Supplementary Information

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

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Materials. All of the DNA strands were purchased from Integrated DNA Technologies (IDT, USA) and thiol-modified siRNA was obtained from Bioneer Corporation (Daejeon, Republic of Korea). Sequences of the oligonucleotides are listed in Table S1. The T4 DNA ligase and PstI restriction endonuclease were purchased from New England Biolabs (NEB, UK). The RepliPHI^{TM} phi29 DNA polymerase was obtained from Epicentre Technologies Corporation (Madison, USA).

Methods

Preparation of circular DNA templates. 40 pmol of single-stranded DNA templates with a phosphate group at a 5′ end and 120 pmol of primers were annealed in 36 μL 1 X reaction buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP) at 95 °C for 5 min and cooled to 10 °C at a rate of - 1.5 °C per 1 sec using a thermal cycler (Bio-Rad T100^{TM}, Germany). After annealing, 4 μL T4 DNA ligases (400 U/μL) were added and incubated at 20 °C for 12 h. The enzymes were inactivated by heating at 80 °C for 10 min. The resultant solution was directly used in the following rolling circular amplification reaction.

Rolling circular amplification (RCA) of circular DNA templates. The circular DNA templates (5 μL) were mixed with 4 μL 10X reaction buffer (400 mM Tris-HCl (pH 7.5), 500 mM KCl, 100 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, and 40 mM DTT), 25 μL 25 mM dNTPs, and 3 μL RepliPHI^{TM} phi29 DNA polymerase (100 U/μL). The mixture was incubated at 30 °C for 18 h and stopped by heating at 80 °C for 20 min.

Site-specific cleavage and self-assembly. The RCA products (40 μL) were mixed with 8 μL 10X NEBuffer 3 (500 mM Tris-HCl (pH 7.9), 100 mM $MgCl_2$, 1000 mM NaCl, and 10 mM DTT), and 30 μL PstI restriction enzymes (30 U/μL), and then incubated at 37 °C for 15 h. After inactivation by heating at 95 °C for 10 min, the resultant solution was performed by cooling from at 95 °C to 10 °C at a rate of - 0.5 °C per 1 min. The desired products, Y-shaped DNA nanostructures, were obtained by polyacrylamide gel electrophoresis (PAGE) purification and ethanol precipitation. To prepare Y-shaped DNA/siRNA nanostructures, the resultant Y-shaped DNA nanostructures were incubated with siRNA-FA conjugates in 1X phosphate buffered saline (PBS) at room temperature for 2 h.

PAGE analysis. All products were verified by 10 % PAGE in 1 X Tris-borate-EDTA (TBE) buffers at room temperature. For denaturing analysis, the circular DNA templates and cleaved DNA oligomers were dissolved in 8 M urea buffer and then performed from 95 °C for 5 min to 50 °C at a rate of - 1 °C per 1 min. The resultant samples were analyzed using 10 % PAGE at 50 °C. The gel was stained with SYBR® Gold (Invitrogen, USA) and visualized using a Gel Doc™ EZ (Bio-Rad).

Determination of amplification factors and generation factors. Amplified products from RCA solutions were obtained by ethanol precipitation. The resultant pellets were dissolved in 40 μL DEPC-treated DW. 1 μL the resultant solutions was diluted by a 50-fold and OD_{260} was measured to be 3.78 in a 1 cm path length. The extinction coefficient of Y-shaped DNA templates was 1,414,900 L/mole·cm. Based on the Beer-Lamber law, the concentration of amplified products was calculated as follow: OD_{260} (3.78)/Ext.Coefficient (1,414,900 L/mole·cm) x path length (1 cm) = 2.67 μM. The amount of final amplified products was 5340 pmol (2.67 μM x 50 x 40 μL). It means that 5 pmole DNA templates were used to amplify a 1068-fold complementary DNA template. Thus, amplification factor was 1068. To quantify a yield of self-assembled Y-shaped DNA, 5340 pmole of amplified products were processed in 80 μL cleaved/self-assembled process. 1 μL (66.75 pmole) of the resultant solution was run on a 10 % PAGE and the band intensity of expected Y-shaped DNA was estimated to be 19.2 % by Image Lab software (Bio-Rad). The amount of final products of Y-shaped DNA nanostructures was 1125.28 pmole (66.75 pmole x 0.214 x 80) from 5 pmole Y-shaped DNA templates. Thus, generation factor was 205.1.

