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ATP Responsive DNA Nanogels Grown on Biocompatible Branches for Anticancer Drug

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Experimental section

1. Materials

A549 tumor cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell culture media and reagents were all purchased from KeyGEN, Inc. (Jiangsu, China). The ATP aptamer (ACC TGG GGG AGT ATT GCG GAG GAA GGT, sequence from 5' to 3'), the DNA chains with carboxyl modification at the 5' terminal (5'-(COOH) Oligo DNA), including DNA_1 (CTC TCT CTC TTT ACC TTC CTC CGC) and DNA_2 (ACT CCC CCA GGT AAA GAG AGA GAG) were purchased from TaKaRa Inc. (Dalian, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), 1-hydroxypyrrolidine-2, 5-dione and cellulose dialysis bags (MWCO = 1000 Da) were purchased from Aladdin Co., Ltd. (Shanghai, China). Carboxymethyl chitosan (CMCS, 65% carboxymethyl substitution) were purchased from Fankewei Co., Ltd. (Shanghai, China). Doxorubicin hydrochloride was purchased from Meilun Biotech Co., Ltd (Dalian, China). Dimethyl sulfoxide (DMSO) and ammonia solution (28%) were obtained from Sinopharm Chemical Reagent Co., Ltd. All chemicals and solvents were of analytical grade.

2. Methods

2.1 *Synthesis and characterization of CMCS-DNA copolymers*

The carboxymethyl chitosan-grafted-ssDNA copolymers were synthesized by the following two steps. In the first step, DNA_1 (100.76 nmol, Mw 7065) and DNA_2 (71.86 nmoL, Mw 7377) both chemically bonded with resin were dissolved respectively in 1 mL of 2-(N-morpholino) ethanesulfonic acid buffer (MES, 0.1 M, pH = 4.9) with N-hydroxysuccinimide (NHS, Mw 115.09, 8.6 μmoL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) (Mw 155.24, 6.4 μmoL). The activation reaction of the ssDNA carboxylic acid groups was carried out in a thermal shaker (Hangzhou allsheng instrusment Co., Ltd, China) for 2 h at room temperature. Secondly, the carboxymethyl chitosan (Mw ≈ 4500, 3.3 μmoL) was added to the reactor and was dissolved in the above mixed solution at room temperature under 1200 rpm shaking for 24 h in the thermal shaker to enable the formation of CMCS-DNA copolymers. The excess reagents were removed by rinsing with MES buffer (5 mL). The carboxymethyl chitosan-grafted-ssDNA on solid support was cleaved from the resin by adding 1.0 mL of a 28% ammonia solution. This reaction was performed in the same thermal shaker and maintained at 40 °C for 24 h. The copolymers were then filtered to remove the resin, and the resin was further washed with water.

Carboxymethyl chitosan-grafted-ssDNA copolymers subsequently underwent dialysis (dialysis membrane, MWCO = 1000 Da) against TM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH = 8.0) at room temperature for 12 to 14 h (600 mL) to remove any residual reagents, also for protecting groups. Then, the copolymers were lyophilized using a freeze dryer (Beijing Boyikang Laboratory Instrument Co. Ltd, Beijing, China).

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) were used to characterize the CMCS-DNA copolymer. The copolymer was dissolved in an aqueous solution of acetonitrile (70/30 acetonitrile/H_2O, *v/v* ratio) using 2, 5-dihydroxybenzoic acid (DHB) as matrix (Sigma Aldrich, Buchs, Switzerland). Besides, the chemical structures of CMCS-DNA copolymers were verified using Fourier transform infrared spectroscopy (FTIR, TENSOR 27, Bruker Corporation, Germany).

2.2 Preparation and characterization of NGs@DOX and NGs without DOX

DOX itself has red fluorescence, for this reason, the prepared DOX-loaded CMCS-ATP apt-DNA nanogels can be indicated by the fluorescence quenching of DOX after encapsulated in the CMCS-ATP apt-DNA nanogels. The NGs@DOX was prepared by the co-incubation of CMCS-DNA_1, CMCS-DNA_2, ATP aptamer and DOX solution. DOX (18.56 μg/mL, 0.5 mL) in TM buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH = 8.0) was added together with CMCS-DNA_1 (16 μM, 0.5 mL), CMCS-DNA_2 (16 μM, 0.5 mL) and ATP aptamer (32 μM, 0.5 mL) and incubated at room temperature for 17 h. Followed by column chromatography to remove the free DOX, the DOX@CMCS-ATP apt-DNA nanogels were completely prepared. The fluorescence spectra of DOX were scanned at an excitation wavelength of 471 nm by the fluorescence microplate reader (RF-5301 pc, Shimadzu, Japan). Subsequently, the NGs without DOX were prepared using a similar procedure in the absence of DOX. Then determine the encapsulation efficiency of DOX. The morphologies of the nanogels were examined by transmission

electron microscopy (TEM) (Hitachi, Japan) with an accelerating voltage of 200 kV. The samples were dropped onto a 400 mesh copper grid, and air-dried before analysis.

