Supporting Information for

DNA Dendrimer-Based Nanocarriers for Targeted Co-Delivery and Controlled Release of Multiple Chemotherapeutic Drugs.

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Materials

Tris-HCl-EDTA, 1 M Hepes buffer, doxorubicin hydrochloride, Fusitin dihydrochloric acid, N, N'-methylene diacrylamide, TAE buffer, TBE buffer, Sodium chloride (NaCl), Magnesium chloride ($MgCl_2$), acrylamide, sucrose gel loading buffer I, Agarose, DMEM (high sugar) culture-medium, Extra grade Fetal Bovine Serum (FBS), penicillin/streptomycin solution (sterile), PBS buffer, trypsin, dimethyl sulfoxide (DMSO) were purchased from Shanghai BioEngineering Co., LTD. N, N, N', N'-tetramethylenediamine was purchased from Shanghai Bider Medical Technology Co., LTD. Green cell membrane dye, Hoechost 33342, purchased from Thermo Fisher Scientific. Cell Counting Kit was purchased from Shanghai Seven Sea Futai Biotechnology Co., LTD. The deionized water used in the experiment was obtained from the Millipore deionized water system and filtered through a 0.22 μm microporous filter membrane to remove bacteria. The DNA oligonucleotide sequences were synthesized by Shengong Bioengineering (Shanghai) Co., Ltd. and purified by high performance liquid chromatography (HPLC). The DNA sequence is in Table S1.

Methods

Gel electrophoresis

Agarose gel electrophoresis method for the synthesized DNA dendrimers of different generations (G0, G1, G2, DDN, Apt-DDN, etc.), agarose gel electrophoresis method was used for preliminary analysis. The specific operation is as follows:

Weigh 1.5 g agarose in a 250 mL conical flask, add 100 mL 1 × TAE buffer (40 mM Tris-Acetate; 1 mM EDTA; pH 8.0-8.6), put in a magnetic stirrer, stir and boil on a heated magnetic stirrer to obtain a boiling colorless and transparent agarose solution. After allowing the solution to cool slightly, add 1 μL of ethidium bromide and mix thoroughly. Pour the mixture into a plastic mold, insert a comb of suitable pore size, and allow it to cool and solidify to form a 1.5% agarose gel.

After the gel is formed, pull out the comb and load the gel plate into the JY-SPDT horizontal electrophoresis tank. Inject 1 × TAE buffer into the tank to cover the gel surface by 2-3 mm. Inject 6 μL (5 μL DNA + 1 μL 6× sucrose gel loading buffer) of the sample to be electrophoresed into the gel holes. Close the lid and turn on the power. Perform electrophoresis at a voltage of 100 V for 20 minutes. Then take photos, observe and analyze on the gel electrophoresis imager.

In order to characterize the stability of the synthesized DNA dendritic macromolecules under physiological conditions, 20 nM 20 μL Apt-DDN was incubated in PBS buffer at 37 ℃ for 2 h, and then the samples before and after incubation were loaded into 1.5 % agarose gel and run in a horizontal electrophoresis tank for 20 minutes (Run buffer: 1 × TAE; Operating voltage: 120 V), and finally take pictures on the gel electrophoresis imager for observation and analysis.

Atomic Force microscopy (AFM)

