Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Floxuridine-containing Nucleic Acid Nanogels for Anticancer Drug Delivery

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MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich unless otherwise specified and used without further purification. 3-((Bis(diisopropylamino)phosphino)oxy)propanenitrile (95%, Ark Pharm, Inc.), 1H-tetrazole (98%, Adamas), 4,4'-dimethoxytrityl chloride (DMT-Cl, 97%, Shanghai Shaoyuan Co. Ltd), floxuridine (**F,** Ark Pharm, Inc.) were used as received. Solvents, such as dichloromethane (DCM), pyridine (Py), acetonitrile (MeCN), and methanol (HPLC grade) were purchased from Tansoole-reagent, China, and were used without further purification. All DNA sequences were synthesized on an ABI 3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA), and the synthesis reagents were purchased from Shanghai Lingjiang Industrial Development Co., Ltd. DuraScribe® T7 Transcription Kit (Epicentre, Madison, WI), Pyrophosphatase, ATP, CTP, and GTP (ThermoScientific, USA), and 5-FdUTP (Jena Bioscience) were used as received. Three RNA strands (Yb'-1, Yb'-2 and Yb'-3) were synthesized by Sangon biotech (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA Laboratories GmbH. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma-Aldrich. Alexa fluor® 488 Annexin V/dead cell apoptosis assay kit were purchased from Invitrogen. Ultrapure water was used in all experiments. Clear polystyrene tissue culture treated 24-well and 96-well plates were obtained from Corning Costar. All other reagents and solvents were obtained from the domestic suppliers and used as received.

All oligonucleotide sequences are listed in the table below. Floxuridine is denoted as **F** in the sequences.

Table S1. The detailed information of DNA sequences

Name	Detailed sequence information
Ya-1	5'-GGG AGA **F**GC **F**G**F** CC**F** AAC CA**F** GAC CG**F** C-3'
Ya-2	5'-GGG AGA **F**GG ACG G**F**C A**F**G **F**AC **F**AG A**F**C A-3'
Ya-3	5'-GGG AGA **F**G**F** GA**F** C**F**A G**F**A G**FF** AGG ACA G-3'

Yb-1	5’-CA**F** C**F**C CCA **F**CC GCA **F**GA CA**F** **F**CG CCG **F**-3’
Yb-2	5’-CA**F** C**F**C CCA CGG CGA A**F**G ACC GAA **F**CA G-3’
Yb-3	5’-CA**F** C**F**C CCC **F**GA **FF**C GG**F** **F**CA **F**GC GGA **F**-3’
FAM-labeled Yb-1	5’-FAM-CA**F** C**F**C CCA **F**CC GCA **F**GA CA**F** **F**CG CCG **F**-3’
FAM-labeled Yb-2	5’-FAM-CA**F** C**F**C CCA CGG CGA A**F**G ACC GAA **F**CA G-3’
FAM-labeled Yb-3	5’-FAM-CA**F** C**F**C CCC **F**GA **FF**C GG**F** **F**CA **F**GC GGA **F**-3’

Table S2. The detailed information of RNA sequences

Name	Detailed sequence information
Ya’-1	5’-GGG AGA **F**GC **F**G**F** CC**F** AAC CA**F** GAC CG**F** C-3’
Ya’-2	5’-GGG AGA **F**GG ACG G**F**C A**F**G **F**AC **F**AG A**F**C A-3’
Ya’-3	5’-GGG AGA **F**G**F** GA**F** C**F**A G**F**A G**FF** AGG ACA G-3’
Yb’-1	5’-CAU CUC CCA UCC GCA UGA CAU UCG CCG U-3’
Yb’-2	5’-CAU CUC CCA CGG CGA AUG ACC GAA UCA G-3’
Yb’-3	5’-CAU CUC CCC UGA UUC GGU UCA UGC GGA U-3’
FAM-labeled Yb’-1	5’-FAM-CAU CUC CCA UCC GCA UGA CAU UCG CCG U-3’
FAM-labeled Yb’-2	5’-FAM-CAU CUC CCA CGG CGA AUG ACC GAA UCA G-3’
FAM-labeled Yb’-3	5’-FAM-CAU CUC CCC UGA UUC GGU UCA UGC GGA U-3’

Measurements.