AFM imaging. The AFM samples (1 μL, 1 μM) were dissolved in 30 μL buffer (40 mM HEPES-HCl (pH 7) and 10 mM $NiCl_2$), and deposited onto freshly cleaved mica (Pelco Mica sheets, Ted Pella Corp.). After 30 min incubation, the mica surface was rinsed with DEPC-treated deionized water (DW) and immediately dried using nitrogen gas. The resultant samples were scanned in non-contact mode on a Park NX-10 ADM (Park Systems Corp. Korea) with NC-NCH tips (Park Systems Corp. Korea)

Gene silencing studies. GFP-overexpressing KB cells were cultured in RPMI medium (10 % FBS, 1 % Glutamax) without folic acid for at least a week to overexpress FR on KB cells. For gene silencing test, GFP-KB cells were seeded at 3.0×10^5 cells/well in 6-well culture plates. After 24 h incubation, two different siRNA concentrations (50 and 100 nM) of Y-shaped nanostructure were treated to the cells for 24 h in normal growth medium with 10% serum. The GFP expression of GFP-KB cells was measured by FACSCalibur system (BD biosciences) and the GFP gene silencing was analyzed using the flowJo program. Fluorescence images of non-treated cells (negative control) and cells treated with Y-shaped DNA/FA-siRNA nanostructures (100 nM) were obtained by the Zeiss Axiovert 200 microscope (Carl Zeiss).

Table S1. List of oligonucleotides sequences used in this study.

Strands	Sequences (5′→ 3′)
159 nt Y-DNA	Pho-GCTACCTCGCCTTGAAGCCTACATCGTCACGGCGGGCGGGCAAGGCCCCTGCAGC TACCTCGCCTTGAAGCCTTGCCCGCCCGCGCGCCCCGGAAAGGCCCCTGCAGCTACCT CGCCTTGAAGCCTTTCCGGGGCGCCGTGACGATGTAGGCCCCTGCA
53 nt Y_1 DNA	Pho-GCTACCTCGCCTTGAAGCCTACATCGTCACGGCGGGCGGGCAAGGCCCCTGCA
53 nt Y_2 DNA	Pho-GCTACCTCGCCTTGAAGCCTTGCCCGCCCGCGCGCCCCGGAAAGGCCCCTGCA
53 nt Y_3 DNA	Pho-GCTACCT CGCCTTGAAGCCTTTCCGGGGCGCCGTGACGATGTAGGCCCCTGCA
Primers (20 nt)	TTCAAGGCGAGGTAGCTGCAGGG
AS GFP siRNA	rArArGrUrCrGrUrGrCrUrGrCrUrUrCrArUrGrUTTTGCAGCTACCTCGCCTTGAA
SS GFP siRNA	rArCrArUrGrArArGrCrArGrCrArCrGrArCrUrUTT

The underlines indicate a primer binding site that is included in a PstI restriction enzyme (■). Pho: a phosphate group; r: ribonucleotide sequences.