2.3 *In vitro ATP-responsive release of DOX*

The model drug, DOX, was used as an indicator to explore the ATP-responsive dissociation of CMCS-ATP apt-DNA nanogels because of its red fluorescence. The fluorescence quenching and recovery measurements were applied on the basis of the interaction between nanogels and DOX. Actually, we adjusted the ATP concentrations (0.1, 0.2, 0.3, 8, 12, and 14 mM) in drug release assay by adding the PBS buffer solutions (pH = 6.5) containing different concentrations of ATP into release mediums. After adding ATP buffer solutions into the DOX-loaded nanogels at 37 °C and incubated for a certain time, the fluorescence intensities of DOX released from the nanogels were measured by the fluorescence microplate reader (RF-5301 pc, Shimadzu, Japan), at an excitation wavelength of 471 nm with an emission wavelength of 559 nm. The DOX release fluorescence curve were plotted to analyze the *in vitro* ATP-responsive release of DOX.

2.4 *Cellular uptake*

A549 cells were seeded in 24-well plates with a density of 2×10^4 cells per well and the cells were cultured in 500 μL of RPMI 1640 medium for 24 h to allow cell attachment. After that, the medium solution was replaced by 500 μL fresh RPMI 1640

medium of free DOX and DOX-loaded nanogels at equivalent DOX concentration were added into each well and the cells were incubated with the test solutions for 1, 2, 3 and 4 h. Then, the cells were rinsed three times with PBS buffer before observation under a fluorescence microscope (Olympus IX 51, Osaka, Japan).

In order to quantify the cell uptake of free DOX and DOX-loaded nanogels, A549 cells were cultured in 24-well plates at a density of 6×10^4 cells per well for 24 h. After incubation with RPMI 1640 medium, the medium (500 μL) of free DOX and DOX-loaded nanogels with equivalent DOX concentration (4 μM) replace the culture medium and the blank fresh media (500 μL) was added to the cells as control group. The cells were incubated with test solutions for another 4 h at 37 °C and then detected via flow cytometry by using a fluorescence-activated cell sorting FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.5 *Intracellular ATP-responsive release of DOX*

Briefly, A549 cells (6×10^4 cells per well) were seeded in 24-well plates. After culture for 24 h, the cells were incubated with DOX solutions and CMCS-ATP-apt-DNA nanogels (4 μM DOX concentration) at 37 °C for 3 h. The excessive DOX and DOX-loaded nanogels were removed and the cells were incubated with the fresh FBS free culture medium at 37 °C with or without IAA (Iodine acetic acid, 180 μM) for additional 2 h. IAA was used to inhibit ATP production in the cells. The cells were washed by ice-cold PBS buffer three times and harvested. The fluorescence intensity

of DOX in the cells was measured using fluorescence-activated cell sorting FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.6 *In vitro cytotoxicity studies of DOX loaded NGs*

A549 cells (a human non-small cell lung cancer cell line) were cultured in culture flasks containing RPMI 1640 medium and 10% (*v/v*) fetal bovine serum (FBS) with 10000 U/mL of penicillin/streptomycin under an atmosphere of 5% CO_2 and 90% relative humidity in an incubator (Thermo Scientific, America) at 37 °C. The cells were sub-cultivated approximately at 80% confluence using 0.25% trypsin for enzymatic detachment.

To evaluate the anti-tumor efficiency of DOX-loaded nanogels, the cytotoxicities of DOX and DOX-loaded nanogels (NGs@DOX) were evaluated by an (3-(4, S-dimethyl-2-thiazoyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. To evaluate the biocompatibility of the native nanogels, solutions of DOX-free CMCS-ATP apt-DNA nanogels in RPMI 1640 medium (pH 7.4) at different concentrations were used as negative controls. Briefly, the A549 cells (5×10^3 cells per well) were seeded in 96-well plates and incubated with 100 μL RPMI 1640 medium for 24 h. On the following day, the cells were exposed to the DOX solution and DOX-loaded nanogels with different concentrations of DOX (200 μL of fresh medium solutions containing known equivalent DOX concentrations of free DOX and NGs@DOX) for 48 h in the incubator before the MTT assay. Then, 20 μL of MTT solution (5 mg/mL in PBS buffer) was

added into each well for the MTT assay. After 4 h of incubation at 37 °C, the medium was removed, and the cells were mixed with 150 μL of dimethyl sulphoxide to dissolve the insoluble form crystals. The absorbance at 570 nm was measured via the Enzyme Immunoassay Instrument (ELx800, BioTek Instruments, Winooski, VT) and the relative cell viability (%) was calculated according to the obtained absorbance data.