Nuclear magnetic resonance (NMR). ^{19}F NMR spectra were recorded on Bruker AVANCEIII 400 spectrometer with deuterium oxide (D_2O) as a solvent.

Ultraviolet-visible absorption (UV-vis). The UV-vis absorption of the sample solutions was measured by using a Thermo Electron-EV300 UV-vis spectrophotometer at room temperature. The slit-width was set as 1 nm with a scan speed of 480 nm/min.

Dynamic light scattering (DLS). DLS measurements were performed by a Zetasizer Nano ZS90 (Malvern Instruments Ltd.) equipped with a 125 mW laser at 25 °C. The scattering angle was kept at 173° and the wavelength was set as 633 nm during the whole experiment.

Atomic force microscopic (AFM). AFM images were obtained by using a

Dimension FastScan Bio AFM in tapping mode to collect height.

Methods

Synthesis of F-containing RNA sequences Ya'-1, Ya'-2, Ya'-3

F-containing RNA sequences were synthesized according to a previously reported method.[1] Generally, by using 5-FdUTP monomer instead of natural UTP monomer during the *in vitro* transcription process, a T7 RNA polymerase variant Y639F will incorporate 5-FdUTP into RNA strands, in which the natural uridine nucleosides can be completely replaced by 5-FdUTP. In a typical *in vitro* transcription reaction, T7 RNA polymerase Y639F for the synthesis of RNA sequences was added to the transcription reactions containing template DNA (1 μg), ATP (5 mM), CTP (5 mM), GTP (5 mM) and 5-FdUTP (5 mM), 10 mM DTT and reaction buffers. Subsequently, the reaction solution was incubated for 6 h at 37°C. During the *in vitro* transcription process, **F**-modified monomer 5-FdUTP was used to replace the UTP in the reaction buffer. Therefore, the obtained ssRNAs contained therapeutic **F**s at the places that uridines were supposed to be in the sequences. Thereafter, **F**-containing RNA sequences was purified by denaturing polyacrylamide gel electrophoresis (PAGE) and excised from the gel and electroeluted. Subsequently, the eluted RNA was precipitated with chilled ethanol overnight at -20°C. After centrifugation, the pellet was washed and dissolved in RNase free water and stored at -80°C.

First, the floxuridine incorporation in the RNA was demonstrated by ^{19}F NMR spectroscopies, which were recorded on a Bruker AVIII400 overnight. As shown in Figure S1, for free floxuridine, there is only one sharp peak in the ^{19}F NMR spectra (in D_2O), indicating only one kind of 'F' in floxuridine. When **F** was incorporated in RNA strands, multiple peaks appeared in Ya'-1, Ya'-2, and Ya'-3 in D_2O with varied chemical shift compared to free floxuridine, suggesting the different chemical environment of 'F's in the RNA strands. As such, we are quite sure that floxuridine has been successfully incorporated into the RNA strands.

Moreover, denaturing PAGE analysis for **F**-containing Ya'-1, Ya'-2, and Ya'-3, were also performed in a gel that contained 20% acrylamide (19:1, acrylamide/bisacrylamide), employing normal **U**-containing Ya'-1, Ya'-2, Ya'-3 (containing normal uridine in the sequence instead of **F**) as controls. As shown in Figure S2, **F**-containing ssRNA Ya'-1, Ya'-2, and Ya'-3 appeared as sharp bands on the 20% denature PAGE gel with the same mobility as normal strands containing uridine, which indicated the successful synthesis of **F**-containing ssRNAs.

Table S3. The double-stranded template DNA for *in vitro* transcription of **F**-containing Ya'-1, Ya'-2, and Ya'-3. Note: Blue part in the template is the promoter sequence. Green arrow indicates the start point of transcription from 5' to 3'.

