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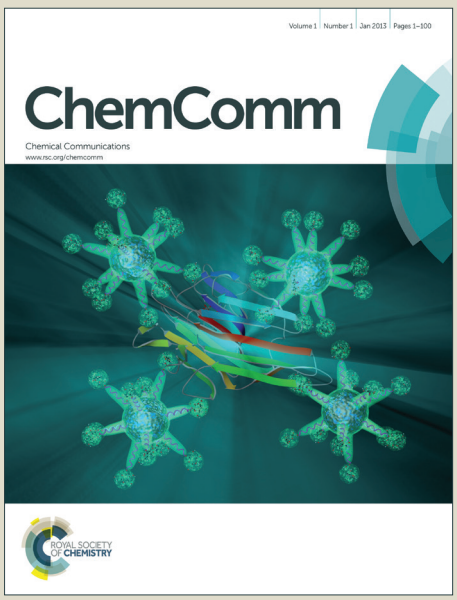

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Self-assembled DNA Nanostructures by Rolling Circle Amplification for the Delivery of siRNA Conjugates**

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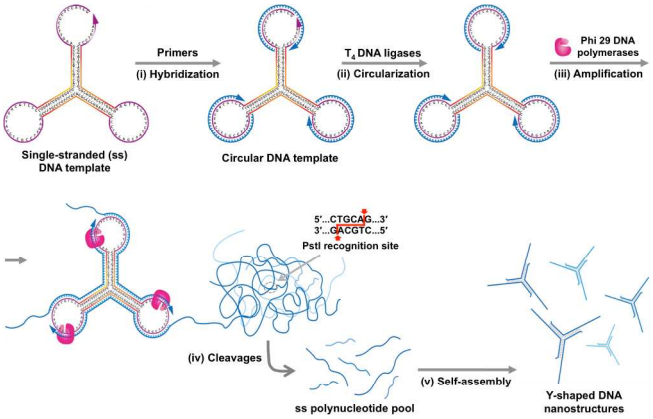
Inspired by the isothermal enzymatic process of rolling circle amplification (RCA) of DNA strands, we have developed a system to achieve more than a 200-fold increase in the synthesis of DNA nanostructures using a single-stranded circular DNA template. The amplified DNA nanostructures have shown efficient delivery of folic acid (FA) conjugated siRNAs into KB cells with a dose dependent gene silencing.

One of the main drawbacks of small interfering RNA (siRNA)-mediated gene therapy is to develop effective and safe delivery systems that can transport exogenous siRNA into cells.[1] To date, a variety of materials such as synthetic polymers, lipids, and inorganic nanoparticles have been extensively exploited as promising carriers for siRNA delivery.[2] Despite of successful *in vitro* gene regulation using the aforementioned carriers, there still remains many critical issues for clinical applications of RNA interference (RNAi) therapeutics due to undesirable immune response and toxicity of delivery carriers.[3] Therefore it is critical to utilize biocompatible and biodegradable molecules for siRNA delivery. Recently, molecularly self-assembled DNA nanostructures have been employed as drug delivery carriers because of their suitable sizes, well-defined structures, and facile preparation.[4] For example, a wide range of two- and three-dimensional DNA nanostructures such as tetrahedrons, nanotubes, and nanocubes has shown superior intracellular drug delivery efficiency as compared to the conventional materials.[5] However, the construction of DNA nanostructures often requires multiple long synthetic DNA strands, which is very costly to prepare the DNA nanostructures for practical applications. It is also difficult to make long DNA strands with over 200 nucleotides (nt) via chemical synthesis.

Rolling circle amplification (RCA) is a simple and robust technique that can continuously elongate complementary single-stranded (ss) DNA around the circular ss DNA templates under isothermal condition.[6] This process includes the recognition and binding of Phi29 DNA polymerase with a short double-stranded (ds) DNA region on the circular ss DNA by primer hybridization. DNA polymerization around the closed circular template can generate more than 70 k nt long ss DNA molecules.[7] This elongated ss DNA products from RCA have been widely utilized as an excellent scaffold to periodically assemble various small molecules such as proteins, gold nanoparticles, and fluorescent dyes.[8]



Schemes 1. Schematic illustration of the stepwise approach for the preparation of three-dimensional Y-shaped DNA (Y-DNA) nanostructures via subsequent enzymatic reactions.