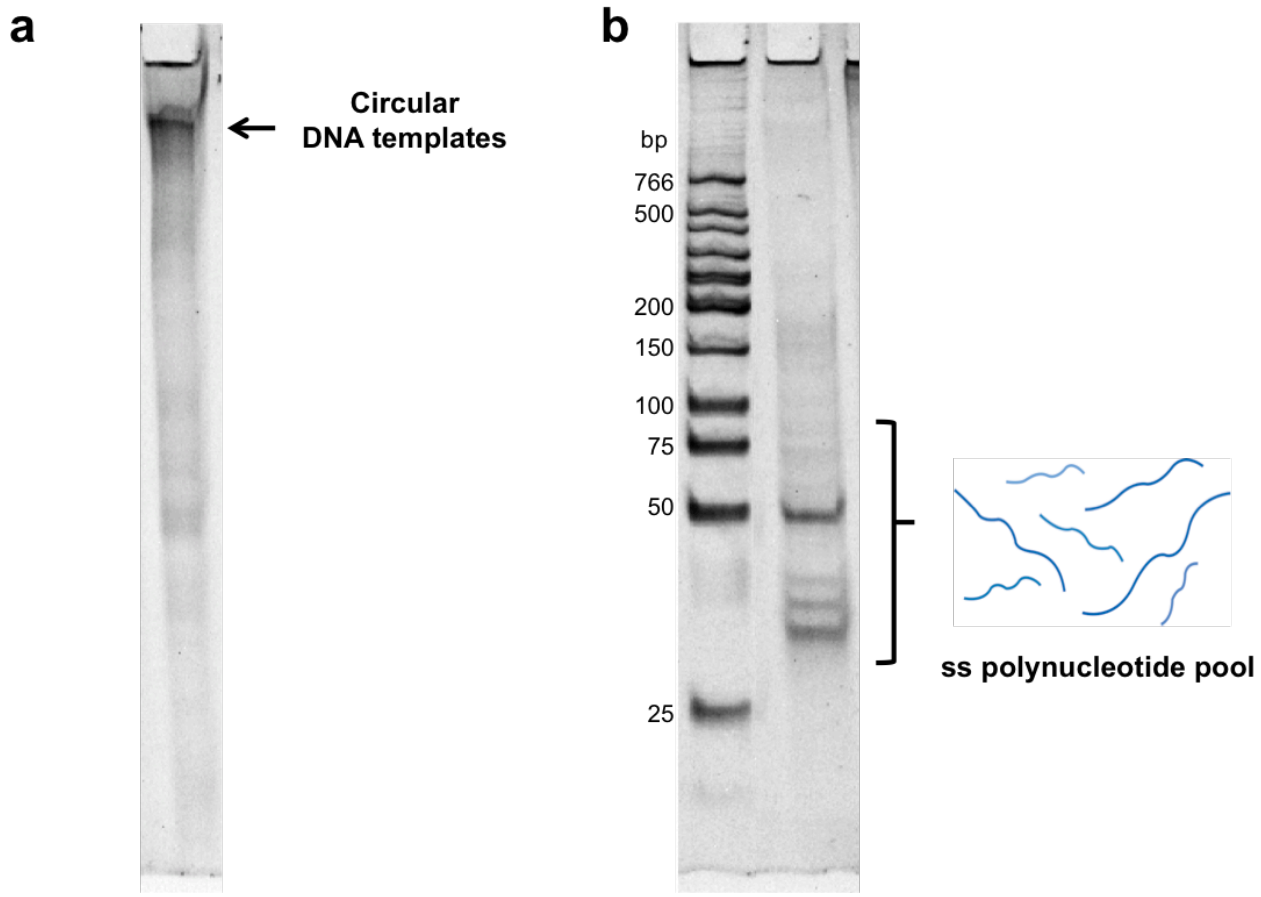

Figure S1. 10 % PAGE analysis of (a) circular DNA templates and (b) enzymatically cleaved amplified DNA products under strong denaturing condition (8 M urea buffer). L is a DNA ladder.

