol (DTT), 0.2 mg/mL nuclease free bovine serum albumin (BRL), and mixed with 1–2 μL of 1.25 μM γ-^{32}P-ATP (10 mCi/mL) and 2 units of polynucleotide kinase (Boehringer) for 45 min–2 h at 37 °C; radioactive labeling is followed by addition of unlabeled ATP to 1 mM, and incubation proceeds for another 10 min. The reaction is stopped by phenol/chloroform extraction and ethanol precipitation of DNA.

C. Ligations. Ligations are performed in the kination buffer, which is brought to 66 μM ATP. T4 polynucleotide ligase (U.S. Biochemical) (20–40 units) is added, and the ligation proceeds at 16–22 °C for 16–18 h. The reaction is stopped by washing the solid support five times at 65 °C. A 10–20-fold excess of DNA is added for each growing object on the support.

D. Restriction Endonuclease Digestions. Restriction enzymes are purchased from New England Biolabs and used in buffers suggested by the supplier, but usually in much larger quantities and for much longer times than recommended for unbranched DNA molecules.[20] The reactions are stopped by washing the solid support five times at 50 °C; the lower temperature is used because the product is no longer covalently closed.

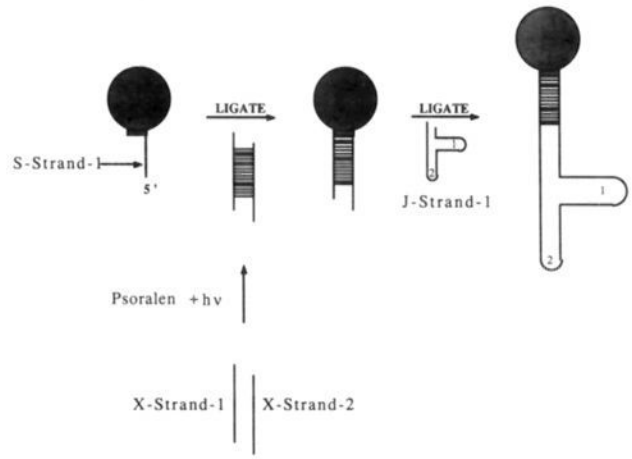
E. Exonuclease III Treatment. Exonuclease III (U.S. Biochemical) digestion is performed in a buffer containing 50 mM Tris·HCl, pH 8.0, 10 mM 2-mercaptoethanol and 5 mM $MgCl_2$ at 37 °C. The reaction is stopped by washing the solid support five times at 65 °C.

Results

Preparation of the Solid Support: A. General Strategy. The preparation of the starting solid-support is outlined in Scheme II. The Teflon-based support is used as a solid-support for the normal 3′ → 5′ synthesis of S-Strand-1, which remains attached to it after deprotection. This strand is hybridized to a psoralen-cross-linked duplex composed of strands X-Strand-1 and X-Strand-2. J-Strand-1 is then ligated on the exposed sticky end of the X-Strand duplex. Cross-linking the X-Strand duplex renders topologically-bonded molecules ligated to it resistant to denaturing conditions.

B. The Solid-Support Material. S-Strand-1 is synthesized directly on the Teflon-based support described by Lohrmann et al.[27] It is key that this floss-like material does not bind strands

Scheme II. Preparation of the Support[a]



[a] The first stage is the synthesis of S-Strand-1 directly on the Teflon-based support. X-Strand-1, partially complementary to this strand, is psoralen-cross-linked to X-Strand-2, which is then ligated to S-Strand-1, to form a nondenaturable double-helical base for the synthesis. This duplex is then ligated to J-Strand-1 to complete preparation of the support.