2.7 *Statistical analysis*

Each experiment was performed in triplicate. The obtained data were expressed as the mean value ± standard deviation and analyzed by unpaired Student's t-test. $*p < 0.05$ and $^{\#}p < 0.05$ were considered statistically significant, $**p < 0.01$ and $^{\#\#}p < 0.01$ were extremely significant.

Supporting data

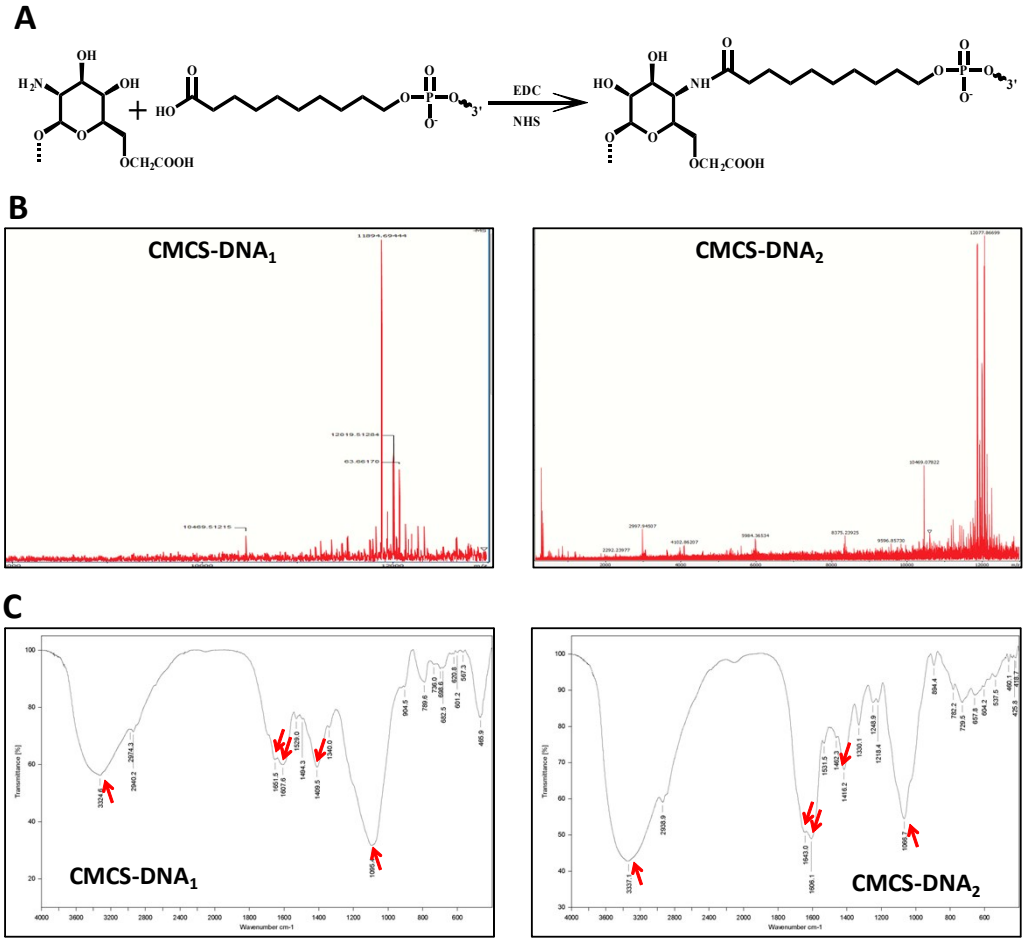

Figure S1. (A) Formula of CMCS grafted single strand DNA_1 or DNA_2. MALDI-TOF-MS (B) and FTIR (C) spectrum of CMCS-DNA_1 and CMCS-DNA_2.