RNA	Sequences
Ya'-1 template	5'-**TAATACGACTCACTATAGGGAGA**TGCTGTCCTAACCATGACCGTC-3' 3'-ATTATGCTGAGTGATATCCCTCTACGACAGGATTGGTACTGGCAG-5'
Ya'-1	**5'-GGGAGAFGCFGFCCFAACCAFGACCGFC-3'**
Ya'-2 template	5'-**TAATACGACTCACTATAGGGAGA**TGGACGGTCATGTACTAGATCA-3' 3'-ATTATGCTGAGTGATATCCCTCTACCTGCCAGTACATGATCTAGT-5'
Ya'-2	**5'-GGGAGAFGGACGGFCAFGFACFAGAFCA-3'**
Ya'-3 template	5'-**TAATACGACTCACTATAGGGAGA**TGTGATCTAGTAGTTAGGACAG-3' 3'-ATTATGCTGAGTGATATCCCTCTACACTAGATCATCAATCCTGTC-5'
Ya'-3	**5'-GGGAGAFGFGAFCFAGFAGFFAGGACAG-3'**

Formation of F-containing DNA nanogels, F-containing RNA nanogels, F-containing DNA nanogels labeled with FAM, F-containing RNA nanogels labeled with FAM

Stoichiometric amounts of the ssDNA strands for the Y-shaped motifs Ya, Yb, FAM-labeled Yb, Ya', Yb', and FAM-labeled Yb' were separately added to the eppendorf (EP) tubes with a buffer solution containing tris-acetic-EDTA-Mg^{2+} (TAE/Mg^{2+})

buffer. Subsequently, the mixture of Ya-1, Ya-2, and Ya-3 were slowly cooled from 95°C to room temperature over 24 h to form the desired Ya building motifs. Similar procedure was followed for Yb-1, Yb-2, and Yb-3 to form Yb motifs. Besides, the mixture of Ya'-1, Ya'-2, and Ya'-3 were slowly cooled from 70°C to room temperature over 24 h to obtain Ya' motifs. Similar procedure was followed for Yb'-1, Yb'-2, and Yb'-3 to form Yb' motifs, FAM-labeled Yb-1, FAM-labeled Yb-2 and FAM-labeled Yb-3 to form FAM-labeled Yb motifs, FAM-labeled Yb'-1, FAM-labeled Yb'-2 and FAM-labeled Yb'-3 to form FAM-labeled Yb' motifs.

For the preparation of **F**-containing DNA nanogels, stoichiometric quantities of Ya and Yb were mixed together in a TAE/Mg^{2+} buffer, and the solution was heated to 45°C and slowly cooled down to 25°C over 24 h to form DNA nanogels with a concentration of 500 nM. Similarly, **F**-containing RNA nanogels (Ya' mixed with Yb'), **F**-containing DNA nanogels labeled with FAM (Ya mixed with FAM-labeled Yb), **F**-containing RNA nanogels labeled with FAM (Ya' mixed with FAM-labeled Yb') were obtained in the similar way.

Gel electrophoresis

Denaturing PAGE analysis for **F**-containing Ya'-1, Ya'-2, and Ya'-3 were performed in a gel that contained 20% acrylamide (19:1, acrylamide/bisacrylamide), employing the normal uridine (**U**)-containing Ya'-1, Ya'-2, Ya'-3 RNA as controls. Both the gel and running buffer contained 8 M urea, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (1×TBE buffer). The gel was run on a FB-VE10-1 electrophoresis unit (Fisher Biotech) at 4 ^{o}C and followed by staining with ethidium bromide (EB, Sigma) and scanned.

Native PAGE analyses for the Y-shaped motifs (Ya, Yb, Ya', and Yb') were performed in a gel containing 6% acrylamide (19:1, acrylamide/bisacrylamide) and TAE/Mg^{2+} buffer. Each sample solution was loaded into the gel and then run on a FB-VE10-1 electrophoresis unit (Fisher Biotech, 120V, constant voltage) with TAE/Mg^{2+} buffer. After electrophoresis, imaging was carried out by EB staining and UV

illumination.