Each generation of DNA dendrimers was characterized by atomic force microscopy. The specific method is as follows: each generation of DNA dendrimers was prepared into a 20 μL atomic force microscope imaging sample with a mass of about 750 ng, containing 5 mM $MgCl_2$, 10 mM Tris-HCl buffer, and pH 7.0. Subsequently, 20 μL of the sample solution was deposited on the surface of a new layer of mica sheet and allowed to adsorb for 5 minutes. The surface of the mica sheet was then gently rinsed with deionized water for about 15 seconds, blown dry with an ear bulb, and then placed in a vacuum dryer for 30 minutes. After drying, the sample was scanned using the tapping mode of the atomic force microscope and the results were analyzed. We used NanoScope Analysis to perform particle analysis, and the results showed that the average particle size of G0 was about 14.398±0.65 nm, the average particle size of G1 was about 25.153±1.15 nm, the average particle size of G2 was about 41.671±1.90 nm, and the average particle size of Apt-DDN was about 53.246±2.43 nm (**Fig. S3**). As the number of layers of DNA dendrimers increased, the particle size of the nanoparticles also increased, which further confirmed the successful synthesis of the DNA dendrimer drug delivery system. In addition, it can be seen from the figure that the single-layer Y-shaped DNA is the highest, while the height of the multi-layer DNA dendrimer is reduced. There may be many reasons for this phenomenon. The pressure of the needle tip, the adsorption of DNA by the mica sheet, and the dehydration of DNA may all cause changes in the height of the DNA nanoparticles.

Fluorescence analysis

In this experiment, RF-5301PC fluorescence spectrometer from Shimadzu Company in Japan was used to measure the fluorescence intensity of DNA dendritic macromolecules loaded with doxorubicin under 469 nm excitation light source, and recorded every one nanometer from 350 nm to 700 nm. The excitation slit and emission slit were set at 10 nm.

We reacted the drug-loaded Apt-DDN in PBS buffer and different concentrations of GSH solution at 37 ℃ for 2 hours, and then performed fluorescence spectroscopy analysis. In the experiment, the concentration of doxorubicin was 0.2 mM, the concentration of DNA dendrimer was 85 nM, and all samples were diluted 3 times for fluorescence spectroscopy analysis. The fluorescence intensity of doxorubicin not mixed with DNA dendrimers as the control group was the highest.

In order to determine the optimal loading capacity of dendritic macromolecules on doxorubicin, Apt-DDN and doxorubicin were mixed in different proportions. Specifically, the mass ratio of Apt-DDN and doxorubicin in the control solution remained unchanged. 0.55, 1.38, 2.76 and 5.52 were gently shaken at room temperature for 6 h to make adriamycin fully embedded in the DNA double helix structure, and then their fluorescence intensity was detected respectively.

The target drug release rate is calculated using the following formula based on fluorescence intensity:

$$Target\ drug\ release\ rate = \frac{(Target\ fluorescence\ intensity\ level) - (Dox - Apt - DDN\ fluo}{(Dox\ fluorescence\ intensity\ level) - (Dox - Apt - DDN\ fluor}$$

Cell culture

Cell recovery

The cells were removed from the liquid nitrogen tank and gently shaken in a 37 ℃ water bath for 1-2 minutes to resuscitate them. All the cell fluids were transferred to a centrifuge tube containing 9 mL DMEM medium in a biosafety cabinet, centrifuged at 1500 rpm for 5 minutes, and then the supernatant was sucked up. Then 3 mL DMEM medium containing 10% FBS and 1% dicarboxylate solution (cyanostreptomycin mixed solution) was added, and the cell suspension was transferred into a 25 cm^2 cell culture bottle and cultured in a 37 ℃ cell culture chamber containing 5 % carbon dioxide.

Cell passage

The well-grown Hela cells (Nanjing Kebai Biotechnology Co., Ltd.) were removed from the cell incubator and cultured under an inverted biological microscope when the number of cells in the visual field was greater than 70 % of the entire visual field area. To be specific: The cell culture bottle to be passed was disinfected and put into the biosafety cabinet, the original medium was sucked out, the cells were cleaned three times with PBS, 1mL 0.25 % trypsin was added to the cell culture bottle, and the cell was put into the cell culture box for 2-3 minutes, and the cells fell off the wall of the cell culture bottle under the action of trypsin. Then 6-10 mL culture medium (depending on the number of cells) was added to the biosafety cabinet and evenly divided into 3 empty cell culture bottles.

Under normal circumstances, the culture medium is changed every day, and the specific operations are as follows: Remove the cells from the cell incubator, disinfect the cell culture bottle with 70 % alcohol, and quickly put into the biosafety cabinet; The original medium was sucked, the cells were cleaned three times with PBS buffer preheated in 37 ℃ water bath, and then 3 mL of medium was added and cultured in a 37 ℃ cell incubator containing 5 % carbon dioxide.