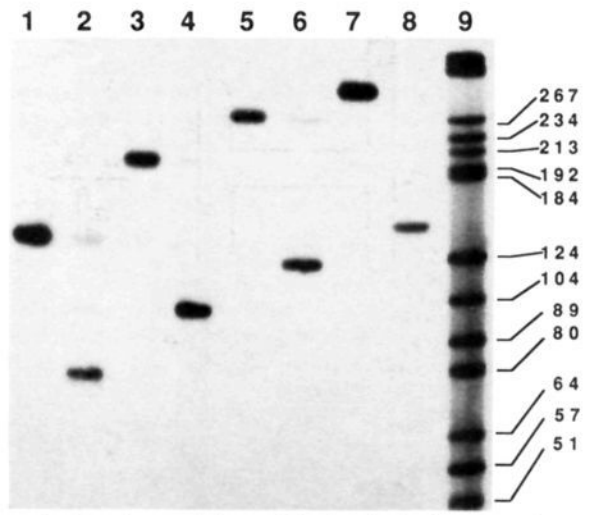


Figure 3. Stepwise restriction and ligation in the formation of a quadrilateral. This is an autoradiogram of an 8% denaturing gel, illustrating the demonstration synthesis of a quadrilateral, as cartooned in Scheme I. Lane 9 contains a series of length markers. Lanes 1–8 contain aliquots of the successive products of the synthesis that have been released from the solid support, containing the L-Strand linker, by digesting with BsmA I: Lane 1 contains released J-Strand-1, lane 2 contains the digestion product after restriction with AlwN I, lane 3 contains the ligation product of adding J-Strand-3 to the material in lane 2, lane 4 contains the Bgl I restriction product of the material in lane 3, lane 5 contains the ligation product of adding J-Strand-2, lane 6 contains the AlwN I digestion product of the material in lane 5, lane 7 contains the ligation product of adding another copy of J-Strand-3, and lane 8 contains the Bgl I digestion product of this material. Each strand, including J-Strand-1 is 5′ labeled. The entire single-stranded molecule is visible on this autoradiogram in the ligation bands. However, the only portion of the growing molecule visible upon restriction is that part seen in Scheme I that traces counterclockwise around the molecule, starting from the left side of the support; the part on the right side is unlabeled until the final cyclization ligation (Figure 6) joins the 5′ label of J-Strand-1 to the outer portion of the quadrilateral made up of the 3′ ends of J-Strand-2 and J-Strand-3.

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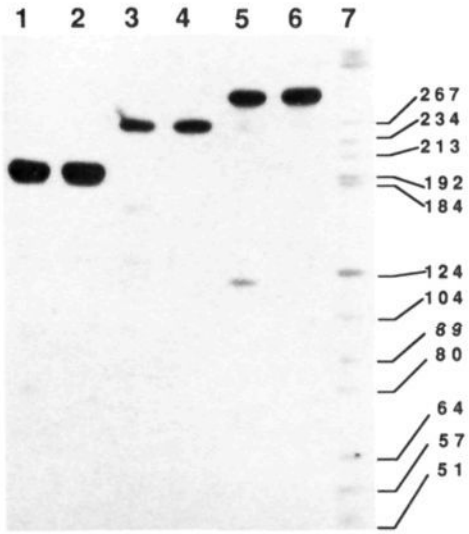

Figure 4. Exonuclease III digestion of ligation failures on the support. This is an autoradiogram of an 8% denaturing gel. Lane 7 contains length markers. Lanes 1, 3, and 5 contain the raw ligation products of the synthesis: lane 1 contains the ligation products of adding a single junction (J-Strand-3) to the AlwN I restriction product J-Strand-1, lane 3 contains the products of adding J-Strand-2 to the Bgl I restriction product of the material in lane 1, and lane 5 contains the products of adding J-Strand-3 to the AlwN I restriction product of the material in lane 3. Lanes 2, 4, and 6 illustrate the removal of imperfectly ligated materials by treatment with exonuclease III for 30–60 min on the support. The disappearance of the major contaminating materials is evident.

in a nonspecific fashion: Virtually all added radioactive DNA is released from the support by 2 h of restriction endonuclease digestion.

C. Psoralen Cross-Linking. Cross-linking the X-Strands keeps the growing structure covalently-closed and topologically-bonded at the nongrowing end. Approximately half of the starting material is cross-linked by the procedure described above, and the purified yield is about 20% of the total starting material. Treatment with exonuclease III for 1 h does not yield very short products, suggesting that cross-linking blocks the action of the enzyme (data not shown). The digested duplex contains 50–60 nucleotides, indicating that psoralen binds near the 3′ ends of both strands.

