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Synthesis and characterization of topologically linked single-stranded DNA rings

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Abstract

We investigated the synthesis of linked-ring DNAs by two DNA-ligation-based methods. In the first method, two DNA oligonucleotides were associated through a duplex segment of more than a full helical turn. Circularization of the entwined oligonucleotides by T4 DNA ligase resulted in two linked-ring DNAs with a total yield of ~40%. In the second method, a DNA oligonucleotide was circularized over a circular DNA template, resulting in the formation of ~10% linked-ring product. The circular nature of linked-ring DNAs was verified with exonuclease digestion and the existence of topological linkages was demonstrated by analyzing the electrophoretic mobility pattern of DNA products obtained from the digestion of each linked-ring DNA using specific restriction endonucleases. A linked-ring DNA library in which one of the two rings contained random-sequence nucleotides was also constructed and tested for compatibility with in vitro selection.

Keywords: DNA ligation; T4 DNA ligase; In vitro selection; Deoxyribozyme; DNAzyme

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1. Introduction

DNA is best known for its double-helical character and its universal role in living organisms as a reservoir for genetic information. When liberated from its helical structure confinement, DNA is also capable of forming complex tertiary structures and performing binding and catalytic functions. DNA molecules with binding abilities are called DNA aptamers while DNA molecules with catalytic abilities are known as deoxyribozymes, DNAzymes, or DNA enzymes [1–3]. Various deoxyribozymes have been made to catalyze reactions including RNA cleavage [4], RNA ligation [5], DNA ligation [6], DNA cleavage [7], DNA phosphorylation [8], DNA adenylation [9] and porphyrin metallation [10]. DNA enzymes are regarded as valuable molecular tools and can be used for a variety of applications. For example, fluorescence-signaling deoxyribozyme reporters have been engineered for the detection of metal ions [11], ATP [12], nucleic acids [13], and even pH changes [14]. However, most known deoxyribozymes are significantly less efficient than protein enzymes. This is reflected by the fact that the catalytic rates (k_{cat}) exhibited by the very best deoxyribozymes are still several orders of magnitude smaller than their protein counterparts [15,16]. Therefore, creating DNA enzymes with faster catalytic rates is one of the challenges faced by deoxyribozyme engineers.

Nearly all known deoxyribozymes are small in size, typically containing a few dozen nucleotides. In addition, all deoxyribozymes are single-chain catalysts and often use simple Watson–Crick helical elements or guanine-quartets in their tertiary folds [17]. The relatively moderate catalytic rate seen with existing deoxyribozymes may correlate with their diminutive size and structural simplicity. This speculation draws indirect support from ribozyme studies. It is well established that ribozymes with complex catalytic functions or large catalytic rates are huge RNA molecules. These include naturally occurring group I, group II, and RNase P ribozymes, and an artificial ribozyme known as "class I ligase" [18–21]. The latter ribozyme, catalyzing template-directed RNA ligation with a remarkable k_{cat} of more than $1\,s^{-1}$, was originally isolated from a random-sequence RNA pool composed of RNA molecules nearly 300 nt [22]. To date, almost all deoxyribozymes have been created using libraries that typically contained linear DNA molecules of 100 nucleotides or less. Therefore, it is conceivable that efficient deoxyribozymes might be obtained from larger, more complex DNA libraries.

We believe assemblies made of two topologically linked single-stranded DNA rings (denoted linked-ring DNAs in this study) can offer more complex structures than linear DNA molecules, and using linked-ring DNA libraries to conduct in vitro selection might lead to the isolation of catalytic DNAs with new or improved functions. To explore topologically linked DNA molecules for in vitro selection, we first need to establish an effective method for converting linear DNA oligonucleotides into desirable ring structures with a high yield. Of particular interest is finding enzymatic ways to produce linked circular DNA molecules. There are two groups of DNA manipulating enzymes that could potentially be exploited for this purpose: topoisomerases and DNA ligases. Topoisomerases and related DNA recombination enzymes are known to act on circular DNA substrates to produce different types of

knots or catenanes [23–25]. However, since these enzymes usually produce a series of knotted and catenated products, it is difficult to control their activity to obtain a single desirable linked-ring product in good yield. For this reason, topoisomerases are not particularly suited for controlled linked-ring DNA construction. DNA ligases are known to perform high-efficiency template-directed DNA ligation by joining an acceptor DNA to a 5′-phosphorylated donor DNA when both molecules are properly juxtaposed within a duplex [26]. DNA ligases have been used to construct complex DNA structures (including a DNA cube [27] and Borromean rings [28]) from "sticky-end" DNA molecules [29]. Since it is easy to control templated DNA ligation, we speculated that it should be possible to produce topologically linked DNA rings in good yield by DNA ligation. In this report, we will describe two simple methods for constructing such DNA ring structures through T4 DNA ligase-mediated ligation of synthetic DNA oligonucleotides.

2. Materials and methods

2.1. Materials

Synthetic oligonucleotides were prepared by automated DNA synthesis using standard cyanoethylphosphoramidite chemistry (Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University) and purified by 10% preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE). Purified oligonucleotides were dissolved in water and their concentrations were determined spectroscopically and calculated using Biopolymer Calculator program (accessed at http://paris.chem.yale.edu/extinct.frames.html). All chemical reagents were purchased from Sigma. T4 DNA ligase, T4 polynucleotide kinase, T4 DNA polymerase, and restriction enzymes were purchased from MBI Fermentas.

2.2. DNA phosphorylation using PNK and [γ-^{32}P]ATP

DNA molecules were labeled at the 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase (T4 PNK) using the manufacturer-supplied protocol. To ensure that all donor DNA molecules contain the 5′ phosphate required for ligation, T4 PNK-mediated end-labeling solution containing 2.5 μCi/μL [γ-^{32}P]ATP was further incubated with 1 mM ATP for 30 min at 37 °C. The phosphorylated DNA was purified by 10% preparative denaturing PAGE.