Herein we demonstrate the large-scale synthesis of self-assembled DNA nanostructures for siRNA delivery by employing two consecutive enzymatic reactions, RCA and site-specific restriction cleavage. In this report, we have carefully examined how much of self-assembled Y-shaped DNA (Y-DNA) nanostructures can be produced by RCA process as well as confirmed the enhanced gene silencing activity of the resultant Y-DNA nanostructures when delivering the FA-siRNA conjugates. To obtain the large-scale production of well-defined DNA nanostructures, we have designed a

series of distinct processes (Scheme 1). The single-stranded (ss) 159 nt DNA templates are designed to self-assemble into Y-DNA nanostructures with three-way junction in a core and three hairpin loops in arms. The three arms contain two closed loops and one open loop with a nick, which encode a complementary sequence to the short DNA oligomers (20 nt) termed as a primer. This loops also contains a restriction site (6 nt) for endonuclease PstI enzymes. The 5′-phosphate group and 3′-hydroxyl group in the open loop can be placed in the close proximity to each other by annealing with a primer, which can improve DNA ligation by T4 DNA ligases. The circular Y-DNA templates serve as the substrates for RCA reaction. In the presence of Phi29 DNA polymerases, the 3′-end of the primers is continually extended around the circular Y-DNA templates at the constant temperature. This elongated DNA strands contain repeated units of complementary Y-DNA templates. In addition, three primers-binding sites in circular Y-DNA templates can also provide the enhanced amplification rate of DNA strands in three directions. Notably, the elongated ss DNA product can be folded randomly into ds structures via inter- and intra-molecular self-assembly, inducing the hybridization of palindromic PstI sequences (CTGCAG). It is possible to site-specifically recognize and cleave the elongated ss DNA by PstI endonuclease without addition of complementary ss DNA strands, usually called as helper strands. After the treatment with PstI enzymes, the elongated ss DNA is efficiently cleaved to release individual fragments with various nucleotide lengths, named as a DNA polynucleotide pool. After proper thermal annealing process, the DNA pool can self-assemble to generate Y-DNA nanostructures, which are exactly complementary to the initial Y-DNA templates. The resultant Y-DNA nanostructures are composed of Y-shaped ds DNA junction in a core and three ss overhangs (20 nt) in arms. The sticky overhangs in the Y-DNA can provide a stable and effective hybridization of siRNA molecules with complementary overhangs, thus allowing three ds siRNAs to bind per a Y-DNA nanostructure.

To confirm the construction of Y-DNA nanostructures from a DNA polynucleotide pool, each step was verified by polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 1). The annealed products (Fig. 1, line 3) between ss primers (Fig. 1, line 1) and Y-DNA templates (Fig. 1, line 2) showed a clear band shift, which migrated less than the Y-DNA templates. After ligation reaction, the bands of Y-DNA/primers were further shifted, suggesting the conformation of closed circular Y-DNA templates (Fig. 1, line 4). A clear band of the circular DNA templates was observed in the presence of a strong denaturant (8 M urea), which measured over 95 % yields of desired circular DNA templates (Fig. S1a). The RCA products showed much stronger band intensity in the sample-loading zone due to the increased molecular weight (Fig 1, line 5). It was clear that Phi29 DNA polymerases could efficiently unwind the ds DNA junctions to amplify the Y-DNA templates. In our study, 1 pmole of circular Y-DNA templates can be amplified approximately 1068 pmole of complementary DNA templates in the elongated polynucleotides. The elongated DNA molecules were site-specifically degraded by PstI enzymes to regenerate ss DNA fragments with different lengths, which were verified using 8 M urea solution in gel electrophoresis (Fig. S1b). The resultant DNA strands were assembled to form hybridized products with different molecular weights (Fig 1. line 6). Among of them, the major bands of expected Y-DNA nanostructures were eluted from the gels to successfully obtain final products via PAGE purification and ethanol precipitation (Fig. 1, line 7). Under optimal condition, we were able to produce more than 213 pmole of self-assembled Y-DNA nanostructures using 1 pmole of Y-DNA templates, which was estimated using ultraviolet spectrophotometers (UV) and gel densitometry (Supplementary Information).