D. Attachment of the Cross-Linked Duplex to the Support. The first opportunity to control the "loading" of the support with active groups comes when the cross-linked duplex is ligated to the support. By trityl estimation, 4300 pmol of DNA are loaded with 80 pmol of the cross-linked duplex. The attachment is largely finished after 8 h and yields 70 pmol loaded duplex after 16 h (data not shown). The specificity of DNA binding obviates the need to "cap" or destroy free S-Strand-1 sites.

E. Ligation of the First Branched Group to the Support. Attachment of the first junction (J-Strand-1) provides a second opportunity to limit the nearest neighbor contacts of growing objects. J-Strand-1 (20 pmol) is ligated to the support in approximately 85% yield, estimated from radioactivity. Thus, 17 pmol of active support is attached to a field containing 4300 pmol S-Strand-1 starting chains. Approximately one potential site in 16 is occupied in each direction of a hypothetical two-dimensional growing surface. This is a ratio that may need to be modified when large objects are constructed.

Restriction and Ligation on the Support. The test molecule synthesized as a demonstration of this methodology is a quadrilateral. The route to its synthesis is illustrated in Scheme I. The basic procedure is the successive restriction of, and ligation to, the restriction site labeled 1. The site may be an interrupted site for two different enzymes, as long as the exposed nucleotides are the same. In fact, we have alternated between restriction with AlwN I and restriction with Bgl I. In each case, the 3′ overhang G-T-G is exposed; it coheres with, and is ligated to, a molecule of J-Strand-2 or J-Strand-3 with a 3′ C-A-C overhang. The progress of this synthesis is illustrated in alternating lanes in Figure