2.3. DNA ligation for linked-ring formation

DNA ligation reactions were performed typically in a 20-μL volume with all oligonucleotides and their ligation templates present in stoichiometric equivalents at a concentration of 0.3 μM. The oligonucleotide mixture in the 1× ligase buffer (supplied as 10× buffer by the manufacturer) was heated at 90 °C for 1 min and cooled to room temperature, followed by the addition of 1 U of T4 DNA ligase. After

incubation at room temperature for ~16 h, the reaction was quenched by the addition of an equal volume of 2× PAGE loading buffer (16 M urea, 5 mM Tris–borate, pH 8.3 at 23 °C, 0.3 M sucrose, 50 mM EDTA, 0.02% w/v xylene cyanol, and 0.02% w/v bromophenol blue), and 10 μL of the resulting mixture was loaded onto a 10% denaturing PAGE for analysis. The resolved DNA bands were quantitated using a Molecular Dynamics Storm 820 Phosphorimager with ImageQuant software.

2.4. Nuclease digestion with T4 DNA polymerase

Ligation reactions were performed as described above except at 2× scale. DNA was recovered by ethanol precipitation and dissolved in water. The 5× reaction buffer [335 mM Tris–HCl, pH 8.8 at 25 °C, 33 mM $MgCl_2$, 5 mM DTT, and 84 mM $(NH_4)_2SO_4$] was added to the mixture and the digestion was initiated with the addition of 5 U of T4 DNA polymerase (or water as control). The mixture was incubated at 37 °C for 16 h and the reaction was quenched with the addition of an equal volume of 2× PAGE loading buffer. Ten microliters was used for PAGE analysis.

2.5. Restriction digestion

Ligation reactions were performed as described above except at 20× scale. DNA was recovered by ethanol precipitation and purified by 10% preparative denaturing PAGE. Restriction digestion reactions were carried out in a volume of 10 μL at a concentration of approximately 0.2 μM DNA, with a 10-fold excess of the appropriate restriction template. The mixture was heated at 90 °C for 1 min and cooled to room temperature prior to the addition of the 10× buffer (supplied by the vendor) and 10 U of the restriction endonuclease. The reaction was incubated at 37 °C for 2.5 h and quenched with an equal volume of the 2× PAGE loading buffer. Ten microliters was used for PAGE analysis.

2.6. DNA preparation for MALDI-TOF mass spectrometry experiment

Ligation reactions and product purification were performed as described above except at 700× scale. The counter-ion in DNA was switched from Na^+ to NH_4^+ using a 10 K nanosep centrifugal concentrator (Pall Filtron) according to the manufacturer's specifications. The sample was first concentrated, and then washed three times with 200 μL of 50 mM ammonium acetate, followed by three washes (200 μL each) with H_2O. The final sample was dried and dissolved in 5 μL of water. The sample was analyzed by MALDI-TOF mass spectrometry at McMaster MS Facility Center.

2.7. In vitro selection

The selection scheme and sequences are shown in Fig. 5. The initial round used 100 pmol of 5′-phosphorylated ON5 (~1% ^{32}P-labeled) and was performed under the following conditions: 500 nM each of linear ON5, circular ON4, and linear ON6, and 0.25 U/μL T4 DNA ligase. Prior to the addition of the ligase, the reaction

was heated at 90 °C for 1 min and cooled to room temperature. After the addition of the ligase, the reaction was incubated at room temperature for 30 min. The DNA was precipitated with ethanol and analyzed by denaturing PAGE. The linked-ring band was excised and eluted, precipitated and washed with ethanol, and then resuspended in water. The amplification of selected sequences was achieved using two PCR steps. In the first PCR, selected linked rings that contained ON5 were amplified with primers P1 and P2 (P1 introduced an extra 23 nt to the 5′-end of ON5). PCR amplification was performed by thermocycling of 94 °C (30 s), 50 °C (45 s), and 72 °C (45 s).

A small fraction (~1/20th) of the first PCR product was used as a template for the second PCR, which used primers P2 and P3. [α-^{32}P]dGTP was added as a radiolabel. P3 is a ribo-terminated primer; the purpose of the second PCR was to introduce the ribonucleotide linkage so that single-stranded DNA could be generated by alkaline hydrolysis. The PCR was performed as described above. The amplified DNA was precipitated in ethanol, resuspended in 90 μL of 0.25 M NaOH, and heated at 90 °C for 10 min to cleave the ribonucleotide linkage. The solution was neutralized with 10 μL of 3 M sodium acetate (pH 5.5), and the DNA was precipitated in ethanol. The 100-nt single-stranded DNA was isolated by denaturing PAGE. The purified DNA was phosphorylated at its 5′-end with PNK and ATP, precipitated, and washed with ethanol. This DNA was used to initiate the next round of selection after quantification using the standard spectroscopic method described above.

The above selection and amplification procedures were repeated for three more rounds with an identical procedure as described for the first round, except that the ligation time was reduced from 30 to 5 min. After completion of the final round of selection, the DNA pool was cloned and sequenced using previously described protocols [12].

3. Results and discussion

3.1. Generation of linked-ring DNAs by simultaneous closure of two linear oligonucleotides entwined through a central helical region

In this report, we describe two ways of exploring T4 DNA ligase to make linked-ring DNAs from two synthetic oligonucleotides. The first approach is to simultaneously close the ends of two linear DNA molecules intertwined through a central complementary region (Fig. 1A; denoted double circularization in this study). The second approach is to make one ring first and then use it as a template to circularize the second ring (denoted single circularization). It is worth commenting that other approaches can also be devised to make linked-ring DNAs. For example, simultaneous closure of two linear oligonucleotides each containing a sequence as the template for the circularization of the other could also produce linked-ring structures without the use of any external template. Creating linked-ring DNAs through non-enzymatic approaches are also possible, as methods for chemically linking the ends of two DNA oligonucleotides in close spatial proximity have been demonstrated [30,31].