Cell storage

The cells that are not used for the time being can be frozen to facilitate subsequent use. The specific operations are as follows: First, the cells that need to be frozen are digested with trypsin; Then the suspended cell fluid was transferred to a centrifuge tube containing 9 mL culture medium at 2000 rpm for 5 minutes, the supernatant was sucked out, and the bottom-sunk cells were dispersed with 1 mL culture medium containing 10 % DMSO and 10 % FBS, and all of them were transferred to the cell freezing tube. Place in a cell freezing program cooling box and store in a -20℃ medical low temperature box for 12 hours, and put the cell freezing tube into a liquid nitrogen tank the next day for storage.

Anti-cancer drug-loaded DNA Dendrimer-based Nanocarrier

Anti-cancer drug-loaded Apt-DDN

Doxorubicin-loaded Apt-DDN

(1) Weigh out 11.6 mg of doxorubicin hydrochloride, add to 5 mL of deionized water, and vortex until fully dissolved to prepare a 4 mM orange-red clear solution of doxorubicin hydrochloride. Seal and store at 4 ℃ for future use.

(2) Conduct toxicity tests on tumor cells using dendritic DNA macromolecules loaded with varying amounts of anticancer drugs to establish a concentration ratio of dendritic DNA macromolecules to anticancer drugs at 1:2000 for subsequent experiments.

(3) Following the determined concentration ratio, mix 95 μL of 90 nM Apt-DDN with 5 μL of 4 mM anticancer drug doxorubicin, and incubate with gentle shaking at room temperature for 6 hours to allow for complete intercalation of doxorubicin into the DNA double helix.

Netropsin-loaded Apt-DDN

(1) Weigh out 20 mg of netropsin dihydrochloride dihydrate, add to 10 mL of deionized water, and vortex until fully dissolved to prepare a 4 mM colorless and transparent netropsin solution. Seal and store at ℃ for future use.

(2) Mix the 95 μL 90 NM Apt-DDN and 5 μL 4 mm anticancer drug spindle mixing. Incubate with gentle shaking at room temperature for 12 hours to allow for complete intercalation of actinomycin into the minor grooves of the DNA double helix.

Doxorubicin/Netropsin-loaded APT-DDN

(1) Configure 4 mm orange-red clarion hydrochloride solution and 4 mm colorless transparent Netropsin solution at 4 ℃ for later use.

(2) Pay 90 μL 90 nm Apt-DDN and 5 μL 4 mm Doxorubicin, 5 μL 4 mm Netropsin, and incubate with gentle shaking at room temperature for 12 hours to ensure thorough intercalation of the drug into the DNA double helix and minor grooves.

Cell viability tests

Firstly, the well-grown Hela cell line was digested and separated with trypsin, and then an appropriate amount of medium was added. Then the cell suspension was inoculated into 96-well plates (100 μL/well), with about 5000 cells per well, and cultivate for 12 hours. After the cells were attached to the wall, drug vectors were added into the 96-well cell culture plates respectively. The corresponding amount of Cell culture solution, drugs, and Cell Counting Kit solution were added but the holes without cells were used as blank control (in order to prevent evaporation, a circle around the 96-well plate was added with PBS for moisturizing). Three duplicate holes were set in each group, and the culture was continued for 48 h.

Cell Counting Kit solution (10 μL) was added to each well and incubated in the cell incubator for 2 h. Then the absorbance (OD) was measured with a full-wavelength enzyme-label at the main wavelength of 450 nm and the secondary wavelength of 650 nm, and the values were expressed as three independently repeated mean ± standard deviation. Cell viability was converted from OD value by the following formula:

$$Cell\ survival\ rate\% = \frac{Experimental\ group\ OD\ value}{Control\ group\ OD\ value} \times 100\%$$

Cell growth inhibition rate %= (1- cell survival rate %) ×100%

Then, with drug dose as the horizontal coordinate and cell survival rate as the vertical coordinate, the cell viability histogram was drawn and analyzed.