To further verify the cleaved DNA products as well as the self-assembled Y-DNA structures, we designed an additional RCA template to fully analyze the process of amplification and self-assembly (Fig. S2). The components of ss 159 nt Y-DNA templates can be amplified and re-assembled together. Three individual ss DNA oligomers with a 53 nt have clearly confirmed the step-wise assembly of complementary Y-DNA structures (Fig. S3). It is noticeable that one of major cleaved bands showing on near 50 bp is the longer ss DNA products with unmatched palindromic PstI sequences. This bright band remained stable under denaturing

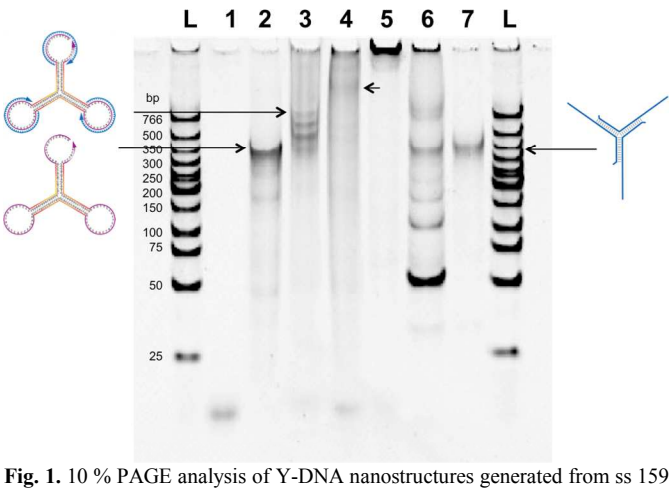


Fig. 1. 10 % PAGE analysis of Y-DNA nanostructures generated from ss 159 nt Y-DNA templates. Line 1: primers; line 2: Y-DNA templates; line 3: hybridized Y-DNA/primers products; line 4: circular Y-DNA templates; line 5: RCA products; line 6: cleaved/self-assembled products; line 7: purified Y-DNA products via gel elution. L is a DNA ladder.

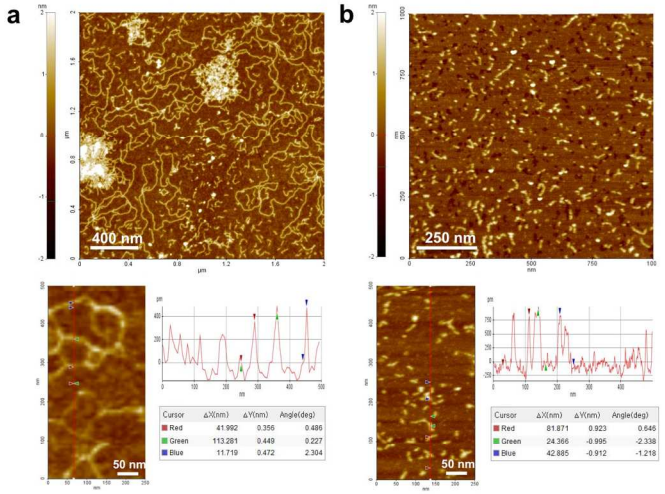


Fig. 2. AFM images (upper panel) and selection analysis (lower panel) of (a) elongated RCA products amplified from circular Y-DNA templates and (b) cleaved/self-assembled RCA products after incubation with restriction enzymes and thermal annealing.

conditions confirming that they were not the hybridized products.