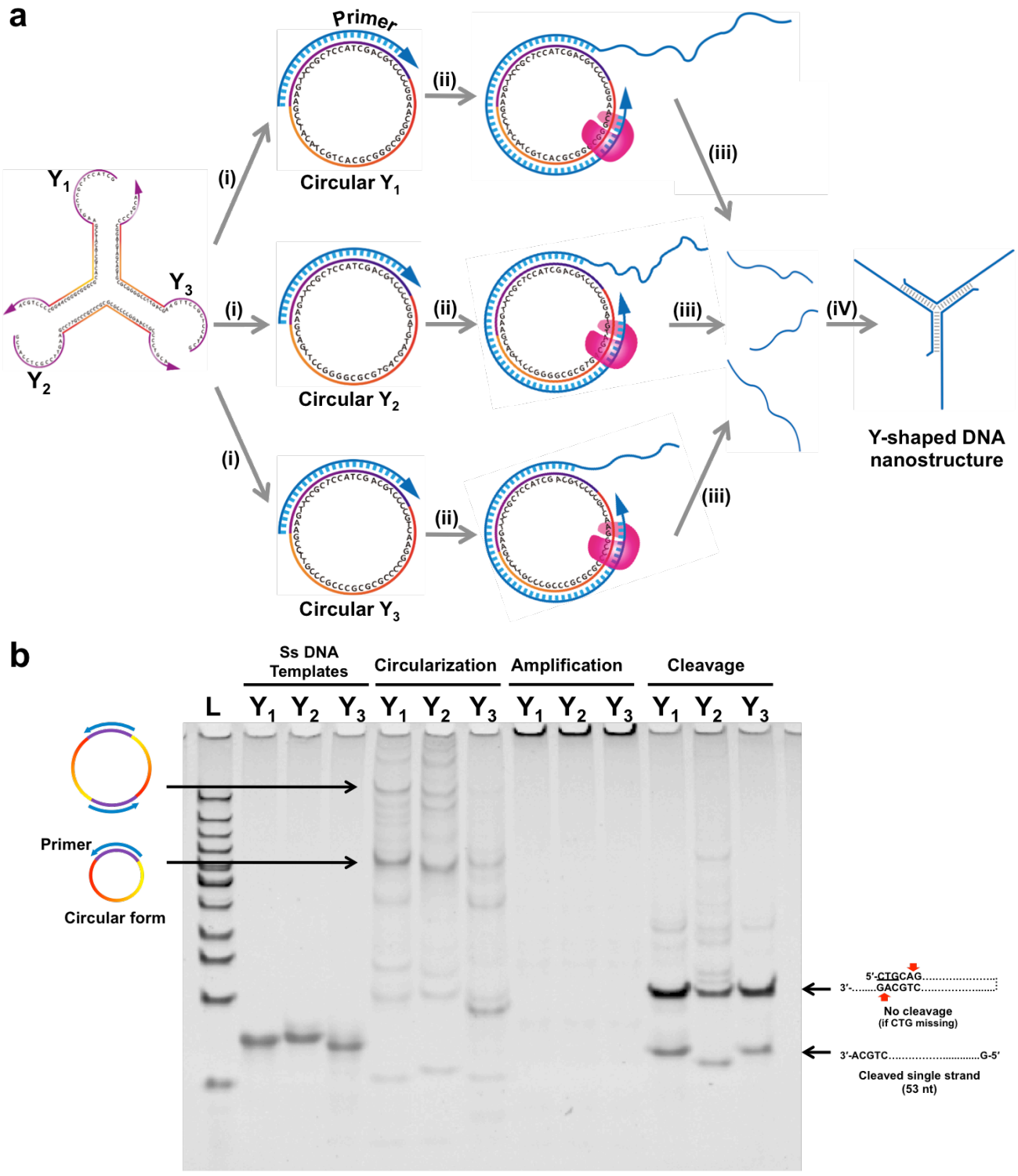

Figure S2. (a) Scheme for the construction of three-dimensional Y-DNA nanostructures from three individual DNA oligomers via RCA and restriction enzyme reactions. (i) Circularization by T4 DNA ligases (ii) Elongation by RCA reactions (iii) Site-specific cleavages by restriction enzymes (iv) Programmable molecular self-assembly. (b) 10 % PAGE analysis to verify the generation of single-stranded (ss) 53 nt DNA oligomers with complementary to ss DNA templates via RCA and restriction enzyme reactions.

