Supplemental Information

Noncanonical self-assembly of multifunctional DNA nanoflowers for biomedical applications

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Supplemental Experimental Section

DNA Preparation. All DNA synthesis reagents were purchased from Glen Research (Sterling, VA), and all DNA probes (see sequences in Table S1) were synthesized on an ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) based on solid-state phosphoramidite chemistry at a 1 μmol scale. FITC or phosphate was coupled on the 5'- ends of primers and templates (see Table S1 for sequences), if applicable. DNA sequences were deprotected according to manufacturer's guidance. Deprotected DNA was further purified with reversed-phase HPLC (ProStar, Varian, Walnut Creek, CA, USA) on a C-18 column using 0.1 M triethylamine acetate (TEAA, Glen Research Corp.) and acetonitrile (Sigma Aldrich, St. Louis, MO) as the eluent. The collected DNA products were dried and detritylated by dissolving and incubating DNA products in 200 μL 80% acetic acid for 20 minutes. The detritylated DNA product was precipitated with NaCl (3 M, 25 μL) and ethanol (600 μL). UV-Vis measurements were performed with a Cary Bio-100 UV/Vis spectrometer (Varian) for DNA quantification.

Cell culture. Cell lines CCRF-CEM (Human T-cell ALL), Ramos (Human B-cell Burkitt's lymphoma), and HeLa cells (Human cervical carcinoma) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (heat inactivated, GIBCO) and 100 IU/mL penicillin-streptomycin (Cellgro) at 37 °C in a humid atmosphere with 5% CO_2. Cell density was determined using a hemocytometer prior to each experiment.

Agarose gel electrophoresis. The sizes of DNA template, primer, and NFs were estimated by agarose gel electrophoresis using an agarose gel (2%) for 40 min (100 V). The gel was stained with Ethidium Bromide (EB) and imaged under UV irradiation.

Characterization of NFs. To examine NFs using scanning electron microscopy, the products were deposited on silicone matrices, dried, and coated with Au, followed by observation on an S-4800 scanning electron microscope (HITACHI, Japan). Additionally, NFs were characterized using transmission electron microscopy on an F-2010 TEM microscope (JEOL, Japan) at a working voltage of 100 kV. Atomic force microscopy of samples was performed on a Nanoscope IIIa (Veeco, Santa Barbara, CA) using tapping mode in ambient air. Dynamic light

scattering on a Nano-zs90 Zetasizer (Malvern Instruments Ltd, UK) was used for size determination, and polarized light and an Optipho-2 polarizing microscope (Nikon, Japan) were used for polarized light microscopy. Fluorescent NFs were observed under a DM6000 B fluorescence microscope (Leica Microsystems, Germany).

Bioimaging of intracellular behaviors of NFs and Dox delivered by NFs. Bioimaging was performed using confocal laser scanning microscopy (CLSM) on a Leica TCS SP5 confocal microscope (Leica Microsystems Inc., Exton, PA) in DIC mode. An Ar laser (for FITC and Dox) and He-Ne laser (for Cy5) were used for excitation. Cells (2×10^5) in buffer (200 μL, 4.5 g/L glucose and 5 mM $MgCl_2$ in Dulbecco's PBS) were incubated with NFs (10 μL equivalent NF reaction solution) or NF-Dox complexes (2 μM Dox equivalent) in a cell culture incubator for 2 h, followed by washing with washing buffer (1 mL) twice and addition of Dulbecco's PBS (200 μL) before imaging.

Drug loading into NFs. Doxorubicin (Dox) (1 mM) was incubated with the DNA $\mathbf{NF_{0.2}s}$ (from 20 μL raw NF products) dispersed in 100 μL Dulbecco's PBS (Sigma Aldrich, St. Louis, MO) at room temperature for 24 h, followed by centrifugation at 10 000 rpm for 15 min. The free Dox in the supernatant were isolated and quantified by measuring the absorption of Dox at 480 nm on a Cary Bio-100 UV/Vis spectrometer (Varian). The Dox loading amount into $\mathbf{NF_{0.2}s}$ was calculated as shown in **Equation S1**.

$$\text{Loading amount} = \text{Total Dox amount} - \text{Dox amount in supernatant} \quad \textbf{[S1]}$$

The precipitate (NF-Dox complexes) was then dispersed in Dulbecco's PBS (100 μL).