The morphology of RCA as well as cleaved/self-assembled products was investigated by atomic force microscopy (AFM). As shown in Fig. 2a, the RCA products exhibited a long linear DNA strand in the micron range confirming the successful elongation of complementary ss DNA molecules from the circular Y-DNA templates. The measured height of elongated ss DNA was about 0.45 ± 0.09 nm (Fig. 2a, lower panel), which is consistent with previous reports (0.35 ± 0.05 nm) as compared to 0.84 ± 0.05 nm of the ds DNA strands in a non-contact mode.[9] It should be noted that several aggregation was also observed in AFM images of RCA products due to inter- and intra-hybridization of the elongated strands. After cleaved/self-assembled process, the RCA products were self-assembled to nano-sized globular shapes as shown in AFM image (Fig. 2b). These structures indicated the self-assembled Y-DNA nanostructures and the height of well-dispersed globular structures was approximately 1.04 ± 0.12 nm (Fig. 2b, lower panel), suggesting the formation of Y-DNA junction as compared to that of linear cleaved ss DNA oligomers (0.47 ± 0.07 nm).

To investigate whether the Y-DNA nanostructures can enhance the delivery of FA-siRNA conjugates into target cells, FA-siRNA with a 1,4-bis(3-aminopropyl)piperazine linker was used. The FA-siRNA conjugates readily hybridized to the Y-DNA nanostructures via sticky-end assembly (Fig. 3a). The gene silencing efficiency was tested by transfecting Y-FA-siRNA nanostructures into GFP-KB cells. The enhanced gene silencing of Y-FA-siRNA nanostructures was evaluated against that of FA-siRNA conjugates (Fig. 3b and c). The GFP expression was decreased approximately 25 % and 40 % at a siRNA dose of 50 nM and 100 nM. However, FA-siRNA conjugates alone (100 nM) did not show any significant gene silencing effect as reported previsouly.[10] The theoretical length of one arm of Y-DNA nanostructures is about 19.04 nm, which suggests that three FA ligands of Y-DNA are located in very close proximity (< ~ 30 nm) to each other (Fig. S4). It is likely that our Y-DNA nanostructures can provide the increased local density of targeting ligands, which may facilitate intracellular delivery through the enhanced interactions between ligands and cell surface receptors.[5]

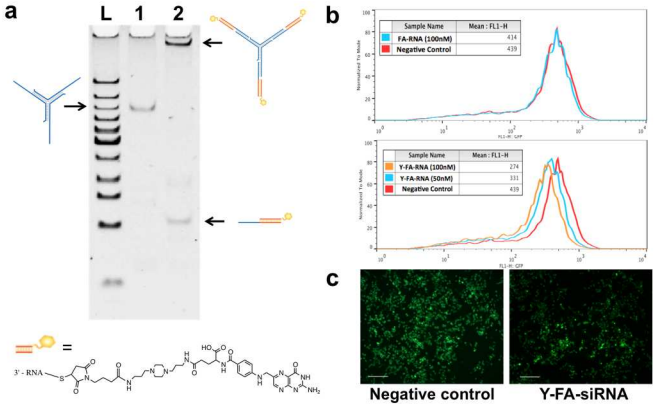


Fig. 3. (a) 10 % PAGE analysis of the hybridization of FA-siRNA conjugates to Y-DNA nanostructures. (b) GFP-KB cells treated with FA-siRNA conjugates alone (upper panel) and Y-FA-siRNA nanostructures (lower panel). (c) Fluorescence microscopy images of GFP-KB cells incubated with Y-FA-siRNA (100 nM).

In conclusion, we demonstrate a facile and reliable synthesis method for the large-scale production of DNA nanostructures for siRNA delivery. In our knowledge, this is the first attempt to use RCA technology and molecular self-assembly to fabricate well-defined DNA nanostructures for siRNA delivery. Our approaches can be widely useful and applicable as a new platform to fabricate large-scale enzymatic and biological synthesis of various DNA nanostructures for therapeutic applications.

Notes and references

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

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