Supplementary Information

Topology-and Linking Number-Controlled Synthesis of Closed 3 Link Chain of Single-Stranded DNA

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Materials and Experiments

DNA oligonucleotides

All PAGE purified oligonucleotides were purchased from Sangon (Shanghai, China).

Assembly of CirDNA^{Triad}

The DNA sequences used for synthesizing the CirDNA^{Triad} are listed in Table S1**.** Three ssDNA pre-rings (0.5 μM) attaching phosphate group at 5′ terminus, ssDNA A, B, and C were mixed together in 1×T4 DNA ligation buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8 @25^{o}C) to a total volume of 20 μL. The sample was putted onto a PCR machine to incubate 3 min at 90^{o}C for opening the undesired secondary strictures. Then, after cooling down to 25^{o}C at the rate of 0.1^{o}C/s, three corresponding splints (1 μM) were added and incubated at 25^{o}C for 20 min. Finally, T4 DNA ligase (5 U/μL, 5 U) was added and the ligation was carried out at 25^{o}C for 2 h.

For preparing single ring, only the ssDNA pre-ring and corresponding splint were added, and the sample was treated as described above. For fabricating [2] catenane, the two ssDNAs and the corresponding splints were added. For preparing [2] catenane in the presence of the third pre-ring strand, all the three ssDNA pre-rings were mixed together, but only two splints for circularizing the two catenaned pre-rings were added. Other conditions were the same as CirDNA^{Triad}

Digestion of CirDNA^{Triad} by restriction enzymes

For digestion by Bfa I, the 1×CutSmart Reaction Buffer (50 mM CH_3COOK, 20 mM Tris-CH_3COOH, 10 mM CH_3COOMg, 100 μg/ml BSA, pH 7.9 @25°C) was used, and the digestion was carried out at 37^{o}C for 1 h; For digestion by Rsa I, the digestion was carried out in 1×CutSmart Reaction Buffer at 37^{o}C for 15 min; For digestion by Sau3A, however, 1×NEBuffer1.1 (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 100 μg/ml BSA, pH 7 @25°C) was used, and the digestion was carried out at 37^{o}C for 1 h. All the digestion sites were designed to be present at hybridization part between the splint and the circular DNA.

The protocol is described as follows. Mixing the preparing products of CirDNA^{Triad} (0.2 μM) and 5 U restriction enzyme into corresponding 1×reaction buffer, followed by adding water to 20 μL, and incubated on a PCR machine at 37^{o}C for a certain time..

Gel electrophoresis

All reaction products (DNA rings, CirDNA^{Triad}, and digestion results) were analyzed on 11% denaturing polyacrylamide gel electrophoresis (dPAGE). The gels were run in 1×TBE buffer (90 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.0 @25°C) in room temperature. DNA bands were stained by SYBR Green II for 10min and detected by the charge-coupled device camera with IS-100 digital imaging software (Alpha Innotec, San Leandro, CA).

Scanning the purified CirDNA^{Triad} by AFM

After cutting the bands of CirDNA^{Triad} from the gel, DNA samples were purified by gel extraction kit (D2500-01-Gel Extraction Kit (50)). The concentration of the product was determined by a Nanodrop.

To prepare the sample on mica, 1 M $MgCl_2$ water solution was used to treat the smooth surface of mica for 1 min, and washed it with water. This procedure was repeated three times. Then, 2 μL sample of 2 nM was dropped onto the mica and dried in the air for 10 min, followed by washing the non-combined samples with water and drying in the air. The scanning was operated in AFM (AGILENT 5400) with tapping mode of the probe (Vecco) in air.

Name	Sequences (5'-3')	Length (nt)
ssDNA A	PO_4-GAGATTTTTTTTTTTTTTGGCTTGGGTCCGTAGTTTTTT TTTTTTTCGCTA	52
ssDNA B	PO_4-ACTGACTTTTTTTTTTTTTTTACTACGGACTGGATGGTT TTTTTTTTTTTTCGTCGT	58
ssDNA C	PO_4-AGGTAGTTTTTTTTTTTTTTTTTCCATCCAGCCCAAGC CTTTTTTTTTTTTTTTTTTGATCG	64
ssDNA A'	PO_4-GAGATTTTTTTTTTTTTCGGAGCTGTTGATACAGTTTTT TTTTTTTCGCTA	52
ssDNA B'	PO_4-ACTGACTTTTTTTTTTTTTTACTGTATCTTCTCTGAATTT TTTTTTTTTTTCGTCGT	58
ssDNA C'	PO_4-AGGTAGTTTTTTTTTTTTTTTTTATTCAGAGTTCAGCTC CGTTTTTTTTTTTTTTTTTGATCG	64
Splint A	AATCTCTAGCGA	12
Splint B	GTCAGTACGACG	12
Splint C	CTACCTCGATCA	12