Targeted drug delivery using NFs. The cytotoxicity of NFs, free drug, or drug-NF complexes were evaluated using CellTiter 96 cell proliferation assay (Promega, Madison, WI, USA). Cells (5×10^4 CEM or Ramos, or 5×10^3 HeLa cells/well) were treated with NFs, free Dox, or Dox- NF complexes in FBS-free medium. After incubation for 2 h in a cell culture incubator, supernatant medium was removed, and fresh medium (10% FBS, 200 μL) was added for further cell growth (48 h). Then medium was again removed, and CellTiter reagent (20 μL) diluted in fresh FBS-free medium (100 μL) was added to each well and incubated for 1-2 h. The

absorbance (490 nm) was recorded using a microplate reader (Tecan Safire microplate reader, AG, Switzerland). Cell viability was determined according to the manufacturer's description.

Supplemental Figures

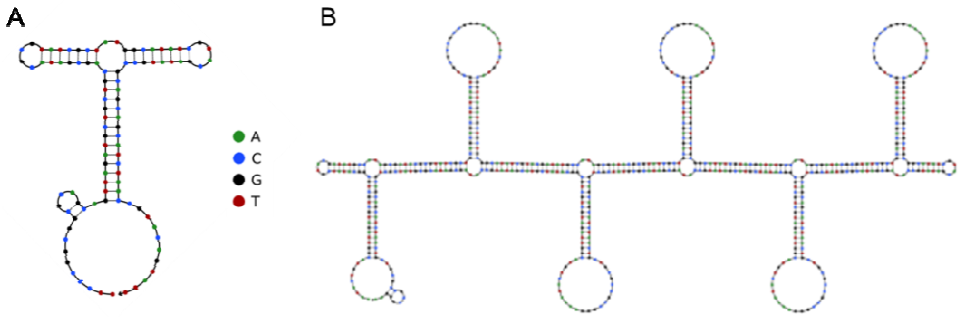

Figure S1. Predicted secondary structures of the linear template (**T-1**) with 3-way junction structure (*A*) and octameric concatemer RCR products (*B*) with branched aptamer structures protruding and aligning on alternative sides and many dsDNA (for drug loading) on the backbone and stem. Structures were predicted using the Nupack software[1].

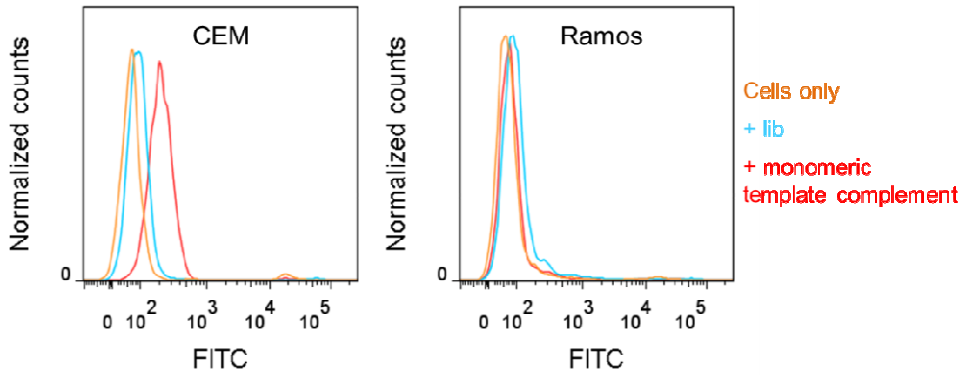

Figure S2. Flow cytometry data showing selective recognition ability of monomeric template complement to target CEM cells, as an example, but not to nontarget Ramos cells.

