DNA Replication

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Rolling-Circle Amplification of a DNA Nanojunction**

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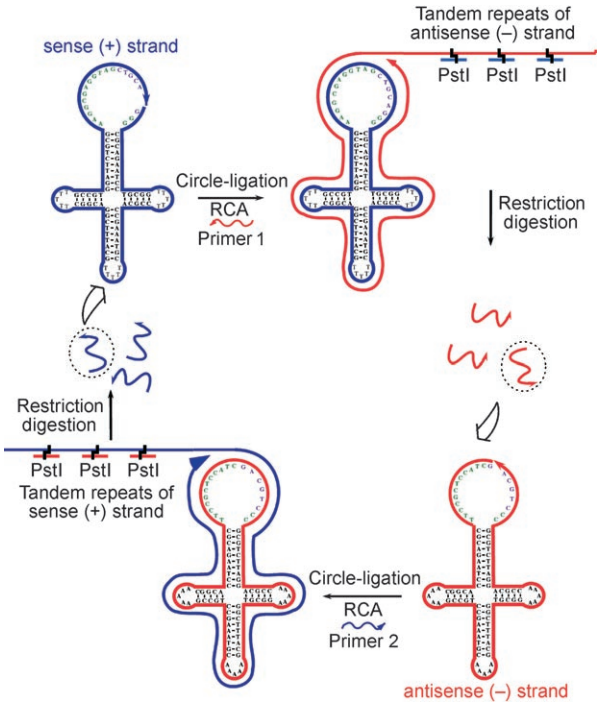
The field of structural DNA nanotechnology has seen exciting progress in recent years in using branched DNA junctions as building blocks for nanoconstruction.[1,2] One limiting factor in scaling up this technology is the cost of synthesizing DNA oligonucleotides. Thus, an immediate challenge is to develop efficient strategies to replicate branched DNA nanojunctions. However, the presence of a junction structure and the topology of the branched DNA nanostructure make it difficult for polymerase enzymes to pass through, preventing PCR from being a viable approach for this purpose. Prior outstanding efforts toward replicating DNA nanostructures involved either applying chemical copying mechanisms[3] or generating a long scaffolding DNA strand (through a plasmid DNA cloning method) that can fold into a unique nanostructure[4] through the assistance of added smaller synthetic DNA oligonucleotides. To date, the whole structure of branched DNA has not been replicated by using only enzymatic reactions.

Rolling-circle amplification (RCA) is an isothermal enzymatic DNA replication process that uses certain DNA polymerases to generate long single-stranded DNA (ssDNA) with tandem repeats of the complementary sequence of a single-stranded circular DNA template.[5] Such a process is initiated by the hybridization of a linear DNA primer to the circular DNA template. In the presence of dNTP, ϕ29 DNA polymerase adds nucleotides to the 3′ end of the primer strand and replicates the DNA around the template circle continuously with the preceding strand displaced from the template. The linear ssDNA product synthesized by RCA consists of several hundred tandem repeats of the template sequence. Recently, Mao and co-workers[6] and Simmel and co-workers[7] have used RCA to obtain long linear DNA strands to organize metallic nanoparticles into one-dimensional arrays with well-defined interparticle distances.

Previous studies demonstrated that the ϕ29 DNA polymerase can unwind DNA duplexes efficiently and is capable of strand displacement for processive DNA synthesis on a circular DNA template.[8–11] It has been shown that the ϕ29 DNA polymerase can overcome certain local topological constraints, such as circular padlock DNA probes hybridized with a linear DNA target or catenated to a circular DNA strand. The DNA strands causing the topological constraints can be displaced when ϕ29 DNA polymerase processes along the circle template. As the circle DNA templates themselves do not contain stable secondary structures or topological constraints, it is still not clear if RCA can work effectively on a circular template that has a stable secondary structures.

In this study, we chose a four-arm DNA junction as a target to replicate by using RCA. We aim to address the following two critical questions: 1) can RCA replicate a DNA junction that contains a specific secondary structure and topology, and 2) how efficient would the replication be? Our data suggest that this DNA polymerase can readily overcome the stable Holliday junction structure in the single-stranded circular template and replicate the DNA nanostructure efficiently.

Scheme 1 illustrates the amplification cycle of the four-arm DNA strand by using the RCA strategy. This 93-nucleotide ssDNA, termed as the sense (+) strand, folds into a Holliday four-way junction.[12] This cloverleaf-shaped structure contains two 5-base-pair (bp) arms, a 9-bp arm, and



Scheme 1. Schematic diagram of the DNA nanojunction amplification cycle.