Table S1. Sequences used to fabricate CirDNA^{Triad} in this study. PO_4 indicates the oligonucleotide was decorated with phosphate group for DNA ligation. The DNA sequences in the same underline mean the complementary regions between two adjacent ssDNA in CirDNA^{Triad}. ssDNA A', B', and C' indicate the sequences for preparing CirDNA^{Triad} added TT spacers in the center.

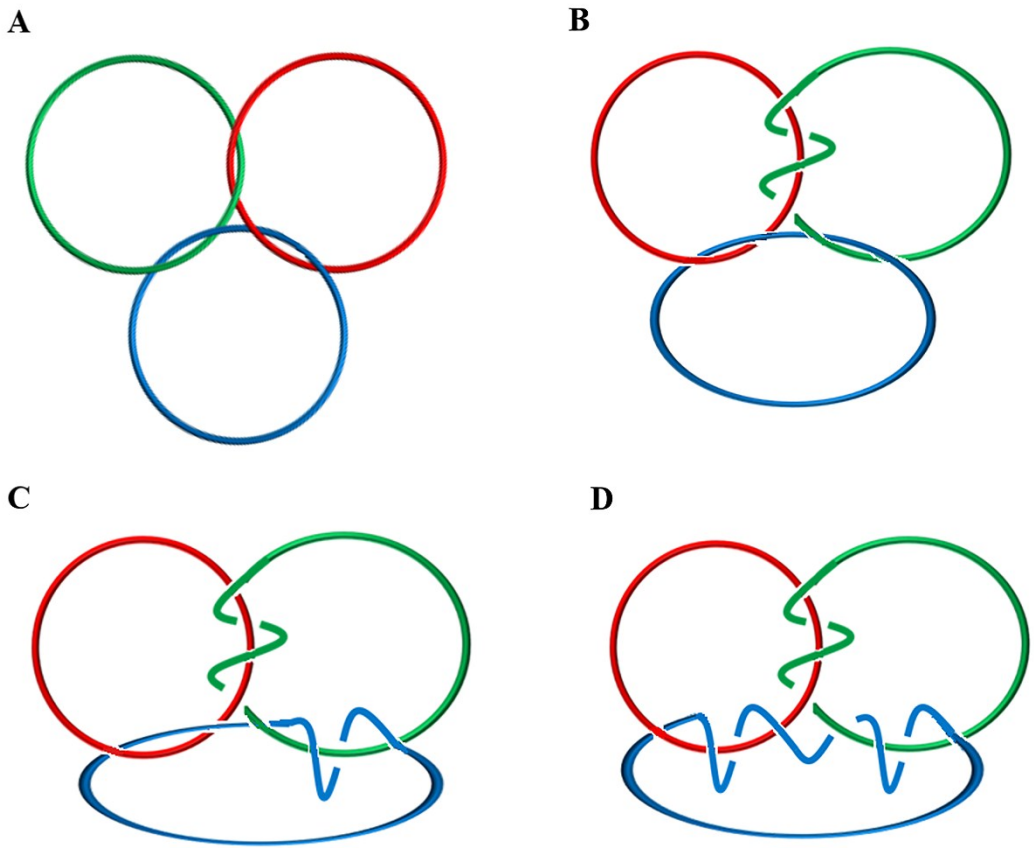

Figure S1. Isomers of CirDNA^{Triad}. (A) CirDNA^{Triad} synthesized in this study, the Lk between each two adjacent rings was strictly controlled in 1. (B) Isomeric CirDNA^{Triad} with one Lk2 and two Lk1, there are totally three types according to the different position of Lk2 and Lk1. (C) CirDNA^{Triad} containing two Lk2 and one Lk1 has three isomers with different sites of Lk1 and Lk2. (D) Lk2 CirDNA^{Triad} isomer that each two neighboring rings are restrained in Lk2.