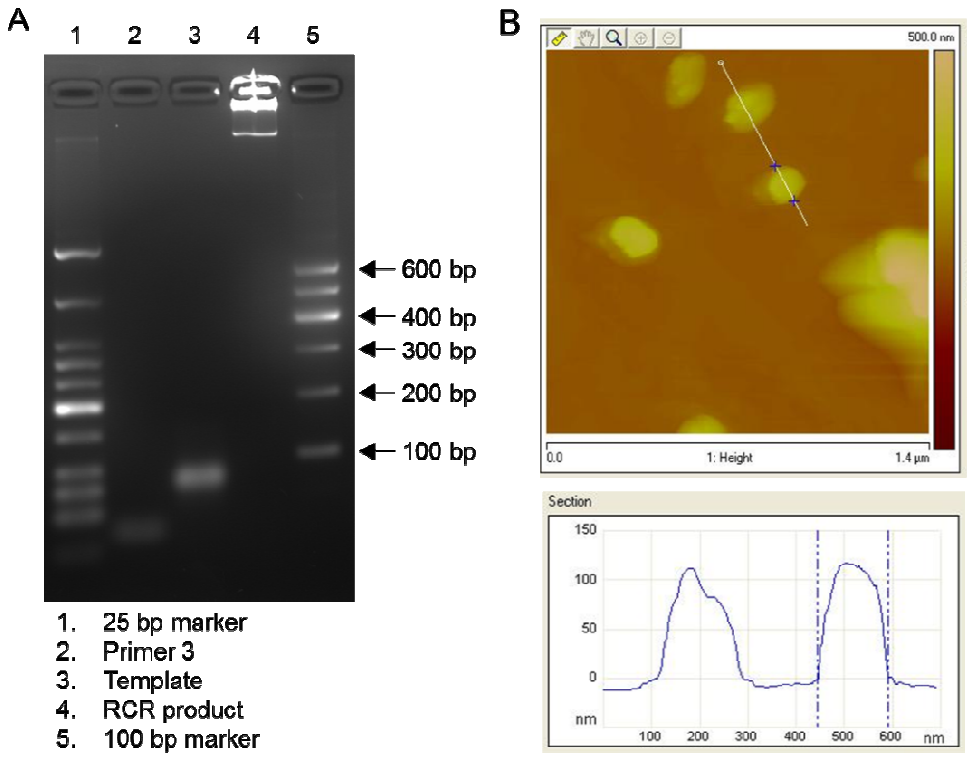

Figure S3. (*A*) An image of agarose gel (2%) electrophoresis indicating the elongation of DNA through RCR. (*B*) An AFM image displaying monodisperse **NFs** and the size determination.

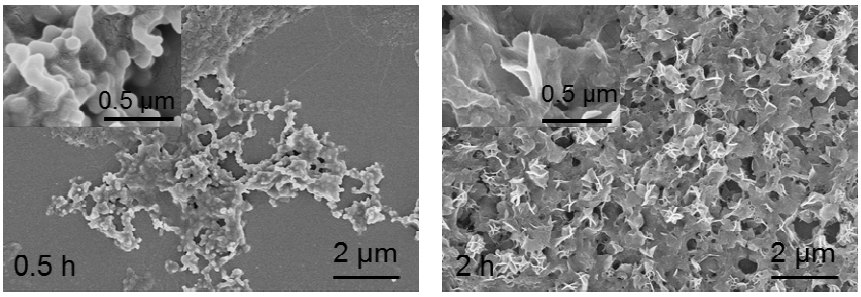

Figure S4. SEM images of RCR products from $\mathbf{RCR_{0.5}}$ and $\mathbf{RCR_{2}}$.

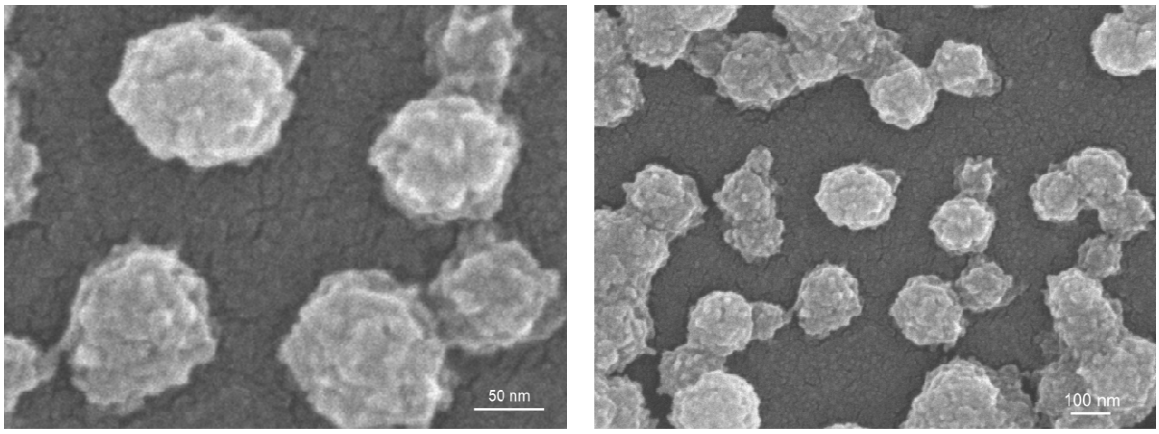

Figure S5. SEM images of sonicated NF particles from **RCR_{24}**. The hierarchical structures on the surfaces of these sonicated DNA particles reflect the internal structures and the high density of DNA in the original NFs.