Confocal microscopic analysis

DNA dendritic macromolecules were mixed with the anti-cancer drug doxorubicin and gently shaken for 6 h to form Dox-Apt-DDN complex, which was then co-cultured with Hela cells. To be specific: First, the well-grown Hela cells were digested with trypsin, added into 10 mL medium, shook evenly, and inoculated into 2 confocal dishes (1 mL cell suspension in each confocal dish), cultivate for 12 hours, the cells were glued to the wall, and then cleaned with PBS three times. Doxorubicin solution and Dox-Apt-DDN solution with equal quality of doxorubicin were added to confocal small dishes respectively, and cultured at 37 ℃ for 2 h, the cells were cleaned three times with PBS, and 1 mL colorless DMEM medium was added. Then the distribution of doxorubicin in the cells was observed and analyzed using confocal microscopy.

Experimental Results

Oligonucleotide sequences information

Table S1. Oligonucleotide sequences information used in this study

Oligonucleotide name	Oligonucleotide base sequence (from 5' to 3' ends)
G0-1	**GCTAGGCATCACT** -S-S- GCACGACATCGAC ATGAGAGTGTGTC
G0-2	**GCTAGGCATCACT** -S-S- GACACACTCTCAT TCTGCCAAGCGTC
G0-3	**GCTAGGCATCACT** -S-S- GACGCTTGGCAGA GTCGATGTCGTGC
Y1-1	**TAGCTGGCTCATC** -S-S- CAGTCTACGCTCA CGGACATGCAAGC
Y1-2	**TAGCTGGCTCATC** -S-S- GCTTGCATGTCCG TCAGTTGTGCATC
Y1-3	**AGTGATGCCTAGC** -S-S- GATGCACAACTGA TGAGCGTAGACTG
Y2-1	**CAACAGCGTGACT** -S-S- TGCTGCAAGCTGC CTGGACTGCTTGA
Y2-2	CAACAGCGTGACT -S-S- GCTCACAGATGCA GCAGCTTGCAGCA
Y2-3	**GATGAGCCAGCTA** -S-S- TCAAGCAGTCCAG TGCATCTGTGAGC
MUC1	**AGTCACGCTGTTG** CACGTAGCCGTTACGTACTT GCAGTTGATCCTTTGGATACCCTGG GCTCGAGTAACGGCTACGTG
VEGF	**AGTCACGCTGTTG** CACGTAGCCGTTACGTACTT GCACTCTGTGGGGGTGGACGGGCCGGGT GCTCGAGTAACGGCTACGTG
P-gp	**AGTCACGCTGTTG** -S-S- TTCAAGATCCATC CCGACCTCGCG

Note: Bold letters of the same color are complementary sequences that connect adjacent layers. The [-s-s-] in the middle of the base represents the disulfide bond modification, and the specific modification chemical structure is shown in the figure below.

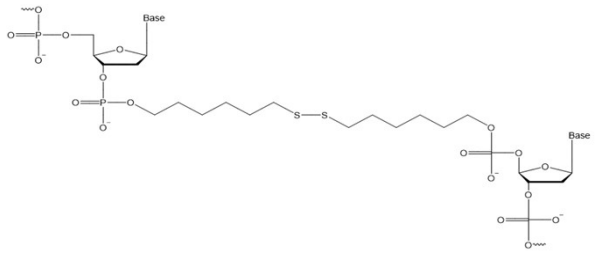

Figure S1. Schematic of disulfide bond modification positions.