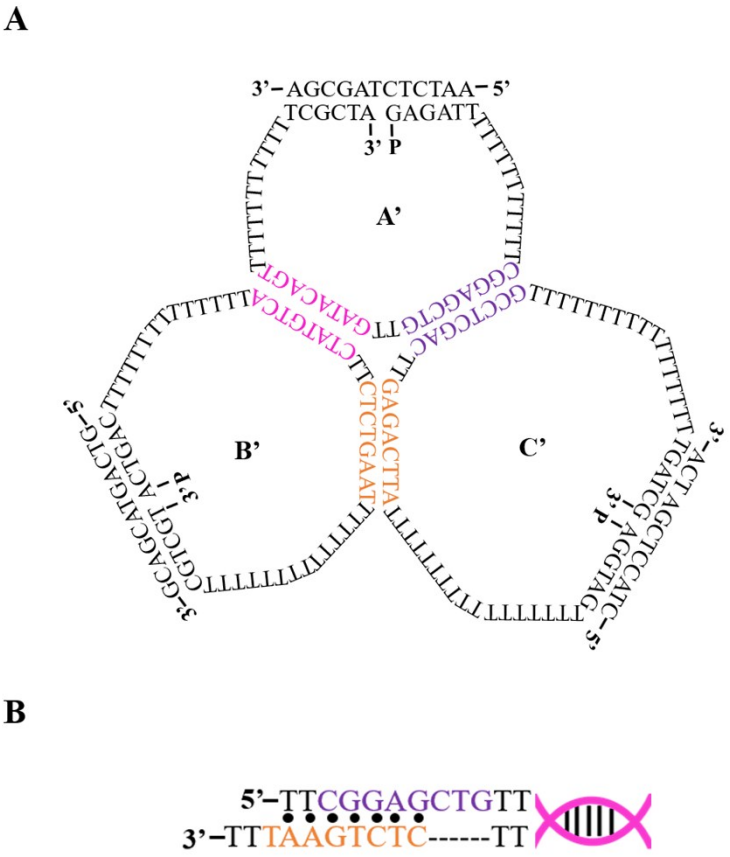

Figure S2. Sequences information of CirDNA^{Triad} containing TT spacers in the center. (A) Three pre-rings are A' (52 nt), B' (58 nt), and C' (64 nt). TT spacer was placed between the complementary regions in the three pre-rings to expand the space in the center of CirDNA^{Triad} and facilitate the conjugation of three pre-rings. (B) Scheme of the complementary base pairs above the 8-bp complementary region between A' and B'.