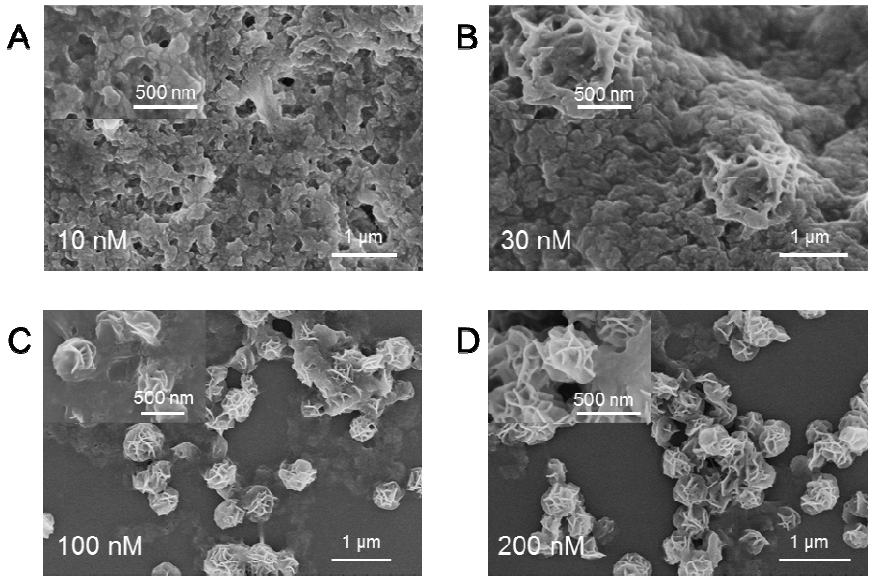

Figure S6. SEM images of products from RCR using a series of increasing template concentrations. Results indicate that NFs started to be formed with template concentrations up to 100 nM (RCR: 10 h).

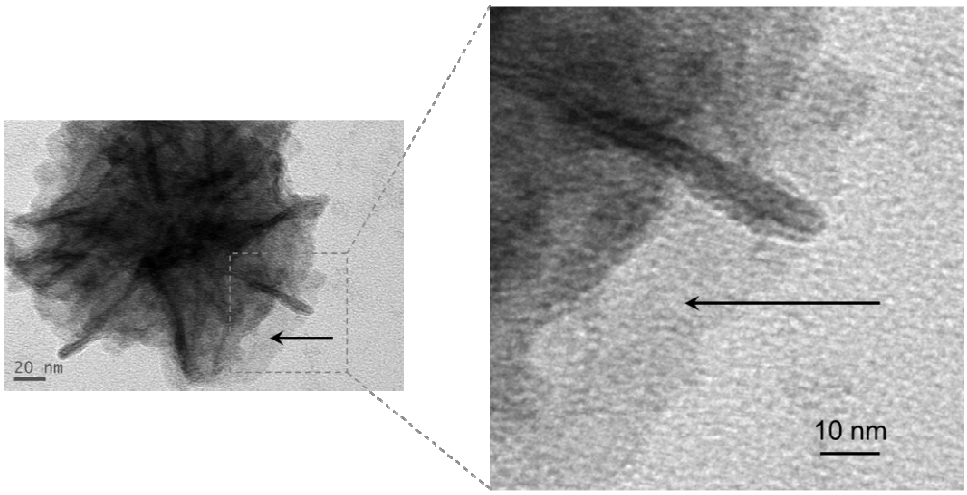

Figure S7. TEM images of **$NF_{0.2}$s** displaying ultrathin sheet sections (indicated by arrows).