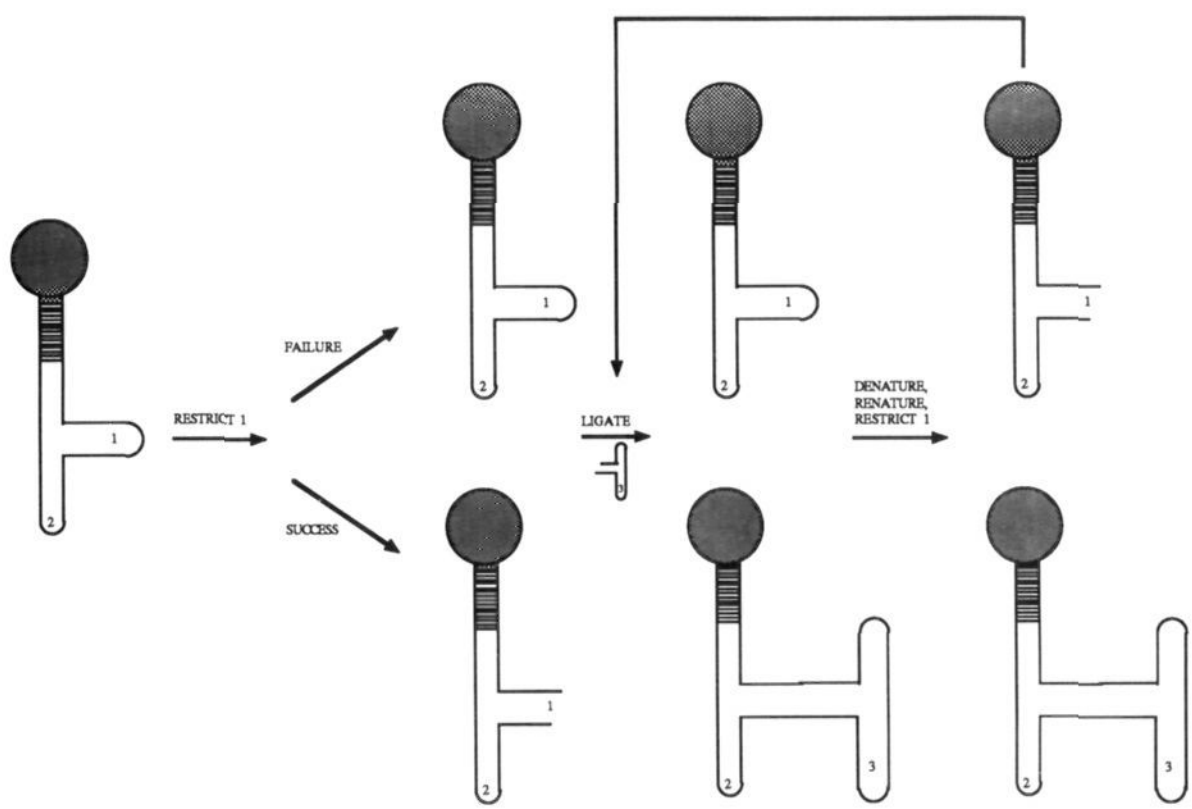

Figure 5. The strategy of a restriction rescue procedure. The rescue procedure is illustrated for the first restriction and ligation. After restricting at site 1, the successful molecule is ligated to a second junction containing site 3. It cannot contain site 1. Those molecules that fail to be restricted are then subjected to denaturation–renaturation, so as to expose and/or form their sites more perfectly. This procedure does not harm the successfully ligated molecules. At this point, one can then restrict at site 1 again and add more of the strand containing site 3.

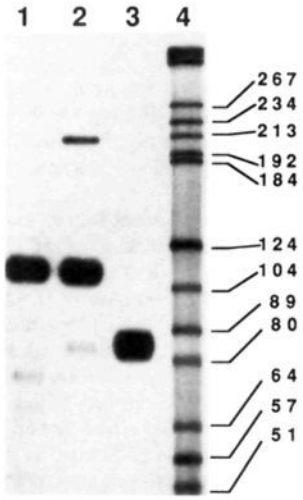

Figure 6. Cyclization on the support. This is an autoradiogram of an 8% denaturing polyacrylamide gel. Lane 4 contains a series of length markers. Lane 3 contains the digestion products of the complete addition product (three additions, four total junctions) that have been restricted by PflM I and Bgl I. This double digestion liberates an 82-mer linear strand (the eventual inside strand of the quadrilateral) which is visible as an intensely labeled band. The intensity is a result of the band having been labeled (statistically) three times, once each addition. A short labeled linear molecule, corresponding to the 5′ end of J-Strand-1 upstream from the PflM I restriction site (2 in Scheme I) is too short to be seen on this gel. The bulk of the length of the outside strand is unlabeled. Lane 2 illustrates the cyclization reaction, an 82-mer circle, whose mobility can be calibrated against standards;[18,32] virtually all of the material has cyclized. This lane also contains a 206-mer linear strand, corresponding to the unannealed outside strand of the quadrilateral. Note that this strand is only labeled once, and that the label is much older than the label in the cyclic strand. Lane 1 contains the results of treating this material with exonuclease III. The linear is seen to be susceptible to the enzyme, but the cyclic molecule is unaffected.

3. This denaturing gel shows the labeled single strand liberated when an aliquot of the material is cleaved from the support. Lane 1 corresponds to unrestricted starting support (with J-Strand-1 attached), the post-restriction lanes (2, 4, and 6) contain the outer strand (Scheme I), and lane 8 contains the results of cleaving the four-junction molecule only at site 1 but not at site 2. The post-ligation lanes (3, 5, and 7) contain the entire single-stranded molecule. Each ligation step is followed by treatment with exonuclease III, to destroy unligated material. It is evident from this gel that it is possible both to restrict and to ligate when the growing object is attached to the solid support.

Exonucleolytic Digestion of Failure Products. Purification of target product from failure products is a major problem with complex objects. For example, a great deal of experimentation has been necessary to determine conditions whereby the six-cycle cube-like molecule may be separated from the corresponding five-cycle failure product.[20,30] The problem is addressed in oligonucleotide synthesis by means of the capping reaction:[21] A chain that fails to elongate in a given cycle is capped with acetate so that it never extends further. We have sought to include a similar aid to purification during synthesis, namely exonuclease III digestion of unligated strands. Figure 4 illustrates the efficacy of exonuclease III treatment after each ligation step. The final product is virtually pure, despite the transient appearance of failure products.