Non-denaturing gels were used to check the formation of the nucleic acid nanogels. For the characterization of DNA nanogels, 0.8% (w/w) agarose gels were run on a FB-VE10-1 electrophoresis unit (90 V, constant voltage) with TAE/Mg^{2+} running buffer. Similarly, the formation of RNA nanogels was analyzed by 1.5% (w/w) agarose gel electrophoresis analysis. After electrophoresis, the gels were stained by EB and scanned.

AFM imaging

A little drop of solution containing nucleic acid nanogels was spotted onto freshly cleaved mica surface (Electron Microscopy Sciences) and incubated for 10 s to allow absorbing onto the substrate. Subsequently, the sample drop was washed off by 30 μl magnesium acetate solution (2 mM), and dried by compressed air. Nanogel samples were then imaged with a Dimension FastScan Bio AFM in a tapping mode in the air.

Stability assay in the presence of fetal bovine serum

The stability of nucleic acid nanogels in physiological condition was characterized by PAGE electrophoresis. DNA and RNA nanogels were incubated with phosphate buffered saline with 10% (v/v) fetal bovine serum at 37^{o}C for 1 h, respectively. These obtained final samples were then loaded on 10% native PAGE gel, which was run at 100 V in 1×TAE/Mg^{2+} buffer.

Cell culture

HeLa cells (a human cervical cancer cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (FBS) and antibiotics (50 units mL^{-1} penicillin and 50 units mL^{-1} streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2.

Cellular uptake studies of nucleic acid nanogels

Confocal laser scanning microcopy (CLSM) and flow cytometry were used to characterize the cell uptake behaviors of nucleic acid nanogels. For CLSM studies,

HeLa cells were seeded in 24-well culture plates at a density of 3.0×10^4 cells per well and cultured for 24 h. Then the medium was replaced with medium containing FAM-labeled DNA nanogels, FAM-labeled RNA nanogels, ssDNA (FAM-labeled Yb-1) or ssRNA (FAM-labeled Yb'-1) at a concentration of 4 μM in OptiMEM (Life Technologies, Inc.) for 20 h, respectively. Then the culture medium was removed and the cells were rinsed with PBS three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were stained with Hoechst 33342 fluorescence dye for 15 min. Finally, the slices were mounted and observed with a laser scanning confocal microscope (Leica TCS SP8 STED 3X).

For flow cytometry, HeLa cells were seeded in 24-well culture plates at a density of 3.0×10^4 cells per well and cultured for 24 h. Then the medium was replaced with medium containing FAM-labeled DNA nanogels, FAM-labeled RNA nanogels, ssDNA (FAM-labeled Yb-1) or ssRNA (FAM-labeled Yb'-1) at a concentration of 4 μM in OptiMEM for 20 h, respectively. Thereafter, the culture medium was removed and the cells were washed with PBS three times and then harvested. The amounts of intracellular fluorescent signal of FAM-labeled DNA nanogels and FAM-labeled RNA nanogels were quantified using BD Accuri™ C6 flow cytometer, indicating the amount of nucleic acid nanogels internalized by HeLa cells.

Nuclease digestion assay.

As a promising DDS, the abilities of **F**-containing nucleic acid nanogels to release their toxic cargoes were characterized by PAGE electrophoresis according to the previously reported method.[2] For instance, after cell internalization, DNA nanogels can be digested by DNase II to release therapeutic agents and inhibit cell proliferation. To demonstrate their degradation, DNA nanogels were incubated with 20 U/mL DNase II at 37 ^{o}C for 24 h. Similarly, the digestion of RNA nanogels was simulated by incubation with RNase (10 U) in the same time. These obtained final samples were then loaded on 10% native PAGE gel, which was run at 100 V in TAE/Mg^{2+} buffer.