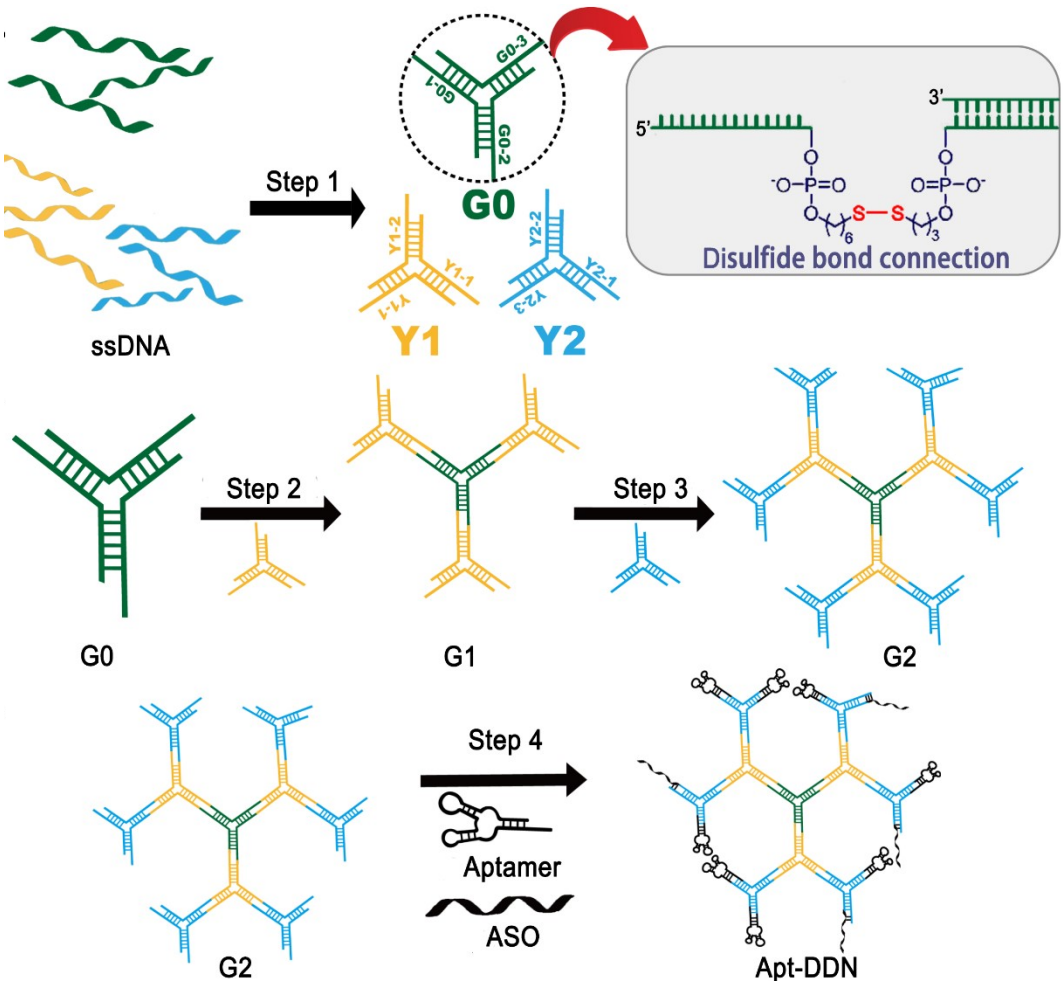

Figure S2. Schematic of the synthesis of surface-functionalized DNA dendrimers

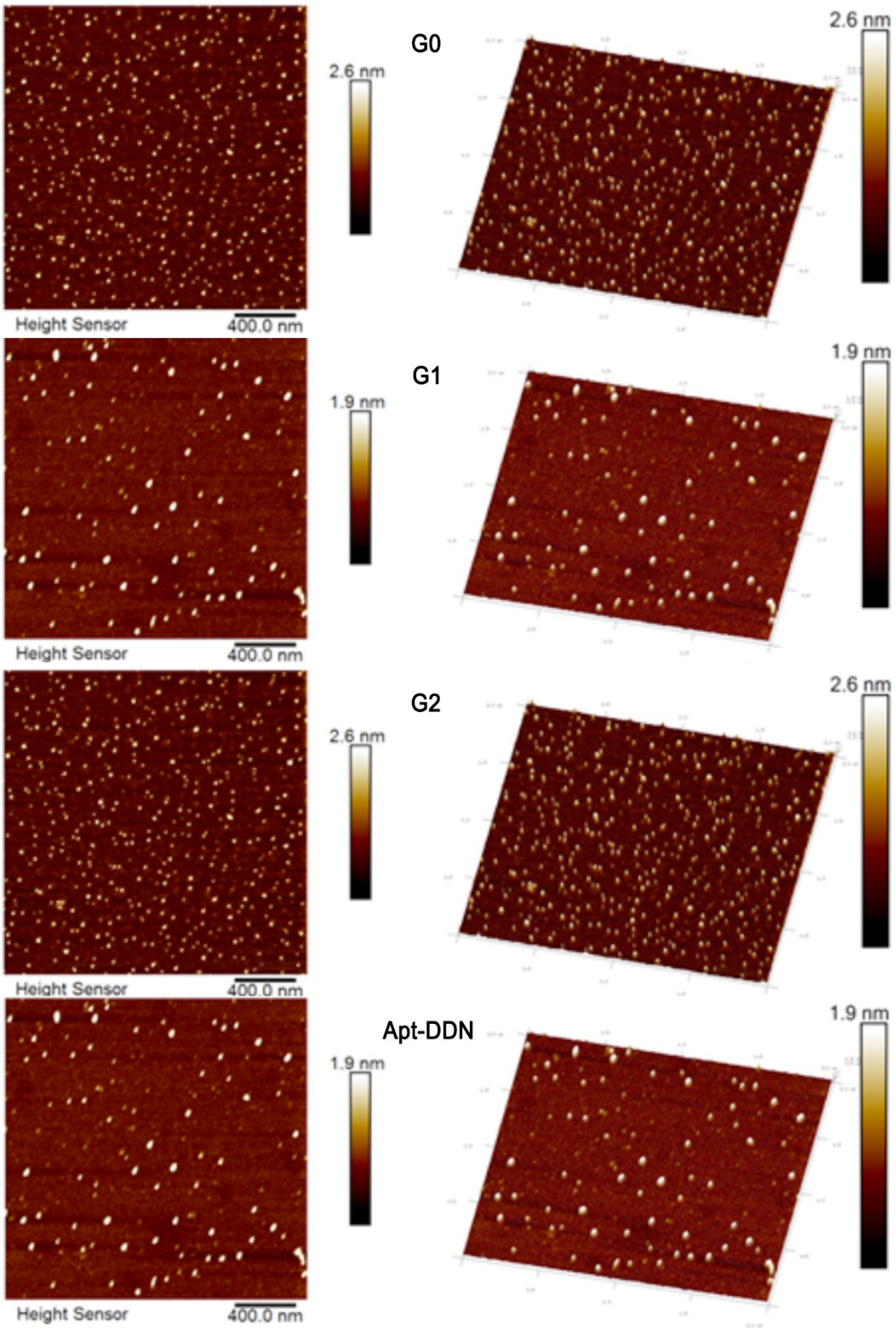

Figure S3. Atomic force microscopic imaging of the DNA-based nanostructures G0, G1, G2, and Apt-DDN by tapping mode.

Table S2. Aptamer functionalized DNA dendrimer synthesis information

Oligonucleotides name	Number of bases	GC content (%GC)	Denaturation temperature (TM)	Molecular mass (MW)
G0-1	39	53.8	69.3	12345.2
G0-2	39	53.8	69.6	12176.1
G0-3	39	59.0	72.6	12368.2
Y1-1	39	56.4	71.1	12241.2
Y1-2	39	53.8	70.9	12260.2
Y1-3	39	51.3	68.7	12409.3
Y2-1	39	56.4	72.9	12312.2
Y2-2	39	56.4	72.9	12299.2
Y2-3	39	53.8	69.6	12345.2
MUC1	78	53.8	77.3	24041.6
VEGF	81	61.7	82.2	25136.2
P-gp	37	56.8	71.3	11589.7