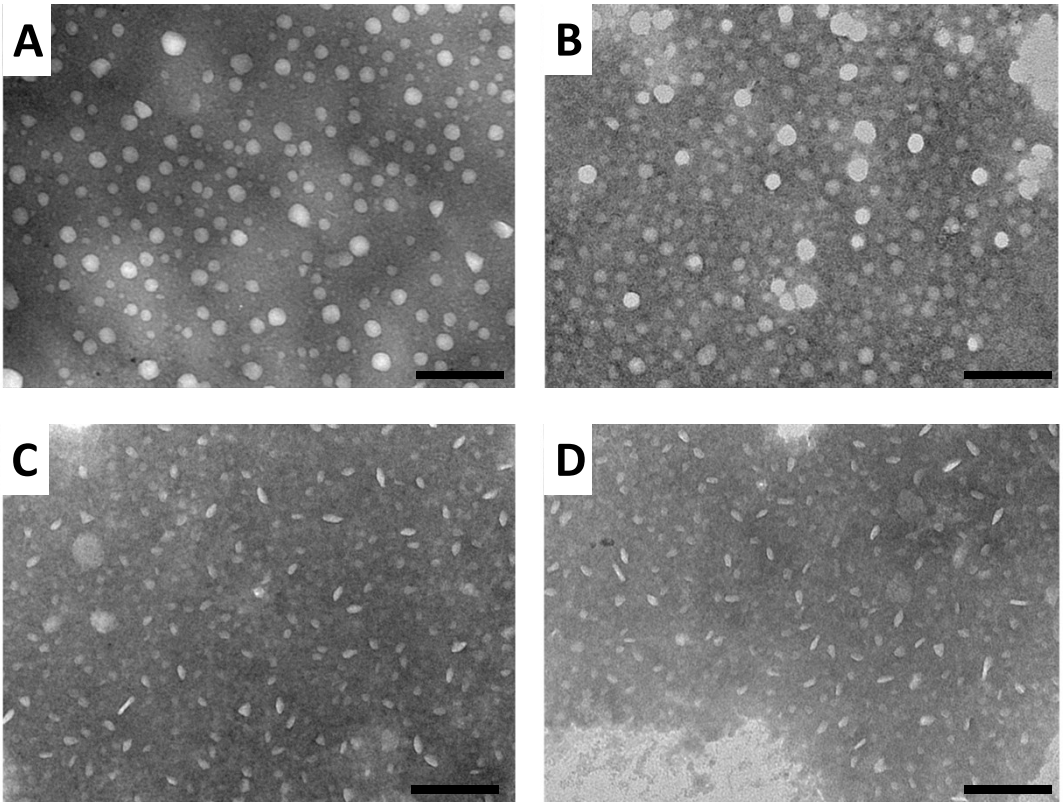

Figure S2. TEM images of NGs@DOX with or without ATP at different pH. (A) pH 6.5, without ATP; (B) pH7.4, without ATP; (C) pH 6.5, with 10 mM ATP; (D) pH 7.4, with 10 mM ATP. Bar = 200 nm.

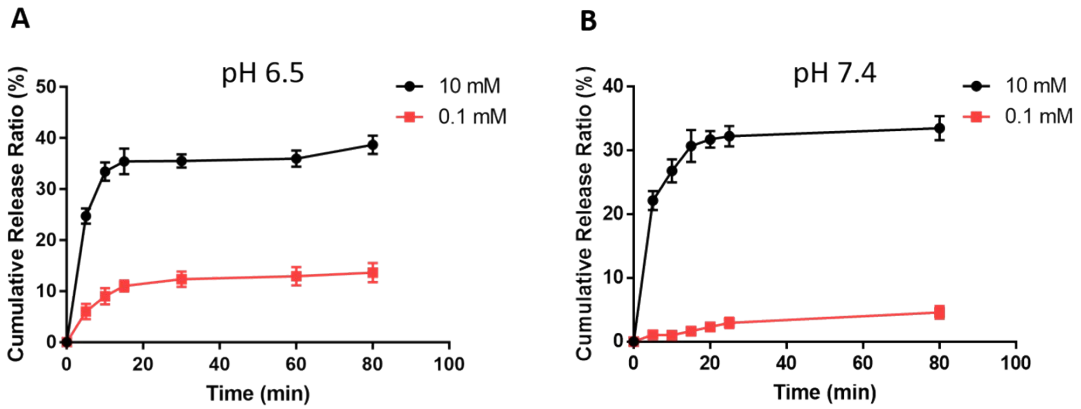

Figure S3. The accumulative release percentage of DOX from NGs@DOX at different ATP concentrations (10 and 0.1 mM) and different pH values (pH 6.5 and 7.4).