***In vitro* anticancer effects of nucleic acid nanogels**

The HeLa cells were chosen to evaluate the anticancer effects of nucleic acid nanogels by MTT assay, respectively. Meanwhile, the free drug **F**, **F**-containing ssDNA (Ya-1) and **F**-containing ssRNA (Ya'-1) were used as controls. The cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 200 μL medium. After 24 h incubation, the cells obtained 70-80% confluence. Then the culture medium was removed and replaced with 200 μL fresh medium containing serial dilutions of **F**-containing DNA nanogels, **F**-containing RNA nanogels, free drug **F**, **F**-containing ssDNA (Ya-1) and **F**-containing ssRNA (Ya'-1), respectively. The cells without any treatment were set as control. Then, HeLa cells were cultured for another 72 h at 37 °C under 5% CO_2 and further incubated with the addition of 20 μL of MTT assay stock solution (5 mg mL^{-1} in PBS) for another 4 h. The culture medium was removed and 200 μL DMSO was added into each well to dissolve the formazan crystals. Afterwards, the plates were vibrated for 10 min and the absorbance of each well was measured in a BioTek® Synergy H4 at a wavelength of 490 nm.

Cell apoptosis assay

HeLa cells were seeded in 24-well plates at a density of 3.0×10^4 cells per well and cultured for 24 h. Then the HeLa cells were treated with **F**-containing DNA nanogels, **F**-containing RNA nanogels, **F**-containing ssDNA (Ya-1), **F**-containing ssRNA (Ya'-1) and free drug **F** at the same concentration in OptiMEM (equivalent **F,** 10 μM) for 48 h. The untreated HeLa cells were used as control. The apoptosis experiments were performed using a combined staining with FITC-Annexin V/PI (FITC, fluorescein isothiocyanate, PI, propidium iodide) flow cytometry assay. Briefly, untreated and treated cells were suspended in annexin binding buffer, with the addition of FITC-labeled Annexin V and PI, and incubated at room temperature for 15 min. After incubation, the samples were analyzed by BD Accuri™ C6 flow cytometer. Finally, the cell populations were analyzed with the FlowJo software.