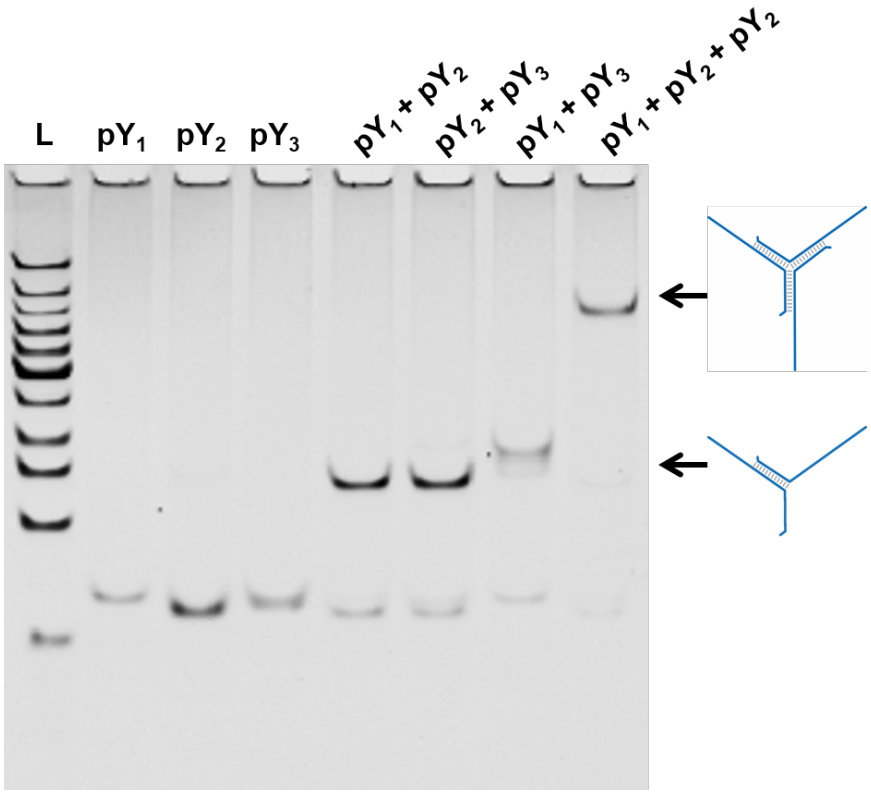

Figure S3. 10 % PAGE analysis to confirm the self-assembly of the purified ss DNA oligomers after RCA and restriction enzyme reactions by individual ss DNA templates (Figure S2). The presumed structures drawn to the right of the gel images. L is a DNA ladder.

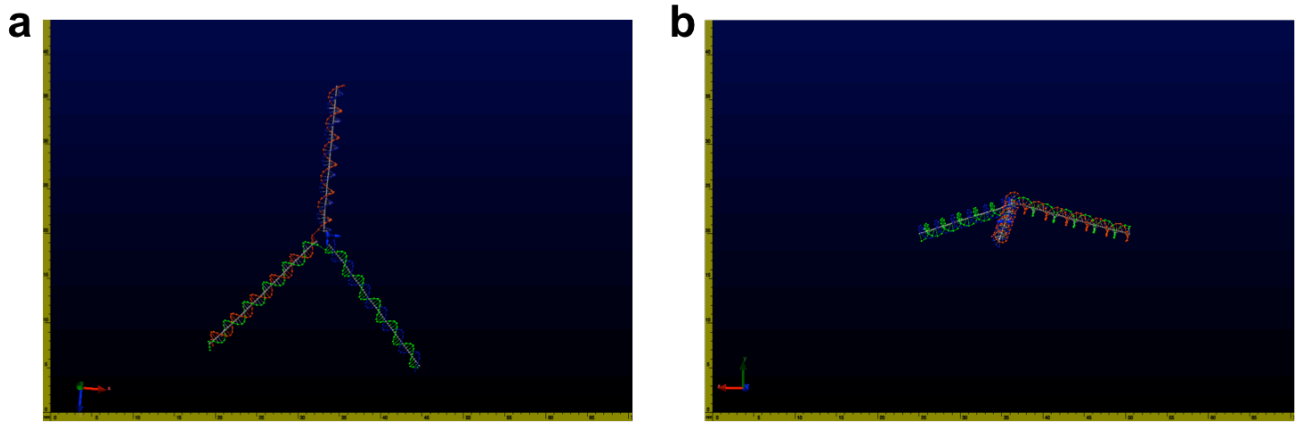

Figure S4. The three-dimensional illustrations of Y-DNA nanostructures using NanoEngineer-1 software. Each DNA arm is 19 nm long and the distance between FA ligands is under 30 nm apart: (a) Upper view and (b) side view.