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a 10-bp arm. The three shorter arms are each enclosed with a TTTTT pentaloop. The 10-bp arm has a 5′ overhang of three nucleotides (nt) and a 3′ overhang of 17 nt. The 5′ and 3′ ends of the DNA can be covalently ligated by a ssDNA ligase, CircLigase (Epicentre Biotech). Once closed, this 20-nt single-stranded loop serves as a primer-binding site for the RCA reaction. This loop also contains a restriction site (purple-colored bases) for the restriction enzyme PstI. To replicate the sense (+) DNA strand, two sequential RCA reactions need to be carried out. In the first phase of the cycle, ϕ29 DNA polymerase synthesizes a linear strand of DNA from the circularized sense (+) strand template following the hybridization of the primer 1 (red). This long ssDNA product consists of tandem repeats of a DNA sequence that is complementary to the template. To convert these tandem repeats to its monomeric units, the restriction helper strand 1 (a 15-base oligonucleotide) was annealed to the DNA product to form cleavable restriction sites for the PstI enzyme along the repeating sequences. After the restriction digestion by the PstI enzyme, individual copies of the antisense (−) DNA strands were released and purified by denaturing PAGE. In the second phase of the cycle, the antisense (−) strands were circularized and used as a template in the next RCA reaction to generate sense (+) DNA strands. The antisense:sense amplification uses primer 2 and restriction helper strand 2 and follows the same procedures described above.

To determine the efficiency of the RCA replication, DNA samples from each step of the amplification cycles were analyzed by denaturing PAGE (Figure 1). The yield of the circularization reaction by CircLigase is near 100% for both the sense (+) and antisense (−) DNA strands (see Figure 1a and 2b, compare lanes 1 and 2). The long DNA products amplified by RCA reactions (Figure 1a and b, lane 3) were digested by PstI enzyme to give rise to monomers of the linear DNA nanojunction with an expected size (lane 4). The amplification yield of RCA is about 12-fold for the sense:antisense reaction, and about 200-fold for the antisense:sense reaction. The difference in amplification efficiency is speculated to result from the difference in the sequence between the sense (+) and antisense (−) strands. The overall yield could be further improved by optimizing the digestion conditions as a fraction of DNA products were not fully digested (Figure 1b, lane 4). Different restriction enzymes could also be chosen to achieve higher digestion efficiency. Under optimal condition, one full cycle of two sequential RCA reactions is expected to amplify the DNA sense strands at least 2400-fold.

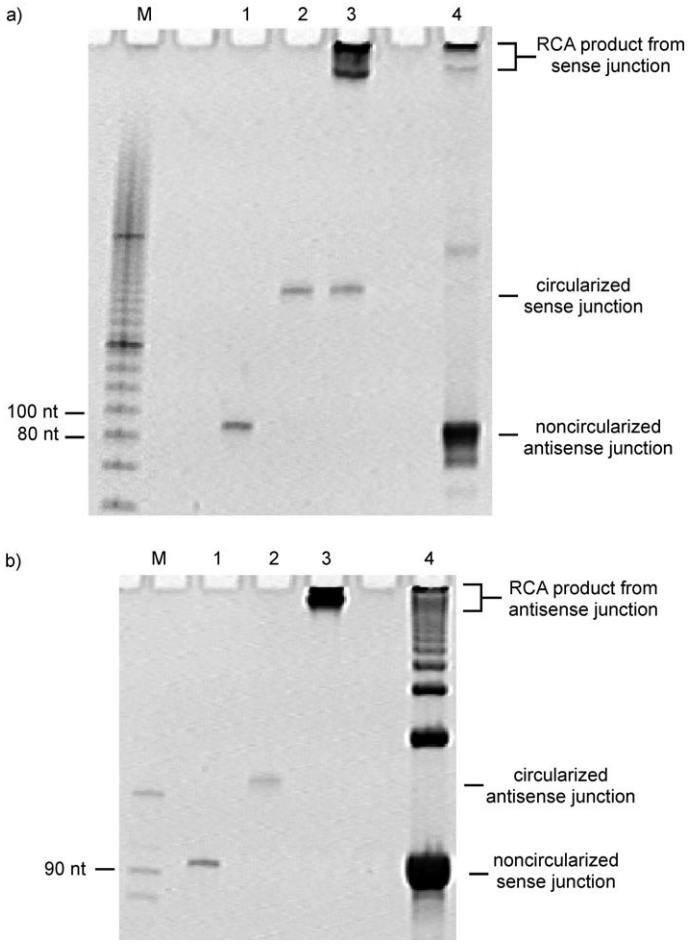

Figure 1. Amplification efficiency of DNA nanojunction through RCA. a) The first phase of the RCA cycle. Lane M: 20-bp ladder; lane 1: linear sense strand (LSS; 1 pmol); lane 2: after circle ligation; lane 3: after RCA; lane 4: after PstI digestion. Lanes 2–4 each contain products started from LSS (1 pmol). The major band in lane 4 was cut and eluted, yielding linear antisense strand (LAS, 12.3 pmol). b) The second phase of the RCA cycle. Lane M: 10-nt ssDNA ladder; lane 1: LAS (2 pmol); lane 2: after circle ligation; lane 3: after RCA products; lane 4; after PstI digestion. Lanes 2 and 4 each contain products that started from LAS (2 pmol), and lane 3 contains products from LAS (0.5 pmol). The major band in lane 4 was cut and eluted, yielding LSS (420 pmol).