Supplementary Figures

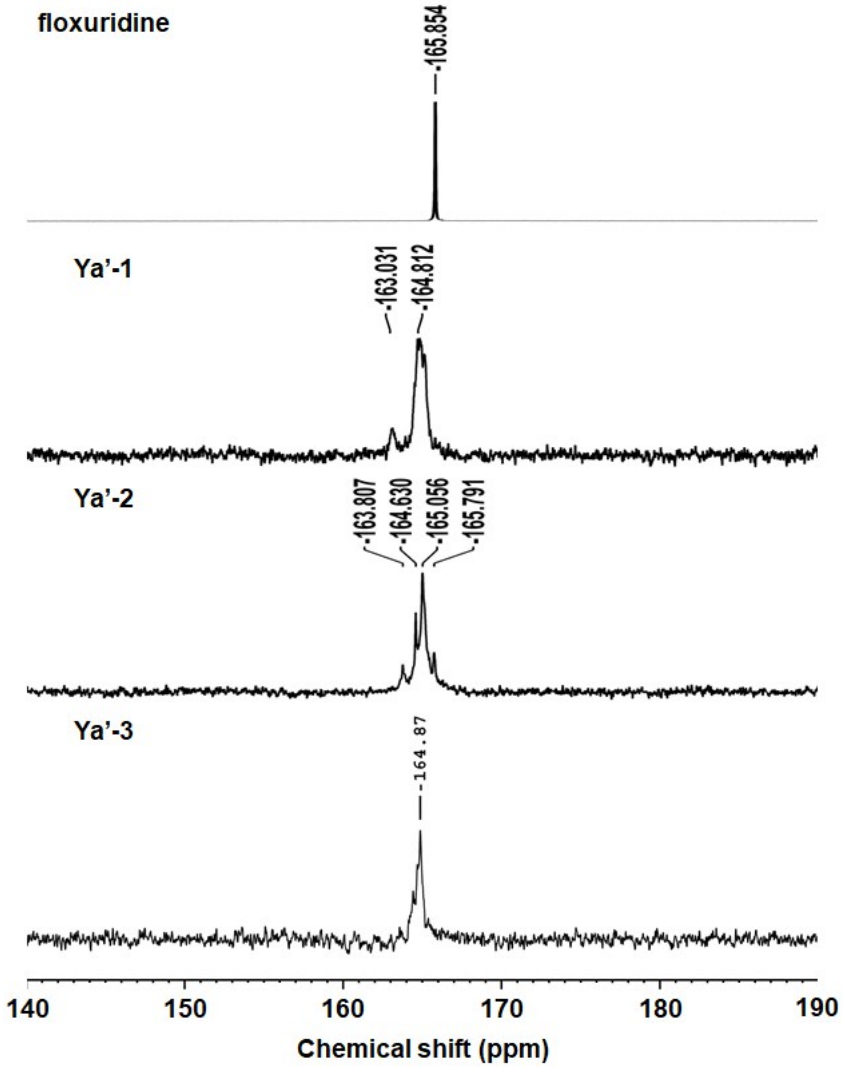

Figure S1. ^{19}F NMR spectra of free floxuridine, **F**-containing Ya'-1, Ya'-2, Ya'-3 in D_2O.

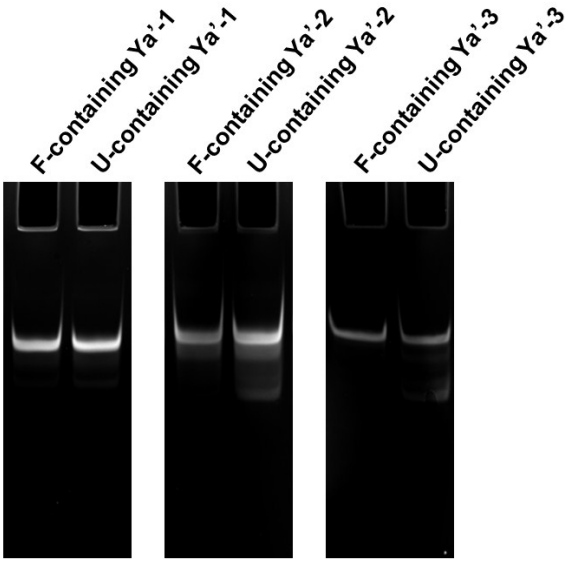

Figure S2. 20% Denaturing polyacrylamide gel electrophoretic analysis of the **F**-containing Ya'-1, Ya'-2, Ya'-3. While uridine (**U**)-containing Ya'-1, Ya'-2, Ya'-3 normal RNA strands were used as controls.

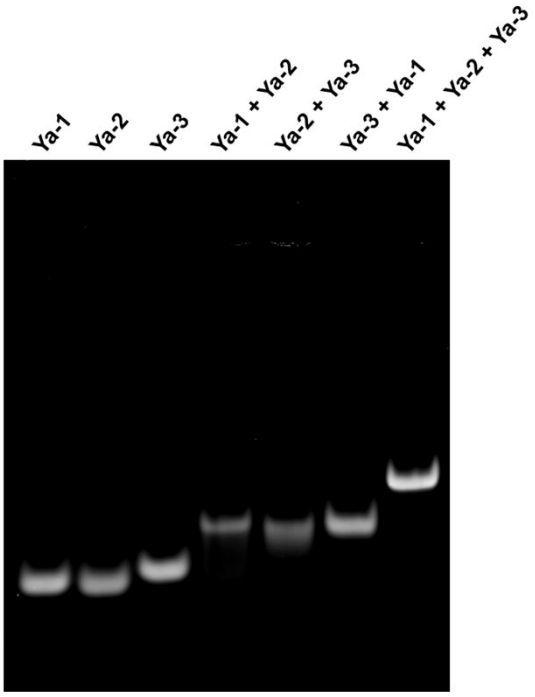

Figure S3. Characterization of the formation of Ya analyzed by 6% non-denaturing PAGE electrophoresis. Lanes 1-3 are single-stranded DNAs including Ya-1, Ya-2, and Ya-3. Lanes 4-6 are the partial assemblies containing only two strands, Ya-1+Ya-2, Ya-2+Ya-3, and Ya-3+Ya-1. Lane 7 is Y-motif, Ya.