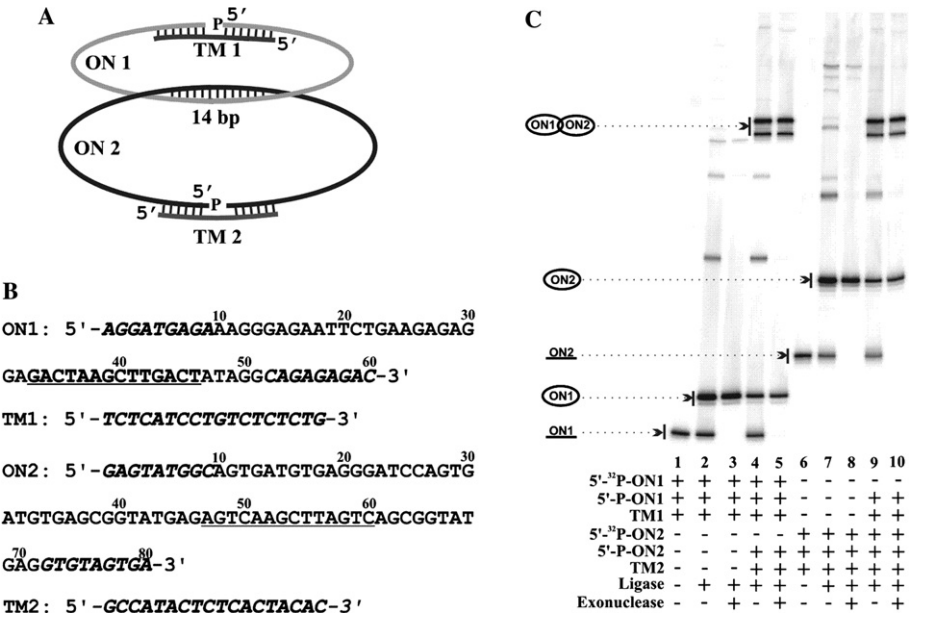


Fig. 1. Linked-ring formation by double circularization. (A) DNA ligation scheme used for making linked-ring DNAs. ON1 and ON2 are designed to form a central duplex. ON1 and ON2 can be closed by DNA ligation using TM1 and TM2 as templates, respectively. (B) DNA sequences of ON1, ON2, TM1, and TM2. The underlined nucleotides in ON1 are complementary to those in ON2. The nucleotides at both 5′ and 3′ ends (shown in italic) of ON1 and ON2 are designed to anneal with TM1 and TM2, respectively, for DNA circularization. (C) Results of DNA ligation and exonuclease treatment. 5′-Phosphorylated ON1 (20% ^{32}P-labeled) was incubated with TM1 in the absence (lane 1) and presence (lane 2) of T4 DNA ligase, and in the presence of T4 DNA ligase followed by exonuclease treatment (lane 3). 5′-Phosphorylated ON1 (20% ^{32}P-labeled) was incubated with non-radioactive 5′-phosphorylated ON2, TM1, and TM2 in the presence of T4 DNA ligase for linked-ring formation (lane 4), and the ligated mixture was treated with exonuclease (lane 5). The experiments shown in lanes 1–5 were performed again by replacing ON1 and TM1 with ON2 and TM2 (lanes 6–10). The DNA samples were analyzed by denaturing 10% PAGE and the identities of main DNA bands were putatively assigned based on the gel mobility and exonuclease protection analysis. These species are indicated in the figure with linear or circular symbols.

Our preliminary assessments showed that the double circularization approach was more effective (with a yield of ~40%) than the single circularization method (with a yield of less than 10%). For this reason, we chose to initially focus on the double circularization method. Two oligonucleotides, 60-nt ON1 and 80-nt ON2 (Fig. 1B), were arbitrarily chosen for our examination. ON1 can be circularized over 18-nt template oligonucleotide TM1 (Fig. 1B, which aligns the 5′ and 3′ ends of the same ON1 molecule, shown in italic, to form a nicked duplex for DNA ligation) and ON2 can be circularized over 18-nt TM2 (Fig. 1B). Since TM1 (or TM2) can also align the 5′ end of one ON1 (or ON2) molecule next to the 3′ end of another, it is expected that DNA ligation could also generate a series of ligation by-products including linear

dimers, linear trimers, linear tetramers, as well as circular dimers, circular trimers, and so on. Indeed, analysis of the ligation mixture of ON1 (lane 2, Fig. 1C) and ON2 (lane 7) by denaturing polyacrylamide gel electrophoresis (PAGE) revealed many ligated DNA products. The major product (~70%) of each reaction was identified as circular ON1 (lane 3) or circular ON2 (lane 8) following a treatment with T4 DNA polymerase (circular DNA is resistant to T4 DNA polymerase's 3′–5′ exonuclease activity, while linear DNA is not). For simplicity, we will subsequently refer to treatment with T4 DNA polymerase in this manner as 'exonuclease treatment.' All non-circular ligation by-products will not be identified and discussed throughout this report.

Two DNA ligation reactions (lanes 4 and 9) were performed to derive linked ON1–ON2. Each ligation reaction used 5′-phosphorylated ON1, 5′-phosphorylated ON2, TM1, and TM2, but used a different source of radioactive DNA: 5′-^{32}P-labeled-ON1 for the first reaction (lane 4) and 5′-^{32}P-labeled-ON2 for the second (lane 9). After DNA ligation, each mixture was subjected to exonuclease treatment (lanes 5 and 10, respectively). Three circular DNA bands were observed for each reaction, one of which (the bottom DNA band) was identified on the basis of gel mobility as either circular ON1 (lane 5, the first reaction) or circular ON2 (lane 10, the second reaction) and the other two (the top two DNA bands in both lane 5 and lane 10) were suspected to be linked-ring DNA species. Since 5′-^{32}P-labeled-ON1 and -ON2 were incorporated separately for each reaction, it became apparent that both of the suspected linked-ring DNA species contained a circular ON1 and a circular ON2.

3.2. Characterization of linked-ring DNAs

An *Eco*RI site and a *Bam*HI site were incorporated into ON1 and ON2, respectively (Fig. 2A). Therefore, digestion of the linked-ring DNAs with restriction enzymes would confirm the existence of the topological linkage.

Two short oligonucleotides, designated as TMA and TMB, were used to convert circular forms of ON1 and ON2 into duplex substrates for the two restriction enzymes. As expected, when purified circular ON1 was digested with *Eco*RI in the presence of TMA, linear ON1 was produced (lane 4, Fig. 2B; lanes 1, 2, and 3 contained linear ON1, undigested circular ON1, and circular ON1 treated by *Bam*HI). Similarly, circular ON2 was converted into linear ON2 by *Bam*HI (lane 8) but not by *Eco*RI (lane 7) in the presence of TMB (lanes 5 and 6 showed linear and circular ON2, respectively).