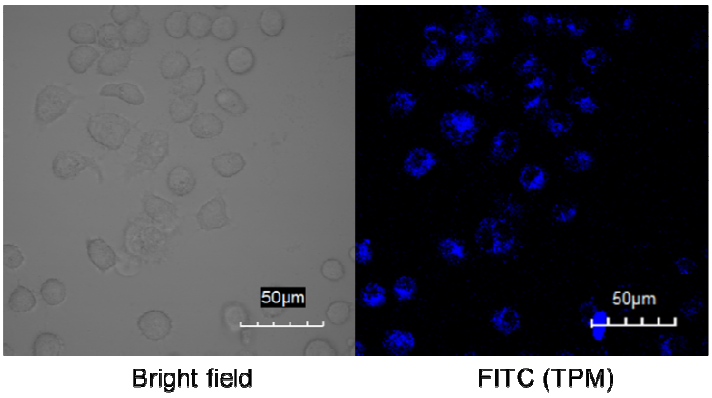

Figure S8. Two-photon microscopy (TPM) images displaying intracellular FITC signal in HeLa cells treated with **$NF_{0.2}$s** incorporated with FITC and sgc8, following internalization of NFs after incubation at 37 °C for 2 h.

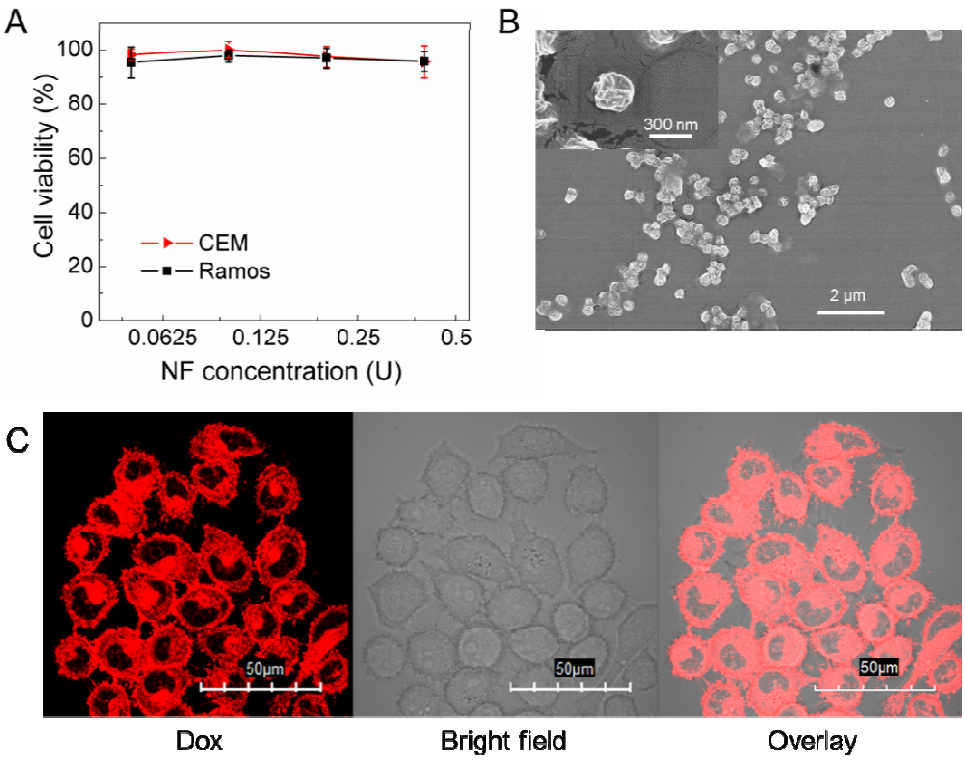

Figure S9. (*A*) MTS assay results verifying the biocompatibility of **$NF_{0.2}$s** in both CEM cells and Ramos cells (1 U NF corresponds to the NF concentration for 10 μM Dox in **$NF_{0.2}$-Dox**). (*B*) SEM images of **$NF_{0.2}$s** loaded with Dox (**NF-Dox**). (*C*) Confocal microscopy images displaying Dox distribution in HeLa cells treated with NF-Dox (2 μM Dox equivalent) for 2 h.

Supplemental Tables

Table S1. Sequences of DNA probes. FITC was labeled at the 5'-ends, if applicable.

	Sequences (5'-3')
T-1 (RCR template 1, complementary drug loading site-Sgc8)	Phosphate- TTCCCGGCGGCGCAGCAGTTAGATGCTGCTGCAGCGATACGCGTATCGC TATGGCATATCGTACGATATGCCGCAGCAGCATCTAACCGTACAGTATT
T-2 (RCR template 2)	Phosphate- TTCCCGGCGGCGCAGCAGTTAGATTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAACCGTACAGTATT
Primer	TCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA
Sgc8	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA

References

1. Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, *et al.* (2010) NUPACK: Analysis and design of nucleic acid systems. *J Comput Chem* 32(1): 170-173.