Three control experiments were performed to confirm the expected formation of the four-way junction in the amplified DNA nanostructure. First, proper folding of the DNA strand was tested by a gel-mobility shift assay (Figure 2a). The DNA with a nanojunction showed a slower gel mobility compared with linear DNA with the same length but a random sequence, suggesting that the strand folds into a stable secondary structure. Next, we performed an Endo VII enzyme digestion experiment to detect the Holliday-junction structure in the DNA products. Endo VII is an endonuclease that specifically cleaves DNA with a Holliday-junction structure at well-defined positions.[12] The ^{32}P-labeled 5′-end DNA strand was cleaved by Endo VII at the Holliday-junction structure and gave rise to the correct bands corresponding to the expected 29/30-nt ^{32}P-labeled products for the sense strand and 39/40-nt products for the antisense strand (see Figure 2b and the Supporting Information for the cleavage sites). This result supports the theory that RCA-replicated DNA nanojunctions can fold into Holliday-junction structures as expected. The melting curve of the DNA products provides another line of evidence for a stable secondary structure in the DNA nanojunction. The sense junction exhibits a melting temperature at 77 °C (Figure 2c), which is consistent with the melting temperature that was predicted by using Mfold[14] based on this particular sequence.

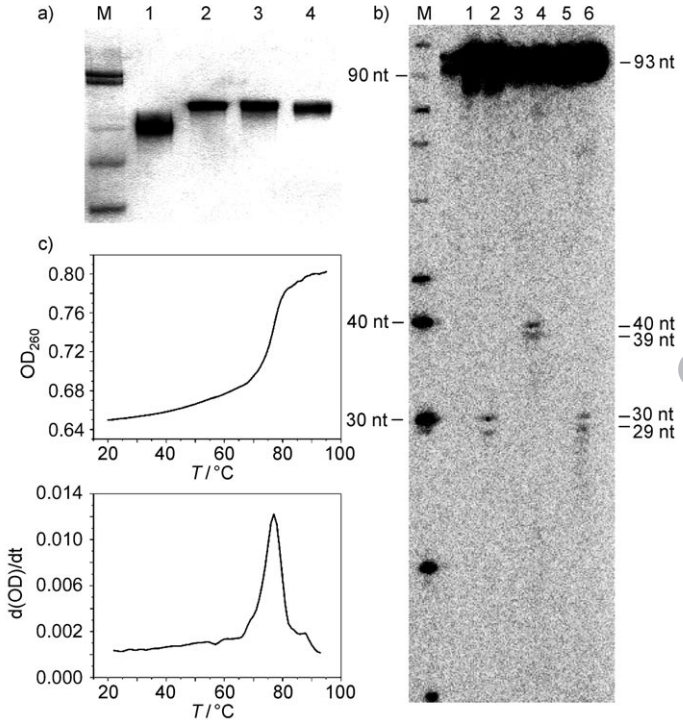

Figure 2. Analysis of the Holliday-junction structure of the DNA nanojunction. a) Native PAGE analysis of the DNA nanojunction. Lane M: 10-nt ssDNA ladder; lane 1: annealed random sequenced 93 mer ssDNA; lane 2: annealed LSS; lane 3: annealed LAS; lane 4: annealed LSS (final product after the complete replication cycle). b) Endonuclease cleavage of DNA with a Holliday junction. DNA samples treated with T4 Endonuclease VII (lane 2: LSS; lane 4: LAS; lane 6: LSS (final product)) or without T4 Endonuclease VII (lane 1, LSS; lane 3: LAS; lane 5: LSS (final product)). Lane M: 10-nt ssDNA ladder. c) Melting-curve profile of the sense junction (top) and the differential melting curve (bottom). OD = optical density.

We expect that the RCA reaction strategy could be employed to replicate and amplify other DNA nanostructures with different structures and more complicated topology (see the examples in the Supporting Information). The replication concept utilized herein is straightforward for single-stranded templates that can be easily circularized. A variety of ssDNA nanostructures can be designed by utilizing paranemic-crossover (PX) DNA structures.[15] Even periodic 2D structures could possibly be achieved by PX cohesion[16] after replicating DNA nanostructures. Multistranded structures can also be replicated separately for each strand and then combined together.

To compare the replication efficiency of the RCA reaction with that of PCR, we have tested asymmetric PCR on the sense strand (see the Supporting Information). Owing to the secondary structure, significant amounts of truncated products can also be seen, thus the amplification efficiency (approximately five times more of the correct-length product) is lower. Most DNA nanostructures constructed so far contain strands both with secondary structures and strands without secondary structures. It is possible to combine RCA with PCR for multistranded DNA nanoarchitectures.[17]

In summary, we have successfully demonstrated the use of RCA as a strategy to amplify a four-arm branched nanojunction. This is the first time that a four-arm junction has been replicated by using an enzymatic reaction. The fact that a nanojunction can be replicated by the RCA opens a new avenue for large-scale enzymatic synthesis of DNA nanoconstruction.

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