When the two purified ON1–ON2 linked-ring DNAs (in which both ON1 and ON2 contained a ^{32}P label) were treated with *Bam*HI in the presence of TMB (lanes 14 and 17, with lanes 13 and 16 being non-digestion controls), circular ON1 and linear ON2 were produced as expected. Similarly, treatment of the two purified linked-ring products with *Eco*RI in the presence of TMA produced the expected linear ON1 and circular ON2 (lanes 15 and 18). These experiments indicated that the suspected linked-ring DNAs were composed of circular ON1 as well as circular ON2, and linearization of one strand resulted in the release of one linear DNA and one circular DNA.

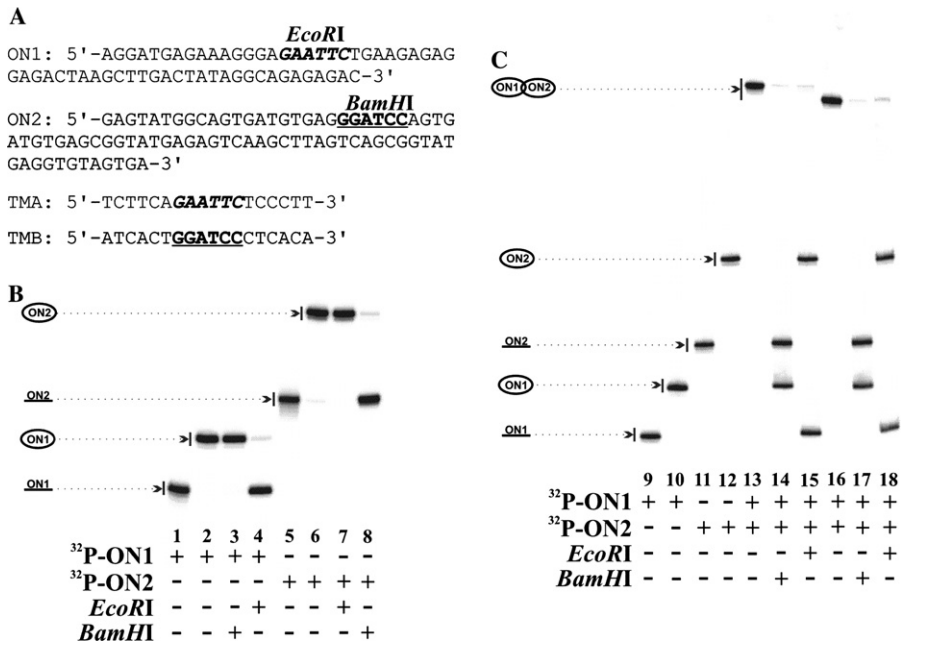


Fig. 2. Linearization of circular oligonucleotides by restriction endonucleases. (A) ON1, ON2, and related TMA and TMB sequences used for restriction digestion assays. TMA is complementary to ON1 at the *Eco*RI site (shown in italic) and TMB to ON2 at the *Bam*HI site (underlined). (B) Linearization of purified circular ON1 and ON2. Both ON1 (lanes 1–4) and ON2 (lanes 5–8) were circularized with their respective templates and purified by gel electrophoresis. The purified circular ON1 was mixed with TMA and then treated without restriction enzyme (lane 2), with *Bam*HI (lane 3), and *Eco*RI (lane 4). Linear ON1 served as a control (lane 1). Similar treatment was performed for ON2 (lanes 5–8). (C) Linearization of linked DNA rings. The two suspected linked ON1–ON2 products (both ^{32}P-labeled) were purified by PAGE. The slower-migrating band was treated without enzyme and template (lane 13), with TMB and *BamH*I (lane 14), and with TMA and *Eco*RI (lane 15). The faster-migrating band was treated the same way (lanes 16–18). Purified linear ON1 (lane 9), circular ON1 (lane 10), linear ON2 (lane 11), and circular ON2 (lane 12) served as the markers.

When each of the purified ON1–ON2 species was re-analyzed on denaturing PAGE (lanes 13 and 16, Fig. 2C), only a single DNA band was observed. This rules out the possibility that these two DNA species were two slowly interchanging conformations of the same DNA structure, as purification and recovery process was carried out over several days. Moreover, since there were no DNA products other than the expected circular or linear form of ON1 or ON2 after restriction digestions, we can conclude that both linked-ring DNAs contained circular ON1 and circular ON2 in a ratio of 1:1. Furthermore, analysis by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) revealed the same molecular weight for both species (which also matched the calculated value of 44.0 kDa). These analyses provided strong evidence to support that the two higher-order DNA molecules were two linked-ring DNA isomers, each composed of one ON1 and one ON2 and differing only in topological properties.

An explanation for the observation of two linked-ring DNA products is given in Fig. 3. The two oligonucleotides engage each other through the designed helical interaction while the two arms of each DNA are not confined to any particular configuration and have the freedom to sample all available conformations. The 5′-phosphate-containing arm (“head” in Fig. 3) and the 3′-OH-containing arm (“tail”) of the second DNA molecule can adopt three different orientations relative to the first molecule. Orientation A will give rise to the formation of a linked ring with two topological crosses, orientation B will lead to the formation of a single linked-ring product with one topological cross while orientation C results in two separate rings. We believe that the linked-ring DNAs with one and two topological crosses correspond to the slow-moving and fast-moving DNA bands observed on denaturing PAGE, respectively. This is because more topological crosses should help compress a linked-ring structure leading to increased gel mobility.

3.3. The number of base pairs in the central linking region and the yield of linked-ring formation

A series of ON1 analogs (ON1BP8–13, Fig. 4A) were generated to determine the influence of the number of base pairs in the linking region (8–13 base pairs covered by ON1BP8–13, respectively) on the yield of linked-ring formation (data shown in Fig. 4B).