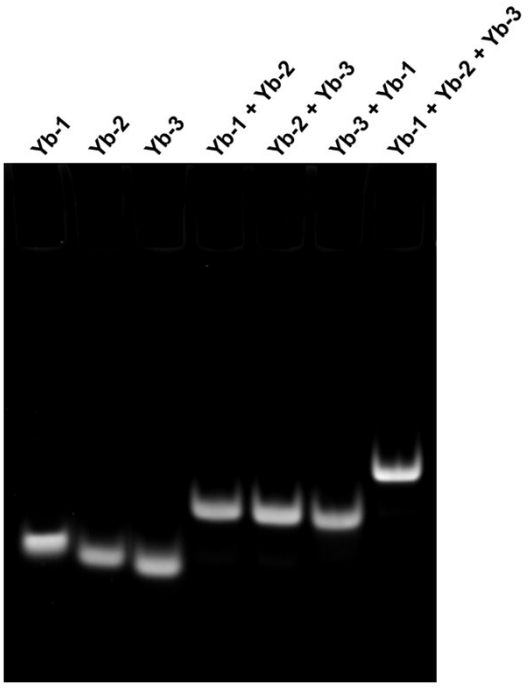

Figure S4. Characterization of the formation of Yb analyzed by 6% non-denaturing PAGE electrophoresis. Lanes 1-3 are single-stranded DNAs including Yb-1, Yb-2, and Yb-3. Lanes 4-6 are the partial assemblies containing only two strands, Yb-1+Yb-2, Yb-2+Yb-3, and Yb-3+Yb-1. Lane 7 is Y-motif, Yb.

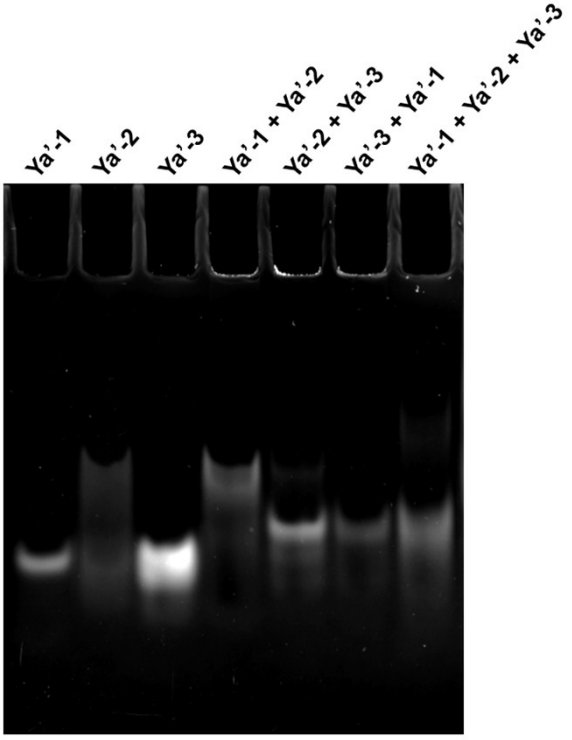

Figure S5. Characterization of the formation of Ya' analyzed by 6% non-denaturing PAGE electrophoresis. Lanes 1-3 are single-stranded DNAs including **F**-containing Ya'-1, Ya'-2, and Ya'-3. Lanes 4-6 are the partial assemblies containing only two strands, **F**-containing Ya'-1+Ya'-2, Ya'-2+Ya'-3, and Ya'-3+Ya'-1. Lane 7 is **F**-

containing Y-motif, Ya'. Note that, Ya'-2 exhibits a smear band which migrate slower than Ya'-1, and Ya'-3 despite of their same sequence length, which may be ascribed to the formation of secondary structure of Ya'-2. For the similar reason, partial assemblies of Ya'-1+Ya'-2 also migrate slower than those of Ya'-2+Ya'-3 and Ya'-3+Ya'-1.