Rescue of Failed Restriction Endonuclease Digestions. Restriction is not always completely successful on the support. However, heat denaturation of the molecular arrangement on the support will often liberate or properly form the target restriction site. The basis for this "rescue" procedure is illustrated in Figure 5. Rescue must be performed after ligating closed molecules that are restricted successfully. Yields can be increased markedly if this procedure is followed. Two growth enzymes are necessary to perform rescues, although their exposed nucleotides may be the same (Figure 5); if only one enzyme is used, heterogeneous products will result. In one rescue experiment, five successive restriction cycles leave 52%, 39%, 23%, 21%, and 10% total unrestricted hairpin. The synthesis shown in Figure 3 does not include the rescue operation, but restriction there has been performed with three times the normal amount of restriction enzyme.

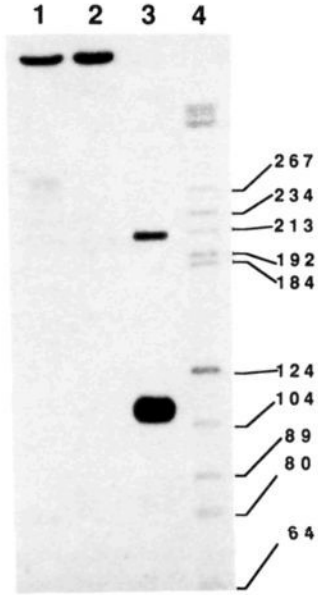

Figure 7. Annealing the final product. This is an autoradiogram of an 8% polyacrylamide denaturing gel. The radioactivity has not been scaled between lanes on this gel. Lane 4 contains a series of length markers. Lane 3 contains the unannealed product of the final cyclization of the quadrilateral. Lane 2 contains the annealed material, and lane 1 contains the product of treating this material with exonuclease III, to demonstrate that it is largely cyclic material. This ligation is necessarily performed in solution, using ligation protocols standard for this system.[20]

Cyclization on the Support. One of the key features of this procedure is the ease with which it is possible to close cyclic molecules on the support. Figure 6 illustrates that the two overlapping hairpins (indicated 1 and 2 in Scheme I) can be restricted and then ligated together. No other closed side products result. Furthermore, the extent to which the central strand is cyclized appears to be largely complete. The total yield of the quadrilateral is about 60% of the starting material, for an average addition step yield of about 84%.

Annealing the Complete Product. When the target molecule is released from the support, it contains an open double helical end. This end is annealed with a hairpin, in order to create an intact molecule that can be treated vigorously. Figure 7 illustrates the closure of the end released from the support. The value of the restriction-site-removal strategy in performing the enzymatic steps is highlighted by our experience with this step. Releasing the complete quadrilateral from the support by using the Fok I site on J-Strand-1 yields material that cannot be annealed. This problem can be remedied by the insertion of a linker (L-Strand-1 and L-Strand-2) containing a BsmA I site between the support and J-Strand-1. BsmA I digestion results in cleavage at the same position, but trace amounts of BsmA I cannot harm the annealed quadrilateral as Fok I can. Thus, our inability to remove all traces of Fok I, whose restriction site is on the quadrilateral, prevents annealing if that enzyme is used to release the product. We experience very little success when ligating restricted hairpins in solution without this strategy (unpublished).