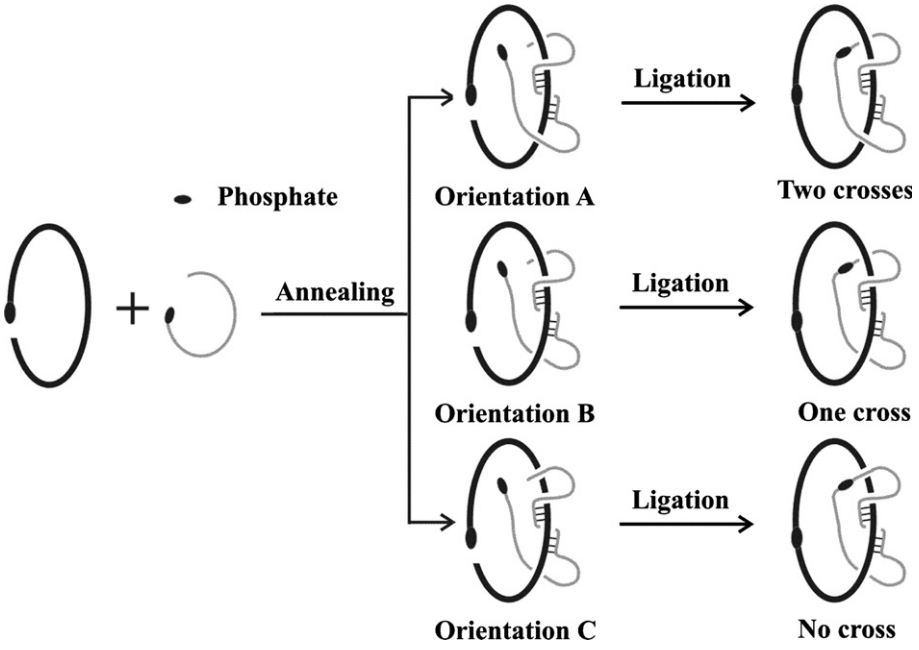


Fig. 3. An explanation for the topological outcome of the double circularization approach. Orientations A, B, and C lead to a linked-ring DNA with two topological crosses, a linked-ring DNA with one topological cross, and two separate rings, respectively.

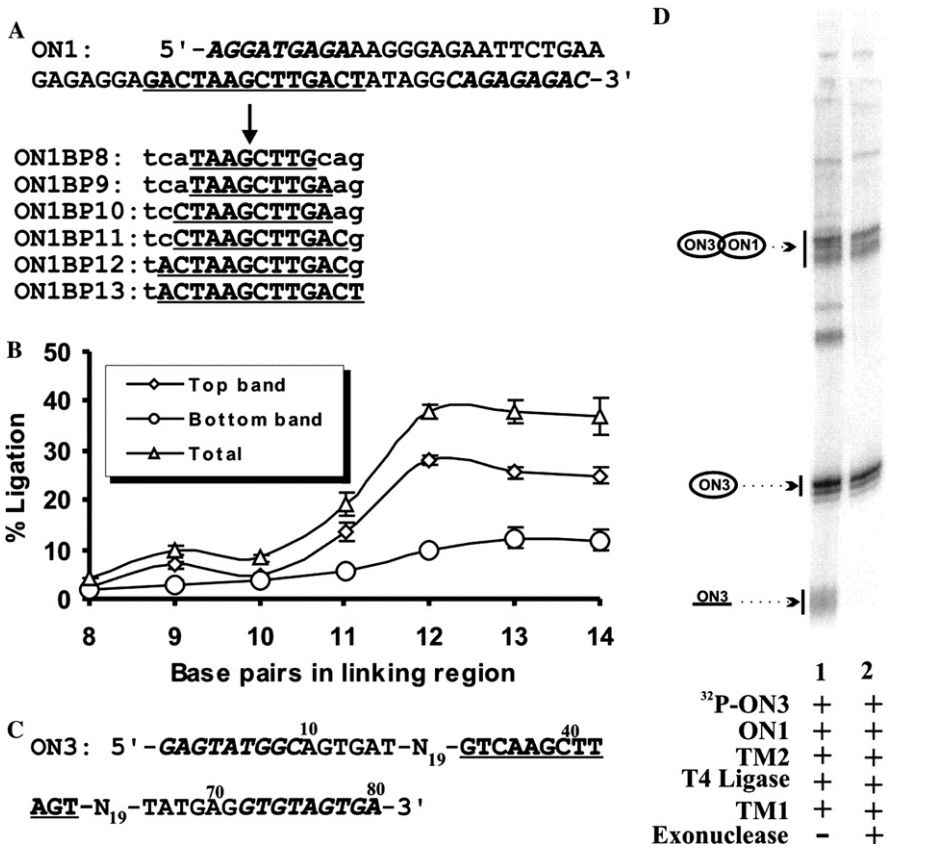


Fig. 4. Influence on linked-ring formation by the length of the linking helical region and by the presence of random-sequence nucleotides. (A) Sequences of the oligonucleotides used for the assessment of the length influence. Six mutated ON1 molecules, ON1BP8–13, were designed to form 8–13 base pairs with ON2, whereas ON1 forms 14 base pairs with ON2. The mutated nucleotides relative to ON1 are shown in lower case and complementary regions are underlined. (B) Yield of linked-ring formation versus base pairs in the linking helical region. The percentage conversion of linear ON2 into fast-moving linked-ring (circles) and slow-moving linked-ring (diamonds) in the presence of ON1BP8–13 and ON1 was measured from PAGE analysis. The total linked-ring formation (triangles) was taken as the combined percentage. Four repeats were carried out and the average values are plotted with error bars indicated. (C) The sequence of ON3. ON3 contains two stretches of random-sequence nucleotides (indicated by N_{19}) that sandwich a central 12-nt region designed to form a duplex with ON1 for linked-ring formation. (D) Formation of ON1–ON3 linked rings. Lane 2 was the DNA ligation sample in lane 1 after exonuclease digestion.

ON2 (5′-^{32}P-labeled) and each ON1 were annealed at 15 °C for 16 h prior to the addition of T4 DNA ligase to improve the likelihood of duplex formation between ON1 and ON2. Low-yield linked-ring DNA products (10% or less) were produced when the helical region contained 10 base pairs or less. The yield of the linked ring was significantly increased when the helical segment was enlarged from 10 to 11 base pairs, and again from 11 to 12 base pairs. The addition of base pairs beyond 12 did

not increase the reaction yield any further. Therefore, to obtain high-yield linked-ring DNAs, a minimum of 12 base pairs are required.

3.4. Generation of a linked-ring DNA library using an oligonucleotide containing random-sequence nucleotides

ON3 (Fig. 4C) is an 80-nt oligonucleotide that can form a 12-bp duplex with ON1, and its annealing region is centered between two 19-nt random-sequence domains. We studied the double circularization of ON1 and ON3 to determine whether an oligonucleotide containing random-sequence nucleotides could be circularized.