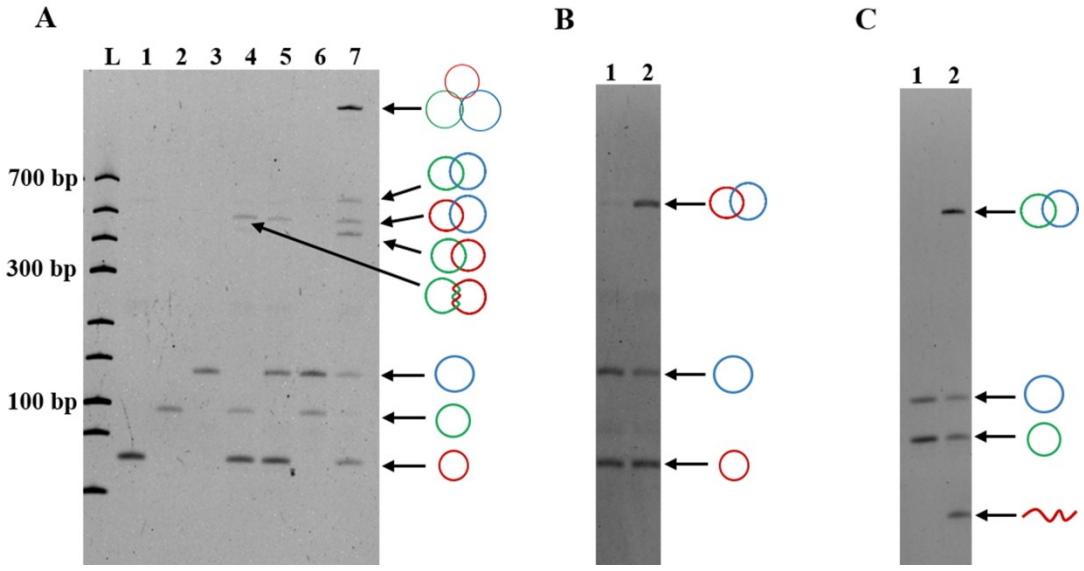

Figure S3. Fabrication results of CirDNA^{Triad} with three strands attaching TT spacers. (A) 11% dPAGE. Lane 1, the A' ring, prepared from pre-ring A'; lane 2, the B' ring; lane 3, the C' ring; lane 4, [2] catenane A'B', prepared from pre-rings A' and B'; lane 5, [2] catenane C'A'; lane 6, DNA [2] catenane B'C'; lane 7, products of catenation of the rings A', B', and C'. Lane L is dsDNA ladder. (B) 11% dPAGE. Catenation of pre-rings A' and C' to [2] catenane in the presence (lane 2) and the absence (lane 1) of pre-ring B'. In this case, only the splints for the closure of pre-rings A' and C' were employed for the T4 ligase reactions. (C) 11% dPAGE. Preparation results of [2] catenane B'C' with (lane 2) or without (lane 1) the pre-ring A' when only splints of pre-ring B' and C' were added before T4 ligase ligations.

For improving the yield of CirDNA^{Triad}, we inserted two thymines (TT spacer) in each strand between the complementary portions in the three pre-rings to promote the yield of CirDNA^{Triad}. The sequences used for the conformation of the CirDNA^{Triad} are shown in Table S1 and Figure S2, and the preparation results were monitored by 11% dPAGE in Figure S3A (lane 7).

The CirDNA^{Triad} added TT spacers in the center still had the slowest mobility in the gel, and the yield of this assembly improved from 32 mol% (no TT spacers, Fig. 4A) to 54 mol% (calculated by ring B; lane 7, Fig. S3A). The TT spacers probably expanded the space in the middle of Y-DNA and make three pre-rings easier to combine with each other. The other faint bands were the byproducts of three DNA [2] catenanes (A'B', B'C', and A'C') in the upper middle of the gel and three singe rings (A', B' and C') in the bottom of the gel. No detectable non-circularized pre-rings visualized in the gel.

Three single rings (lane 1-3) and three DNA [2] catenanes (lane 4-6) were synthesized as the references for CirDNA^{Triad}. Unexpectedly, the isomer of Lk2 DNA [2] catenane A'B' was the dominant catenaned product in lane 4. After checking the

sequences information of pre-ring A' and B', we discovered that there were extra 7-bp complementary part above the 8-bp complementary region we designed originally (Figure S2B). The catenation product of totally 15-bp hybridized portion between two catenaned rings was mainly Lk2 DNA [2] catenane, which is in accordance with our previous study. Interestingly, three DNA [2] catenanes of CirDNA^{Triad} prepared products in lane 7 all demonstrated only one band of Lk1 in the gel. These indicated that our method could efficiently control the Lk between each two pre-rings in CirDNA^{Triad} to 1.

It should be noted that the yields of DNA [2] catenane A'C' and B'C' in the absence of the third pre-ring were very low, especially for B'C', almost no detectable band monitored in the gel (Figure S3A, lane 5-6; Figure S3B, lane 1; Figure S3C, lane 1). However, when we added the third pre-ring into the catenaned system, the catenation efficiency was promoted largely (Figure S3A, lane 7; Figure S3B, lane 2; Figure S3C, lane 2). In Figure S3B, for example, in the absence of pre-ring B', the band of DNA [2] catenane A'C' was very faint (lane 1), main products were single ring A' and C'. When preparing DNA [2] catenane A'C' with the assistance of pre-ring C' by using only two splints for closing the pre-ring A' and C', we got a satisfactory yield in lane 2. In order to receive high yield of the catenation, scaffolding pre-ring B' is vital, especially for the short complementary region (8-bp) to obtain Lk1. These results illustrated that each pre-ring in CirDNA^{Triad} could work as scaffold for drawing close of the other two pre-rings and improving the hybridization efficiency between them.

In conclusion, each of the three pre-rings in CirDNA^{Triad} acts as scaffold to control the Lk of the other two pre-rings to 1, and promote the hybridization of the other two ssDNA. Accompanied with TT spacers, the yield of Lk1 CirDNA^{Triad} is further increased.