Discussion

The solid-support synthesis system developed here allows a much greater degree of control over the construction of DNA objects than the solution techniques described earlier.[4,18-20] We are able to target the synthesis of a given edge and then form it from a

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restricted hairpin, by either addition or by cyclization. Addition requires restriction endonuclease digestion of only a single hairpin, followed by addition of a junction, whereas cyclization requires restriction of two adjacent hairpins, followed by ligation of their overlaps. The procedure we describe removes the sensitivity of the products to the concentration of the reactants. In so doing, we have returned the apparently convenient scheme of addressing sticky ends with their complements[1,2] to a methodology that works for flexible branched DNA molecules as well as for linear molecules. Restriction site elimination removes sensitivity of the ligation step to contaminating traces of restriction enzymes. In addition, failure to restrict is demonstrated to be a remediable problem. Purification is facilitated (at the cost of yield) by exonuclease III digestion of ligation failures on the support. The overall efficiency here (60% yield) is much greater than in the solution synthesis of a comparable quadrilateral (15%).[4]

It is evident from the presentation here that the success of each step is assayed by gel electrophoretic analysis of an aliquot of the growing material. This procedure is adequate here, but it would be desirable to have a reliable assay that depends upon the analysis of protecting groups (restricted hairpins), in the same way that trityl analysis is used to monitor oligonucleotide synthesis.[21] We find that hybridizing liberated hairpins with an excess of labeled perfect complement, followed by analysis on a native gel, is not a reliable means for estimating the extent of restriction. Incorporation of well-defined radioactive or nonradioactive label in the hairpin itself is probably necessary to eliminate aliquot analysis.

Attachment to the growing support implies the presence of an umbilical arm. The polygon built here has four external arms, and the annealed umbilical arm is merely one of them. Polygons or polyhedra that are designed to be attached to each other require external arms at some junction vertices, in addition to those necessary for their internal connectivities. Thus, the extra arms concomitant with this technology are not likely to be a major problem for this application. Nevertheless, it is possible to eliminate the extra arm, if one designs the umbilical arm to be half of the last edge to close: One can restrict the arm forming the other half of its edge, wash, release from the support, and perform the last ligation in solution. This strategy will eliminate extra external arms, if they are unwanted.

The quadrilateral is synthesized from individual junctions. Each junction is covalently closed, except for the sticky end, and it is ligated under native conditions. There is no apparent reason why this methodology need be limited to the addition of junctions. In principle, one should be able to combine polygons in the same way, in order to create polyhedra. It should also be possible to combine polyhedra with individual sticky ends to create two-dimensional and perhaps three-dimensional periodic arrays (crystal nuclei).[1-3] The starting materials (individual polygons or polyhedra) could also be synthesized by the solid-state methodology described here. Whereas DNA objects are fundamentally specific polycatenated strands,[20] this methodology should also be applicable to the construction of polycatenated polymers.

If one wishes to use starting materials that are closed and more complex than the individually synthesized strands employed here, it is important to have as many restriction sites available as possible; product immunity requires that each edge built from a type A enzyme uses two enzymes, although each edge built from type B enzymes needs only a single enzyme. A major advantage of the solid-support technique is that growing objects are isolated from each other. As noted above, this means that one need not worry about intermolecular interactions between growing objects, thereby virtually eliminating multimeric byproducts at each stage of addition or cyclization. In addition to increasing the specificity of products, the isolation of individual objects permits symmetric sticky ends to be used as type A pairs for cyclizations and (with reduced efficiency) for additions. A new set of restriction enzyme groups becomes available for use as virtual type A pairs, when this is the case. Examples of groups of symmetric-site-cleaving restriction enzymes with different specificities that produce identical tetranucleotide sticky ends are [Bgl II, Bcl I and BamH I], [Nhe I, AvrII, Xba I, and Spe I], [Mlu I and BssH II], [Xma I and Bsp MII], [Xho I and Sal I], [Nco I and BspH I], and [Pst I and Nsi I]. In principle, even pairs of restriction endonucleases that produce blunt ends can be used this way.

Building macroscopic objects in this fashion will eventually become quite tedious. In this regard, it is worth noting that the solid support used here is in fact a support that is satisfactory for use in an automated synthetic procedure.[27] We do not describe any synthetic steps that appear to have an absolute requirement for human intervention. The recent development of chemical catalysis[31] of comparable efficiency to enzymatic catalysis of DNA ligation is an encouraging result that may simplify the automated synthesis of DNA-based geometrical objects. Automation is expected to hasten the construction of the highly complex DNA objects that have been proposed.[1-8]

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