First, we performed the double circularization on an analytical scale (Fig. 4C) using 0.5 pmol of ON1 and 0.5 pmol of ON3. Linked-ring DNAs were produced at a yield of ~40%, which was consistent with the yield observed for ON1–ON2 (Fig. 4D, lane 1). We then performed the same reaction on a preparative scale using 1000 pmol of ON3 and 1000 pmol of ON1. After DNA ligation, exonuclease digestion, and PAGE purification, 246 pmol of purified linked-ring products were obtained, accounting for ~25% conversion. Therefore, it seems practical to use the described approach to construct linked-ring DNA libraries. We further found that the resulting linked-ring DNA can be amplified by polymerase chain reaction (PCR) and the single-stranded DNA molecules converted from the double-stranded PCR product can be re-circularized into linked-ring DNAs (data not shown). This suggests that it is possible to conduct in vitro selection using linked-ring DNA libraries.

3.5. Generation of a linked-ring DNA library using single circularization method

Initially, we observed that the single circularization method (i.e., circularization of a linear oligonucleotide over a circular DNA template) could also generate linked-ring DNA products, but at a relatively low yield (less than 10%, data not shown). Interestingly, we observed only one linked-ring band rather than two linked-ring bands as seen with the double circularization method described above. Based on this observation, we decided to investigate whether a DNA library could be made by the single circularization method.

Two new DNA oligonucleotides, ON4 and ON5, were used in this assessment (Fig. 5B). ON4 contained 86 nucleotides, and circularization of linear ON4 in the presence of 19-nt template TM3 and T4 DNA ligase produced circular ON4 at ~70% yield (data not shown). ON5 contained 70 centrally located random-sequence nucleotides, flanked on each side by 15 fixed-sequence nucleotides (Fig. 5B). We found that linear ON5 (5′-^{32}P-labeled) can be converted into circular ON5 in the presence of 19-nt TM4 and T4 DNA ligase with a yield of ~70% (Fig. 5C, lane 1; the circular ON5 was identified by exonuclease digestion; data not shown). ON6 was a 50-nt oligonucleotide complementary to part of the non-annealing region of ON4 (underlined nucleotides, Fig. 5B). Its inclusion in the above ligation mixture was to prevent the tertiary folding of circular ON4, which may prevent

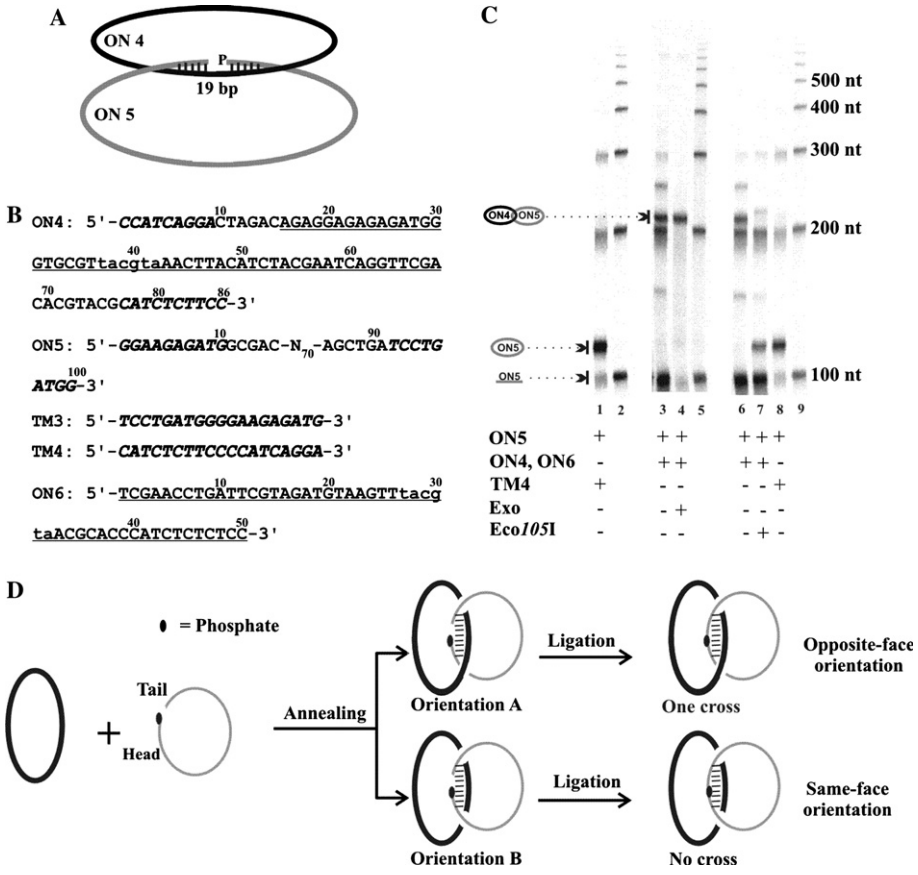


Fig. 5. Linked ON4–ON5 ring formation by circular DNA templated ligation. (A) DNA ligation scheme. ON4 is a pre-made circular DNA, which can engage the two ends of ON5 for duplex formation. (B) DNA sequences of linear ON4, ON5, ON6, TM3, and TM4. The italicized nucleotides in ON4 (or TM4) can juxtapose the two ends of ON5 (also italicized) for DNA ligation. TM3 is the DNA template to make circular ON4. TM4 is the template for making circular ON5. Both ON4 and ON6 contain an *Eco*105I site (in small letters) for restriction digestion. ON5 contains 70-nt random-sequence domain (indicated by N_{70}). (C) Results of DNA ligation, exonuclease treatment and restriction digestion. 5′-Phosphorylated ON5 (20% ^{32}P-labeled) was circularized by T4 DNA ligase in the presence of TM4 (lane 1 and lane 8), or circular ON4 (lane 3 and lane 6). The latter mixture was treated with exonuclease (lane 4) or *Eco*105I endonuclease (lane 7). Lanes 2, 5, and 9 contained 100-nt single-stranded DNA markers. (D) An explanation of topological outcomes of single circularization. Orientation A (“opposite-face orientation”) will result in a linked-ring DNA with one topological cross and Orientation B (“same-face orientation”) will generate two separate rings.

proper annealing of ON5 and ON4. Circularization of linear ON5 (5′-^{32}P-labeled) in the presence of non-radioactive circular ON4 and linear ON6 at 1:1:1 ratio led to the generation of linked ON4–ON5 at ~10% yield (lane 3). Linked-ring DNA could still be obtained in the absence of ON6, but the yield dropped to ~5% (data not shown).