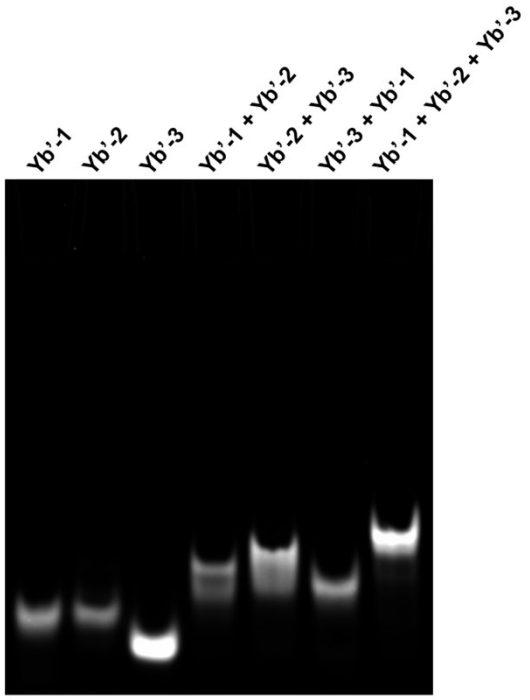

Figure S6. Characterization of the formation of Yb' analyzed by 6% non-denaturing PAGE electrophoresis. Lanes 1-3 are single-stranded DNAs including Yb'-1, Yb'-2, and Yb'-3. Lanes 4-6 are the partial assemblies containing only two strands, Yb'-1+Yb'-2, Yb'-2+Yb'-3, and Yb'-3+Yb'-1. Lane 7 is Y-motif, Yb'.

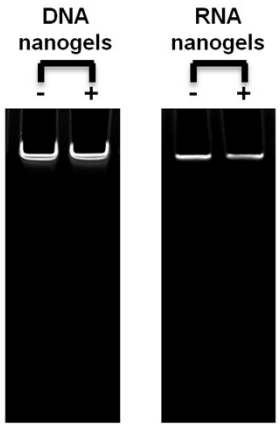

Figure S7. Native PAGE of DNA and RNA nanogels after incubation in phosphate buffered saline with 10% (v/v) fetal bovine serum, which showed their good stability in the mimicked physiological condition. (The '-' and '+' denote the absence or presence of fetal bovine serum).

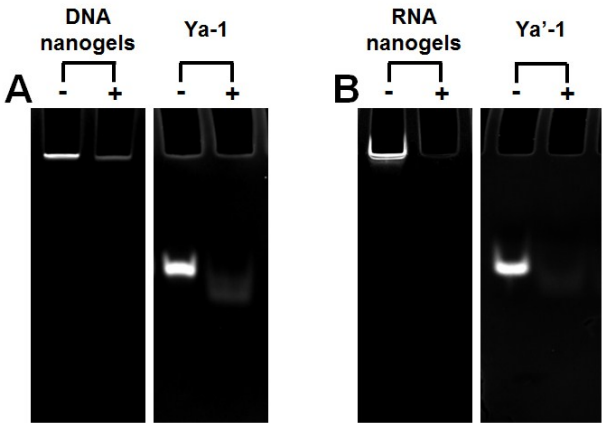

Figure S8. Native PAGE of DNA nanogels, **F**-containing Ya-1, RNA nanogels, and **F**-containing Ya'-1 digested by DNase II or RNase, respectively. (A) DNase II degrades DNA nanogels and Ya-1, as inferred from the absence of digestion products (The '-' and '+' denote the absence or presence of DNase II). (B) RNase degrades RNA nanogels and **F**-containing Ya'-1 (The '-' and '+' denote the absence or presence of RNase).

References

1. S. Kruspe and U. Hahn, *Angew. Chem., Int. Ed.*, 2014, **53**, 10541-10544.
2. Q. Mou, Y. Ma, G. Pan, B. Xue, D. Yan, C. Zhang and X. Zhu, *Angew. Chem., Int. Ed.*, 2017, **129**, 12702-12706.