The linked ON4–ON5 was identified by exonuclease digestion (comparing lane 3 and lane 4). When the above ligation mixture (lane 3) was treated with the exonuclease, only one higher-order DNA band remained (lane 4). The existence of the topological linkage was confirmed by digestion with the restriction endonuclease *Eco*105I (comparing lanes 6–8). Since ON4 was designed to contain an *Eco*105I restriction site (Fig. 5B, indicated by the small letters), treatment of the linked ON4–ON5 structure with *Eco*105I should yield circular ON5 and linear ON4. When the above ligation mixture (lane 3, also re-run as lane 6) was digested with the restriction enzyme (lane 7), the suspected linked-ring DNA band disappeared and a new DNA band having the same gel mobility of circular ON5 (lane 8, a re-run of lane 1) was observed, suggesting that *Eco*105I linearized ON4 and freed circular ON5.

The above experiment indicated that the single circularization method could also be used to construct a linked-ring DNA library. Although the yield (~10%) by this method was significantly lower than that observed for the double circularization method (~40% conversion), the resulting library should still have sufficient sequence diversity. For example, a typical automated DNA synthesis at 0.2 μmol scale usually produces more than 5000 pmol of a linear oligonucleotide of ~100 nt after PAGE purification. This can be converted into 500 pmol of linked-ring product by the single circularization method, accounting for 3×10^{14} molecules. Such a DNA pool affords sufficient sequence diversity for in vitro selection.

The topological outcomes of the single circularization approach are speculated using a theoretic scheme shown in Fig. 5D. The 5′-phosphate-containing head and the 3′-OH-containing tail of the linear DNA molecule can adopt two orientations relative to the circular template. Orientation A (“opposite-face orientation”) would give rise to the formation of a linked ring with one topological cross while Orientation B (“same-face orientation”) would lead to the formation of a non-linked ring.

3.6. In vitro selection of ON5 variants capable of facilitating fast and high-yield linked-ring formation

To demonstrate that linked-ring DNA libraries are compatible with in vitro selection, we carried out a simple in vitro selection experiment to isolate ON5 sequences that were capable of facilitating fast and high-yield linked-ring DNA formation in the presence of ON4. We speculated that even though circularization of ON5 over circular ON4 could only produce ~10% linked-ring DNA in a 20-h incubation, many ON5 members had the capability to facilitate fast and high-yield linked-ring formation.

We designed an in vitro selection scheme as shown in Fig. 6A. In Step I, ON5 is incubated with T4 DNA ligase and circular ON4 for a short period of time (30 min for the first round, 5 min for the selection rounds thereafter). After purification by denaturing PAGE (Step II), the linked-ring DNA was amplified by two consecutive PCRs (Steps III and IV); the second PCR used a ribo-terminated primer to generate double-stranded DNAs with a single ribonucleotide linkage. Such a DNA product was digested under alkaline conditions to regenerate single-stranded ON5

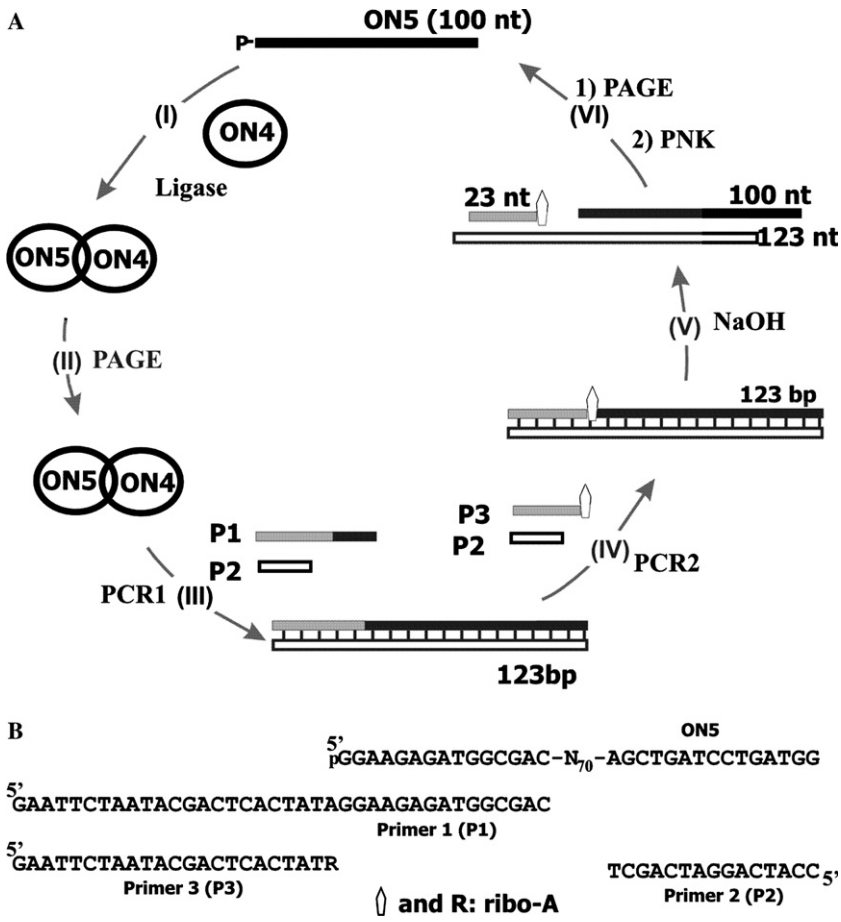


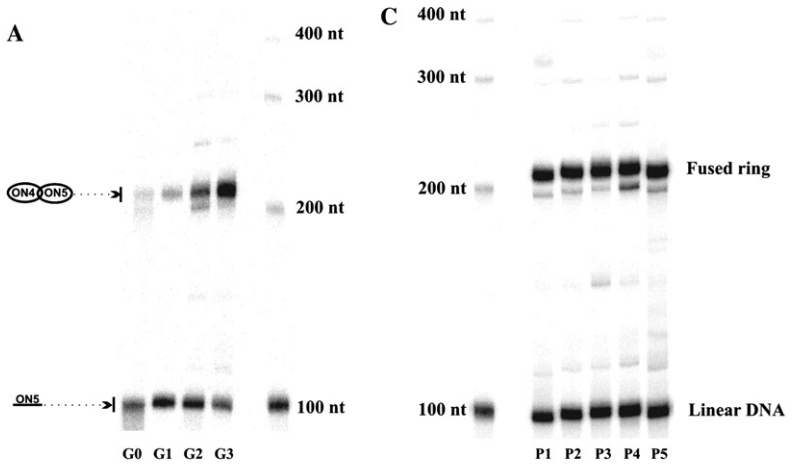
Fig. 6. Selecting DNA sequences with the ability to form high-yield linked-rings. (A) A six-step selection scheme. Step I, ON5 is treated with T4 DNA ligase in the presence of circular ON4; Step II, purification of linked ON4–ON5 by denaturing PAGE; Step III, amplification of selected ON5 by PCR using P1 and P2; Step IV, re-amplification of ON5 by PCR using P2 and P3 to introduce a cleavable ribonucleotide linkage in the double-stranded DNA; Step V, alkaline digestion of the second PCR product to regenerate single-stranded ON5; and Step VI, PAGE purification and DNA phosphorylation of the selected ON5 sequences, which are then used to initiate the next round of selection. (B) Sequences of the DNA library (ON5) and DNA primers (P1–P3) used for in vitro selection.

molecules (Step V). After PAGE purification and DNA phosphorylation (Step VI), the new ON5 variants were used to initiate the next round of selection. The selection cycle is repeated until the yield of linked-ring formation reaches a plateau.

We used 100 pmol of ON5 and 100 pmol of circular ON4 for the first round of selection (G0). After three rounds of selective amplification, the enriched population (G3) was found to be capable of producing linked-ring DNA at greater than 70%

yield (Fig. 7A) in 5 min. The selected bands were shown to be exonuclease resistant (data not shown). More selection rounds did not increase the yield any further (data not shown).

The linked-ring product from G3 was cloned and five sequences were determined (their random domain sequences are shown in Fig. 7B). All five sequences showed an ability to form linked-ring structures that were exonuclease resistant at high yields (over 50%) in a 5-min incubation time (Fig. 7C). Although the precise reasons for the fast and high-yield linked-ring formation by the enriched ON5 sequences (such as DNA1–DNA5, Fig. 7C) are unclear and beyond the scope of the current investigation, we speculate that each selected molecule has an unusual ability to align the two ends of its own sequence onto the circular DNA template for DNA ligation. The successful enrichment of desired DNA molecules for fast and high-yield linked-ring DNA formation indicates that linked-ring DNA libraries are compatible with in vitro selection.



B

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DNA1      10        20        30        40        50        60        70
  GGAAGAGTATGGCAAGTTTAGTGAGACCGCGGACCGTAGGGGGGAGTGTGTCACGGTCGCGTTGGTG
DNA2
  GTATGGGTGGTACAATCGGGCGCCCCGTATATCGCAATGTTAGTTATTTTATGTTAATAGGTGGTTATG
DNA3
  GGTGGAAATAGTCAGCCGATGGGACTCGATGAATTAACCGTTCTCTATGTACTGGTCTTAGTTTTGTATG
DNA4
  GGGGAGCGTAGTAGGGGCCGAGAGCGGCATGCGACGGCACTTTGTTGACGGACGTATTTCAATTGGGTG
DNA5
  AGATGGTGCTACCCTACCCGCGGGGGCAGCCCGTTGGAGATTGTTTTGTAGTCAGTTATTTGGCATGGTG
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Fig. 7. In vitro selection results. (A) The linked-ring formation ability of ON5 before in vitro selection (labeled as G0) and after one, two, and three rounds of in vitro selection (labeled G1, G2, and G3, respectively). (B) Sequences of five selected DNA molecules, DNA1–DNA5. Only the original 70-nt random-sequence domain (N70 in Fig. 6B) is shown. (C) Linked-ring formation behavior of DNA1–DNA5. Each DNA molecule was circularized by T4 DNA ligase over the circular DNA template ON4.

4. Concluding remarks

We have shown above that topologically linked single-stranded DNA rings can be produced at high yields from two synthetic DNA oligonucleotides using T4 DNA ligase-mediated DNA ligation. We have also demonstrated that linked-ring DNA assemblies in which one DNA strand contains random-sequence nucleotides can be used to conduct in vitro selection experiments. Although our case study was directed at the isolation of DNA sequences that can facilitate T4 DNA ligase-mediated circularization over a circular DNA template to produce a linked-ring structure, it is also conceivable that linked-ring DNA libraries can be exploited to conduct in vitro selection experiments to search for new functional DNA molecules with desirable catalytic or binding capabilities.

Using linked-ring DNA libraries could offer several advantages over the use of linear DNA libraries. First, linking two or more DNA molecules into a ring structure will not only increase the size of DNA considerably, but could also give rise to the formation of tertiary structures with substantially increased complexity. Second, the linked-ring approach could facilitate the exploration of useful chemical functionalities for DNA catalysis. To do so, the fixed-sequence DNA ring can be designed to contain one or several kinds of chemical functionalities that are foreign to DNA while the other ring contains random-sequence nucleotides to provide the sequence diversity. The new functional groups need to be introduced only once during the automated DNA synthesis without the involvement of tailor-made DNA polymerases [32]. Arguably, the functional groups can also be placed in the constant sequence regions of a linear DNA library to achieve the same goal; however, linked-ring assembly should offer more complex tertiary structures for improved catalytic function. It is also possible to place a new functionality on a short oligonucleotide, which is then presented to the DNA library by DNA hybridization. However, simple hybridization does not ensure that they will be in close proximity to the catalyst, as a catalytic DNA molecule may fold in such a manner that precludes hybridization of the oligonucleotide containing this functionality.

A third advantage is the potential to engineer bifunctional, or even multifunctional deoxyribozymes, where different rings perform different catalytic functions. Similarly, the linked-ring approach may also be used to create complex aptamers with multiple binding sites for different targets. Furthermore, almost all current in vitro selection protocols for the isolation of DNA catalysts require covalent attachment of the substrate to the DNA library; the linked-ring approach could then offer an alternative way of linking the substrate to the library by non-covalent means yet still allow for the identification of active molecules upon substrate modification.

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